



Roots and Gates: Evolutionary Origins and Transport Mechanisms of the Amt/Mep/Rh Ammonium Transport Superfamily

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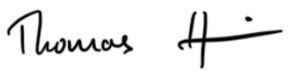
A thesis submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy at the University of Strathclyde.

April 2026

Declaration

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Acknowledgments

Science is a collaborative exercise, and it was against my instincts to change the 'wes and 'ours to 'ls and 'mys in this thesis. A huge part of this was my primary supervisor Dr Arnaud Javelle. His hard work, guidance and belief in me has made this PhD possible. Dr Leighton Pritchard, has also been integral, and I cannot overstate how grateful I am for his help especially since he was drafted in after I had started my PhD to advise with the phylogenetics. Since this is the only section, they cannot comment on (I think?) it can be an indication of how hard they worked, especially Arnaud, to get the rest of the thesis readable. I am also grateful to Prof Paul Hoskisson, my second supervisor, for providing feedback and funding.

My thanks also go to Dr Gordon Williamson and Dr Adriana Bizior. Gordon for sparking my interest for the ammonium transporters by being so great when I was doing my undergraduate project and Adriana for helping me so much when I started the PhD. I would also like to thank Dr Angelika Kiepas for being so much help with the phylogeny. Thanks also to Peter Henderson, I am sorry you have inherited problems with the SURFE²R but I am sure you will take it in your stride.

To everyone else on floor 6 I really appreciate your company over the years I was in the lab, and it would not have been nearly so good without it.

I would like to also thank my mum, Anna, and dad, Mark, for their encouragement. Mum, you started me down the path of biology in your lessons at school and I don't regret a moment. Dad your research has always been an interest and a mystery and it's nice to have a glimpse into academia and the world you inhabit. Thanks to my brothers Adam and Johnny, Adam for inspiring me with your hard work and Johnny because your job will always be better than mine.

My thanks also to my girlfriend, Agáta, your support and cooking for me throughout this PhD has certainly made it possible. Although I'll never be a real doctor like you, I can still try to pretend.

Finally, I would like to thank my dog Esme. Although you will likely never read this, your company has meant a lot to me. You may have not been much help with the contents of this thesis and your insistence of snoozing on my lap meant that a lot of this document was written with one hand, your cheerful presence has made my life better in every way.

Publications

The full publications can be found in the Appendix section.

One paper was published with work from my undergraduate project

Williamson G, Brito AS, Bizior A, Tamburrino G, Dias Mirandela G, **Harris T**, Hoskisson PA, Zachariae U, Marini AM, Boeckstaens M, Javelle A. Coexistence of Ammonium Transporter and Channel Mechanisms in Amt-Mep-Rh Twin-His Variants Impairs the Filamentation Signaling Capacity of Fungal Mep2 Transceptors. *mBio*. 2022 Apr 26;13(2):e0291321. doi: 10.1128/mbio.02913-21

Three review articles were published during the course of this PhD

Bizior A, Williamson G, **Harris T**, Hoskisson PA, Javelle A. Prokaryotic ammonium transporters: what has three decades of research revealed? *Microbiology (Reading)*. 2023 Jul;169(7):001360. doi: 10.1099/mic.0.001360.

Williamson G, Bizior A, **Harris T**, Pritchard L, Hoskisson PA, Javelle A. Biological ammonium transporters from the Amt/Mep/Rh superfamily: mechanism, energetics, and technical limitations. *Biosci Rep*. 2024 Jan 31;44(1):BSR20211209. doi: 10.1042/BSR20211209.

Williamson G, **Harris T**, Bizior A, Hoskisson PA, Pritchard L, Javelle A. Biological ammonium transporters: evolution and diversification. *FEBS J*. 2024 Sep;291(17):3786-3810. doi: 10.1111/febs.17059.

The results from Chapter 3 will form the core of a paper which is currently in preparation

Harris T, Pritchard L, Javelle A. 'Ammonium transporter superfamily evolutionary insights' manuscript in preparation

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List of abbreviations

°C	Degrees Celsius
A solution	Activating solution
Å	Angstrom
AeAmt1	<i>Aedes aegypti</i> Ammonium transporter 1
AfAmt1	<i>Archaeoglobus fulgidus</i> ammonium transporter 1
AgAmt	<i>Anopheles gambiae</i> ammonium transporter
AgRh50a	<i>Anopheles gambiae</i> Rhesus 50 a
AgRh50b	<i>Anopheles gambiae</i> Rhesus 50 b
Amt	Ammonium transporter
AOB	Ammonia oxidising bacteria
CaMep2	<i>Candida albicans</i> Methylamine permease 2
CF	5-carboxy fluorescein
CHO	Chinese Hamster Ovary
CryoEM	Cryogenic electron microscopy
CV	Column Volume
DDM	dodecyl-β-maltopyranoside
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EcAmtB	<i>Escherichia coli</i> ammonium transporter
g	<i>x gravity</i>
HGT	Horizontal gene transfer
HEK	Human embryonic kidney cells
HK	Histidine kinase
ICL	Intracellular loop
IMAC	Immobilised Metal Affinity Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISO	Inside out
KDa	KiloDalton
Km	Michaelis constant
Ks-Amt5	<i>Kuenenia stuttgartiensis</i> ammonium transporter 5
LB	Lysogeny Broth

LDAO	lauryldecylamine oxide
LPR	Lipid to Protein Ratio
LUCA	Last universal common ancestor
MDS	Molecular Dynamic Simulation
MeA	Methylammonium
Mep	Methylammonium permease
ML	Maximum likelihood
MP	Maximum parsimony
nA	NanoAmpere
NA solution	Non-activating solution
NeRh50	Nitrosomonas europaea Rh50
nF	NanoFaraday
nS	Nanoseconds
OD	Optical density
OHS	Overhydrated stomatocytosis
PAGE	Polyacrylamide gel electrophoresis
PAS	Per-Arnt-Sim
PDB	Protein Database
pdi	Polydispersity index
pH	Power of hydrogen
PMSF	Phenylmethylsulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
RBC	Red blood cell
RF	Robinson Foulds
RNA	Ribonucleic acid
Rsat	Saturation Constant
Rsol	Solubilisation Constant
RSO	Right side out
Rh	Rhesus
RMSD	Root Mean Square Deviation
ScMep2	<i>Saccharomyces cerevisiae</i> Methylaminepermease 2
SdAmR	<i>Shewanella denitrificans</i> Ammonium receptor
SDS	Sodium Dodecyl Sulphate

SEC	Size Exclusion Chromatography
SMALP	Styrene maleic acid lipid particles
SSME	Solid Supported Membrane Electrophysiology
TBE	Transfer bootstrap expectation
TEMED	Tetramethylethylenediamine
TMH	Transmembrane helix
TM	Transmembrane
TOL	Tree of life
Tris	Tris(hydroxymethyl)aminomethane
WT	Wild type
QM/MM	Quantum mechanics/ molecular mechanics

Abstract

Ammonium transport across biological membranes is essential for life and is mediated by the Amt/Mep/Rh superfamily of membrane proteins, which are conserved across all domains of life. Despite a shared overall fold and key structural features, these proteins exhibit diverse physiological roles, functioning as high-affinity ammonium transporters, bidirectional detoxifiers, or non-transporting sensors. While individual subfamilies have been studied, the evolutionary and functional relationships among them remain incompletely understood. The overall goal of this project was to investigate this superfamily of transporters as a whole by generating a phylogenetic tree, developing a method of measuring the activity of proteins without the need to purify and reconstitute them and assess the function of a ubiquitously conserved feature, the Phe Gate.

A root is proposed for the phylogenetic tree of ammonium transporters between those proteins that have a Glutamate residue at the entrance to an ammonium binding pocket and those that have Tyrosine residue. Because there were frequent pairings of archaeal and bacterial proteins, this tree gave evidence to the hypothesis that LUCA had ammonium transporters. This is at odds with previous research in predicting LUCA's genome and constitutes an interesting find. The tree was also used to investigate other features of the protein's family such as the occurrence of Amt's fused to other enzymatic domains where it was found that in this tree, they only occur in proteins found in the Glu group. It was also found that the Twin His motif (HH) variation where the first His is changed for a Glu (EH), previously only seen in fungal proteins, is more common with an archaeal group sitting at the base of the fungal group containing a mixture of EH and HH proteins. The Phe Gate was found to be totally conserved.

To circumvent the requirement for protein purification and reconstitution, a functional assay was developed by generating vesicles directly from *E. coli* cells overexpressing EcAmtB, with ammonium transport activity assessed using solid-supported membrane electrophysiology (SSME). This approach reliably detected transporter-mediated ammonium flux, demonstrating the assay's viability for functional analysis in a near-native membrane context.

Despite its strict evolutionary conservation across the Amt/Mep/Rh superfamily, the functional role of the phenylalanine gate (Phe Gate) remains unresolved. Previous studies have proposed potential roles in substrate selectivity or facilitating deprotonation during transport. To investigate its function, Phe Gate variants of EcAmtB and *NeRh50* were analysed using SSME. These variants exhibited a complete loss of transport activity without any alteration in potassium ion selectivity. These findings indicate that the Phe Gate is not merely a passive structural feature or selectivity filter but plays an essential mechanistic role in enabling ammonium transport. Overall, this work has increased our knowledge of the phylogenetic history of these proteins and sets the foundation for future work to be able to measure the activity of a greater diversity of ammonium transporters.

Chapter 1: Introduction

1 Chapter 1: Introduction

Nitrogen is the fourth most prevalent element in biological systems (Frieden, 1972) and serves as a crucial component of DNA, RNA, and proteins. Consequently, nitrogen availability often limits the growth rate of organisms. Despite this, nitrogen is abundant in the Earth's atmosphere, with N_2 (dinitrogen) comprising approximately 70% of it. However, N_2 is chemically inert and, therefore, biologically inaccessible to most organisms, except for certain bacteria and archaea capable of fixing N_2 into biologically usable NH_3 (ammonia). Ammonia, as the most common nitrogen species after N_2 , is the preferred nitrogen source for bacteria, fungi, and plants. Therefore, transporting ammonia across biological membranes is a critical physiological function for all organisms. Due to its small size and uncharged nature, ammonia can diffuse across membranes; however, NH_3 is in equilibrium with its charged form, NH_4^+ . Given that the pKa of the NH_4^+/NH_3 equilibrium is 9.25, 99% of total ammonium exists as NH_4^+ at physiological pH, which cannot readily diffuse across membranes. Despite this, organisms can accumulate ammonium, even against concentration gradients, suggesting the existence of specialised ammonium translocators (Kleiner, 1981, 1985).

1.1 Ammonium transporter discovery

The process of ammonium translocation is performed by the Ammonium transporter/ Methylamine permease/ Rhesus protein (Amt/Mep/Rh) superfamily of proteins found across the kingdoms of life.

While investigating a non-specific amino acid permease transporter expressed under nitrogen starvation conditions in *Penicillium chrysogenum*, it was observed that ammonium was the only substrate able to inhibit the permease with a K_i order of magnitude lower the K_m of the transporter for amino-acid, indicating that this protein acts as an ammonium, rather than an amino acid, permease (Benko, Wood and Segel, 1969).

In 1994 two labs simultaneously identified the two first genes encoding for ammonium transporting proteins in *Saccharomyces cerevisiae* named methylamine permease (Mep) as [^{14}C]-methylamine was used as a substrate analogue to characterise the transport system (Marini *et al.*, 1994), and *Arabidopsis thaliana* named ammonium transporter (Amt) (Ninnemann, Jauniaux and Frommer, 1994). Later using

bioinformatics, it was discovered that the Rh50 glycoproteins found in humans and other animals, were distantly related to the Mep and Amt though it was not known if they were also capable of ammonium translocation (A. M. Marini *et al.*, 1997). It was later confirmed using yeast complementation assays and patch clamp electrophysiology in *Xenopus laevis* oocyte expressing Rh proteins that the human Rh proteins RhAG, RhBG and RhCG were capable of ammonium translocation (Marini *et al.*, 2000; Daniel Mak *et al.*, 2006). Since then, it has been established that the three subgroups, Amt, Mep, and Rh, constitute a superfamily of ammonium transporters found in nearly all living organisms. Five ammonia oxidising prokaryotes were found to not code for any ammonium transporters opening the question of how they acquire their ammonium/ammonia (Lehtovirta-Morley *et al.*, 2016).

1.2 Functions of ammonium transporters

Ammonium transporters exhibit functions that extend beyond ammonium scavenging for assimilation. Many organisms can express multiple ammonium transporters, often from distinct Amt/Mep/Rh sub-groups (Matassi, 2017). This diversity implies that each transporter may serve a specific role, and accumulating evidence further supports the idea that distinct transporter members fulfil specialised functions (Williamson *et al.*, 2024). In addition to their role as ammonium scavengers for biosynthetic purposes, Amt/Mep/Rh proteins may also participate in ammonium sensing and detoxification processes.

1.2.1 Ammonium transporters as sensors

The ammonium transporter AmtB in *Escherichia coli* (*EcAmtB*) is also an ammonium sensor protein and is regulated by the P_{II} protein GlnK depending on the concentration of ammonium in the surrounding environment (Javelle *et al.*, 2004; Conroy *et al.*, 2007).

Mep2 in the fungi *Candida albicans* and *S. cerevisiae* also function as a sensor protein triggering the shift from yeast to filamentous growth under low ammonium concentrations conditions (Michael C. Lorenz and Heitman, 1998; Biswas and Morschhäuser, 2005; Brito *et al.*, 2020). This switch has been linked to pathogenicity as the fungi can invade organisms around it (Smith *et al.*, 2003).

Some ammonium transporter proteins possess an additional C-terminal domain that exhibits enzymatic activity (Pflüger *et al.*, 2018; Pflüger *et al.*, 2024). For example, Amt5 from *Candidatus kuenenia stuttgartiensis* possess a C terminal histidine kinase domain (Pflüger *et al.*, 2018). This protein is incapable of transporting ammonium; instead, it undergoes autophosphorylation upon ammonium binding, resembling the function of bacterial sensor kinases in two-component systems. The biological pathway in which this protein is involved is yet to be identified (Pflüger, *et al.*, 2018). The mosquito *Anopheles gambiae* express three ammonium transporters named AgAmt, AgRh50a and AgRh50b (Pitts *et al.*, 2014). AgAmt and AgRh50b are expressed in the antenna of females and are involved in volatile ammonia sensing for prey detection (Pitts *et al.*, 2014).

AmtA in the slime mould *Dictyostelium discoideum* detects ammonium concentrations and regulates the transition between slug and fruiting body development. This process initiates cell culmination and differentiation, resulting in the formation of a base and stalk, with spores positioned at the apex (Singleton, Kirsten and Dinsmore, 2006).

1.2.2 Ammonium transporters as detoxifiers

In animals, ammonium is a toxic byproduct of amino acid metabolism hence must be directly excreted (in ammoniotelic organisms) or converted to the less toxic urea (Bishop *et al.*, 2010). Most fish and crustaceans are ammoniotelic organisms, and the Rh proteins expressed in their gills function as bidirectional transporters essential for maintaining ammonium homeostasis (Weihrauch, Morris and Towle, 2004; Ip and Chew, 2010). To detoxify the blood in humans, ammonium is taken into the red blood cells by RhAG and transported to the kidneys where its level is managed by RhBG and RhCG (Bishop *et al.*, 2010). Rhesus proteins are vital to organism homeostasis with dysregulation being associated with many pathologies, this will be discussed in the next section (Bruce *et al.*, 2009; Stewart *et al.*, 2011).

Amt1a has an important role in brain cavity development in the tunicate *Ciona intestinalis* as ammonium presence affects the viscosity and pH of solution (Marino *et al.*, 2007).

1.2.3 Human/mouse pathologies caused by rhesus malfunction

Overhydrated stomatocytosis (OHS) is a form of haemolytic anaemia caused by the leakage of monovalent cations into red blood cells via the RhAG protein, resulting from mutations in conserved residues critical for the protein ammonium transport function (Bruce *et al.*, 2009; Stewart *et al.*, 2011; Genetet *et al.*, 2012; Deschuyteneer *et al.*, 2013).

When investigating a region of the human chromosome involved in early onset major depressive disorder it was found that single nucleotide polymorphisms (SNPs) were present in the RHCG gene (Verma *et al.*, 2008).

In oesophageal cancers, RhCG is downregulated compared to normal levels. This was found in both high and low risk areas suggesting that it is truly linked to cancer and not merely oesophageal injury (Chen *et al.*, 2002).

Mus musculus Rhcg is also expressed in the testis where it plays a role in keeping the epididymal luminal fluid acidic to stop developing spermatozoa moving during maturation. Rhcg^{-/-} mice had reduced fertility and higher epididymal luminal fluid pH (Biver *et al.*, 2008).

1.3 Structure of ammonium transporters

The first structural analysis of ammonium transport proteins investigated their topology (Thomas, Mullins and Merrick, 2000). Hydropathy plots of thirty known ammonium transporters revealed that most transporters have between eleven and twelve transmembrane helices (TMH) present. Further, a series of PhoA and LacZ fusion with a truncated version of *E. coli* AmtB after each predicated TMH showed experimentally that the protein possesses eleven TMHs (Figure 1.1A) (Thomas, Mullins and Merrick, 2000).

Analytical ultracentrifugation of purified *Ec*AmtB revealed the protein to be a homogenic trimer (Figure 1.1B) (Blakey *et al.*, 2002). The protein was found to have a molecular mass of 90kDa with each monomer having a molecular mass of 30kDa. This was later corroborated using atomic force microscopy (Conroy *et al.*, 2004). It was widely assumed that the determination of the first crystal structure would provide insights into the elusive mechanism of transport.

1.3.1 Crystal structures

In 2004, crystal structure of EcAmtB was independently published by two teams at the high-resolution of 1.35Å and 2.1Å respectively (Khademi *et al.*, 2004; Zheng *et al.*, 2004). The structure shows that each monomer possessed a translocation pore. The pore-lining amino acids were predominantly hydrophobic, creating an environment that prevents the direct translocation of the charged substrate (NH_4^+) through the channel.

Since 2004, seven more ammonium transporters have had their structures solved: *A. fulgidus* Amt-1 (Andrade *et al.*, 2005), *Nitrosomonas europaea* Rh50 (Li *et al.*, 2007; Lupo *et al.*, 2007), *Homo sapiens* RhCG (Gruswitz *et al.*, 2010), *S. cerevisiae* and *C. albicans* Mep2 (Van Den Berg *et al.*, 2016), *Candidatus Kuenenia stuttgartiensis* Amt5 (Pflüger *et al.*, 2018) and *Shewanella denitrificans* Amt1 (Pflüger *et al.*, 2024). The 'core' of the protein consists of eleven membrane spanning helices. The core arrangement is made up of two pseudo-symmetrical bundles between the transmembrane helices 1-5 and 6-10. TMH 11 lies across the structure (Figure 1.1C). The 3D structure of these Amt/Mep/Rh in bacteria, archaea, fungi, and human remains highly conserved (Figure 1.1D).

The structures remained the same when the proteins were crystallised in the presence or absence of ammonium. This structural rigidity may be due to a crystallisation artifact, the protein being stable in certain conformation in the crystal lattice. A better method to keep the protein close to native conditions could be useful in determining if there are any conformational changes associated with the presence/translocation of the substrate. This could be achieved using Cryo-Electron Microscopy (CryoEM), with proteins stabilised in Styrene Maleic Acid Lipid Particles (SMALPs). This approach avoids the use of detergents, preserving the protein in its native lipid environment, which has been shown to be essential for its activity (Dias Mirandela *et al.*, 2018; Mirandela *et al.*, 2019). Preliminary data on this project are presented at the end of this thesis (See Chapter 7 section 7.4.3).

Four different structural features potentially important for the translocation cycle are conserved in these proteins, namely the outer facing vestibule, the "Phe-Gate", the "Twin His" motif and the hydrophobic pore (Figure 1.2). I will begin by outlining these conserved features, followed by a discussion of the specifics of each structure published to date. Since the structure of EcAmtB was the first to be elucidated, it will

be described initially, with subsequent comparisons highlighting differences in the individual structures.

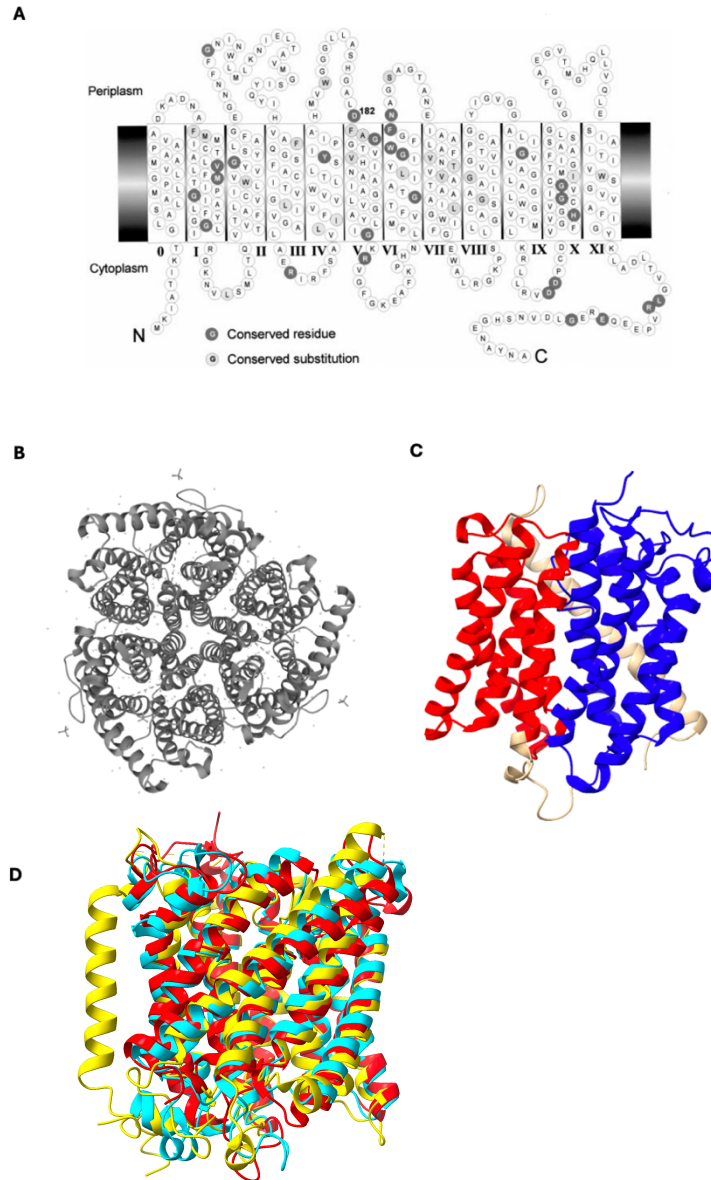


Figure 1.1 Topology and structure of ammonium transporters. A) The topology of the 11 helices in EcAmtB adapted from Thomas *et al* 2000. TMH 0 is labelled as such because it is a signal peptide and is cleaved off following insertion into the membrane. B) The trimeric organisation of the crystal structure of EcAmtB (pdb 1U77 (Khademi *et al.*, 2004)) from above. At the centre of each monomer lies a translocation pore. C) The two pseudo-symmetrical bundles coloured blue (TM regions 1-5) and red (TM regions 6-11) behind can be seen TMH 11 locking the structure together (pdb 1U77 (Khademi *et al.*, 2004)). D) An alignment of three ammonium transporter crystal structures to highlight their structural conservation. Yellow represents RhCG from *H. sapiens* (pdb 3HD6 (Gruswitz *et al.*, 2010)), blue represents Amt-1 from *A. fulgidus* (pdb 2B2I (Andrade *et al.*, 2005)) and red is AmtB from *E. coli* (pdb 1U77 (Khademi *et al.*, 2004)). Despite being from the separate kingdoms of bacteria, archaea and eukaryotes, these proteins share remarkable similarity in their core helical arrangement.

1.3.2 Extracellular vestibule

In the trimer, each monomer has an extracellular vestibule. The site differs between rhesus proteins and the other ammonium transporters and will be discussed later. In EcAmtB, at the bottom of the vestibule four highly conserved residues (F107, W148, D160 and S219) define an NH_4^+ binding site named S1 (Figure 1.2) (Khademi *et al.*, 2004). This S1 site stabilises NH_4^+ via a hydrogen bond with the hydroxyl group of S219 and two cation π interactions with F107 and W148. This makes the binding site highly selective for NH_4^+ over other ions of similar size and valence, such as K^+ or Na^+ , as the number of carbonyl oxygen atoms stabilising ions in solution is a key factor in transporters/channel ion selectivity (Thomas *et al.*, 2007). The monovalent cation TI^+ , which shares a similar size and valence with NH_4^+ , can also be stabilised through tetrahedral coordination, similar to NH_4^+ . As a result, it has been shown to bind to the NH_4^+ binding site of the glutamine synthetase. When co-crystallised with TI^+ , the structure of EcAmtB reveals an anomalous difference electron density map at the TI^+ absorption edge ($\lambda = 0.97822 \text{ \AA}$), located precisely at the S1 site (Javelle *et al.*, 2008). Furthermore, the residues D160, S219 and W148 at the S1 site have been found using electrophysiology, transport assays and molecular dynamic simulations (MDS) to be a crucial for AmtB activity (Lin, Cao and Mo, 2006; Javelle *et al.*, 2008; Williamson *et al.*, 2020). Taken together these findings validate the presence of a specific NH_4^+ binding site at the periplasmic entrance of AmtB.

1.3.3 Phe Gate

Below the S1 binding site, two Phe residues F107 and F215 are stacked together forming the “Phe Gate” (Figure 1.2). The phenyl rings of these residues are positioned in a way that completely obstructs the translocation pathway between S1 and the entrance to the hydrophobic pore (Khademi *et al.*, 2004). It is therefore hypothesised that the two rings must rotate to allow for passage into the pore. In all crystal structures of AmtS and Meps the two Phe rings are co-planar whereas in Rh proteins the first Phe sits perpendicular to the second, making the passage more open (Figure 1.5) (Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005; Li *et al.*, 2007; Lupo *et al.*, 2007; Gruswitz *et al.*, 2010; Van Den Berg *et al.*, 2016; Pflüger *et al.*, 2018; Pflüger *et al.*, 2024). The exact role of the Phe-gate remains unclear and has been one of the focuses of my research, as discussed in Chapter 6.

1.3.4 Twin His Motif and Hydrophobic pore

Following the Phe Gate opens a narrow and highly hydrophobic pore into which protrude two highly conserved hydrophilic residues H168 and H318 named the Twin His motif, a characteristic trait of this family (Figure 1.2) (Javelle *et al.*, 2006). Densities were observed in this area in the presence (Khademi *et al.*, 2004) and absence (Zheng *et al.*, 2004) of ammonium (Figure 1.3A). At a resolution of 1.35Å it is difficult to tell whether these densities correspond to ammonia or a water molecule. MDS and free energy calculations revealed that the Twin His Motif residues are crucial as they connect and stabilise two water wires which explain the densities seen in the first crystal structures (Figure 1.2). The first water wire, called the periplasmic water wire, extends from D160 in the S1 binding site to the Twin His Motif. The second water wire, known as the cytoplasmic water wire, stretches from the Twin His Motif to the cytoplasm (Figure 1.3) (Lamoureux, Klein and Bernèche, 2007; Williamson *et al.*, 2020). This provides a potential explanation for how a proton is translocated through the hydrophobic pore via the Grotthuss mechanism, which I will discuss in more detail in section 0.

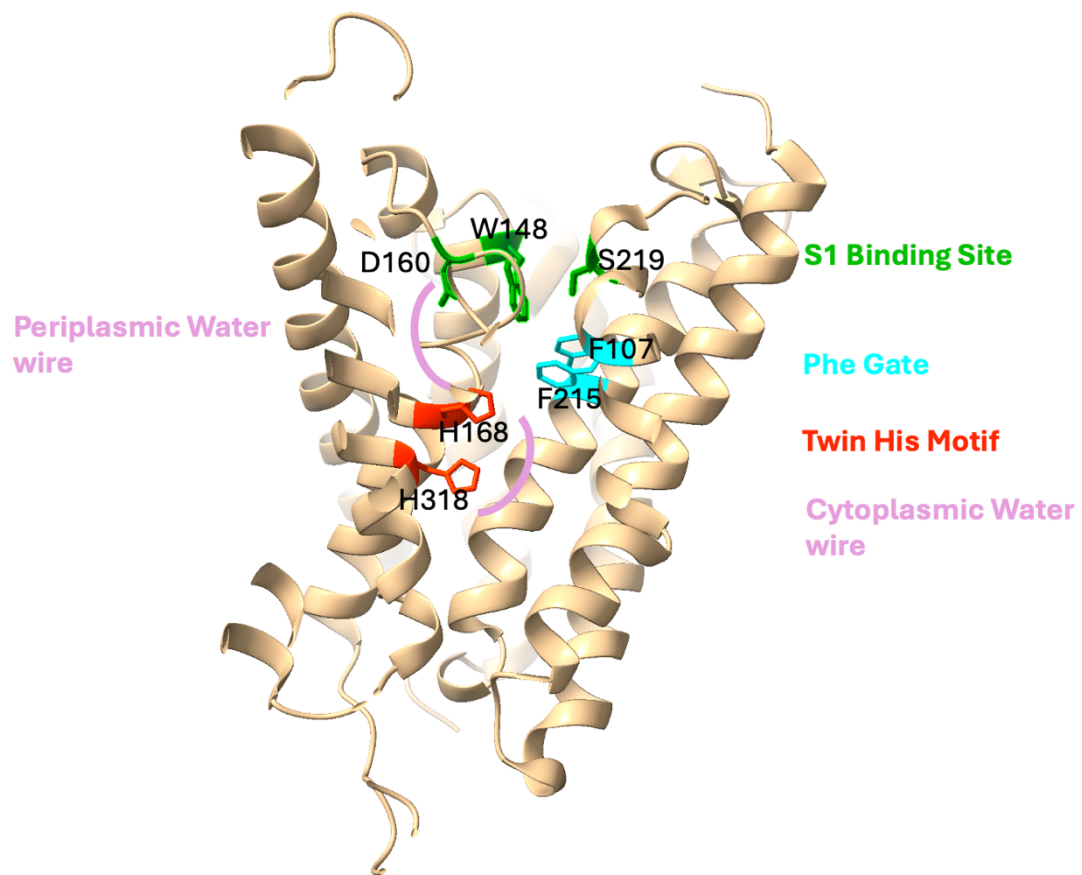


Figure 1.2 **The conserved regions of EcAmtB**. The top of the pore is located at the green S1 binding site, an ammonium ion would approach this from the periplasm to be translocated through the pore, past the Phe gate and the Twin His Motif. Note, this diagram excludes helices to give a better view of the translocation pore and the residues therein. Protein pdb ID 1U77 (Khademi *et al.*, 2004).

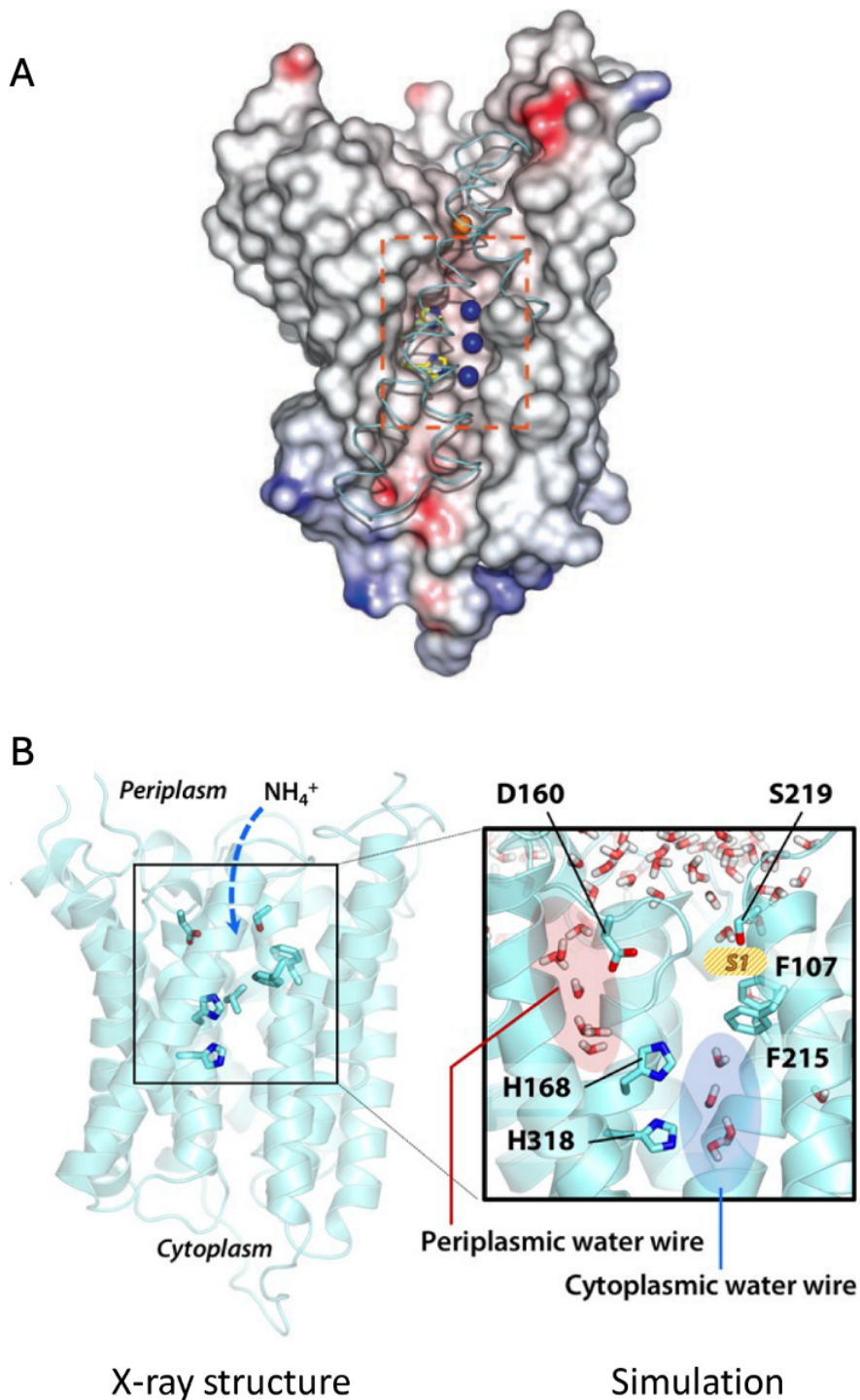


Figure 1.3 **Two water wires are formed in the pore of EcAmtB.** A) Densities were seen in the pore of the first structures of EcAmtB, these were thought to be ammonia, but it was acknowledged that, given their size, it is difficult to tell the difference between ammonia and water. B) In MDS it was seen that water molecules will arrange themselves into two water wires that traverse the pore. Figure adapted from Khademi *et al* 2004 and Williamson *et al* 2020.

1.3.5 *A. fulgidus* Amt-1

The archaeon *A. fulgidus* encodes three Amts, and the structures of all three were elucidated in 2005 (Andrade *et al.*, 2005). The proteins are homotrimeric and each monomer possesses eleven transmembrane helices with two pseudo-symmetrical helical bundles (Figure 1.8) (Andrade *et al.*, 2005). The two rings of the Twin His Motif were almost coplanar which allowed for a stronger hydrogen bond between the N δ atoms. The archaeal protein also had several factors to make it more flexible. A large hydrophobic cavity that filled with xenon derivatives was found at the centre of the structure. This structural feature is conserved in EcAmtB and is characteristic of active transporters, where it has been hypothesised to facilitate the structural rearrangement of transmembrane helix 5 (TMH5) that may pivot on two conserved glycine residues located at its base. The amide nitrogen atoms of these glycine residues are bound to D159 (EcAmtB D160). This Asp residue has been shown to be of critical importance in EcAmtB activity (Javelle *et al.*, 2004; Williamson *et al.*, 2020).

1.3.6 *N. europaea* Rh50

Using x-ray crystallography two groups were able to solve the structure of NeRh50 protein to a resolution of 1.3Å (Li *et al.*, 2007; Lupo *et al.*, 2007). NeRh50 is also a homotrimer with the same pseudo-symmetrical helical bundles seen in other Amt/Rh proteins (Figure 1.8). It differs in the extracellular binding site, the Phe gate, possess a putative CO₂ binding site and an C terminus extension (Figure 1.4). In the periplasmic binding site of Rh50, only one of the three highly conserved residues that constitute the S1 binding site in Amts is present: the phenylalanine (Phe) residue, which also forms part of the Phe gate. The rest of the space in the vestibule is taken up by water molecules. Unlike the parallel rings found in the Phe gate of EcAmtB, F86 is perpendicular to F194. This opens the constriction and allows a water molecule around 2Å deeper into the pore. A gap located above the first histidine of the Twin His motif, at the periphery of the F194 site, is formed by G251, a residue smaller than the equivalent T273 in EcAmtB. This gap is further enlarged due to the increased physical separation of the helices. In this gap two more water molecules are found. It was hypothesised that the transduction of substrate would still require tilting of F194 (Lupo *et al.*, 2007).

A CO₂ binding site was identified in the cytoplasmic vestibule (Li *et al.*, 2007), but to date, there is no experimental evidence supporting the transport of CO₂ by rhesus protein. The C-terminal extension forms a helix that bundles beneath the protein, interacting with the helices of neighbouring monomers (Figure 1.4). This structural feature is hypothesised to be involved in regulating NeRh50 activity, though no definitive evidence has been found to date.

1.3.7 *H. sapiens* RhCG

The crystal structure of RhCG, expressed in the basal and apical membranes in kidney epithelial cells, was elucidated in 2010 (Gruswitz *et al.*, 2010). It was found that, like previous members of the family, the protein was trimeric and exhibited the same pseudo-symmetrical helical bundles (Figure 1.8).

The protein also contained an additional TMH, compared to other Amt/Rh structures, located just before helices 1-11, and was therefore named M0. EcAmtB and NeRh50 also have a helix at this location, identified as a signal peptide cleaved off as the protein is inserted into the membrane (Khademi *et al.*, 2004; Lupo *et al.*, 2007). M0 interact with M2 via a hydrogen bond between residues Q10 and Q101. In all Rh proteins with this extra helix these Gln residues are conserved.

The protein also had the conserved extracellular Asp residue (D177), the Phe gate and the hydrophobic pore with the Twin His Motif arrangement, but they differ in several ways.

RhCG, like NeRh50, lacks the full S1 binding site found in EcAmtB and AfAmt-1, but has the acidic residue E166, located at the bottom of the periplasmic vestibule which is hypothesised to be involved in ammonium binding (E146 in NeRh50) (Gruswitz *et al.*, 2010).

Like NeRh50 but unlike EcAmtB and AfAmt-1, the first Phe F130 of the Phe Gate is rotated 90° to the other (Figure 1.5). A possible explanation for this conformation is that the nearby D129 residue interacts with F130, locking it in this perpendicular conformation. F235 appears to be flexible, as small voids on either side of the ring potentially provide space for it to move. (Figure 1.5).

Another difference is the presence of a pocket running diagonally from the cytoplasmic vestibule to the centre of the lipid bilayer termed the shunt. The shunt is hydrophilic at the vestibule but becoming more hydrophobic towards the lipid. The authors suggest

this could be an alternative pathway for ammonium entry and the delivery of ammonia to the lipid bilayer.

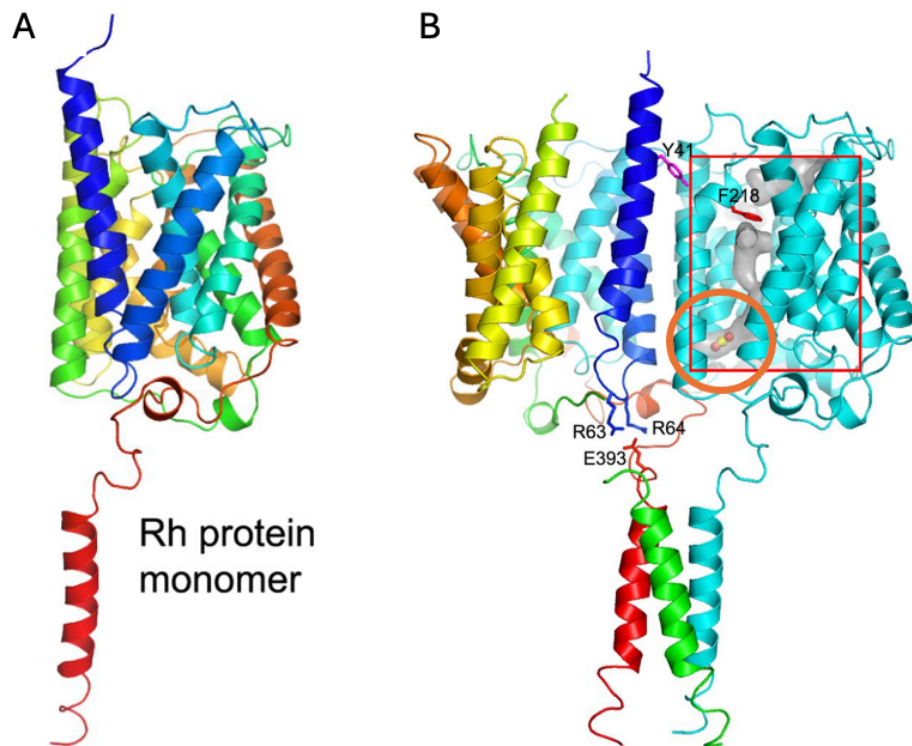


Figure 1.4 **Structure of NeRh50.** The NeRH50 protein with the elongated C terminal tail hanging below the protein shown as a monomer (A) and a trimer (B). In the trimer the pore is highlighted on the cyan monomer in the red box and the CO₂ binding pocket is circled in orange. Figure adapted from Li *et al* 2007.

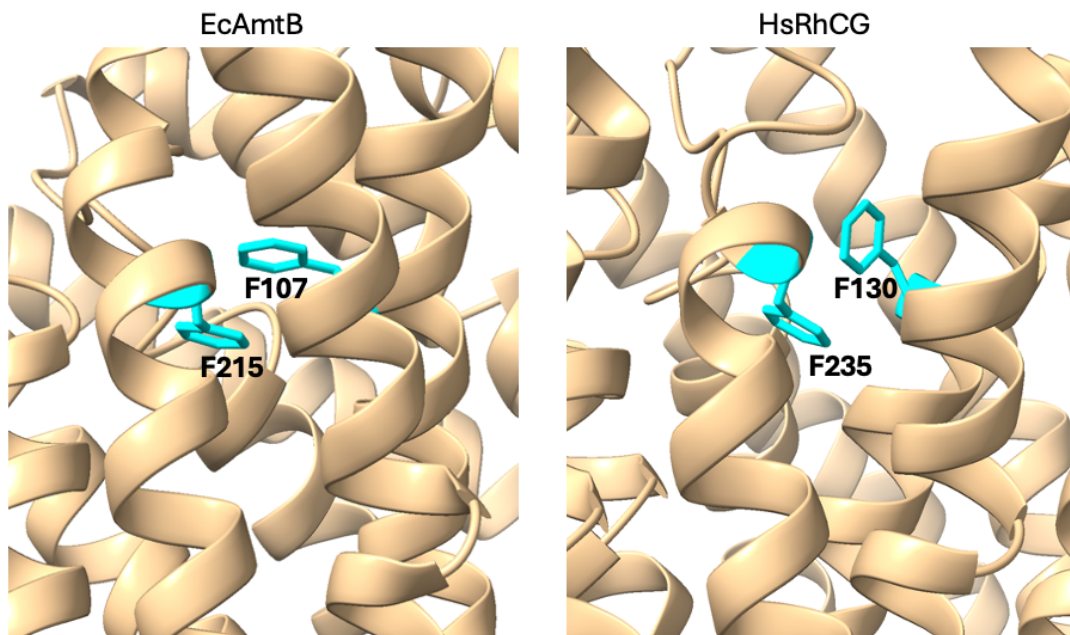


Figure 1.5 Different orientations of the first Phe in the Phe Gate. In EcAmtB (pdb 3c1g (Javelle *et al.*, 2008) (brown) and other amts the two Phe (blue) lie parallel to each other blocking the pore. In HsRhCG (pdb 3hd6 (Gruswitz *et al.*, 2010)) (brown) and other Rhesus proteins the Phe gate (blue) is different. The first Phe lies perpendicular to the other which opens the pore slightly more. Figure created by author.

1.3.8 *Candida albicans* and *Saccharomyces cerevisiae* Mep2

The Mep2 proteins from *C. albicans* and *S. cerevisiae* crystal structures were published simultaneously (Van Den Berg *et al.*, 2016). The structure of these proteins was similar to that of EcAmtB (Figure 1.8). The proteins were homotrimers, each monomer containing eleven TMH arranged in two pseudo-symmetric bundles around a central pore. The proteins have the same binding site, hydrophobic pore, Phe gate and Twin His Motif identified in the structure of the bacterial AmtS. However, the Mep2 proteins differ from Amt and Rh proteins in that the channel is blocked by two distinct structural arrangements: 1) The cytoplasmic end of TMH 2 shifts upward, altering the position of intracellular loop 1 (ICL1), which allows the residue Y49 in CaMep2 to form a hydrogen bond with H342 in the pore, effectively blocking it (Figure 1.6). 2) The ICL3 is also shifted 10 Å upward, positioning it across the cytoplasmic opening of the pore and further blocking it (Figure 1.6). These specific structural arrangements in Mep2-type proteins are hypothesised to be involved in the regulation of transport, ensuring that the pore is blocked under nitrogen-sufficient conditions.

The N-terminus is 20-25 amino acids longer than that of bacterial proteins. This extra length allows the N-terminus of one monomer to interact with the extracellular loop 5 of the neighbouring monomer. While deletion of this region had no effect on protein activity, as assessed by yeast complementation, it is involved in the regulation of Mep2 activity through phosphorylation by Npr1 (Boeckstaens *et al.*, 2014; Van Den Berg *et al.*, 2016).

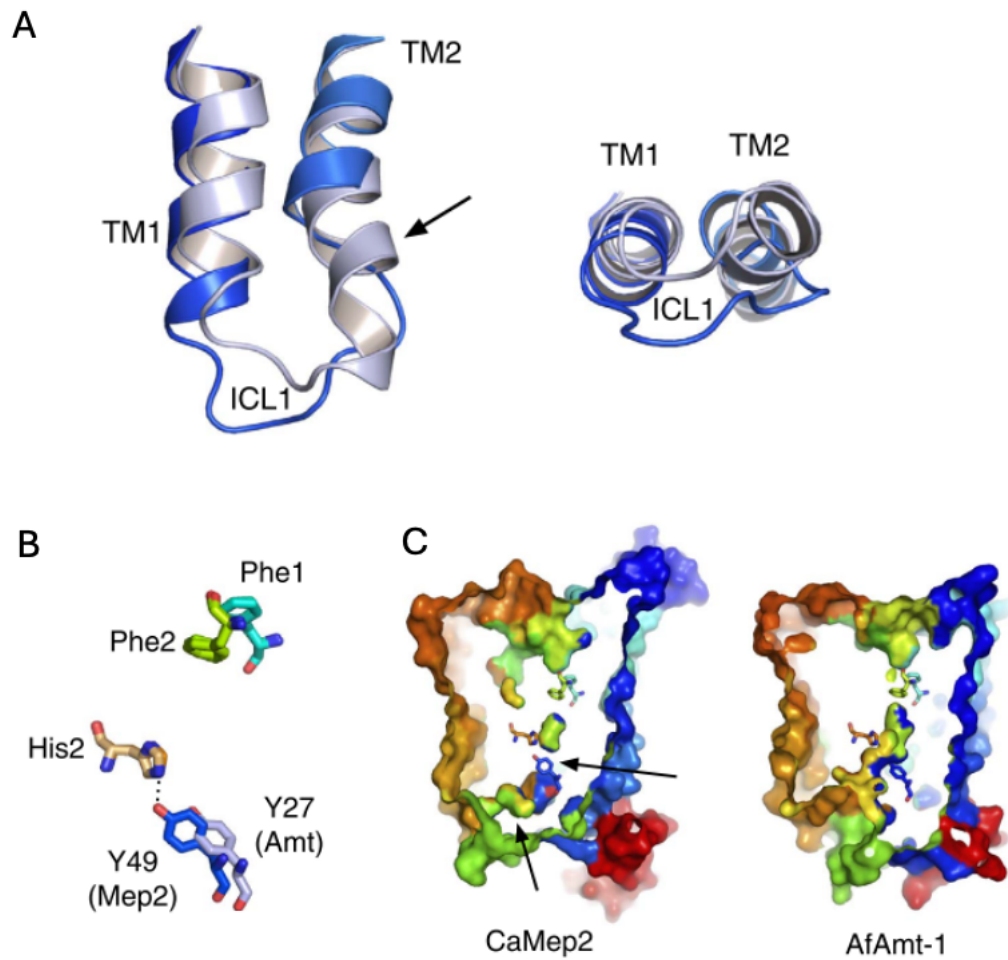


Figure 1.6 **Comparison between the translocation pores of CaMep2 and AfAmt-1.** A) Ribbon structure showing the differences in ICL1 arrangement between AfAmt-1 (silver) and CaMep2 (blue). B) The result of the conformation change brings Y49 closer to His2 in CaMep2 causing a bond to be formed. C) The bond formed causes the pore of CaMep2 to be blocked while AfAmt-1 is open. Figure adapted from Van Den Berg *et al* 2016.

1.3.9 *Candidatus kuenenia stuttgartiensis* Amt5

Candidatus kuenenia stuttgartiensis is an Anaerobic Ammonium Oxidation (anammox) bacterium. The anammox reaction is an energy-generating process in which NH_4^+ is anaerobically oxidized by NO_2^- to form N_2 and H_2O released into the atmosphere.

Hence, ammonium transport is very important to this bacterium which encodes seven Amt orthologues. In 2018 the structure of Amt5 from this bacterium (KsAmt5) was solved (Pflüger *et al.*, 2018). KsAmt5 is a highly unusual Amt as its C-terminal tail is fused with a histidine kinase (HK) domain. Although the HK domain organization of Ks-Amt5 resembles that of histidine kinases from bacterial sensor kinases in two-component systems, the protein lacks other common domains such as Per-Arnt-Sim (PAS), Histidine kinases, Adenyl cyclases, Methyl-accepting proteins, or Src homology three and cysteine rich domains. These domains typically link the sensor and transducer mechanistically. The HK domain of KsAmt5 was too flexible for its structure to be resolved.

The overall structure of the Amt domain of KsAmt5 is similar to that of other known Amt/Mep/Rh orthologues (Figure 1.8). Eleven helices span the membrane, helices 1-5 and 6-10 form pseudo-symmetrical bundles. The protein contains an S1 binding site, the Phe gate, and the Twin His motif. However, the structure of KsAmt5 reveals that its hydrophobic pore differs from other Amt/Mep/Rh proteins in two key ways: 1) the pore is blocked by several residues with bulkier side chains, and 2) the residue H326 in the Twin His motif is linked via hydrogen bonding across two putative water molecules, forming an unprecedented cation-binding site 10 Å below the membrane boundary. These structural features may explain why, although the protein can bind NH_4^+ , it cannot complete the full translocation cycle, as shown by microscale thermophoresis and electrophysiology on liposomes containing purified KsAmt5 (Pflüger *et al.*, 2018). It was suggested that ammonium ions passage through the S1 site to then be directed to a cation-binding site located in the pore, which triggers the HK domain to auto-phosphorylate the His406 residue (Figure 1.7). This phosphorylation mechanism was not fully elucidated, but a potential structural change was proposed whereby the longer than usual intracellular loop between M5 and M6 could provide enough flexibility for a transient conformational change.

1.3.10 *S. denitrificans* Amt1

SdAmt1 is another example of an ammonium transporter fused with an active C-terminal domain, in this case diguanylate cyclase (Pflüger *et al.*, 2024). The overall structure is highly similar to other ammonium transporters (Figure 1.8). The arrangement of the water wire between Twin His motif and the ammonium binding sites found in KsAmt5 were also found in SdAmt1 and termed N1/N2 sites (Figure 1.7). This suggests evolution from a common ancestor. Electrophysiological measurements showed that SdAmt1 does not transport ammonium but simply binds it, like KsAmt5 (Pflüger *et al.*, 2024).

1.3.11 Is the translocated substrate NH_3 or NH_4^+ ?

Since NH_4^+ is much more abundant than NH_3 in the environment at physiological pH, it was hypothesised that NH_4^+ must be the recruited substrate for these proteins. In *Synechococcus* sp, an uptake assay revealed that ammonium concentrations could reach up to 1000x external concentrations (Kleiner, 1985). Other advanced computer simulations, which included ammonium transport, assimilation, and regulation in *E. coli*, also concluded that active uptake is necessary to maintain physiological concentrations (Maeda *et al.*, 2019).

It was surprising when the first structure of an ammonium transporter revealed the highly hydrophobic nature of the pore, which would prevent the charged NH_4^+ species from passing through (Khademi *et al.*, 2004; Zheng *et al.*, 2004). This sparked a renewed heated debate regarding the transported substrate (NH_3 versus NH_4^+) and the overall nature of the translocation mechanism, (whether it functions as a channel or a transporter).

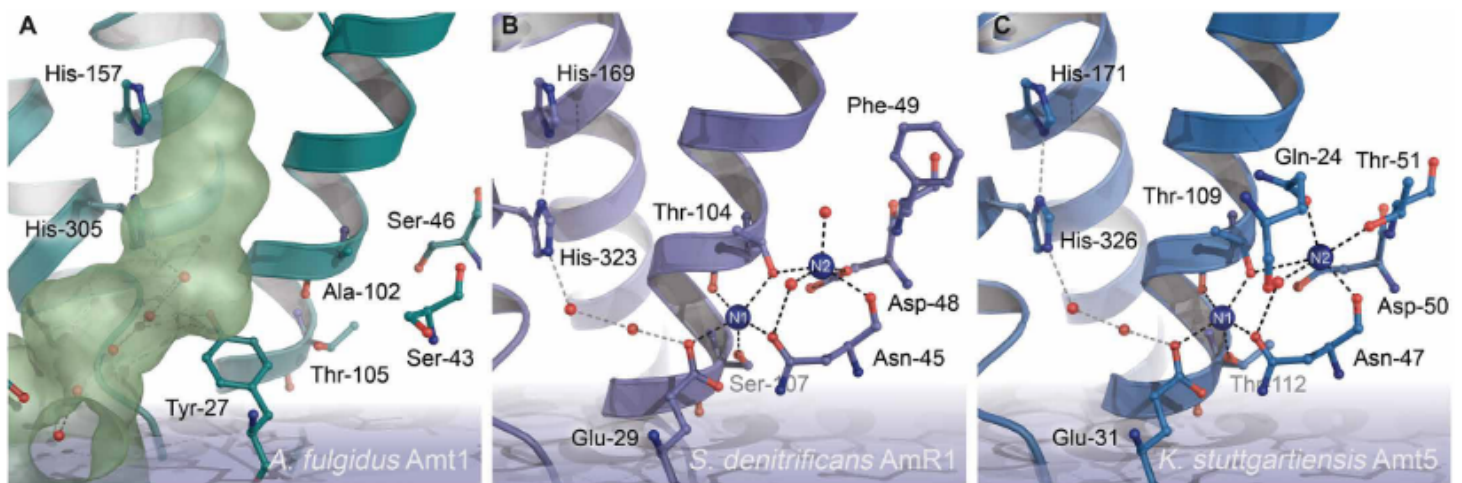


Figure 1.7 **Locations of the N1 and N2 ammonium binding sites in SdAmt1 and KsAmt5.** A) AfAmt1 has an unobstructed pore as shown in green. There is no ammonium binding site located in the pore. B) Bulkier side chains in SdAmt1 block the pore and a water wire, represented by red balls, leads from H323 to the N1/N2 ammonium binding sites. C) The same pore occlusion and water wire arrangement is seen in the other ammonium sensor KsAmt5. Figure adapted from Pflüger *et al* 2024.

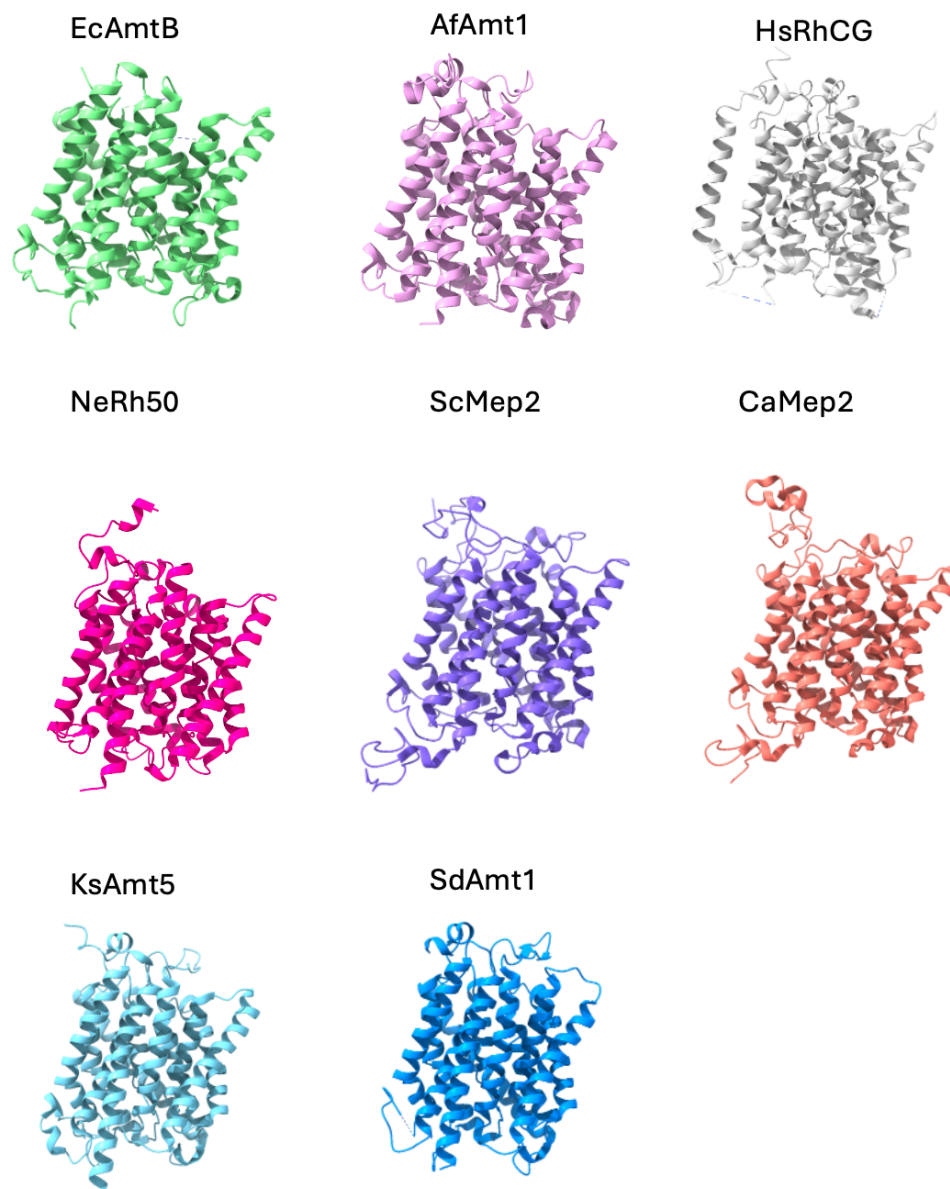


Figure 1.8 A comparison of all solved ammonium transporter structures. The conserved arrangement of helices can clearly be seen with differences between the inter helical loops notable. HsRhCG is the only protein with the extra TMH 0 which can be seen on the left side. EcAmtB (*E. coli* AmtB pdb 3c1g (Javelle *et al.*, 2008)), AfAmt1 (*A. fulgidus* Amt1 pdb 2b2i (Andrade *et al.*, 2005)), HsRhCG (*H. sapiens* RhCG pdg 3hd6 (Gruswitz *et al.*, 2010)), NeRh50 (*N. europaea* pdb 3b9w (Lupo *et al.*, 2007)), ScMep2 (*S. cerevisiae* Mep2 pdb 5aex), CaMep2 (*C. albicans* Mep2 pdb 5af1) (Van Den Berg *et al.*, 2016), KsAmt5 (*Candidatus kuenenia stuttgartiensis* AmtB pdb 6eu6) (Pflüger *et al.*, 2018), SdAmt1 (*S. denitrificans* Amt1 pdb 8qj4) (Pflüger *et al.*, 2024),.

1.4 Translocation mechanism in Amt/Mep/Rh proteins

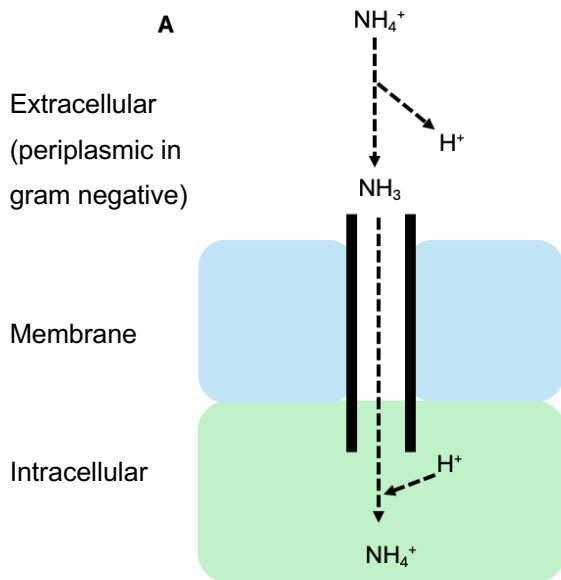
1.4.1 Proposed mechanisms

There were several hypotheses concerning the mechanisms of ammonium translocation through Amt/Mep/Rh protein. Before the publication of the EcAmtB structure, most researchers believed that ammonium transporters likely transported NH_4^+ electrogenically. After the discovery of the hydrophobic nature of EcAmtB in 2004 (Khademi *et al.*, 2004) the situation became less clear. Given the hydrophobic nature of the pore, it is suggested that translocation begins with the deprotonation of NH_4^+ to allow for NH_3 transport. Depending on the fate of the proton following deprotonation the mechanism could be 1) an electroneutral NH_3 transport with the proton being lost to the environment, 2) electroneutral $\text{NH}_3+\text{H}^+/\text{H}^+$ antiport or 3) electrogenic NH_3/H^+ symport (Figure 1.9).

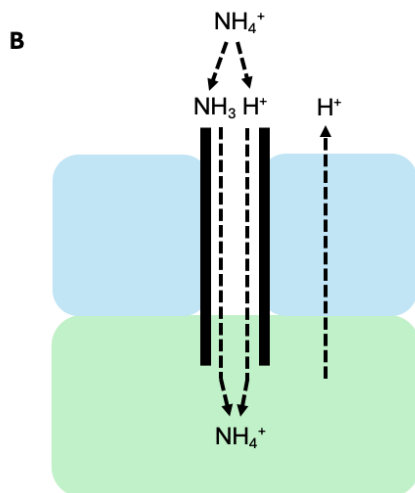
The electroneutral NH_3 transporter mechanism was initially supported by a fluorescence-based assay that measured the influx of ammonia into proteoliposomes containing EcAmtB by monitoring the pH-sensitive fluorescence of 5-carboxy fluorescein (CF) (Khademi *et al.*, 2004). When CF-loaded proteoliposomes were mixed with NH_4Cl , the internal pH increased, indicating the influx of NH_3 . However, these results have never been successfully reproduced despite extensive efforts, raising doubts about the validity of the initial conclusion (Javelle *et al.*, 2007). Patch clamp electrophysiology of Amt1;1 from *Lycopersicon esculentum* (tomatoes) expressed in *Xenopus* oocytes revealed the first electrogenic member of the family (Ludewig, Von Wiren and Frommer, 2002). Since, using Solid Supported Membrane Electrophysiology (SSME) it has been found that Amt-1 and Amt-3 from *A. fulgidus*, AmtB from *E. coli* and Rh50 from *N. europaea* all present electrogenic activity (Wacker *et al.*, 2014; Williamson *et al.*, 2020).

In addition to its ability to import ammonium, RhAG is capable of bi-directional translocation, which is a crucial feature for a protein involved in maintaining blood acid-base homeostasis (Marini *et al.*, 2000). Yeast complementation assays also suggested that NeRh50 could translocate ammonium bidirectionally. Overall, bi-directional translocation seems to be a trait of the Rh proteins.

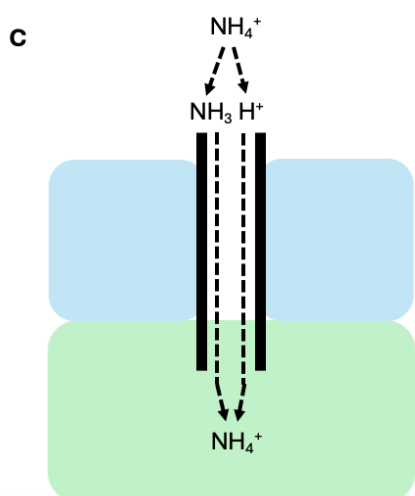
In our lab we have elucidated the transport mechanism of EcAmtB which will be described in the following section along with other findings.



Electroneutral transport: Ammonium is recruited to the extracellular vestibule and deprotonated. The proton is lost to the environment and ammonia diffuses through the pore. In the cytoplasm it is re-protonated.



Electroneutral transport: Ammonium is recruited to the extracellular vestibule and deprotonated. The hydrogen ion and ammonia are translocated separately through the pore. To counter this, the cell expels a hydrogen ion.



Electrogenic transport: Ammonium is recruited to the extracellular vestibule and deprotonated. The hydrogen ion and ammonia are translocated separately through the pore where they are rejoined to make ammonium. This leads to a net import of a charge.

Figure 1.9 A diagram showing different hypothesis of ammonium translocation through the pore. A) and B) are electroneutral transport while C) is electrogenic.

1.4.2 Introduction to Solid Supported Membrane Electrophysiology

Solid-supported membrane electrophysiology (SSME) was central to characterising ammonium transporter activity and to elucidating the mechanism of substrate translocation. Because this technique is used extensively throughout this thesis, it is introduced here. In SSME, gold-plated sensors are coated with a lipid monolayer, onto which proteoliposomes are adsorbed (Figure 1.10A). The sensors are positioned within a Faraday cage to minimise electrical noise and allow sensitive amplification of the signal, while alternating buffer solutions can be applied in a controlled manner. The method is based on capacitive coupling between two electrodes, a reference electrode in solution and a measuring electrode formed by the gold surface at the base of the sensor, which are maintained at the same potential (Figure 1.10A).

Each measurement begins with the sensor equilibrated in non-activating solution for 1 s (Figure 1.10B). The sensor is then pulse with activating solution for 1 s (Figure 1.10C), which initiates substrate transport by the proteins embedded in the proteoliposomes. In the present case, the transported substrate is the charged species NH_4^+ . Its movement alters the charge distribution at the sensor surface, causing the gold electrode to become more positively charged. To restore electrical balance, electrons flow from the reference electrode to the measuring electrode (Figure 1.10D). This transient flow of electrons is detected as a current by the amperemeter. The resulting trace typically shows an initial current peak, corresponding to the maximal transport rate, followed by a decay as transport slows due to the buildup of membrane potential across the proteoliposome membrane (Figure 1.10E).

After 1 s, the activating solution is replaced with non-activating solution (Figure 1.10F). This leads to an inverse current peak, reflecting the movement of previously accumulated NH_4^+ away from the membrane system (Figure 1.10G). For clarity, traces are typically displayed only over the ~1.0–1.25 s interval, as this corresponds to the period during which the sensor responds to the activating buffer.

1.4.3 Translocation mechanism in EcAmtB

1.4.3.1 Ammonium Binding

Ammonium is recruited to the periplasmic S1 binding site. This was shown by crystallising EcAmtB in the presence of thallium (Tl^+) (Javelle *et al.*, 2008). Tl^+ was used because 1) its size is similar to NH_4^+ , 2) its position in a crystal structure can be

accurately identified in an anomalous difference electron density map, 3) it can be stabilised, like NH_4^+ , by a tetrahedral coordination and 4) it had also been seen to bind to the active site of glutamine synthase (Liaw, Kuo, And and Eisenberg, 1995). When co-crystallised with Ti^+ , a Ti^+ ion was clearly identified at the S1 position in the crystal structure of EcAmtB, validating the presence of a cationic NH_4^+ (Javelle *et al.*, 2008). At the S1 position, NH_4^+ is stabilised via a hydrogen bond with the conserved S219 and cation π interactions with the residues F108, W148 and a water molecule present in the vestibule. This makes for a highly selective NH_4^+ binding site which discriminates between other similarly size molecules and cations. Using methylamine uptake assays, it was found that single F108A and triple S219A/F108A/W148A EcAmtB variant proteins had WT activity. Single W148A and S219A variant, however, had 2.5- and 4-times higher activity than the WT (Javelle *et al.*, 2007). This contradicted computational analysis that indicated S219 may deprotonate ammonium and thus be critical (Ishikita and Knapp, 2007). The increase in activity in the W148A and S219A variant indicated that the binding of the substrate is a rate limiting step in the translocation mechanism (Javelle *et al.*, 2008).

1.4.3.2 Ammonium translocation

It has been recently reviewed that numerous methods used to measure the activity of ammonium transporters (^{14}C -methylamine transport assay, electrophysiology in oocyte expressing Amt/Rh proteins, monitoring internal or external pH of cells after an ammonium pulse) have their flaws and often the same method can yield conflicting results (reviewed in Williamson *et al.*, 2023 and references therein). In our lab we therefore developed an *in vitro* method using SSME on a SURFE²R N1 machine made by Nanion to measure the activity of our proteins (Bazzone *et al.*, 2013; Bazzone, Barthmes and Fendler, 2017; Mirandela *et al.*, 2019). A sensor with a gold electrode at the base is coated with a single layer of lipid. Proteoliposomes or vesicles containing our protein are fixed to this layer and the sensor is placed into a faraday cage to amplify signal. The N1 machine then rapidly pulses the sensor with an activating solution that contains our substrate. If the charged substrate is transported, we measure an electric signal.

We have therefore purified and reconstituted EcAmtB and NeRh50 into artificial liposomes to measure their activity using SSME. We found that the transport activities of EcAmtB and NeRh50 are electrogenic (Mirandela *et al.*, 2019; Williamson *et al.*,

2020). Noteworthy, using the same approach, the activity of Amt1 and Amt3 from *A. fulgidus* were found to be electrogenic as well (Wacker *et al.*, 2014).

This is counter intuitive given that the pore of the protein is hydrophobic. Researchers have hypothesised that transport of NH_4^+ could be achieved after NH_4^+ deprotonation. Hence, NH_4^+ binds to the binding site where it is deprotonated and the uncharged NH_3 can diffuse through the pore unhindered. For the transport activity to be electrogenic the proton needs to be also translated in, but how this hydrophobic pore allowed the passage of a charge was unclear.

Using MDS, two water wires linking the S1 binding site to the cytoplasmic vestibule of EcAmtB were identified. These two water wires are bridged via the H168 in the Twin-His motif (Lamoureux, Klein and Bernèche, 2007; Williamson *et al.*, 2020). It was therefore hypothesised that the water wires translocated the proton to the cytoplasm side via a Grothuss mechanism. This *in silico* hypothesis was substantiated experimentally by measuring EcAmtB activity in deuterated water (D_2O) instead of normal water. Compared to water, D_2O takes 30% more energy to pass a proton between molecules. Thus, like in EcAmtB (Figure 1.11) if more than three waters molecule are involved in the translocation mechanism, the protein should be inactive when measured in D_2O . Incubating EcAmtB in D_2O completely abolished the transport activity proving the crucial role of the water wires in the translocation mechanism (Williamson *et al.*, 2020). Finally the proton re-joins NH_3 to form NH_4^+ on the cytoplasmic side of the protein (Figure 1.11).

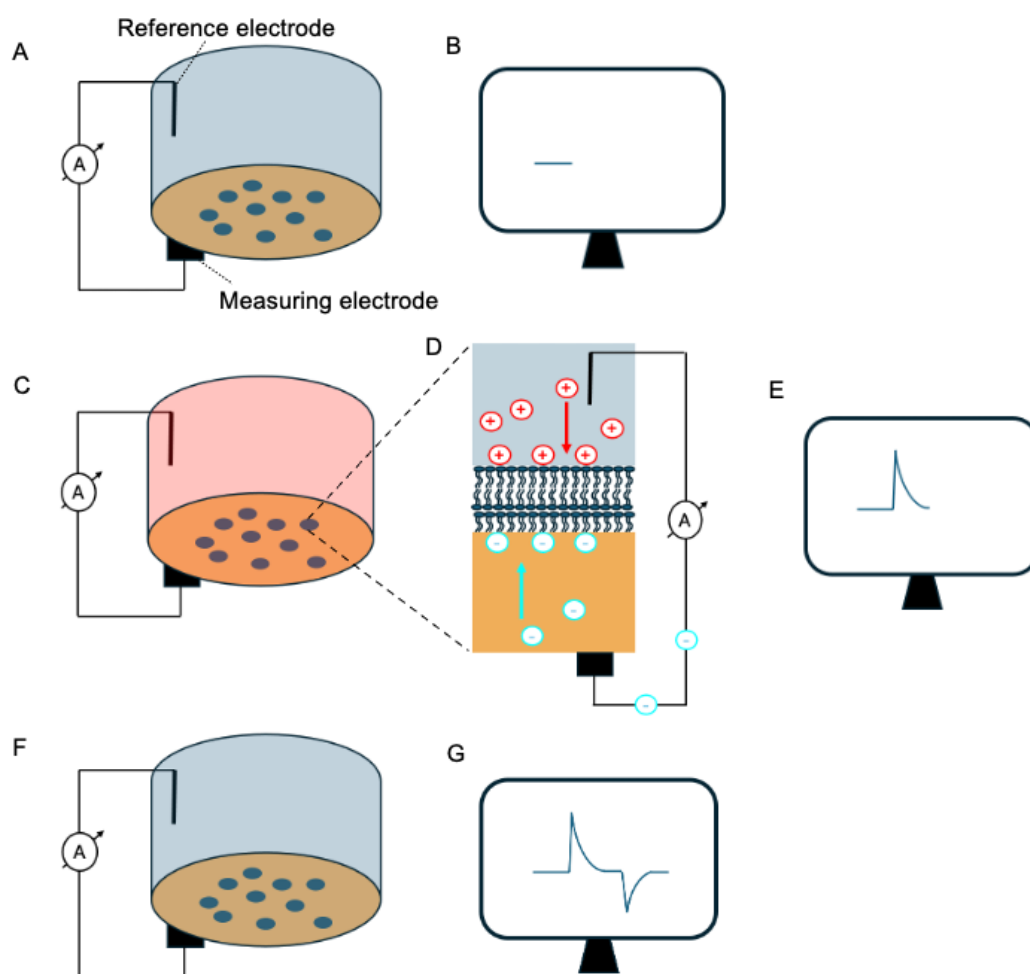


Figure 1.10 **Schematic of an SSME measurement.** A) proteoliposomes are affixed to the gold electrode of the sensor, the proteoliposomes are bathed in NA solution, the same buffer as their contents. The measuring electrode and the reference electrode are at the same charge. B) For the first second the solution is not exchanged, and no charge should be measured. C) NA solution is exchanged with A solution containing the substrate for 1 second. D) The substrate is transported into the proteoliposomes causing the measuring electrode to become more positive. To balance the charge, electrons move to the measuring electrode from the reference electrode through the amperemeter. E) The movement of electrons is measured by the amperemeter and recorded as a peak, initial transport is rapid followed by a declining rate. F) For the third second, A solution is exchanged with NA solution. G) An inverted peak is recorded as the accumulated positive ions exit the liposomes.

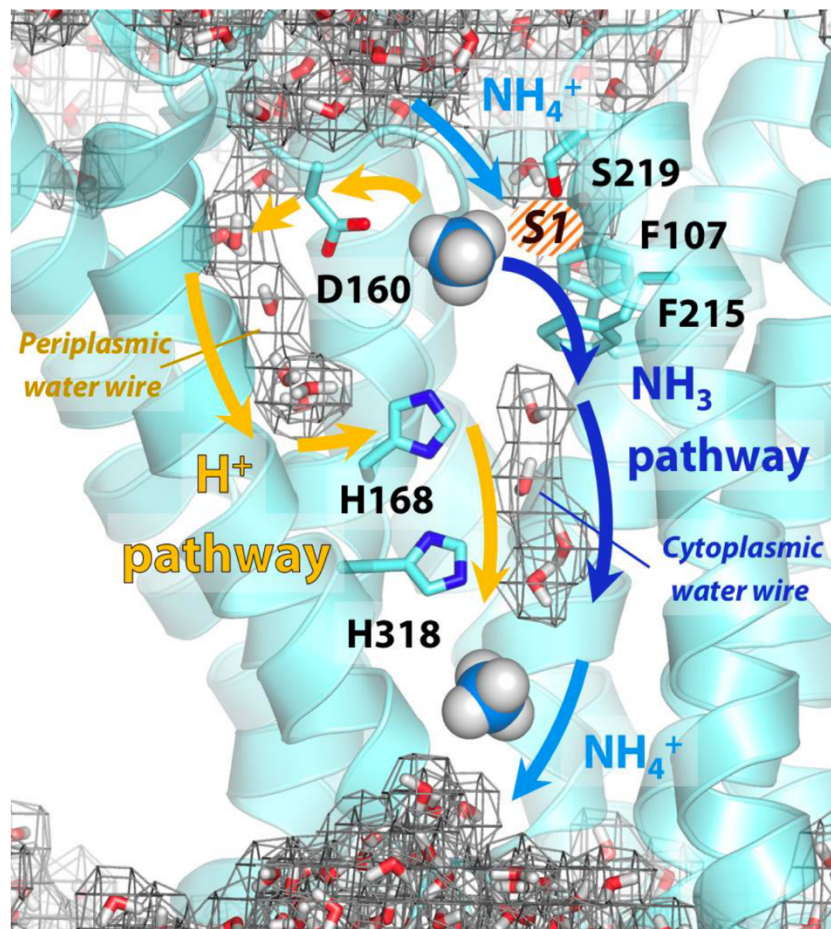


Figure 1.11 **Mechanism of ammonium transport in EcAmtB.** Ammonium binds to the binding site in the cytoplasmic vestibule where it is deprotonated. Uncharged ammonia can then diffuse through the hydrophobic pore while the proton is passed down a water wire through Grothuss mechanism to the twin his motif where it is passed to a second water wire which transports it to the cytoplasm where it is re-joined to ammonia to create ammonium. Thus, a charge can be transported through a hydrophobic pore. Figure from Williamson *et al* 2020.

1.4.4 Fungal Meps

S. cerevisiae and *C. albicans* express three Mep proteins named Mep1-3. They are involved in different biological processes and appear to have different transport mechanisms (Boeckstaens, André and Marini, 2008). Mep2, but not Mep1-3, can induce filamentous growth through sensing the local ammonium concentrations thus acting as a signal transducer. As mentioned, in paragraph 1.2.1, this filamentous growth is closely associated to fungal pathogenicity (Lorenz & Heitman, 1998). It was not known whether the signal for filamentation relies on a signal transduction cascade or a specific Mep2 mechanism of transport, different than for Mep1/3. NH_4^+ is acidic while NH_3 is basic meaning that internal pH change is indicative of the transport of charged NH_4^+ or uncharged NH_3 . In *Xenopus laevis* expressing ScMep1 an ammonium pulse triggered an internal acidification but not in cells expressing ScMep2 suggesting ScMep1 transports NH_4^+ while ScMep2 transports NH_3 (Brito *et al.*, 2020). In Mep1 and Mep3 there is a difference in the highly conserved Twin-His motif: in these proteins the first Histidine is replaced by a Glutamate. Hence, researchers have investigated the impact of changing the first His in Mep2 to a Glu (Boeckstaens, André and Marini, 2008; Williamson *et al.*, 2022). Firstly, it was found that yeast 3Mep Δ cells complemented with Mep2 carrying the H194E variation were still able to grow on low ammonium, suggesting transport capabilities. Secondly, Mep2 H194E variants showed higher transport activity compared to the WT using [^{14}C]-MeA transport assay but lost their ability to signal the transition to filamentous growth on low ammonium concentrations. Using MDS, it has been shown that the same variation introduced in EcAmtB led to the pore becoming filled with water. This water-filled pore enabled the passage of hydrated NH_4^+ without requiring deprotonation while also facilitating the movement of K^+ . Consequently, the substitution of a single histidine in the Twin His motif converts the ammonium transporter into a non-specific ion channel (Williamson *et al.*, 2020, 2022). This mechanistic change induced the loss of Mep2 transducer function. Collectively, these results indicate that the signal from Mep2 to induce filamentation is likely derived from its specific transport mechanism (Williamson *et al.*, 2022).

1.4.5 Mammalian rhesus proteins

The rhesus mechanism of transport has been extensively investigated, and contradicting conclusions have been drawn concerning the transport mechanism and the substrate transported. The methods that have been used are indirect measurement of transport in oocytes expressing Rh proteins by measuring internal pH after an ammonium pulse, two voltage clamp microelectrode or [^{14}C]-MeA transport assays (Table 1.1) (Williamson *et al.*, 2023). Using these methods, no less than seven different mechanisms, each with different energetic characteristics have been hypothesised:

- 1 - NH_4^+ uniport,
- 2 - NH_4^+ deprotonation following by NH_3/H^+ conduction,
- 3 - NH_4^+/H^+ symport,
- 4 - NH_4^+ deprotonation following by NH_3 transport only,
- 5 - NH_3 uniport,
- 6 - CO_2 transport,
- 7 - NH_4^+/H^+ antiport,

Oocytes possess endogenous ammonium-activated channels and thus handle ammonium in an unusual manner compared to the native hosts of the tested transporters (Musa-Aziz *et al.*, 2009). The poorly elucidated behaviour of ammonium handling in oocytes, combined with variations in the use of substrate (ammonium or methylammonium), the specific parameter measured, and the detection method explains how the same basic approach has given rise to such various conclusions on the translocation mechanism of Rh proteins, as exemplified in Table 1.1 for RhBG (Williamson *et al.*, 2023). There is therefore an urgent need to develop an assay that can reproducibly measure the activity of these proteins using SSME. This will be the focus of Chapter 5 in this thesis where I try to create vesicles expressing the protein of interest directly from the cell used for over expression. This will avoid purification and maintain the protein in a lipidic environment and should therefore allow for a greater variety of proteins to be measured.

Table 1.1 Reported transport mechanisms for RhBG when investigated using *X. laevis* oocytes.

Protein	Assay	Conclusion	Reference
Human RhBG	Ammonium pulse associated <i>X. laevis</i> current and pH increase	NH ₄ ⁺ /H ⁺ antiport Electroneutral	(Ludewig, 2004)
Human RhBG	Ammonium pulse associated pH decrease at surface of <i>X. laevis</i> oocytes	NH ₃ uniport Electroneutral	(Geyer <i>et al.</i> , 2013)
Human RhBG	Ammonium pulse associated pH increase in human embryonic kidney (HEK). pH decrease inside HEK when cells loaded with ammonium were pulsed with ammonium free buffer	NH ₃ uniport (bidirectional) Electroneutral	(Zidi-Yahiaoui <i>et al.</i> , 2005)
Mouse Rhbg	Ammonium pulse associated <i>X. laevis</i> current and internal pH decrease	NH ₄ ⁺ uniport Electrogenic	(Nakhoul <i>et al.</i> , 2005)
Mouse Rhbg	Ammonium pulse associated pH decrease in <i>X. laevis</i> oocytes Methylammonium associated pH increase in <i>X. laevis</i> oocytes	NH ₄ ⁺ uniport or Transport of both NH ₃ and NH ₄ ⁺ Electrogenic and Electroneutral	(Nakhoul <i>et al.</i> , 2010)
Mouse Rhbg	Ammonium pulse associated <i>X. laevis</i> current pH affects transport	Transport of both NH ₃ and NH ₄ ⁺ Electrogenic and Electroneutral	(Nakhoul <i>et al.</i> , 2010)
Mouse Rhbg	Ammonium pulse associated <i>X. laevis</i> current	Transport of both NH ₃ and NH ₄ ⁺ Electrogenic and Electroneutral	(Caner <i>et al.</i> , 2015a)
Mouse Rhbg	Ammonium pulse associated <i>X. laevis</i> current Affected by external pH	NH ₄ ⁺ /H ⁺ antiport Electroneutral	(Daniel Mak <i>et al.</i> , 2006)

1.4.6 *N. europaea* Rh50 is electrogenic

Owing to the complexity of working with eukaryotic proteins, bacterial homologues are used to derive a model. The Rh50 protein from *N. europaea* is a good target to study the Rh proteins. Despite previous studies providing evidence for electroneutral transport (Lupo *et al.*, 2007), it has been found using SSME that NeRh50 activity is electrogenic (Williamson *et al.*, 2020). It has also been found that it likely contains two mechanisms of transport; one being associated with deprotonation of NH_4^+ , like in EcAmtB, the other potentially relying on direct translocation of hydrated NH_4^+ through the pore (Bizior, Harris and Javelle in preparation). This is potentially due to the different arrangements of the periplasmic vestibule and hydrophobic pore, essentially making the pore shorter and slightly less hydrophobic allowing more water in and letting ammonium move through (Lupo *et al.*, 2007). This conclusion is supported by an MD simulation study, which reveals that in NeRh50, water molecules can establish a continuous hydrogen-bonded network between the pore entrance and the periplasmic vestibule, facilitating water conduction from the periplasm to the central pore (Hoyhtya, 2020). As a result, hydrated NH_4^+ ions may co-migrate with water molecules through the channel, circumventing deprotonation. This mechanism could account for the observed ~60% reduction in NeRh50 activity when H_2O is replaced with D_2O in SSME buffers (Bizior, Harris, and Javelle, in preparation).

Partly responsible for this are the two perpendicular Phe rings, part of my research presented in Chapter 6.

1.4.7 Can mechanisms be predicted using phylogeny?

All plants studied so far have representatives from two separately clustering groups of ammonium transporters called Amt1 and Amt2. So far, these are the only ammonium transporters to cluster based on mechanism, with Amt1 proteins being electrogenic (Loqué *et al.*, 2009; Ludewig, Von Wiren, *et al.*, 2002) and Amt2 electroneutral (Couturier *et al.*, 2007; Neuhäuser, Dynowski and Ludewig, 2009). It is unclear whether a more extended phylogenetic analysis could allow the Amt/Mep/Rh protein to cluster according to specific transport mechanism.

I aim to elucidate the evolutionary history of the Amt/Mep/Rh superfamily as presented in Chapter 3. Concurrently, the development of a novel transport assay using vesicles that express the protein of interest directly from the overexpression host will facilitate

the evaluation of whether Amt/Mep/Rh proteins phylogenetically cluster in accordance with a mechanistic pattern.

1.5 Evolution of Amt/Mep/Rh genes

1.5.1 Distribution of ammonium transporters

A key aspect of understanding how the conserved structure of Amt/Mep/Rh proteins supports diverse transport mechanisms and physiological roles across different organisms may be the elucidation of their evolution. The current hypothesis splits the family into three groups: Amt, Mep and Rh proteins. The distribution of these proteins show that Amt is found in all domains of life apart from vertebrates, Mep proteins are found in fungi and Rh proteins are found in animals and in some prokaryotes (Figure 1.12). Rh proteins are the only ammonium transporter found in vertebrates, but other organisms can possess multiple copies from different sub families.

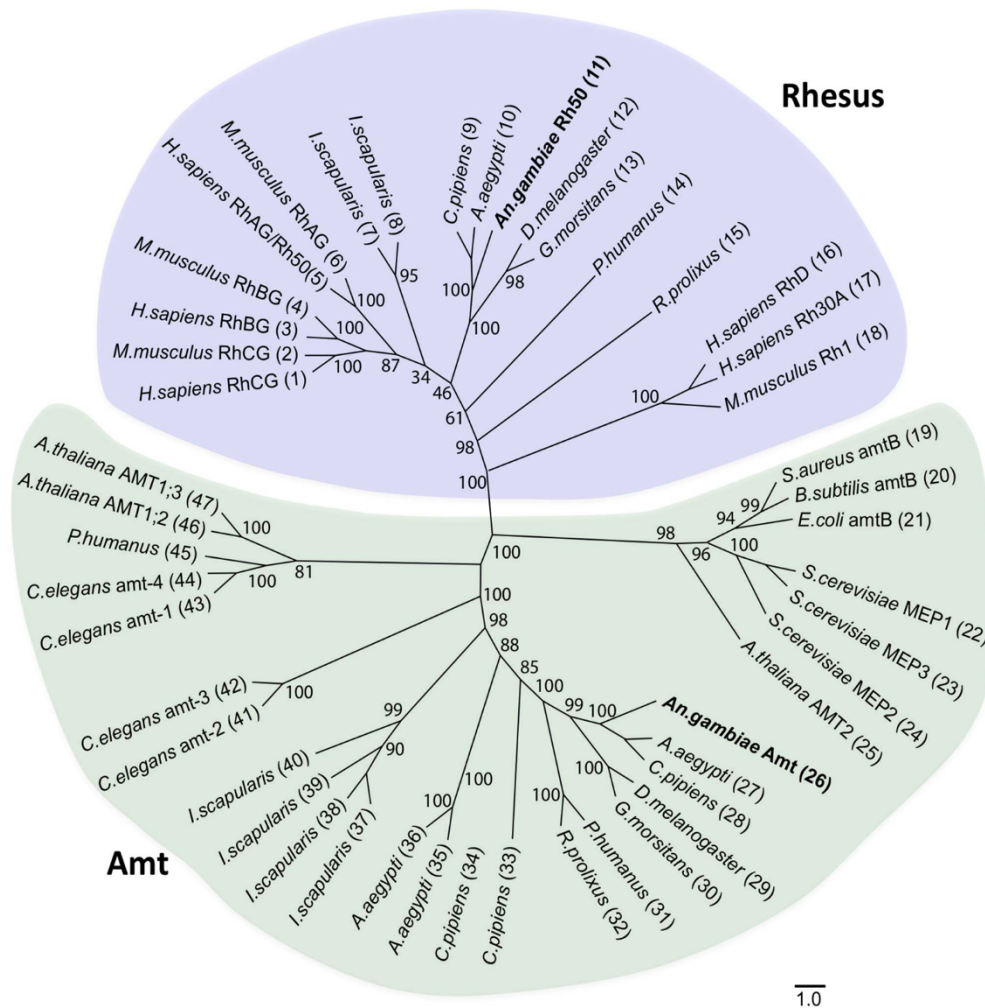


Figure 1.12 **A neighbour joining unrooted phylogenetic tree showing the broad distribution of the three ammonium transporting families.** Rhesus proteins cluster separately to Amt/Mep while Mep seems to be a subgroup of the Amts. Figure adapted from Pitts *et al* 2014.

1.5.2 Origin of ammonium transporters

The presence of, for example, Amt and Rh in the same organism, raises a question on the origin of the subfamilies and their respective physiological role. Reports describing Amt/Mep/Rh protein acting as transporter or sensor and involved in diverse biological processes, including acid–base balance, ammonium distribution, detoxification, development, fertility, olfactory detection, osmoregulation, scavenging, sensing, synaptic physiology are published regularly (reviewed in Williamson *et al.*, 2024 and references therein). Because of the spread of ammonium transporters across all kingdoms of life one can assume that it is an ancient protein likely found in our Last Universal Common Ancestor (LUCA). A fascinating question is whether LUCA had multiple proteins with scavenging/sensory/detoxification functions that were all passed down through vertical descent. Did the following evolutionary pressures on different organisms cause them to lose or retain these functions leading to the distribution we see today? Or, in the beginning, was there only one protein that functionally diverged over time through duplication events and different selection pressures to become the transporting and/or sensory proteins we see today.

Originally, these proteins have been split based on their physiological roles, with the Amt and Mep proteins grouping together as ammonium scavengers and the Rh acting bidirectionally. This can be seen in the distribution of expression with some organisms coding for both Rh/Amt or Amt/Mep suggesting that to be retained, each protein must fulfil a specific function that the other cannot (Huang and Peng, 2005; McDonald and Ward, 2016). In seventeen organisms that code for both Rh and Amt proteins, the average sequence identity between the two protein families is as low as 14% (Huang and Peng, 2005). Despite this, the Amt/Mep/Rh superfamily shares a high degree of structural and functional residue conservation. For example, a structural alignment of NeRh50 and EcAmtB gave a root mean square deviation (rmsd) of 2.2Å (Li *et al.*, 2007). Hence, it is possible that although these proteins have diverged a long time ago, there can be no other structure in nature that can act as ammonium transporter/sensor. Contrarily, other proteins have convergently evolved to fulfil the same function. For instance, flagella (bacteria), cilia (eukaryotes) and archaella (archaea) are all appendages used for motor function and share many common features, but all arising from separate proteins (Beeby *et al.*, 2020).

While the Rh proteins share ~20-27% sequence identity with Amt/Meps proteins (Matassi *et al.*, 1998), their important functional residues identified so far are found in the same order at the same place in each protein (Khademi *et al.*, 2004; Li *et al.*, 2007; Lupo *et al.*, 2007; Gruswitz *et al.*, 2010; Pflüger *et al.*, 2018), something not seen in, for example, the convergently evolved serine proteases in which the functional residues making up the catalytic triad of Ser-His-Asp are in different orders and in different places in the protein sequence (Krem and Di Cera, 2001). This suggests that despite the low sequence similarity among ammonium transport proteins, they all evolved from a common ancestor, allowing for comparative analyses to reconstruct their evolutionary history. A low sequence identity paired with high structural conservation is not unusual for membrane proteins with some also showing <2.5 Å rmsd at below 20% identity (Olivella *et al.*, 2013).

While still not clear, the evolutionary history of Amt/Mep/Rh family appears to be filled with expansion, gene loss and horizontal gene transfer (HGT). Using a manual alignment of nucleotide sequences McDonald *et al* proposed that there had been four occasions of HGT from other organisms into fungi (Tami R. McDonald, Dietrich and Lutzoni, 2012; McDonald and Ward, 2016; Matassi, 2017). The maximum likelihood tree rooted on rhesus proteins suggested that Mep proteins lie broadly between a group of bacterial proteins, here called Mep grade and eukaryotic AmtS found in plants and animals (McDonald, Dietrich and Lutzoni, 2012). The researchers included bacterial ammonium transports into the Mep classification which left plant AmtS, the only group postulated to be electrogenic, for the eukaryotes.

When all members of the superfamily are used to build a phylogenetic tree, the rhesus proteins are used to root it (Kitano and Saitou, 2000; McDonald, Dietrich and Lutzoni, 2012; McDonald and Ward, 2016). Kitano and Saitou 2000 however, use midpoint rooting which placed the Rh proteins at the root. These results indicate that an ancient duplication event sent the rhesus proteins on their own separate evolutionary pathway through vertical descent, while all prokaryotic, bacterial and archaeal Amt proteins, evolved through a separate vertical descent. It has been acknowledged that it was difficult to discern the correct root (Kitano and Saitou, 2000). The following sections will discuss the phylogenetic studies conducted on each individual subfamily published to date.

1.5.3 Amt group

Amts have been investigated with a focus on those in plants (Couturier *et al.*, 2007; McDonald and Ward, 2016). The first investigation of plant AmtS was carried out to deduce the phylogenetics of the 14 AmtS expressed in poplar trees (Couturier *et al.*, 2007). Since the number of ammonium transporters in the tree was high it was thought that they would all be fulfilling different functions, being specifically expressed in different tissues and therefore occupy a greater spread of the evolutionary tree. This study aligned protein sequences from higher plants and used neighbour joining to generate a tree. The ammonium transporters were split into two distinct groups, Amt1 and Amt2. The activities of Amt2 and Amt1 are postulated to be electrogenic and electroneutral respectively and all plants studied so far have representatives from both groups (Couturier *et al.*, 2007; McDonald and Ward, 2016).

McDonald and Ward investigated the evolution of electrogenic transport in ammonium transporters (McDonald and Ward, 2016). The maximum likelihood tree produced by manual alignment of nucleotides was rooted with Rh proteins. The topology revealed a basal Mep grade comprising fungal, bacterial, archaeal, and plant proteins. At the top of the phylogenetic tree, eukaryotic AmtS were positioned, with a distinct Amt1 clade consisting exclusively of plant proteins, arranged in a pattern consistent with a speciation tree. Amt2 clade contained mainly animal proteins from marine creatures with a handful of plants most basal being the algae *Asterochloris*. Amt3 clade was broadly the same as Amt2 in that there were animal sequences with some plants being basal. At that time, electrogenic transporters had only been identified in plants and archaea (Loqué *et al.*, 2009; Neuhäuser, Dynowski, and Ludewig, 2014; Wacker *et al.*, 2014). This study suggested that all land plant sequences within the Amt1 clades were likely electrogenic, although it remained unclear whether proteins from earlier-diverged lineages would also exhibit this property (Figure 1.13)

Another investigation into plant ammonium transporters did not yield such clear distinctions between Amt1 and Amt2 but there was no overlap between the groups (Dai *et al.*, 2023).

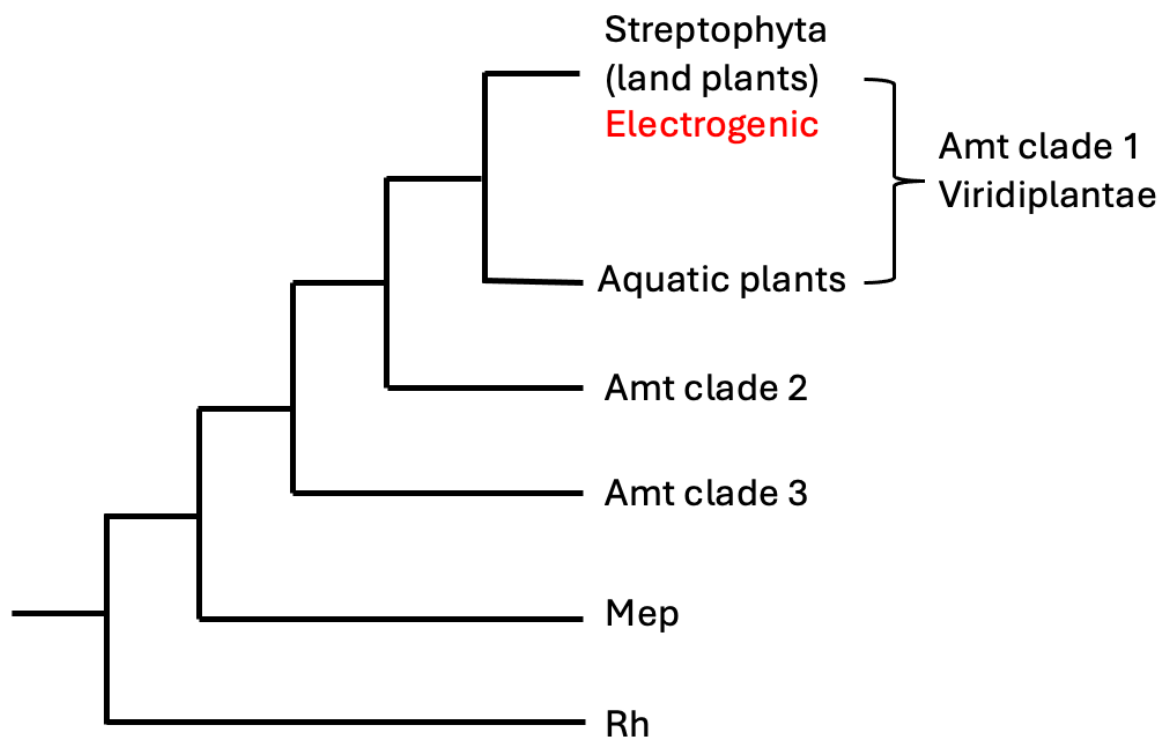


Figure 1.13 **Ammonium transport evolution from McDonald *et al* 2016**. The tree is rooted on Rh proteins. The streptophyta group at the top of the tree has members that are electrogenic leading the authors to conclude that the rest of this group electrogenic and this had evolved after the divergence of land and aquatic plants. There was not enough evidence for the nature of transport for the other groups for the researchers to conclude further. Figure adapted from McDonald *et al* 2016.

1.5.4 Mep group

Only one study has investigated the origins of the Mep proteins (McDonald, Dietrich and Lutzoni, 2012). Of the four ammonium transporting proteins found in the genome of the fungal partner in the lichen *Cladonia grayi*, two were closely related to plant ammonium transporters, but not those of the green algal symbiont. The researchers attempted to deduce the origin of these plant-like proteins. Using a manual alignment of 513 nucleotide sequences containing a mix of Amt, Mep and Rh proteins, a Maximum Likelihood (ML) tree was generated. The tree was rooted with the Rh proteins and displayed a topology very similar to the tree used to investigate the evolution of electrogenic AmtS (McDonald and Ward, 2016). In this phylogenetic tree, all proteins positioned closer to the root than the eukaryotic AmtS were classified as Mep proteins. These MepS proteins were divided in four groups: the Mep grade, Mep α , Mep β and Mep γ (Figure 1.14). The Mep grade contained bacterial sequences and was most basal in the tree. Mep α has putative archaeal origins and contained the plant-like fungal ammonium transporters. In this group were also the plant proteins along with bacterial and archaeal transporters. This suggests the occurrence of HGT events, which the authors proposed to have occurred independently in fungi and plants, approximately 800–700 million years ago and 900–450 million years ago, respectively (McDonald, Dietrich and Lutzoni, 2012). Mep β is mostly bacterial with some eukaryotic organisms present. Mep γ contains most of the fungal ammonium transporting proteins. The acquisition of Mep γ through HGT and subsequent functional displacement of the eukaryotic AmtS found in animals and plants was suggested to be a defining trait of fungal evolution (Tami R. McDonald, Dietrich and Lutzoni, 2012). The authors also hypothesised that nine other separate HGT events occurred in the Mep α and Mep β groups. The Mep grade, the more basal group of bacteria (McDonald, Dietrich, and Lutzoni, 2012), has also been described as part of a broader Amt family, with the MepS representing a subfamily within it (Pitts *et al.*, 2014). The distinction between AmtS and MepS remains a subject of debate. Some classifications divide the AmtS into Amt1 and Amt2, with Mep proteins considered a subgroup of Amt2 (Kitano and Saitou, 2000; Couturier *et al.*, 2007; Pitts *et al.*, 2014; Matassi, 2017). Alternatively, other studies expand the subfamily to include not only fungal ammonium transport proteins but also prokaryotic proteins (McDonald, Dietrich, and Lutzoni, 2012). Depending on the nomenclature applied, some plants, such as *Arabidopsis thaliana* and *Oryza sativa* or

the fungal partner of the lichen *C. grayii* encode both Amt and Mep proteins. (McDonald and Ward, 2016).

Investigations into two Mep proteins isolated from *Aspergillus nidulans*, MeaA (likely homologue of Mep1) and MepA (likely homologue of Mep2) found that MepA may either not transport methylammonium or be capable of bidirectional transport as $\Delta meaA$ strains gained methylammonium resistance (Monahan *et al.*, 2002). A tree was generated using neighbour joining from an alignment of protein sequences. This showed the same topology seen elsewhere with Mep proteins clustering as a subcluster of Amts.

A group of Rh proteins from *Caenorhabditis elegans* and the mammals *H. sapiens*, *Macaca mulatta* (the Rhesus macaque) and *M. musculus* clustered separately (Monahan *et al.*, 2002). No root was placed in the tree.

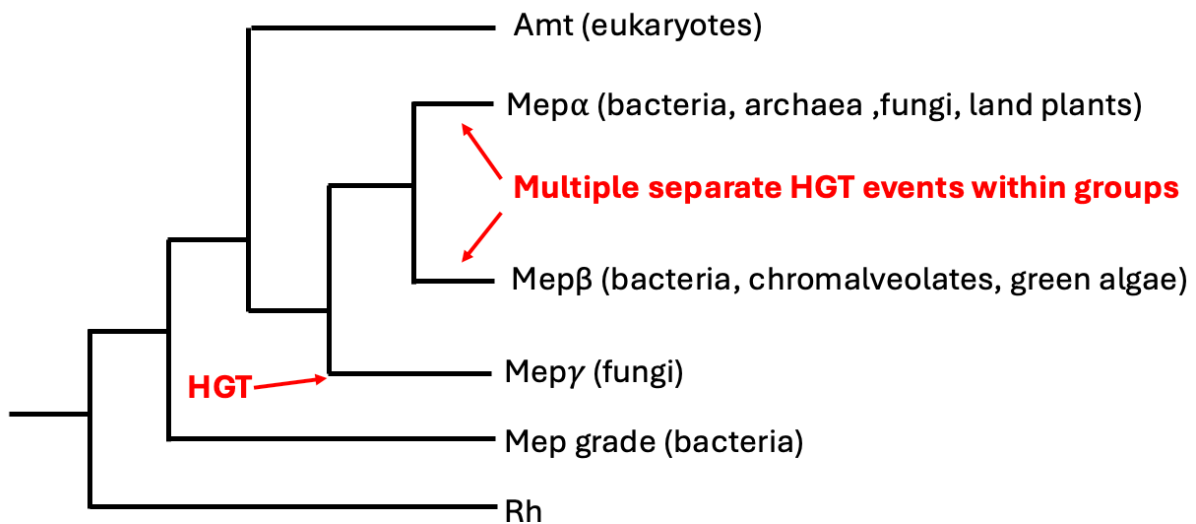


Figure 1.14 **Mep group evolution from McDonald *et al* 2012.** The Mep grade is represented by bacteria while three separate clades, Mep α , Mep β and Mep γ , for the rest of the group these are suggested to have arisen or contain occurrences of HGT events. At the top there are eukaryotic Amt proteins with representatives from animals like mosquitos, plants and other eukaryotes. Once again Rh proteins were used to root this tree. Figure adapted from McDonald *et al* 2012.

1.5.5 Rhesus group

Due to their clinical relevance, the evolution of the Rh group has attracted greater attention and been more extensively studied. (Kitano, Oota and Saitou, 1999; Matassi *et al.*, 1999; Kitano and Saitou, 2000; Huang and Peng, 2005; Cherif-Zahar *et al.*, 2007; Matassi, 2017). Rh was postulated to have arisen from archaea as organisms ranging from humans to prokaryotes including *N. europaea* expressed rh genes (Peng and Huang, 2006).

The archaea *Methanomassiliicoccus luminyensis* and malarial vector *A. gambiae* are examples of organisms expressing both Amt and Rh proteins suggesting that Amt and Rh have co-existed over a long evolutionary period (Huang and Liu, 2001; Huang and Peng, 2005; Pitts *et al.*, 2014; Matassi, 2017).

More detailed examination of Rh evolutionary relationships identified four distinct clusters: non-transporting Rh30 (RhD and RhCE), transporting Rh50 (RhAG, RhBG and RHCG) in vertebrates, and two ancestral clusters – Rhp2 in non-mammalian vertebrates and Rhp1 in microorganisms and invertebrates (Figure 1.15) (Huang and Peng, 2005). Rhp2 proteins are found more basal in the tree than Rhp1 suggesting invertebrates have lost the Rhp2 proteins. It has been proposed that the Rh30 cluster underwent more rapid evolution, suggesting that it may have diverged for tissue-specific functional modifications, while concurrently losing its transport capabilities. This is postulated to have happened either early in fish speciation owing to absence of Rh30 in all organisms below the zebra fish or, using the methods of Rambaut and Bromham, Matassi *et al* calculated that the maximum likelihood divergence was 346-250mya (Rambaut and Bromham, 1998; Matassi *et al.*, 1999; Huang and Peng, 2005). In contrast to Rh30 genes, Rh50 genes have evolved at slower pace (Matassi *et al.*, 1999; Huang and Peng, 2005) and therefore are likely to have a much longer evolutionary history than Rh30. This split could be due to a duplication of an Rh50-like ancestor. Rhp1 is the most diverse cluster and is found in organisms expressing both the Rh and Amt proteins. Species of this group have up to three Rh genes, indicating several gene duplication events (Huang and Peng, 2005).

Two studies suggested that *N. europaea* acquired the *rh50* gene by HGT and it was described as a case of non-orthologous gene displacement (Cherif-Zahar *et al.*, 2007; Matassi, 2017). *Nitrosomonas europaea* is an ammonia-oxidizing bacterium (AOB) commonly found in sewage waters with high ammonium concentrations. However, it

remains unclear whether NeRh50 protein have a scavenging and/or regulatory role (Shrestha *et al.*, 2001).

While the transmembrane helices of Amt/Mep/Rh proteins have a high degree of similarity, the sequences between the helices can vary significantly. This variability suggests that multiple sequence alignments across all three subfamilies may yield poor-quality results and require extensive post-alignment processing to remove gaps (Matassi, 2017). This challenge was addressed in one of the earliest studies on the evolution of this subfamily, where the researchers focused solely on the transmembrane helices of Rh blood group-related genes in vertebrates for their alignment to construct the phylogenetic tree (Kitano and Saitou, 2000). When the authors tried to expand their alignment further by including Amt proteins, the alignment had gaps even in the transmembrane helices. For this reason, and to increase the quality of their alignment, they only included TM helices 4 to 11 in their analysis. The tree that was generated was split into three groups: $\text{amt}\alpha$, $\text{amt}\beta$ and Rh. $\text{Amt}\alpha$ contained the Mep proteins and mainly prokaryotic organisms and the $\text{amt}\beta$ group contained mainly plant Amts (Figure 1.16) (Kitano and Saitou, 2000).

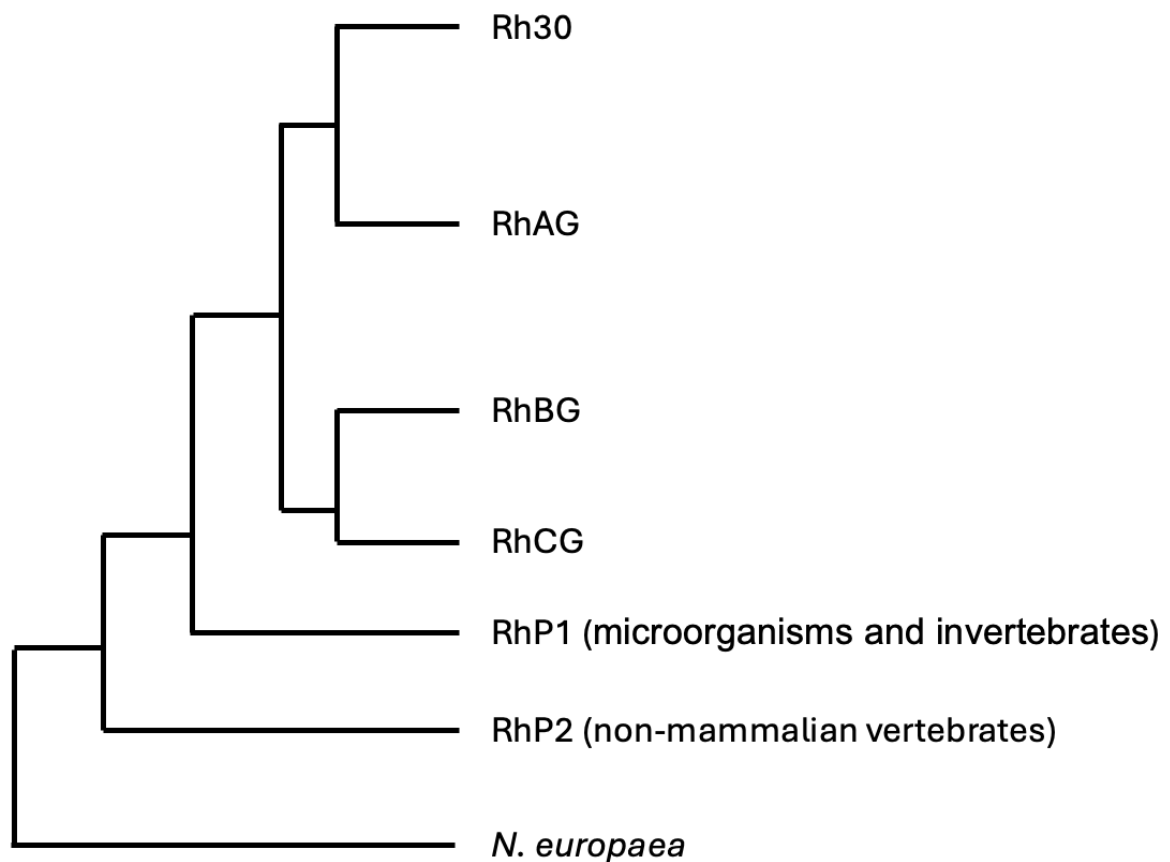


Figure 1.15 Phylogenetic analysis of the Rhesus protein family. The tree is unrooted. There are six groups with *N. europaea* Rh50 lying alone. It is likely that the increase in bacterial rhesus proteins found would mean that NeRh50 would not be alone today. Rh30 at the top is the non-transporting Rh homologue. Figure adapted from Huang and Peng 2005.

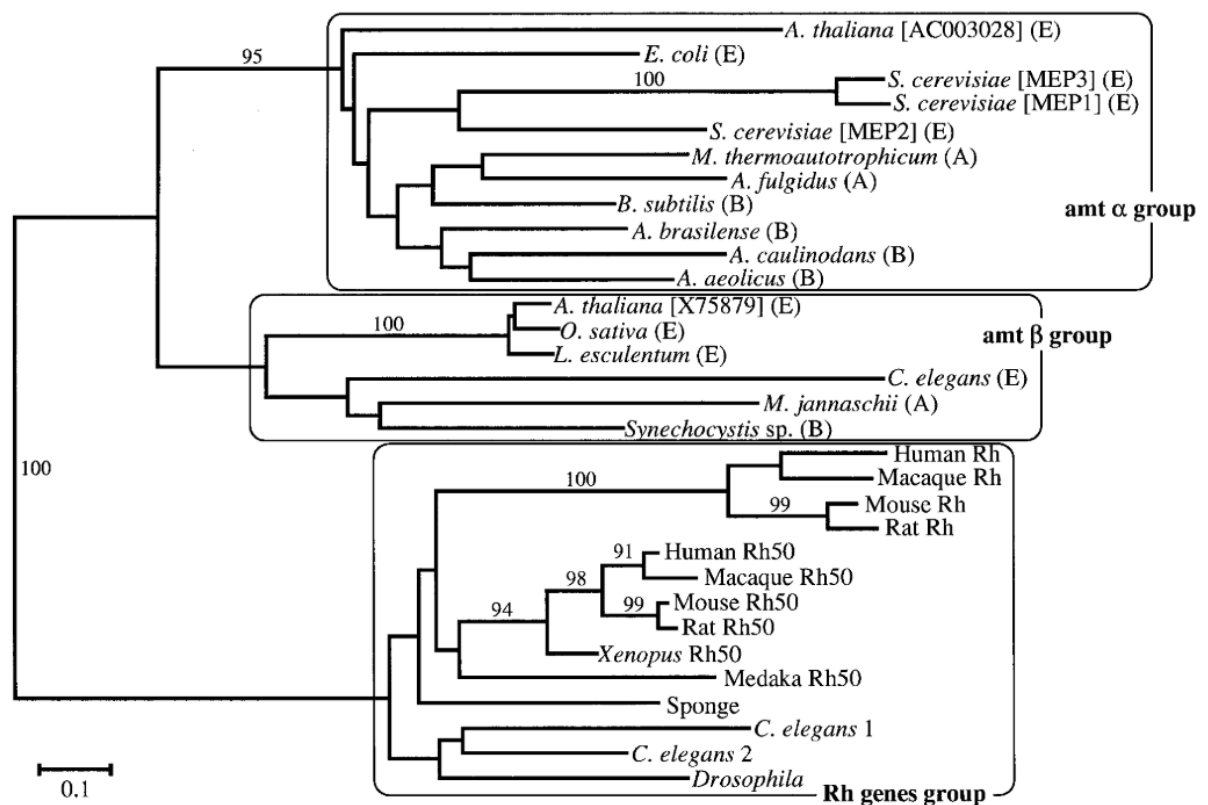


Figure 1.16 **Amt/Mep/Rh phylogenetic tree generated using only TMH regions.** The tree was not rooted but the clustering still reflects what has been found since, Rh proteins together, Mep and other microorganism together and plants together. Figure from Kitano and Saitou 2000.

1.5.6 A new perspective

A comprehensive study specifically focused on the evolution of the entire superfamily is lacking in the literature. When all the groups are included and a root is made, rhesus proteins are used as the root (McDonald, Dietrich and Lutzoni, 2012; Tami R. McDonald and Ward, 2016). This is done with the assumption that since they are found in higher organisms, they are the most different ammonium transporter. Their branch lengths are the longest, indicating that their divergence likely occurred earliest. These trees do not necessarily follow the tree of life and difference of function is not a definitive indicator of more ancient divergence. The Rh30 proteins rapidly evolved from Rh50 meaning their long branch lengths do not reflect divergence time (Matassi *et al.*, 1999). As discussed above, this leaves inconsistencies in the distribution of organisms within the family tree, explained until now with repeated occurrences of HGT. A different root between an archaea and bacteria and the use of more sequences could provide very different interpretations of the same tree.

The presence of ammonium transporting proteins in all domains of life suggests strongly that these proteins are of ancient origin. Life is thought to have arisen in alkaline hydrothermal vents on the ocean floor. These vents are porous allowing concentration of chemicals which is thought to have sped up chemical reactions, much the same as an enzyme (Martin *et al.*, 2008). Modern hydrothermal vents in the Hadean Sea have a pH around 9, close to the pKa of ammonium at 9.25 meaning concentrations of NH_4^+ and NH_3 are roughly equal (Jackson, 2016; Zerkle, 2019). An organism encountering these conditions may have required a protein to export $\text{NH}_4^+/\text{NH}_3$ from the cell in order to maintain homeostasis. As the water surrounding these hydrothermal vents is acidic (Jackson, 2016), an organism exposed to conditions outside these pores may have required a high-affinity transporter to scavenge ammonium from the environment.

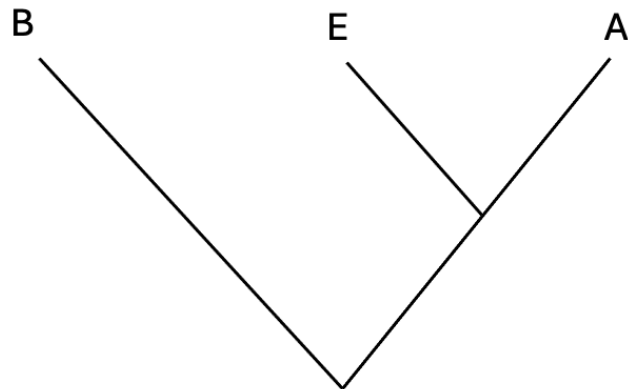
1.5.7 Unravelling the past

The limited availability of sequence data in the past has led some researchers to fail in identifying Rh proteins in bacteria or archaea, resulting in the assumption that Rh proteins emerged only after the evolution of eukaryotes. (Huang and Liu, 2001). Hence, further investigation into the evolution of this protein family would be

significantly facilitated by the substantial increase in available protein and genome sequences from a wider diversity of organisms.

Since Rh proteins are found in all domains of life, we can assume that they share a common ancestor protein that was present in LUCA. It is not known whether LUCA had a single protein that has diverged in terms of function or if it had multiple proteins that had different functions with these being vertically inherited. A comprehensive phylogenetic tree of ammonium transporters looking out for patterns like those in (Figure 1.17) may answer this question. This was one of the focuses of my PhD project and is presented Chapter 3.

A



B

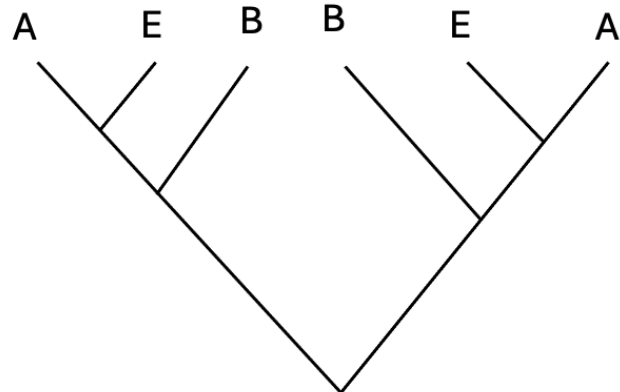


Figure 1.17 **Two possible scenarios for the presence of ammonium transporters in LUCA.** A- Archaea, B- bacteria and E- Eukaryote. A) One protein present that is passed onto ancestors down vertical inheritance. The groups diverge by organism and functions have developed independently. B) Two (or more) functionally different proteins present in LUCA. These are passed onto descendants vertically so there are two groups both with all kingdoms and functionally distinct. There is scope for gene loss, expansion and horizontal transfer to cloud the picture of either of these scenarios.

1.6 Aims

The research project in this PhD aims to address several remaining important questions concerning the Amt/Mep/Rh superfamily of ammonium transporter:

1. I will investigate the evolutionary history of these proteins to better understand their origins and explore whether there is a correlation between phylogeny and mechanism/function.
2. I will develop a method for measuring Amt/Mep/Rh transport activity that eliminates the need for protein purification or reconstitution. This approach will enable the characterisation of the transport mechanisms of specific proteins identified through my phylogenetic analysis, thereby providing a platform to experimentally investigate potential correlations between phylogeny and mechanism/function.
3. I will aim to resolve the outstanding questions regarding the role of the highly conserved "Phe-Gate" within this protein family, using EcAmtB and NeRh50 as model systems.

Chapter 2: Materials and Methods

2 Chapter 2: Materials and methods

2.1 Molecular biology

2.1.1 Strains and Media Preparation

A list of all strains and media compositions used throughout this work can be found in Table 2.1 and Table 2.2 respectively. All media and buffers were filtered through a 0.22 µm pore filter and autoclaved at 121°C for 15 minutes to sterilise.

2.1.2 Preparation of Chemically Competent Cells

A single colony of *E. coli* was used to inoculate 5 mL of LB media in a sterile 20 mL universal and incubated overnight at 37°C at 200rpm. 1 mL of the culture was used to inoculate 100 mL of sterile LB in a 250 mL Erlenmeyer flask and subsequently incubated at 37°C until the OD₆₀₀ reached 0.5.

The culture was transferred to sterile 50 mL centrifuge tubes and rested on ice for 10 minutes to halt the growth. The cells were harvested by centrifugation at 4000 *g* at 4°C for 10 minutes. The cells were gently resuspended in 10 mL of ice-cold sterile 0.1M CaCl and rested on ice for a further 20 minutes. The centrifugation was repeated, and the cells resuspended in 5 mL of ice-cold sterile 0.1M CaCl + 15% glycerol (w/v). The competent cells were dispensed in 200 µL aliquots, flash-frozen in liquid nitrogen and stored at -80°C.

2.1.3 Heat-Shock Transformation of *E. coli*

0.1 µg of plasmid DNA was added to 50 µL of thawed chemically competent cells and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds and transferred to ice. 1000 µL of sterile LB was immediately added and the cells incubated at 37°C for at least 1 hour. Following incubation, 100 µL of culture was spread on selective LB plates.

Table 2.1 Genotype of *E. coli* bacteria strains used.

Strain	Genotype	Reference
DH5 α	F $^{-}$ ϕ 80lacZ Δ M15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (r $_{K}^{-}$, m $_{K}^{+}$) phoA supE44 λ ⁻ thi-1 gyrA96 relA1	(Hanahan, 1983)
C43 (DE3)	F- ompT gal dcm lon hsdSB (rB -mB – λ (DE3)	(Miroux and Walker, 1996)
GT1000	<i>Rbs lacZ::IS gyrA hutCK ΔglnKamtB</i>	(Javelle <i>et al.</i> , 2004)

Table 2.2 Media used in this study

Name of medium	Composition	Reference
Lysogeny Broth	1% (w/v) Tryptone 0.5% (w/v) Yeast Extract 0.5% (w/v) NaCl	(Bertani, 1951)
ZYP-5052	1mM MgSO $_4$; 2% (v/v) 50x5052 5% (v/v) 20xNPS	(Studier, 2005)
ZY	1% (w/v) N-Z Amine 0.5% (w/v) Yeast Extract	(Studier, 2005)
50x5052	25% (w/v) Glycerol 2.5% (w/v) Glucose 10% (w/v) Lactose	(Studier, 2005)
20xNPS	0.5M (NH $_4$) $_2$ SO $_4$ 1M KH $_2$ PO $_4$ 1M Na $_2$ HPO $_4$	(Studier, 2005)

M9 Minimum Media	0.2% (v/v) Glucose 0.2mg/ml Glutamine 1mM MgSO ₄	(Elbing and Brent, 2002)
M9 Minimum Salts	0.6% (w/v) Na ₂ HPO ₄ 0.3% (w/v) KH ₂ PO ₄ 0.05% (w/v) NaCl	(Elbing and Brent, 2002)
Ampicillin	100µg/ml (1:1000 dilution)	
Chloramphenicol	34µg/ml (1:1000 dilution)	

2.1.4 Plasmid Purification

Cells were grown overnight in 5 mL of LB supplemented with the appropriate antibiotic and plasmids purified with a Wizard® Plus SV Miniprep DNA purification system (Promega Corporation), according to the manufacturer's instructions. Briefly, cells were pelleted via centrifugation and chemically lysed under alkaline conditions. Cellular debris was separated via centrifugation and DNA subsequently bound to a provided Promega silica membrane column. RNA and protein contaminants were washed off with a high salt buffer, and the DNA eluted in nuclease-free water. DNA concentration was quantified using Nanodrop 2000.

2.1.5 Site Directed Mutagenesis

Site-directed mutagenesis of the *rh50* and *amtB* genes were performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), according to manufacturer's instruction. Following a PCR mutagenesis, the product was incubated with DpnI restriction digest enzyme for at least 1 hour at 37°C, and transformed into chemically competent *E. coli* cells, DH5α (Table 2.1). DNA was purified using Wizard® Plus SV Miniprep DNA purification system (Promega Corporation), and each construct was checked by Sanger sequencing (Eurofins Genomics). The primers used for the mutagenesis are listed in Table 2.3 and the

template was the either the *rh50* or *amtB* gene cloned into the plasmid pAD7 (*rh50*) or pET22b (*amtB*) (Table 2.4).

Table 2.3 Primers used in this study*

Primer	Direction	Sequence
Mutagenesis:		
EcAmtB F107A	Forward	TCAGGGATCG GCGG CCTGCATTAC
	Reverse	AACGCCACGTGGATATAC
EcAmtB F215A	Forward	TTGGTTTGGC GCGA ACGCCGGGT
	Reverse	CCGATATAGAGAATGGCAG
NeRh50 E146D	Forward	TCTGCTGAAT GATT GGCTGGTGC
	Reverse	TAAACCGGCACGATAAACAATG
NeRh50 E146Q	Forward	TCTGCTGAAT CAGT GGCTGGTGC
	Reverse	TAAACCGGCACGATAAACAATG
NeRh50 F110A	Forward	AGCCACCGGAG GCTTT CCACTCTATATTCTTTTAC
	Reverse	ATTACTGCATCAACGGAG
NeRh50 F218A	Forward	CTGGCCGAGC GCTG CGACTGCAA
	Reverse	AAAAGCCACAGTACCATTGATC

*Nucleotides in bold have been changed to introduce the mutations/restriction sites, *Escherichia coli* (Ec), *Nitrosomonas europaea* (Ne)

Table 2.4 Plasmids used in this study

Plasmid	Description	Reference
pET22b (+)	High copy bacterial expression vector	Novagen
pZheng	pET22b-AmtB(His) ₆	(Zheng <i>et al.</i> , 2004)
pTH6	pET22b-AmtB(His) ₆ ^{F107AF215A}	This study
pAD7	pESV2-Rh50(His) ₆	(Cherif-Zahar <i>et al.</i> , 2007)
pTH1	pESV2-Rh50(His) ₆ ^{E146Q}	This study
pTH2	pESV2-Rh50(His) ₆ ^{E146D}	This study
pTH3	pESV2-Rh50(His) ₆ ^{F110A}	This study
pTH4	pESV2-Rh50(His) ₆ ^{F218A}	This study
pJT5	<i>glnK amtB</i>	(Javelle <i>et al.</i> , 2004)
pJT6E	<i>glnK amtB</i> (His) ₆	(Javelle <i>et al.</i> , 2004)

2.1.6 Overexpression of *E. coli* AmtB and *N. europaea* Rh50

2.1.6.1 *EcAmtB* in pET22b(+) vector

EcAmtB-His6 cloned into the pET22b(+) vector was overexpressed and overproduced in C43(DE3) or GT1000 strain as described previously (Zheng *et al.*, 2004). Briefly, a single transformant colony containing pET22b-AmtB-His6 was used to inoculate 15ml of LB and incubated at 37°C for 6 hours. Following incubation, 1 L of the auto induction medium ZYP-5052, was inoculated at OD₆₀₀ 0.02 and grown at 30°C, 210 rpm in presence of antibiotic for 19 hours.

2.1.6.2 *EcAmtB* in pJT5 and pJT6E vectors

EcAmtB cloned into the pJT5 or pJT6E vectors were over expressed in the GT1000 strain. This involved taking a single transformant colony containing pJT5 or pJT6E, inoculation of 15mL LB and incubation for 6 hours at 37°C. This culture was used to inoculate 1L M9 minimum media and was grown at 30°C 250rpm in presence of antibiotic for 19 hours.

2.1.6.3 *EcAmtB* expression using IPTG

EcAmtB-His6 cloned into the pET22b(+) vector was transformed into the C43 *E. coli* strain. A single colony was used to inoculate 100ml LB and was incubated overnight. This was used to inoculate 1L LB at 37°C to an OD₆₀₀ of 0.05. This culture was grown until it reached an OD₆₀₀ of 0.4-0.5 whereupon it was left to cool for 30 minutes at room temperature. A 50ml sample was taken and IPTG was then added to a concentration of 1 mM and the cultures were incubated at 30°C. Further 50ml samples were taken at 3hr, 6hr and overnight. Cells were harvested from these samples by centrifugation at 4000g for 20mins on a benchtop centrifuge.

2.1.6.4 *NeRh50* in pAD7 vector

Overexpression of *NeRh50* in the pAD7 vector was the same as expression of *EcAmtB* in the pJT5 or pJT6E vectors. 12L of culture was needed to produce enough protein for insertion. The GT1000 strain was used as it lacks its native *amtB* gene. This prevents the situation where Rh50 and AmtB could form trimers together, skewing the measurements.

2.1.7 WT *EcAmtB* and WT *NeRh50* purification

2.1.7.1 Membrane Preparation

The membrane was isolated as described previously (Blakey *et al.*, 2002). Cells were broken by passing the cell suspension three times through a French Press (20 kPSI) followed by centrifugation at 27,000 *g*, 4°C for 30 minutes (JA 25-50, Beckman Avanti JXN-26) to pellet intact cells. Collected supernatant was centrifuged at 205,000 *g*, 4°C for 1 hour (TI45, Beckman Optima XPN-100) to pellet the inner membrane. Membrane pellet was stored at -20°C.

2.1.7.2 Immobilised Metal Affinity Chromatography

EcAmtB and *NeRh50* were purified following the same protocol with one exception. Detergent used for AmtB was N-deodecyl- β -D-maltopyranoside (DDM - 0.03% w/w), whereas for Rh50 n-Dodecyl-N, N-Dimethylamine-N-Oxide (LDAO - 0.09% w/w).

The membrane was solubilised using solubilisation buffer (Table 2.5) to a ratio of 1g/10mL. Appropriate detergent (Table 2.5) was added dropwise to a final concentration of 2% and incubated for 2 hours at 4°C under gentle agitation. To prevent proteins degradation caused by detergent, solubilised membrane was diluted 2-fold with IMAC buffer A (Table 2.5) and non-solubilised material was removed by centrifugation at 27,000 *g*, 4°C for 45 minutes (TI45, Beckman Optima XPN-100).

The sample was loaded on an IMAC column (Hitrap HP 1mL, Ge Healthcare) previously coated with cobalt and equilibrated with IMAC Buffer A containing appropriate detergent (Table 2.5) The His-tag at the C-terminal of protein binds to cobalt present on matrix of IMAC column *via* coordination bond allowing ÄKTA Pure FPLC system to purify the protein of interest. The protein was eluted from IMAC column by gradual increase of imidazole from 40mM to 500mM using IMAC B buffer over 20 column volumes (Table 2.5). Imidazole acts as a competitive inhibitor which displaces the protein bound to cobalt (Bornhorst and Falke, 2000). Protein was eluted into 2mL 96 well plate fraction collector and the absorbance at 280nm was monitored during the purification.

Elution fractions were loaded on a 10% for AmtB and 12.5% for Rh50 sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel (Table 2.7) to monitor the presence of protein. Those which contained the protein were pooled and concentrated to 5 mg/ml by

subsequent centrifugation using MilliporeAmicon® Pro Affinity Concentration Kit Ni-NTA with 100 kDa Amicon® Ultra-0.5 Device.

2.1.7.3 IMAC Column Regeneration

IMAC column was regenerated by sequentially passing 5 column volumes (CV) of water, stripping solution, water, cobalt solution and finally 20% ethanol. The composition of named solutions can be found in Table 2.5.

2.1.7.4 Size Exclusion Chromatography

Size exclusion chromatography (SEC) allows the separation of proteins according to their hydrodynamic property. SEC was performed in SEC buffer containing appropriate detergent (Table 2.) using a Superdex S200 10/300 GL increase (Ge Healthcare) using ÄKTA Pure FPLC system. To confirm the presence and access purity of the protein in the sample, elution fractions were analysed on SDS-PAGE gel (Table 2.7). The sample was concentrated to 5 mg/mL and kept at 4°C.

2.1.7.5 Determination of Protein Concentration

Protein concentration was determined by measuring absorbance at 280nm related to the presence of aromatic amino acids such as tryptophan, tyrosine and cysteine. The molar extinction coefficient depends upon the relative concentrations of these amino-acids and was determined basing on the amino-acid sequence and the ExPASy ProtParam informatics tool (Wilkins *et al.*, 1999). The absorbance was measured using Nano-drop 2000/2000c spectrophotometer (ThermoFisher Scientific) and the concentration was calculated using Beer-Lambert law.

Table 2.5 Buffers used in protein purification and insertion into liposomes

Name of Buffer	Composition
Resuspension buffer	50 mM Tris-HCl pH8 500 mM NaCl 10% (v/v) Glycerol 160 μ M phenylmethylsulphonyl fluoride 10 μ g/ml DNase
Solubilisation buffer	50 mM Tris-HCl pH8 500 mM NaCl 10% (v/v) Glycerol 2% DDM or 2% LDAO
IMAC A buffer	50 mM Tris- HCl pH8 500 mM NaCl 10% (v/v) Glycerol 0.03% DDM or 0.09% LDAO
IMAC B buffer	50 mM Tris- HCl pH8 500 mM NaCl 10% (v/v) Glycerol 500 mM Imidazole 0.03% DDM or 0.09% LDAO
SEC buffer	50 mM Tris-HCl pH7.8 100 mM NaCl 0.03% DDM or 0.09% LDAO
Stripping solution	100 mM EDTA 500 mM NaCl
Cobalt solution	100 mM CoCl ₂
Liposome buffer	300 mM KCl 100 mM potassium phosphate pH7.6

2.1.8 Vesicle Preparation

2.1.8.1 Cell Harvest

This method was taken from (Kaback, 1970). All buffers and solutions used can be found in Table 2.6. Following over expression, cells were harvested by centrifugation at 7000 *g*, 4°C for 20 minutes and washed in wash buffer twice. The pellet was then resuspended in 40 ml vesicle resuspension buffer (Table 2.6), EDTA was added to 10 mM and lysozyme to 0.5 mg/ml and the mixture was stirred for 30 minutes at room temperature to form spheroplasts.

The spheroplasts were then centrifuged at 16,000 *g* for 30 minutes and resuspended in as little as possible of vesicle resuspension buffer 2 using a glass homogeniser. This suspension was poured into 300/500 volume of the hypotonic buffer at 37°C and incubated for 15 minutes. Then EDTA was added to 10 mM and the mixture was incubated for a further 15 minutes. MgSO₄ was then added to a concentration of 15 mM and the mixture was incubated for a final 15 minutes. Following this the mixture was centrifuged at 16,000 *g* for 30 minutes. The pellet was then resuspended in vesicle resuspension buffer 3 at 0°C and 10 mM EDTA. This was spun twice at 800*g* collecting the supernatant. The supernatant was then spun at 45,000 *g* for 45 minutes at 4°C and the pellet was resuspended in vesicle resuspension buffer 3 and washed a further 3 times. Finally, the pellet was resuspended in liposome buffer and frozen at -80°C.

2.1.8.2 Cell Lysis

When cells were not made into spheroplasts, they were lysed prior to loading onto the western blots or SDS PAGE gels. Single colonies of transformed or untransformed cells were used to inoculate 100 mL culture. Following growth overnight the cells were harvested at 7000 *g* and 10 mL lysis buffer (Table 2.6) was used to resuspend the pellet. The samples containing the lysis buffer and pellet were then spun on a rotating wheel for 2 hrs. The lysed cells were then centrifuged at 7000 *g* for 20 mins and the supernatant was collected. The supernatant was used to load the SDS PAGE gels.

Table 2.6 Solutions used for vesicle creation

Solution	Components	pH
Wash buffer	10 mM Tris-HCl	pH 8
Vesicle resuspension buffer	30 mM Tris-HCl 20% sucrose	pH 8
EDTA	250 mM	
Lysozyme	10 mg/ml	
Vesicle resuspension buffer 2	0.1 M potassium phosphate buffer 20% sucrose 20 mM MgSO ₄ DNase 5 mg/ml RNase 5 mg/ml	pH 6.6
Hypotonic buffer	50 mM potassium phosphate buffer	pH6.6
MgSO ₄	1 M	
Vesicle resuspension buffer 3	0.1 M potassium phosphate buffer	pH6.6
Potassium phosphate buffer	KH ₂ PO ₄ 1 M K ₂ HPO ₄ 1 M	pH 6.6
Lysis buffer	DNase 10ug/ml PMSF 4 mM Lysozyme 5mg/ml DDM 2%	No pH adjusting required

2.1.9 Proteoliposome preparation

2.1.9.1 Lipids Preparation

E. coli polar lipids and 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) were mixed at 2:1 ratio and dried using nitrogen gas and vacuum desiccation. Lipids were subsequently rehydrated using liposome buffer (Table 2.7) at 5mg/mL. The mixture of multilamellar liposomes was extruded through a 100 nm pore filter using a Mini-extruder (Avanti Polar Lipids) to achieve a unimodal size distribution of unilamellar liposomes.

2.1.9.2 Insertion of the Membrane Protein into the Liposome

To destabilise the liposomes for optimal protein insertion, Triton X-100 detergent at 25% was used, at a detergent/lipid ratio between the onset of solubilisation (R_{sat}) and the total solubilisation (R_{sol}) of the lipids (Rigaud, Paternostre and Bluzat, 1988; Rigaud, 2002).

Detergent/lipid mixture was incubated at 300 rpm, 25°C for 5 minutes. Following incubation, the protein was added at a lipid: protein ratio (LPR) of 5:1, 10:1, 50:1 and incubated at 300 rpm, 4°C for further 30 minutes. The same procedure was used to prepare protein-free liposomes by replacing the protein with SEC buffer (Table 2.5).

Detergent was removed by incubating with BioBeads SM2-Adsorbent Media, BIO-RAD (~0.2 g BioBeads/500 μ L reconstitution mixture) at 4°C on a rotating wheel for 2 hours, followed by BioBead replacement and overnight incubation at 4°C under rotation.

2.1.9.3 Proteoliposomes Wash by Ultra-centrifugation

Subsequently the proteoliposomes were subjected to three successive washes by ultracentrifugation at 200,000g, 4°C for 1 hour, using liposome buffer. Finally, the proteoliposomes were suspended in liposome buffer (5mg/mL of lipids), aliquoted in 50 μ L, and stored at -80°C.

The size distribution of proteoliposomes was measured *via* dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments).

The quantity of protein inserted in liposomes was assessed *via* SDS-PAGE analysis, 15 μL of proteoliposomes (5mg/ml) containing each variant at a LPR of 5:1, 10:1, or 50:1 (w/w) were run on either a 10% SDS-PAGE gels for AmtB or 12.5% for Rh50 demonstrating that the quantity of protein inserted is similar for all variants and varied according to LPR.

2.1.9.4 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the presence of the protein in elution fractions and to assess the purity of the sample.

For SDS PAGE, polyacrylamide gels were prepared and mounted using a Mini-Protean (Bio-Rad) system. Clamp, gel plates and spaces of the system were assembled into the gel stand. Resolving and stacking gel mix was setup, 10% ammonium persulphate and TEMED were added to the gel mix. The resolving gel mix was poured directly into the assembly gel plates and distilled water was added above the resolving gel mix in plates to keep the surface wet and straight. The gel plates were left at room temperature for 40 min until they solidified. Next, the stacking gel mix was prepared. The mix was poured above the resolving gel (after removing the distilled water) and the appropriate comb was added. The gel was allowed to solidify at room temperature for 40 min. A running buffer was added to SDS-PAGE after the gel was setup.

The protein samples were mixed with the loading buffer, loaded onto the gel and run at $150\text{V}\cdot\text{cm}^{-1}$ for 50 minutes in running buffer. The gel was then stained with Coomassie Blue for 30 minutes and de-stained for 15 minutes. Table 2.7 lists compositions of all the buffers used.

2.1.9.5 Western Blot

The samples were mixed with the loading blue buffer, loaded onto two identical SDS page gels and run at $150\text{V}\cdot\text{cm}^{-1}$ for 60 minutes in running buffer as described before in section 2.5.4. One gel was then stained with Coomassie Blue for 30 minutes and de-stained for 15 minutes. The second gel was equilibrated in transfer buffer for 10 minutes. A $0.45\mu\text{m}$ polyvinylidene difluoride (PVDF) (ThermoFisher SCIENTIFIC)

membrane was activated by immersion in 100% methanol for 1 minute followed by equilibrating it in the transfer buffer for 10 minutes. The sandwich for transferring was assembled as visualised in.

Transfer was carried out at 4 mA/cm³ of the surface area of the membrane: in this case a surface area of (8.5x6.5) = 55.25cm³. This was 30 minutes at 221 mA using semi-dry transfer system (Biometra FastBlot). After completion of the run, the membrane was released and blocked by incubation in a TBS-T 5% non-fat milk buffer for 1 h at room temperature, shaking. Then, the membrane was incubated with 0.1 µg/mL His epitope tag anti-mouse primary mAB antibody (GenScript) in TBS-T 5% non-fat milk buffer overnight at 4°C. The membrane was washed thoroughly with TBS-T. The membrane was incubated with 0.1µg/mL ALEXA FLUOR 790 goat anti-mouse IgG (H+L) secondary antibody (ThermoFisher SCIENTIFIC) in TBS containing 5% non-fat milk at room temperature for 2 hours, shaking. Finally, the membrane was washed thoroughly with TBS-T and the fluorescent bands were detected in the membrane by Li-COR Odyssey Infrared Imager, USA. Table 2.7 lists compositions of all the buffers used.

Table 2.7 SDS-PAGE, western blot and agarose gel buffers

Name of buffer	Composition
SDS PAGE	
10% SDS-PAGE Stacking gel	15% (v/v) 30% Acrylamide 30% (v/v) solution 3 SDS-PAGE 60% (v) H ₂ O 1% (v/v) of 10% APS 0.1% (v/v) TEMED
Solution 2 SDS - PAGE	1.5M Tris-HCl pH8.8 0.3% (w/v) SDS
Solution 3 SDS -PAGE	0.5M Tris-HCl pH6.8 0.3% (w/v) SDS
10x Running Buffer pH 8.3	3% (w/v) Tris-base 14.4% (w/v) Glycine 1% (w/v) SDS
Loading blue 5x	10% (w/v) SDS 30% (v/v) Glycerol 0.02% (w/v) Bromophenol Blue 250mM Tris-HCl pH6.8 5% (v/v) β -mercaptoethanol
Staining Buffer	0.1% (w/v) Coomassie Brilliant Blue 50% (v/v) Methanol 10% (v/v) Acetic acid
Destining Buffer	40% (v/v) Methanol 10% (v/v) Acetic acid 50% (v/v) Distilled water
Liposome Buffer	100 mM potassium phosphate pH7.6 300 mM KCl
Western Blot	
Transfer Buffer	25 mM Tris Base pH 8.3 150 mM glycine 10% (v/v) methanol
TBS	50 mM Tris- HCl pH7.5 150 mM NaCl
TBS-T	50 mM Tris- HCl pH7.5 150 mM NaCl 0.1% (v/v) Tween 20
TBS-T 5% Milk	50 mM Tris- HCl pH7.5 150 mM NaCl 0.1% (v/v) Tween 20 5% (w/v) non-fat milk
Agarose gel	
1-2% Agarose gel	1-2% Agarose TAE buffer 1 μ g/mL Ethidium bromide
TAE Buffer	40 mM Tris-HCl 20mM acetic acid 1 mM EDTA

2.1.10 Solid Supported Membrane Electrophysiology (SSME)

2.1.10.1 *Sensor Preparation*

Sensors (Nanon Technologies, Munich-Germany) were prepared as previously described (Bazzone, Barthmes and Fendler, 2017). Briefly, sensors were coated with 50 μ L of thiol (1-octadecanethiol in isopropanol 0.5mM solution) and incubated for 30 minutes at 4°C. Solution was removed and sensors were rinsed three times with 10mL 2-propanol and 10mL deionised water. Dry sensors were subsequently coated with 1.5 μ L of 1,2-diphytanol-*sn*-glycerol-3-phosphocholine solution and 100 μ L of non-activated (NA) buffer was immediately added to form the solid supported membrane (SSM). Proteoliposomes/protein-free liposomes were defrosted and sonicated (U300H Ultrawave Precision) at 35W for ~10 seconds, diluted 10-fold in NA buffer pH7 (unless stated otherwise) and 10 μ L of solution was applied to the SSM on the sensor. Sensors were then centrifuged at 2000 *g* for 40 minutes and stored at 4°C for a maximum of 48h before electrophysiological measurements.

2.1.10.2 *Single Solution Exchange*

For functional measurements at a fixed concentration, a single solution exchange configuration was used where the order was Non-Activating (NA), Activating (A) and NA buffers. These were injected on the sensor over constant osmolarity at a 200 μ L/s flow rate; only the activating solution contained the ammonium at desired concentration (Bazzone, Barthmes and Fendler, 2017).

The measurements were carried out in triplicate, from two independent purification batches, with 3 measurements recorded for each sensor at room temperature (21°C) using a SURFE²R N1 apparatus (Nanon Technologies). Protein-free liposomes (liposomes not containing the protein) were used as a control. Prior to measurements, the quality of the sensor was checked by measuring the capacitance (15–30nF) and conductance (<5nS). Sensors out-with above ranges were discarded. Details of all activating and non-activating solutions can be found in Table 2.8.

2.1.10.3 *Data Representation and Analysis*

Solid Supported Membrane Electrophysiology (SSME) measures the charge translocation across a membrane in an electric circuit manner. SSME records this as

a transient current which provides two pieces information, namely a pre-steady and a steady state current. The former represents the interaction between the protein and the substrate, whilst the latter represents translocation cycle, which is the interaction and the transport of the substrate (Bazzone, Barthmes and Fendler, 2017).

2.1.10.4 *Decay Time*

To differentiate the signal arising from substrate-protein interaction from signal reflecting full transport cycles, the decay time of the transient current, which is proportional to the velocity of the complete transport cycle, was analysed (Bazzone, Barthmes and Fendler, 2017). The decay time of the transient current was calculated by fitting the raw data with one-phase exponential decay function (equation below) between the maximum height of the peak and the baseline using OriginPro 2018 (OriginLab).

$$y = y_0 + A_1 e^{-x/t_1}$$

The fit was obtained using the Levenberg-Marquardt iteration algorithm, where y is the amplitude in (nA), y_0 is the maximum amplitude, x is the time in seconds, t_1 is the rate constant.

Table 2.8 Solid Supported Membrane Electrophysiology solutions*

	Substrate	Activating	Non-Activating	pH
Standard	Ammonium	100 mM KPho	100 mM KPho	7
		100 mM KCl	300 mM KCl	
		200 mM NH ₄ Cl		
Standard	Potassium	100 mM NaPho	100 mM NaPho	7
		100 mM NaCl	300 mM NaCl	
		200 mM KCl		
Standard	Methylammonium	100 mM KPho	100 mM KPho	7
		100 mM KCl	300 mM KCl	
		200 mM MeACl		
Increased KCl	Ammonium	100 mM KPho	100 mM KPho	7
		300 mM KCl	300 mM KCl	
		200 mM NH ₄ Cl		
Decreasing [NH ₄ Cl]	Ammonium	100 mM KPho	100 mM KPho	7
		100 mM KCl	300 mM KCl	
		100-0.098 mM NH ₄ Cl		
Wacker Solutions	Ammonium	100 mM Tris	100 mM Tris	8
		500 mM KCl	500 mM KCl	
		300 mM NH ₄ Cl	300 mM NaCl	
pH Difference	Proton	100 mM KPho*	100 mM KPho**	*5 **7
		300 mM KCl*	300 mM KCl**	
Tris Buffer, not ionically balanced	Ammonium	100 mM Tris	100 mM Tris	7
		300 mM KCl	300 mM KCl	
		200 mM NH ₄ Cl		
Sodium background	Ammonium	100 mM NaPho	100 mM NaPho	7
		100 mM NaCl	300 mM NaCl	
		200 mM NH ₄ Cl		
Not ionically balanced	Ammonium	100 mM KPho	100 mM KPho	7
		300 mM KCl	300 mM KCl	
		200 mM NH ₄ Cl		

*All solutions adjusted to pH 7 using KOH or NaOH solutions. KPho: potassium phosphate buffer, NaPho sodium phosphate buffer.

2.2 Phylogenetic analysis

2.2.1 Relationship of Proteins with Experimentally Determined Structures

2.2.1.1 Sequence gathering

Full protein sequences for the seven ammonium transporters that have had their structures solved were downloaded from the NCBI website (03/05/2022) (Table 2.9). Owing to the conservation of the core transmembrane region of the protein it was decided to only use this part of the sequence for our alignment (section 1.3.1). The start and end positions for each helix of the seven proteins was found by inspection of the PDB structure and multisequence files were built for each helix (Table 2.10).

Table 2.9 The information for each protein used in this part of the study. This work was done before the most recent structure was published so only seven are included (Pflüger *et al.*, 2024).

Organism	Protein	NCBI Accession	PDB Accession
<i>Escherichia coli</i>	AmtB	CAD6020544.1	3C1G
<i>Nitrosomonas europaea</i>	Rh50	CAD84359.1	3B9W
<i>Candidatus kuenenia stugarttiensis</i>	AmtB	CAJ74453.1	6EU6
<i>Archaeoglobus fulgidus</i>	Amt-1	AAB90264.1	2B2I
<i>Saccharomyces cerevisiae</i>	Mep2	CAA96025.1	5AEX
<i>Candida albicans</i>	Mep2	KAF6070074.1	5AF1
<i>Homo sapiens</i>	RhCG	NP_001307970.1	3HD6

Table 2.10 The start and end positions of each helix from the seven transporters that have had their structures deduced

Organism and protein	Helix 1	Helix 2	Helix 3	Helix 4	Helix 5	Helix 6	Helix 7	Helix 8	Helix 9	Helix 10	Helix 11
<i>Escherichia coli</i> AmtB	28-58	62-91	119-142	146-171	190-204	219-241	246-276	279-295	302-323	332-360	369-403
<i>Nitrosomonas europaea</i> Rh50	33-62	65-90	101-123	127-150	169-191	197-221	224-254	259-275	282-312	319-339	342-370
<i>Candidatus kuenenia stugarttiensis</i> AmtB	1-35	39-68	92-115	120-145	170-186	205-227	233-262	265-281	288-313	321-346	352-386
<i>Archaeoglobus fulgidus</i> Amt-1	2-29	35-64	84-109	113-138	156-172	186-210	213-241	245-261	268-293	300-326	329-367
<i>Saccharomyces cerevisiae</i> Mep2	22-56	64-90	121-143	149-173	193-208	227-251	254-283	290-302	313-337	341-375	388-416
<i>Candida albicans</i> Mep2	24-50	60-86	116-137	143-168	183-202	221-245	248-278	282-298	305-331	335-371	378-408
<i>Homo sapiens</i> RhCG	54-81	84-110	121-143	147-171	184-199	214-236	242-271	278-293	302-333	339-361	384-417

The headers for each sequence were organised with three key features, 1) the NCBI or UniProt accession 2) The start and end coordinates of the helix. 3) The “GN=”, with the corresponding gene feature for UniProt records, or accession for NCBI records. An example header is shown below with key features highlighted. This has the added benefit of being readable by ncfp (10.5281/zenodo.3960233) which is used later (2.2.1.3).

```
>KAF6070074.1/24-50 Ammonium transporter MEP2 [Candida albicans] GN=KAF6070074.1
```

2.2.1.2 Alignment

A multiple sequence alignment was then produced separately for all helix files corresponding to each helix in the TM domain using MAFFT v7.487 using default settings (Kato and Standley, 2013).

```
mafft file.in > file.out
```

2.2.1.3 Obtaining genomic DNA sequence

Using ncfp v0.2.1a1 (10.5281/zenodo.3960233) the nucleotide sequences corresponding to the protein sequence sections were identified using the start and end coordinates in the FASTA header. Ncfp identifies the corresponding coding sequence by traversing NCBI sequence databases using the protein sequence as query.

```
ncfp --unify_seqid -v -s -l helix1.log helix1.fasta helix1.out  
thomas.harris@strath.a.cuk -d caches/new_ncfp -c ncfp_cache
```

Since this command was repeated ten more times, the command

```
--filestem cached --keepcache
```

Was added so the same genomes would be stored in a cache and not have to be re-downloaded every time.

2.2.1.4 Back-threading DNA sequences onto protein alignment

The nucleotide sequences returned by ncfp were back threaded onto the amino acid alignment using tcoffee v12.00.7fb08c2 (Notredame, Higgins and Heringa, 2000). Nucleotide sequence headers were edited manually to match protein sequence seqID accessions, this was done by deleting all information in the headers after the “/” so that all that was left was the accession eg KAF6070074.1.

```
t_coffee -other_pg seq_reformat -in nt-file -in2 alignment-  
file -action +thread_dna_on_prot_aln -output fasta > outfile
```

These nucleotide alignments were concatenated using seqkit v2.4.0 (Shen *et al.*, 2016).

```
seqkit concat helixfile1 helixfile2 ... helixfile11 > outfile
```

A partition file (partition.txt) was manually generated to describe the locations of helix sequence splits. Modeltest-ng v0.1.6 (Darriba *et al.*, 2020) was then used to find the most appropriate evolutionary model for each helix in the concatenated file.

```
modeltest-ng -i helixconcat.fasta -q partition.txt
```

The concatenated nucleotide alignment and the partition file were then used as input for raxml-ng v0.9.0 (Kozlov *et al.*, 2019) to generate a maximum likelihood tree with 100 bootstraps using the seed 1659436425

```
raxml-ng --msa concatenatedalignment.file --model  
partition.file --all --bs-trees 100
```

2.2.2 Ammonium transporter tree using Tree of Life sequences (TOL)

2.2.2.1 Acquisition of UniProt IDs of Ammonium Transporters

All scripts and files used in the next section can be found here <https://doi.org/10.5281/zenodo.15730021>. The organism column was extracted from the supplementary table 1 of Banfield's tree of life (Hug *et al.*, 2016). This file contained all available taxonomic information about the tree taxa but was presented in an inconsistent format. Using the notebook 01_parse_tol.ipynb the names were extracted and cross matched to NCBI's new_taxdump/ranked_lineage.dmp file downloaded on 23/10/2023. This linked genus species names for 1203 organisms from NCBI taxonomy to the TOL tree. Using the 02_sequence_acquisition.ipynb notebook, the names of these organisms were used to search the UniProt database with the word "Ammonium" (13/06/2024) using bioservices v1.10.0 (Cokelaer *et al.*, 2013). This wide sweeping search would return ammonium transporters based on their functional annotations. An example search query would be "*Abiotrophia defectiva* ammonium". The resulting Uniprot accession IDs were downloaded. This method would also return any other proteins that had ammonium in their functional annotations and the following step was designed to filter these proteins out.

2.2.2.2 Extraction of Transmembrane Region Sequences

Still using the 02_sequence_acquisition.ipynb notebook the IDs were used as queries to search UniProt, this time specifically for transmembrane regions (TM) again using bioservices v1.10.0 (Cokelaer *et al.*, 2013).

```
u.search(UniprotID, frmt="tsv", columns='ft_transmem')
```

The start and end coordinates were parsed from this information and used to extract the corresponding sequences from the full protein sequence.

If a protein did not have eleven or twelve predicted helices, it was rejected. If it had twelve, the first helix was ignored. This was done to accommodate rhesus proteins, which have 12 helices (with the extra helix being the first). Eleven different multiple sequence files were generated, containing the structurally corresponding helix from

each sequence. The headers in these files were converted into a form compatible with ncfp as seen in section 2.2.1.1.

2.2.2.3 Identifying coding sequences for each helix

Using the 03_ncfp.ipynb notebook the genomic DNA sequences of the TM region sequences were found as described in section 2.2.1.3.

2.2.2.4 Alignment

The following sections 2.2.2.4 and 2.2.2.5 use the notebook 04_alignment_to_tree_creation.ipynb.

For some proteins the underlying coding sequence could not be found so they were not carried forward in the analysis. The sequences for which the genomic DNA sequence could be found were aligned using MAFFT v7.487 (Kato and Daron M Standley, 2013) with the parameters

```
--op 5 --ep 2.5
```

2.2.2.5 Genomic DNA and back-threading onto alignment

Before the nucleotide sequences generated in section 2.2.2.3 could be back-threaded on the protein alignment the headers needed to be edited so they would match by removing all information after the "/" as described in section 2.2.1.4. The sequences were then back-threaded onto the alignment using tcoffee v12.00.7fb08c2 (Notredame, Higgins and Heringa, 2000) and the command found in section 2.2.1.4. This time the back-threaded sequences were trimmed using trimal's (v1.4.rev15) automatic setting (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009).

```
trimal -in in_file -out out_file -automated1
```

This was because the start and end of the alignments had overhangs that needed to be removed. The sequences were then concatenated, and any duplicate sequences were removed. Due to computational limitations, it was not possible to partition the sequence data so that a separate model could be used for each helix. The evolutionary models for each helix when making the seven-member tree were quite similar

however, so for this reason, no partition file was used and the model used was calculated using the whole sequence. Otherwise, a maximum likelihood phylogenetic tree was generated in the same way as described previously in section 2.2.1.4 using RaxML v0.9.0 (Alexey M. Kozlov *et al.*, 2019) with the seed 1720002030 and the evolutionary model TIM1+G4 as predicted by Modeltest-ng v0.1.6 (Darriba *et al.*, 2020). This time since the tree was much larger than seven members the bootstraps on more basal nodes were very low. Transfer Bootstrap Expectations (TBE) was used to convert the bootstraps.

```
raxml-ng --support --tree 03_bootstrap.raxml.bestTree --bs-trees 03_bootstrap.raxml.bootstraps --bs-metric TBE
```

2.2.2.6 Notebooks

A flow diagram of the order and function of each notebook can be seen below (Figure 2.0.1).

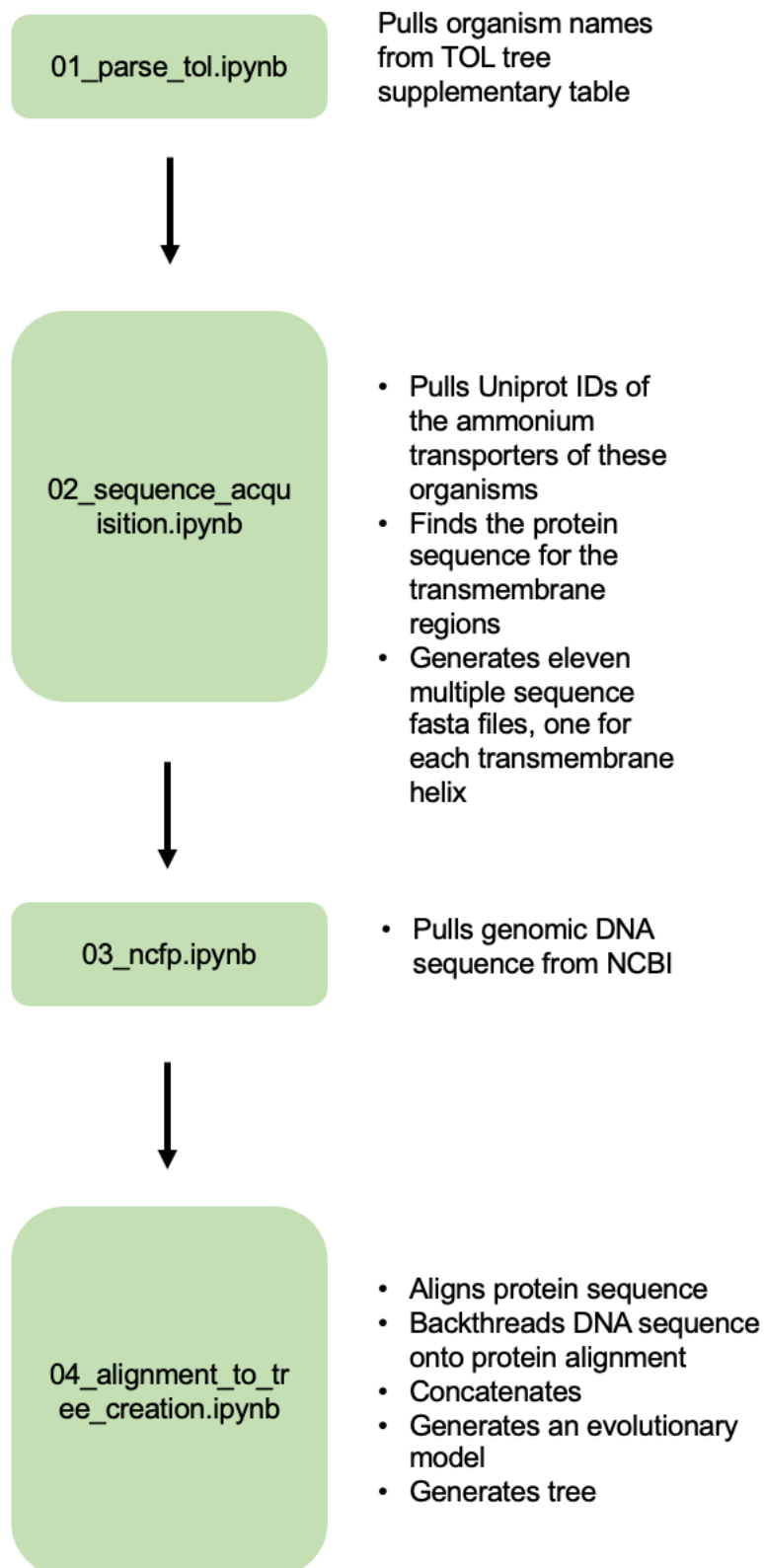


Figure 2.0.1 Flow diagram showing the order and function of each notebook included in the cited repository.

2.2.2.7 Identification of spurious sequences

Having constructed this tree it was noticed that some proteins in it did not have the name “ammonium transporters”. It appeared that the method used to gather proteins in section 2.2.2.1 also managed to include some that were not ammonium transporters, for example a drug resistance protein from *Neurospora crassa* (UniProt ID: Q7S6U7) that has eleven TM helices but for which the alpha fold predicted structure does not look like an ammonium transporter. Proteins that were not annotated with the term “ammonium transport” or similar were investigated by manually checking their UniProt page, the alpha fold structure prediction, and attached publications. Some proteins annotated as “adenylate cyclase” or similar, for example, were seen to have an ammonium transporter domain attached to an extra adenylate cyclase domain, and proteins such as these were retained. Following identification, the spurious proteins were removed from the original helix files. Before a new tree was inferred, we added additional proteins as will be described in sections 2.2.2.8 and 2.2.2.9. A list of the proteins removed in this step can be found below (Table 2.11).

Table 2.11 Table showing proteins found not to be ammonium transporters and therefore removed from tree.

Entry	Protein names	Organism
Q07307	Uric acid-xanthine permease (UAPA transporter)	<i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) (<i>Aspergillus nidulans</i>)
D2VMN0	Predicted protein	<i>Naegleria gruberi</i> (Amoeba)
Q7S6U7	Drug resistance protein	<i>Neurospora crassa</i> (strain ATCC 24698 / 74-OR23-1A / CBS 708.71 / DSM 1257 / FGSC 987)
P0DKG9	High-affinity nitrate transporter 2.1 (OsNRT2.1)	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)
O82811	High-affinity nitrate transporter 2.1 (AtNRT2:1) (Protein ACH1) (Protein LATERAL ROOT INITIATION 1)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9R141	Solute carrier family 22 member 4 (Organic cation/carnitine transporter 1) (Octn1)	<i>Rattus norvegicus</i> (Rat)
Q9Z306	Solute carrier family 22 member 4 (Organic cation/carnitine transporter 1) (Octn1)	<i>Mus musculus</i> (Mouse)
Q9WTN6	Solute carrier family 22 member 21 (Organic cation/carnitine transporter 3) (Solute carrier family 22 member 9)	<i>Mus musculus</i> (Mouse)
Q9Z0E8	Organic cation/carnitine transporter 2 (High-affinity sodium-dependent carnitine cotransporter) (Solute carrier family 22 member 5)	<i>Mus musculus</i> (Mouse)
O70594	Organic cation/carnitine transporter 2 (CT1) (High-affinity sodium-dependent carnitine cotransporter) (Solute carrier family 22 member 5) (UST2r)	<i>Rattus norvegicus</i> (Rat)
P25527	Gamma-aminobutyric acid permease (GABA permease) (4-aminobutyrate carrier) (4-aminobutyrate permease) (Gamma-aminobutyrate permease)	<i>Escherichia coli</i> (strain K12)
C8V329	Purine-cytosine permease fcyB (PCP fcyB) (Cytosine/purine transport protein fcyB) (Fluorocytosine resistance protein fcyB)	<i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) (<i>Aspergillus nidulans</i>)
A0A0G2KTI4	Solute carrier family 12 member 2 (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2) (BSC2) (Na-K-Cl cotransporter 1) (NKCC1)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
P55011	Solute carrier family 12 member 2 (Basolateral Na-K-Cl symporter) (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2) (BSC2) (Na-K-2Cl cotransporter 1) (hNKCC1)	<i>Homo sapiens</i> (Human)

Q9UP95	Solute carrier family 12 member 4 (Electroneutral potassium-chloride cotransporter 1) (Erythroid K-Cl cotransporter 1) (hKCC1)	<i>Homo sapiens</i> (Human)
Q924N4	Solute carrier family 12 member 6 (Electroneutral potassium-chloride cotransporter 3) (K-Cl cotransporter 3)	<i>Mus musculus</i> (Mouse)
Q9UHW9	Solute carrier family 12 member 6 (Electroneutral potassium-chloride cotransporter 3) (K-Cl cotransporter 3)	<i>Homo sapiens</i> (Human)
P29286	Nitrogen fixation regulation protein FixK	<i>Bradyrhizobium diazoefficiens</i> (strain JCM 10833 / BCRC 13528 / IAM 13628 / NBRC 14792 / USDA 110)
Q9Y666	Solute carrier family 12 member 7 (Electroneutral potassium-chloride cotransporter 4) (K-Cl cotransporter 4)	<i>Homo sapiens</i> (Human)
Q9WVL3	Solute carrier family 12 member 7 (Electroneutral potassium-chloride cotransporter 4) (K-Cl cotransporter 4)	<i>Mus musculus</i> (Mouse)
A0A225YUR6	MFS transporter	<i>Cryptococcus neoformans</i> var. <i>grubii</i> c45
Q4WF45	Major facilitator superfamily multidrug transporter mdr3	<i>Aspergillus fumigatus</i> (strain ATCC MYA-4609 / CBS 101355 / FGSC A1100 / Af293) (<i>Neosartorya fumigata</i>)
Q7S343	Major facilitator superfamily (MFS) profile domain-containing protein	<i>Neurospora crassa</i> (strain ATCC 24698 / 74-OR23-1A / CBS 708.71 / DSM 1257 / FGSC 987)
Q6BVC6	DEHA2C03718p	<i>Debaryomyces hansenii</i> (strain ATCC 36239 / CBS 767 / BCRC 21394 / JCM 1990 / NBRC 0083 / IGC 2968) (Yeast) (<i>Torulaspora hansenii</i>)
B9W978	Major facilitator superfamily (MFS) profile domain-containing protein	<i>Candida dubliniensis</i> (strain CD36 / ATCC MYA-646 / CBS 7987 / NCPF 3949 / NRRL Y-17841) (Yeast)
O70577	Solute carrier family 22 member 2 (Organic cation transporter 2) (mOCT2)	<i>Mus musculus</i> (Mouse)
Q9R0W2	Solute carrier family 22 member 2 (Organic cation transporter 2) (rOCT2)	<i>Rattus norvegicus</i> (Rat)
Q7RTM1	Proton channel OTOP1 (Otopetrin-1) (hOtop1)	<i>Homo sapiens</i> (Human)
Q7ZWK8	Proton channel OTOP1 (Otopetrin-1)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)

2.2.2.8 Addition of Review Proteins

The Uniprot ID of the proteins discussed in our recently published reviews (Bizior *et al.*, 2023; Williamson *et al.*, 2023, 2024) were retrieved by manually searching for them. These IDs were inputted into the 02_sequence_acquisition.ipynb notebook to obtain the sequences of their transmembrane regions. The corresponding set of sequences were combined with those found at the end of section 2.2.2.7. A list of these proteins can be found below (Table 2.12). A number of these proteins were lost as a consequence of not having eleven or twelve TM regions or failing to find the genomic DNA sequence as explained in section 2.2.1.3. These proteins were included because their cellular function has been experimentally measured.

Table 2.12 Extra proteins that have been experimentally investigated which that were added to the tree from our recently published review. Proteins that survived the tree making process are highlighted (yellow)

Entry	Protein names	Organism
P54144	Ammonium transporter 1 member 1 (AtAMT1;1)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9SQH9	Ammonium transporter 1 member 3 (AtAMT1;3)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9SVT8	Ammonium transporter 1 member 4 (AtAMT1;4)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9ZPJ8	Ammonium transporter 1 member 2 (AtAMT1;2)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9M6N7	Ammonium transporter 2 (AtAMT2)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
Q9LK16	Putative ammonium transporter 1 member 5 (AtAMT1;5)	<i>Arabidopsis thaliana</i> (Mouse-ear cress)
P58905	Ammonium transporter 1 member 1 (LeAMT1;1)	<i>Solanum lycopersicum</i> (Tomato) (<i>Lycopersicon esculentum</i>)
O04161	Ammonium transporter 1 member 2 (LeAMT1;2)	<i>Solanum lycopersicum</i> (Tomato) (<i>Lycopersicon esculentum</i>)
Q9FVN0	Ammonium transporter 1 member 3 (LeAMT1;3)	<i>Solanum lycopersicum</i> (Tomato) (<i>Lycopersicon esculentum</i>)
Q7XQ12	Ammonium transporter 1 member 1 (OsAMT1;1)	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)
A0A0P0WCF8	Ammonium transporter	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)
Q851M9	Ammonium transporter 3 member 2 (OsAMT3;2)	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)
Q10CV4	Putative ammonium transporter 4 member 1 (OsAMT4;1)	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)
H6UJ51	Ammonium transporter	<i>Pyrus betulifolia</i>
A0A0P0HXD2	Ammonium transporter	<i>Pyrus betulifolia</i>
X5CNT2	Ammonium transporter	<i>Pyrus betulifolia</i>
X5CNT5	Ammonium transporter	<i>Pyrus betulifolia</i>
A0A2K1WV98	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9IGE2	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
A0A2K1RA77	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)

A0A2K1RA81	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
A0A2K2AEV5	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9H8E7	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9HCZ0	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
U5FE59	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9GHA5	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9GRB5	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9HP47	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
A0A2K1WND3	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
A0A2K1Y154	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
A0A2K1ZIV3	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9GRB4	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
B9HSW3	Ammonium transporter	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)
Q82X47	Ammonium transporter family	<i>Nitrosomonas europaea</i> (strain ATCC 19718 / CIP 103999 / KCTC 2705 / NBRC 14298)
Q7T070	Ammonium transporter Rh type B (Rhesus blood group family type B glycoprotein) (Rh family type B glycoprotein) (Rh type B glycoprotein)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)

A0A8M3AW33	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50) (Rhesus blood group family type A glycoprotein)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
Q7T067	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50) (Rhesus blood group family type A glycoprotein)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
A0A0R4IBV5	Ammonium transporter Rh type C-like 2 (Rh family, C glycoprotein b)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
A4QNU4	Rh family, C glycoprotein a (Rh type C glycoprotein2b) (Zgc:162132 protein)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
Q9IAC8	Rh50-like protein	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
A8E4T4	Rh family, C glycoprotein, like 1 (Rhcg1 protein)	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
B0UYU0	Rh50-like protein	<i>Danio rerio</i> (Zebrafish) (<i>Brachydanio rerio</i>)
A6XP98	Ammonia transporter	<i>Kryptolebias marmoratus</i> (Mangrove killifish) (<i>Rivulus marmoratus</i>)
A6XBM2	Rhesus glycoprotein	<i>Kryptolebias marmoratus</i> (Mangrove killifish) (<i>Rivulus marmoratus</i>)
A6XP97	Ammonia transporter	<i>Kryptolebias marmoratus</i> (Mangrove killifish) (<i>Rivulus marmoratus</i>)
A0A1W5PRD1	RhcG	<i>Eptatretus stoutii</i> (Pacific hagfish)
A0A1W5PRA9	RhbG	<i>Eptatretus stoutii</i> (Pacific hagfish)
Q18PF6	Ammonium transporter Rh type B (FRhbg) (Rhesus blood group family type B glycoprotein) (Rh family type B glycoprotein) (Rh type B glycoprotein)	<i>Takifugu rubripes</i> (Japanese pufferfish) (<i>Fugu rubripes</i>)
Q7T3R4	Ammonium transporter Rh type C 2 (FRhcg2) (Rhesus blood group family type C glycoprotein 2) (RHCG-2) (Rh family type C glycoprotein 2) (Rh type C glycoprotein 2)	<i>Takifugu rubripes</i> (Japanese pufferfish) (<i>Fugu rubripes</i>)
Q18PF5	Ammonium transporter Rh type C 1 (FRhcg1) (Rhesus blood group family type C glycoprotein 1) (RHCG-1) (Rh family type C glycoprotein 1) (Rh type C glycoprotein 1)	<i>Takifugu rubripes</i> (Japanese pufferfish) (<i>Fugu rubripes</i>)
Q7T3R8	RHAG-1	<i>Takifugu rubripes</i> (Japanese pufferfish) (<i>Fugu rubripes</i>)
F2YIC4	Rh-like protein	<i>Metacarcinus magister</i> (Dungeness crab) (<i>Cancer magister</i>)
Q95P70	Rh-like protein	<i>Carcinus maenas</i> (Common shore crab) (<i>Green crab</i>)
A0A5B7FN87	Ammonium transporter Rh type B-B	<i>Portunus trituberculatus</i> (Swimming crab) (<i>Neptunus trituberculatus</i>)

A0A0B4M163	Rh protein	<i>Portunus trituberculatus</i> (Swimming crab) (<i>Neptunus trituberculatus</i>)
D0V1X1	Ammonium transporter	<i>Aedes albopictus</i> (Asian tiger mosquito) (<i>Stegomyia albopicta</i>)
A0A1W7R848	Putative ammonium transporter rhbg	<i>Aedes albopictus</i> (Asian tiger mosquito) (<i>Stegomyia albopicta</i>)
S4TCM4	Rhbg	<i>Oreochromis grahami</i>
S4TCV9	Rhcg2	<i>Oreochromis grahami</i>
S4TD66	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50) (Rhesus blood group family type A glycoprotein)	<i>Oreochromis grahami</i>
Q02094	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50) (Erythrocyte plasma membrane 50 kDa glycoprotein) (Rh50A) (Rhesus blood group family type A glycoprotein) (Rh family type A glycoprotein) (Rh type A glycoprotein) (Rhesus blood group-associated ammonia channel) (Rhesus blood group-associated glycoprotein) (CD antigen CD241)	<i>Homo sapiens</i> (Human)
Q9UBD6	Ammonium transporter Rh type C (Rh glycoprotein kidney) (Rhesus blood group family type C glycoprotein) (Rh family type C glycoprotein) (Rh type C glycoprotein) (Tumor-related protein DRC2)	<i>Homo sapiens</i> (Human)
Q9H310	Ammonium transporter Rh type B (Rhesus blood group family type B glycoprotein) (Rh family type B glycoprotein) (Rh type B glycoprotein)	<i>Homo sapiens</i> (Human)
Q8BUX5	Ammonium transporter Rh type B (Rhesus blood group family type B glycoprotein) (Rh family type B glycoprotein) (Rh type B glycoprotein)	<i>Mus musculus</i> (Mouse)
Q9QUT0	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50) (Rhesus blood group family type A glycoprotein) (Rh family type A glycoprotein) (Rh type A glycoprotein) (CD antigen CD241)	<i>Mus musculus</i> (Mouse)
Q9QXP0	Ammonium transporter Rh type C (Rhesus blood group family type C glycoprotein) (Rh family type C glycoprotein) (Rh type C glycoprotein) (Rhesus blood group-associated C glycoprotein) (Rhesus-associated C glycoprotein)	<i>Mus musculus</i> (Mouse)
Q22947	Ammonium transporter AmtB-like domain-containing protein	<i>Caenorhabditis elegans</i>
Q17463	Ammonium transporter AmtB-like domain-containing protein	<i>Caenorhabditis elegans</i>
P54145	Putative ammonium transporter 1	<i>Caenorhabditis elegans</i>
Q21565	Putative ammonium transporter 3	<i>Caenorhabditis elegans</i>
Q20605	Putative ammonium transporter 2	<i>Caenorhabditis elegans</i>
Q17663	Ammonium transporter	<i>Caenorhabditis elegans</i>

Q9BLG3	Ammonium transporter 2	<i>Dictyostelium discoideum</i> (Social amoeba)
Q9BLG4	Ammonium transporter 1	<i>Dictyostelium discoideum</i> (Social amoeba)
Q8MXY0	Ammonium transporter 3	<i>Dictyostelium discoideum</i> (Social amoeba)
Q9NIV0	Rhesus-like glycoprotein A (Rh50-like protein rhgA)	<i>Dictyostelium discoideum</i> (Social amoeba)
Q8I0L6	Rhesus-like glycoprotein B (Rh50-like protein rhgB)	<i>Dictyostelium discoideum</i> (Social amoeba)
Q9VFA9	Ammonium transporter	<i>Drosophila melanogaster</i> (Fruit fly)
A0A6M3Q9I2	Rh50, isoform D	<i>Drosophila melanogaster</i> (Fruit fly)
Q9V3T3	BcDNA.GH03016 (Rh-like protein) (Rh50, isoform A) (Rh50-like protein)	<i>Drosophila melanogaster</i> (Fruit fly)
M9PEN8	Rh50, isoform B (Rh50, isoform C)	<i>Drosophila melanogaster</i> (Fruit fly)
Q7Z1M1	Ammonium transporter	<i>Anopheles gambiae</i> (African malaria mosquito)
Q5BP96	Ammonium transporter 1	<i>Aedes aegypti</i> (Yellowfever mosquito) (<i>Culex aegypti</i>)
Q5BP95	Ammonium transporter 2	<i>Aedes aegypti</i> (Yellowfever mosquito) (<i>Culex aegypti</i>)
A0A6I8TEK7	Ammonium transporter	<i>Aedes aegypti</i> (Yellowfever mosquito) (<i>Culex aegypti</i>)
Q1L727	Ammonium transporter	<i>Aedes aegypti</i> (Yellowfever mosquito) (<i>Culex aegypti</i>)
A0A6I8TEI4	Ammonium transporter	<i>Aedes aegypti</i> (Yellowfever mosquito) (<i>Culex aegypti</i>)
A0A513U888	Ammonium transporter Rh type A	<i>Sinonovacula constricta</i> (Razor clam)
P41948	Ammonium transporter MEP2	<i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) (Baker's yeast)
Q59UP8	Ammonium transporter	<i>Candida albicans</i> (strain SC5314 / ATCC MYA-2876) (Yeast)
A0A2C9CJU7	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
A0A2C9CK98	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
A0A2C9CKL7	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
Q1PX03	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
Q1PX06	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
Q1PX09	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>
Q1Q357	Ammonium transporter	<i>Kuenenia stuttgartiensis</i>

2.2.2.9 *Manual addition of proteins*

Our method of extracting the transmembrane regions and converting the protein sequence back to genomic DNA is similar to the act of protein purification in that the yield drops after each step. In this case many of the review proteins from section 2.2.2.8 (Table 2.12) had failed the filtering steps. Thirty-three of these proteins did not have eleven or twelve transmembrane regions as predicted by TMHMM and reported on UniProt meaning they were discarded in the sequence acquisition step. In a few cases when this was cross referenced against the Alphafold predicted structure eleven helices were observed, in conflict with the lack of eleven as predicted on UniProt. In these cases, the sequence of the missing region/s were manually found based on inspection of the predicted Alphafold structure and added to the correct file. The extra sequences were then added to the respective files of the main dataset. A list of the extra TM regions can be found in the Missed_helices file and in Table 2.13.

Following realignment of protein sequences, backthreading of genomic DNA and concatenation a new tree was generated as described in section 2.2.1.4 using the seed 1721636776 (in case of full replication of the methods) and the evolutionary model TPM1uf+G4 as predicted for the whole sequence by Modeltest-ng v0.1.6 (Darriba *et al.*, 2020). Bootstraps were converted to TBE in the same method as described in section 2.2.2.5.

2.2.2.10 *Removal of spurious proteins*

When the tree generated in the previous section (2.2.2.9) was inspected it was noticed that some of the proteins present were on long branches and had low support. For this reason, these proteins were investigated using UniProt, their alphafold predicted structure and any supporting evidence like published papers. If the protein was deemed suspicious it was removed. In total thirty-seven proteins were removed; these can be found in the file spurious_sequence_to_remove.txt and justifications for each can be found in the file Removal_reasons_spurious. Once this was complete the final tree was generated, and the bootstraps were converted to TBE using the same method as described in section 2.2.2.5 with the seed 1723730411 (in case of full replication of the methods) and the evolutionary model TIM1+G4.

2.2.2.11 Numbers of sequences

The steps used to create this tree meant that the number of sequences reduced following each step (Figure 2.2). The number of sequences that were included in the final tree were of the best quality compared to those initially pulled from UniProt because they had been filtered for the number of transmembrane helices and for the availability of their genomic DNA.

Table 2.13 Manually found missing helices with their start and end coordinates

Organism name	UniProt ID	Missing transmembrane helix	Start-end coordinates
<i>Arabidopsis thaliana</i>	P54144	8	277-308
<i>Arabidopsis thaliana</i>	Q9SQH9	7	281-213
<i>Solanum lycopersicum</i>	Q9FVN0	8	282-297
<i>Pyrus betulifolia</i>	H6UJ51	7	286-316
<i>Pyrus betulifolia</i>	H6UJ51	8	322-336
<i>Pyrus betulifolia</i>	A0A0P0HXD2	7	253-281
<i>Pyrus betulifolia</i>	A0A0P0HXD2	8	287-301
<i>Pyrus betulifolia</i>	A0A0P0HXD2	10	342-359
<i>Populus trichocarpa</i>	B9H8E7	8	274-288
<i>Populus trichocarpa</i>	B9H8E7	9	297-319
<i>Populus trichocarpa</i>	B9GRB5	7	283-313
<i>Populus trichocarpa</i>	B9GRB5	8	319-333
<i>Populus trichocarpa</i>	B9GRB5	10	374-392
<i>Populus trichocarpa</i>	B9HP47	7	249-278
<i>Populus trichocarpa</i>	B9HP47	8	284-298
<i>Populus trichocarpa</i>	A0A2K1Y154	7	255-285
<i>Populus trichocarpa</i>	A0A2K1Y154	8	291-305
<i>Populus trichocarpa</i>	A0A2K1Y154	9	314-337
<i>Populus trichocarpa</i>	A0A2K1Y154	10	346-364
<i>Populus trichocarpa</i>	A0A2K1WND3	10	415-469
<i>Populus trichocarpa</i>	A0A2K1ZIV3	7	284-314
<i>Populus trichocarpa</i>	A0A2K1ZIV3	8	320-330
<i>Populus trichocarpa</i>	A0A2K1ZIV3	9	343-367
<i>Populus trichocarpa</i>	A0A2K1ZIV3	10	375-393
<i>Populus trichocarpa</i>	B9GRB4	7	284-313
<i>Populus trichocarpa</i>	B9GRB4	8	319-333

<i>Populus trichocarpa</i>	B9GRB4	9	342-365
<i>Populus trichocarpa</i>	B9GRB4	10	374-392
<i>Populus trichocarpa</i>	B9HSW3	7	284-313
<i>Populus trichocarpa</i>	B9HSW3	8	319-333
<i>Populus trichocarpa</i>	B9HSW3	9	342-365
<i>Populus trichocarpa</i>	B9HSW3	10	374-392
<i>Danio rerio</i>	A0A0R4IBV5	1	68-93
<i>Danio rerio</i>	A0A0R4IBV5	8	294-307
<i>Danio rerio</i>	Q9IAC8	3	119-139
<i>Danio rerio</i>	Q9IAC8	8	276-292
<i>Danio rerio</i>	A8E4T4	3	125-145
<i>Danio rerio</i>	A8E4T4	8	282-296
<i>Danio rerio</i>	A8E4T4	10	343-363
<i>Danio rerio</i>	B0UYU0	3	119-139
<i>Danio rerio</i>	B0UYU0	8	276-293
<i>Danio rerio</i>	B0UYU0	9	300-328
<i>Aedes albopictus</i>	A0A1W7R848	4	145-169
<i>Aedes albopictus</i>	A0A1W7R848	8	276-293
<i>Aedes albopictus</i>	D0V1X1	4	145-169
<i>Aedes albopictus</i>	D0V1X1	8	276-293
<i>Eptatretus stoutii</i>	A0A1W5PRA9	4	147-172
<i>Eptatretus stoutii</i>	A0A1W5PRA9	8	297-296
<i>Kryptolebias marmoratus</i>	A6XP97	1	68-93
<i>Kryptolebias marmoratus</i>	A6XP97	8	294-308
<i>Kryptolebias marmoratus</i>	A6XP98	7	248-274
<i>Kryptolebias marmoratus</i>	A6XP98	8	282-296
<i>Kryptolebias marmoratus</i>	A6XP98	10	343-363
<i>Sinonovacula constricta</i>	A0A513U888	3	110-130
<i>Sinonovacula constricta</i>	A0A513U888	4	136-161
<i>Sinonovacula constricta</i>	A0A513U888	8	265-282
<i>Aedes aegypti</i>	Q5BP95	4	145-169

<i>Aedes aegypti</i>	Q5BP95	8	276-293
<i>Aedes aegypti</i>	Q5BP96	1	58-78
<i>Aedes aegypti</i>	Q5BP96	8	276-293
<i>Drosophila melanogaster</i>	M9PEN8	1	55-75
<i>Drosophila melanogaster</i>	M9PEN8	3	116-136
<i>Drosophila melanogaster</i>	M9PEN8	8	273-290
<i>Drosophila melanogaster</i>	Q9V3T3	1	55-75
<i>Drosophila melanogaster</i>	Q9V3T3	3	116-136
<i>Drosophila melanogaster</i>	Q9V3T3	8	273-290
<i>Drosophila melanogaster</i>	A0A6M3Q9I2	1	55-76
<i>Drosophila melanogaster</i>	A0A6M3Q9I2	3	116-136
<i>Drosophila melanogaster</i>	A0A6M3Q9I2	8	273-290
<i>Caenorhabditis elegans</i>	Q17663	5	187-205
<i>Caenorhabditis elegans</i>	Q17663	8	294-307
<i>Caenorhabditis elegans</i>	Q17663	10	348-366
<i>Caenorhabditis elegans</i>	Q17463	3	111-131
<i>Caenorhabditis elegans</i>	Q17463	8	267-281
<i>Oreochromis grahami</i>	S4TCV9	3	139-159
<i>Oreochromis grahami</i>	S4TCV9	8	296-310
<i>Oreochromis grahami</i>	S4TCV9	10	357-377
<i>Oreochromis grahami</i>	S4TCM4	7	242-269
<i>Oreochromis grahami</i>	S4TCM4	8	277-294

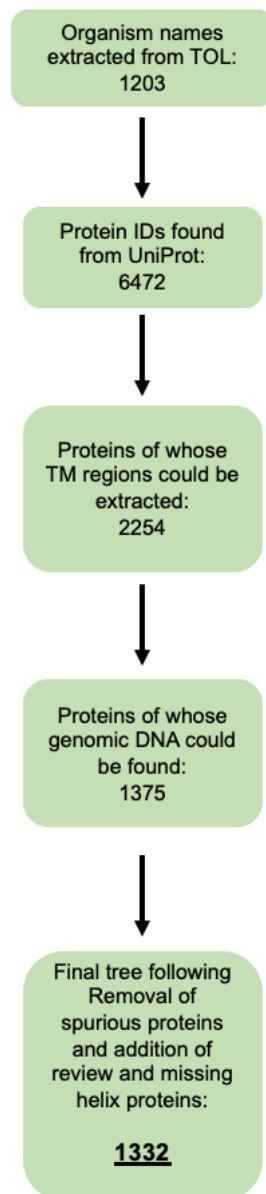


Figure 2.2 **Changes in sequence numbers leading to the final tree.**

2.2.3 Tree annotation

2.2.3.1 *Naming leaves*

The leaves were renamed to show the organism, protein name and unique UniProt ID using the notebook 01_naming_tree.ipynb.

2.2.3.2 *Taxonomic information*

The annotation information was obtained using the notebook 03_makingannotationdf.ipynb. Annotations for each sequence were retrieved from UniProt using the script. This script queries UniProt using the bioservices package to recover the corresponding taxonomy terms for each sequence, and these were stored in the file all_annotations.csv. Functions of the few proteins were added manually from the file review_protein_functions.csv

2.2.3.3 *Amino Acid annotations*

Conserved amino acids found in the sequences and used for tree annotation were added to the annotation file using the notebook 03_makingannotationdf.ipynb.

Owing to the position of some of the relevant amino acids being outside the predicted TM region, the TM sequences were pulled from Uniprot again but extended by 5 amino acids before and after, aligned with mafft v7.487 (Kato and Daron M Standley, 2013) concatenated and loaded into python as a matrix. The positions of relevant amino acids were then recorded manually using the alignment visualisation software Jalview (v2.11.4.1) (Waterhouse *et al.*, 2009), and the relevant columns were pulled from the matrix. This was used to annotate the tree using the notebooks 04_Tree_annotation_midpoint, 05_Tree_annotation_EY_root.ipynb and 06_SN_root_annotation.ipynb for the differently rooted trees

2.2.3.4 *Extra Domains*

For proteins that were annotated as having additional non-TM domains in Pfam, the additional domains and their locations were identified using the script gettingdomainnames.ipynb. Briefly, InterPro was searched (date: 23/02/2025) and the start and end coordinates of each domain was parsed along with the full length of the

protein. This was used to create a visual representation of the full protein length with the Amt domain and extra domain.

2.2.3.5 Rendering trees

Trees were annotated and rendered using notebooks 04_Tree_annotation_middpoint, 05_Tree_annotation_EY_root.ipynb and 06_SN_root_annotation.ipynb for the differently rooted trees and ete3 v3.1.2 (Huerta-Cepas, Serra and Bork, 2016).

2.2.3.6 Notebooks

A flow diagram detailing the order and function of each notebook used to annotate the trees that are included in the GitHub citation can be seen below (Figure 2.0.3).

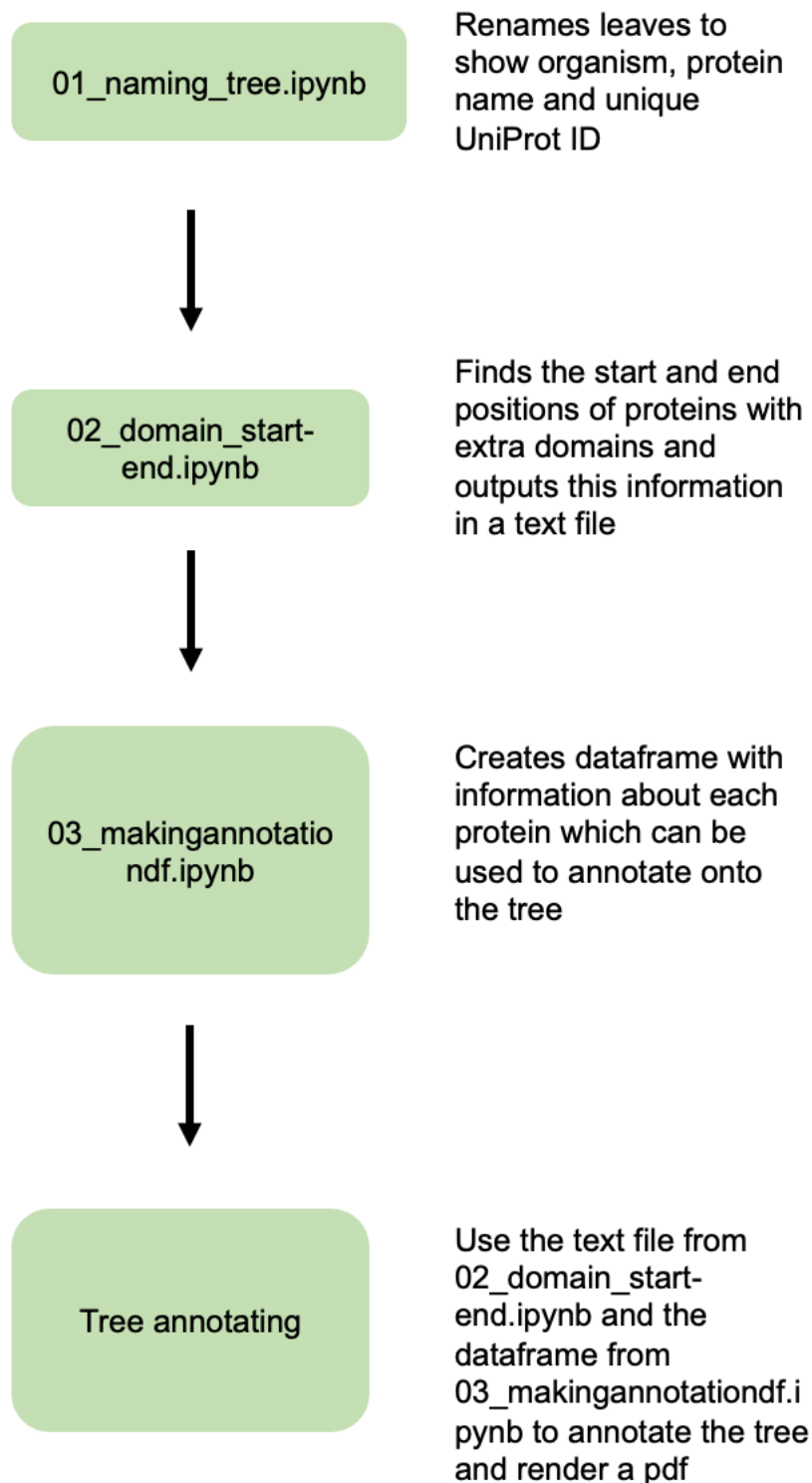


Figure 2.0.3 **Flow diagram showing the order and function of each notebook used to annotate the tree.**

2.2.4 Amt PII

Some proteins in the tree were fused to PII regulators. InterPro categorises 1406 proteins with the domain architecture PF00909 (ammonium transporter) and PF00543 (PII) (04/03/2025). In order to investigate this relationship further, the protein family information was downloaded from InterPro as a tsv file (protein_sequences.tsv) and the accessions were extracted.

2.2.4.1 PII tree

The notebook 01_Getting_PII_domains.ipynb was used to search InterPro and find the start and end coordinates of each domain. Uniprot provide predicted domain annotations, but this was found to not include all the proteins with extra domains. The PII sequences were found by using the start and end coordinates to extract the domain sequence from the full-length protein sequence. The genomic DNA sequences were obtained using the same method as in section 2.2.1.3.

2.2.4.2 Amt TM region tree

The TM regions of the ammonium transporter domains were found using the accessions in the same method as described previously in section 2.2.2.5 using the notebook 02_Getting_amt_TM.ipynb. RaxML was used in tree estimation.

2.2.4.3 Removal of proteins from uncultured organisms and eukaryotic proteins.

Some proteins were found to come from uncultured organisms as indicated in their name on UniProt. Since these sequences are potentially less reliable than those from cultured organisms, they were removed using the notebooks 03_removing_uncultured_Amt.ipynb and 04_removing_uncultured_PII.ipynb. At this point the eukaryotic proteins *Cladocopium goreau* A0A9P1BFQ1 and *Micromonospora olivasterospora* A0A562IDY7 were also removed from the PII files and the only eukaryotic protein *Cladocopium goreau* A0A9P1BFQ1 was removed from the Amt file. This is because they could have been bacterial contamination since there were no other eukaryotic proteins present and the size of the genomes for these two were large at 1Gb+. When assembling genomes of this size, contamination can

become difficult to detect. A list of the proteins that were removed can be found in the file `uncultured_and_euk_to_remove.txt`. The `03_removing_uncultured_Amt.ipynb` and `04_removing_uncultured_PII.ipynb` notebooks were also used to generate a new tree with the models TPM1uf+I+G4 for the Amt tree and SYM+I+G4 for the PII tree. In case of full replication of the methods the seeds 1659012405 and 1744125671 were used for the Amt tree and the PII tree respectively.

The trees were annotated with the kingdom and phylum colours using the notebooks `05_colour_by_kingdom.ipynb` and `06_colour_by_phylum.ipynb`.

Chapter 3: Calculation of phylogenetic tree of the Amt/Mep/Rh superfamily

3 Chapter 3: Calculation of phylogenetic tree of the Amt/Mep/Rh superfamily

The contents of this chapter will form the core of a paper “LUCA had multiple ammonium transporters” Harris T, Pritchard L, Javelle A, in preparation.

3.1 Introduction

Phylogenetic trees are diagrams of inferred evolutionary relationships. There are two main methods to generate phylogenetic trees, clustering and model fitting. There are different ways in which these can be used:

- **Neighbour joining** is a clustering method based around distance measures. It begins with a star like tree that assumes all the sequences are equally distant from the common origin and computes the distances between each sequence in the alignment. When this has been done it identifies the two with the lowest distance score and moves the common ancestor of the two outwards from the centre and solidifies it in the tree. The algorithm then finds the next closest pair of either sequence- sequence, sequence-group or group-group and sets their common ancestor in the tree. It repeats until all sequences have been analysed. Unless an outgroup is supplied this does not give a rooted tree (Saitou and Nei, 1987). How far to place the node away from a group of sequences (branch length) is estimated by taking the distances between the two sequences in the group, the distances the two sequences are from the rest of the sequences and the number of taxa. This method relies entirely on distance so does not accommodate more sophisticated models of evolution or site-specific rates of change but produces a more balanced tree (Gonnet and Scholl, 2011).
- **Maximum parsimony (MP)** is a model fitting method whereby the tree recapitulates the fewest changes between character states (the distinct variations of a trait). MP is looking for a tree that minimises the total count of changes between character states. A matrix is made with the sequences. When a small number of sequences are used the algorithm then generates all possible unrooted trees for n taxa. As this is computationally intense when n is greater than 12 because the number of possible trees is well over 10 million, heuristics are used (Sanderson and Driskell, 2003). This means that for a small number of taxa the smallest score is going to be found but when heuristics are used the smallest score is not guaranteed. This method does not incorporate

an evolutionary clock which can be an advantage when in that you can use a wide range of characters, for example protein sequences, or a disadvantage in the knowing the substitution rate can give a more accurate tree. The method is best for small datasets where the sequences are reasonably well related (Gonnet and Scholl, 2011). Maximum parsimony is also prone to long branch attraction.

- **Maximum likelihood (ML)** is a statistical method for phylogenetic tree reconstruction that employs a search method to find the tree most likely to have generated the observed data, conditioned on an evolutionary model (Stamatakis, 2014; Alexey M Kozlov *et al.*, 2019). It does so by randomly generating multiple trees to cover the search space, called the start tree, of the sequences and taking the inputted multiple sequence alignment and a substitution model and calculating how likely it is that this tree fits this inputted data. The overall likelihood of the alignment is determined by multiplying the likelihoods of each individual site. Small changes to the tree topology are made and likelihood is recalculated. Once a tree with a new highest likelihood score is found it becomes the new best tree and the starting point for the next round of searching. The algorithm will explore the likelihood tree space and find the maximum likelihood. There can be local maxima as well as a true maximum. For reproducibility a seed can be provided which means the search will start at the same location. It optimises the tree using heuristic methods by exploring the space of possible trees. The best, most likely tree is kept. This method requires the calculation of an optimal evolutionary model for the alignment. Bootstrapping is a method that tests the robustness of the best tree by using different start trees and calculating the ML tree from there (Holmes, 2003). This can be done a specified number of times for example 100. If the topology of a node is seen in 70/100 of the new trees the node is given a bootstrap score of 70 (Efron, 1979; Felsenstein, 1985a). Transfer bootstrap expectation (TBE) is a method that recalculates the standard bootstrap to take into account presence of higher taxa from a node rather than the exact position. TBE is a scale of 0-1 and works better at the more basal nodes in larger trees without compromising the critical function of bootstraps to convey robustness (Lemoine *et al.*, 2017).

I chose to use maximum likelihood because, while the Bayesian approaches like MP could give a more accurate tree, the compute time would be prohibitive on a dataset of this size.

Rooting a tree is important and refers to the point at which all tree members diverged from. Although non-reversible substitution models can enable direct inference of rooted trees, their performance can be compromised in deep phylogenies due to limited signal and model violations (Minh *et al.*, 2020; Naser-Khdour *et al.*, 2021). Another method of rooting is to midpoint root a tree whereby the root is placed between to two longest branches. One can also chose a root based on biology.

3.1.1 Evolution of ammonium transporters

The finding that the rhesus proteins in humans were ammonium transporter homologues gave an indication to how widespread this broader family of structurally related proteins are (Marini *et al.*, 2000). Since then, investigations into their history have shown evidence of gene duplication, loss and horizontal transfer (HGT) (McDonald, Dietrich and Lutzoni, 2012; Matassi, 2017). Current nomenclature splits these proteins into Amt/Mep/Rh groups with these names arising from either the substrate used for their first characterisation (ammonium for Amt/ methylammonium for Mep) (Roon *et al.*, 1975; Jayakumar *et al.*, 1989; Marini *et al.*, 1994) or the organism they were first found in (rhesus) (Wiener, 1949).

The initial investigations into the evolutionary origin of the family primarily focused on Rhesus proteins, due to their clinical relevance (Kitano *et al.*, 1998; Kitano, Oota and Saitou, 1999; Kitano and Saitou, 2000). These studies used only the transmembrane regions and found that there were multiple groups of rhesus proteins and the tree topology mirrored speciation. When trees were created with both Amt and rhesus, it was seen in midpoint rooted trees that the rhesus proteins still clustered together separately from the rest and the Amt proteins formed two groups. The root was between the Amt and rhesus protein groups (Kitano and Saitou, 2000).

As discussed in section 1.5.2, other previous studies investigating the phylogenetic history of the ammonium transporter family have focused primarily on individual subfamilies i.e. Amt, Mep, or Rh, based on the assumption that these groups are evolutionarily distinct, despite their structural similarities (McDonald, Dietrich and Lutzoni, 2012; Pitts *et al.*, 2014; McDonald and Ward, 2016; Matassi, 2017). While

these approaches have yielded well-resolved phylogenies within each individual subfamily, they have not clarified the broader evolutionary relationships across the ammonium transporter superfamily. Although the possibility of convergent evolution among the subgroups cannot be entirely excluded, the evidence and arguments presented in section 1.5.2 support the more parsimonious hypothesis that these subfamilies derive from a common ancestral lineage.

Root placement in a phylogenetic tree is crucial because it identifies the most recent common ancestor of all represented taxa. Since I have calculated unrooted trees in this chapter, it is up to the researcher to select an appropriate root based on biological reasoning. In the aforementioned studies, tree rooting was placed using Rhesus proteins as an outgroup, (Kitano and Saitou, 2000; McDonald, Dietrich and Lutzoni, 2012; McDonald and Ward, 2016). The reasoning is not explicitly provided, but it can be assumed to be related to branch length, since the rhesus proteins have the longest relative to the rest of the group. However, long branch lengths could result from several processes: a genuinely long time between common ancestors, inadequate sampling meaning intermediate taxa are not represented, strong selection pressure, the clades are unrelated and have been forced together inappropriately. Faster substitution rates, leading to longer branches, has been seen in the Rh30 antigens when compared to the Rh50 proteins (Matassi *et al.*, 1999). The Rh30 antigen arose from the Rh50 transporters and is found in a complex with Rh50 proteins in the membrane of red blood cells (Matassi *et al.*, 1999). It is plausible that Rh50 proteins also underwent extensive sequence changes from their unidirectional ancestors to evolve into bidirectional transporters. This divergence likely occurred later in the protein family's history, resulting in a longer branch on the tree without being an earlier divergence event. Therefore, while the root of the ammonium transporter superfamily could potentially be the Rhesus proteins, I will also consider alternative options, such as placing the root between more ancient lineages of archaea and bacteria, which would be more consistent with the current Tree of Life.

3.1.2 The conserved structure of ammonium transporters

The conserved structure of the ammonium transporter superfamily is notable (Figure 3.1). This observation suggests two potential explanations: (i) the structure originated once and has since been adapted for diverse functions, or (ii) it evolved independently

multiple times, converging on a similar structural solution. As discussed in section 1.5.2, I believe the former to be the more plausible scenario. The loops between the helices are less conserved and vary in length, probably because the transmembrane core of the protein provides the essential translocation function (Figure 3.1). The "exposed" loops are free to adapt to prevailing conditions, as long as they do not interfere with the core transmembrane function this would effectively make the proteins combinatorially "modular," a recurring principle in evolution. There is the possibility for domain shuffling or recombination to have played a role in the superfamily's history, but this scenario is beyond the scope of this analysis. The lack of sequence conservation can also be observed when attempting to generate a multiple sequence alignment with a number of divergent ammonium transporters, as the alignment becomes populated with large gaps (Figure 3.2). Since I assume the proteins all originated from a common ancestor and the structure of the protein core is the most conserved due to selection pressure to maintain its translocation function, I chose to use only the transmembrane regions of the protein for my alignment, following the approach of Kitano *et al.* (2000) in their study of Rhesus proteins (Kitano and Saitou, 2000). However, unlike their research, I will include all eleven transmembrane regions in my analysis. Given that the transmembrane (TM) regions of ammonium transporters are evolutionarily conserved and that the amino acid residues within these domains occupy structurally equivalent positions across homologous proteins, restricting phylogenetic analyses to TM segments allows for a more accurate and meaningful comparison of evolutionary patterns within the family. In contrast, including the extra-membranous loop regions, whose length and structural variability differ markedly between homologues (Figure 3.1), would introduce alignment ambiguities, as many residues would not correspond to equivalent structural positions. This could degrade alignment quality and potentially obscure conserved features critical for functional or evolutionary interpretation. Therefore, these equivalent structural sites are pinned in the alignment columns. This approach is in common use and has been employed to study, for example, the conserved membrane transport proteins, as the loops between their transmembrane regions (TMs) vary in length and sequence (Dabravolski and Isayenkov, 2022).

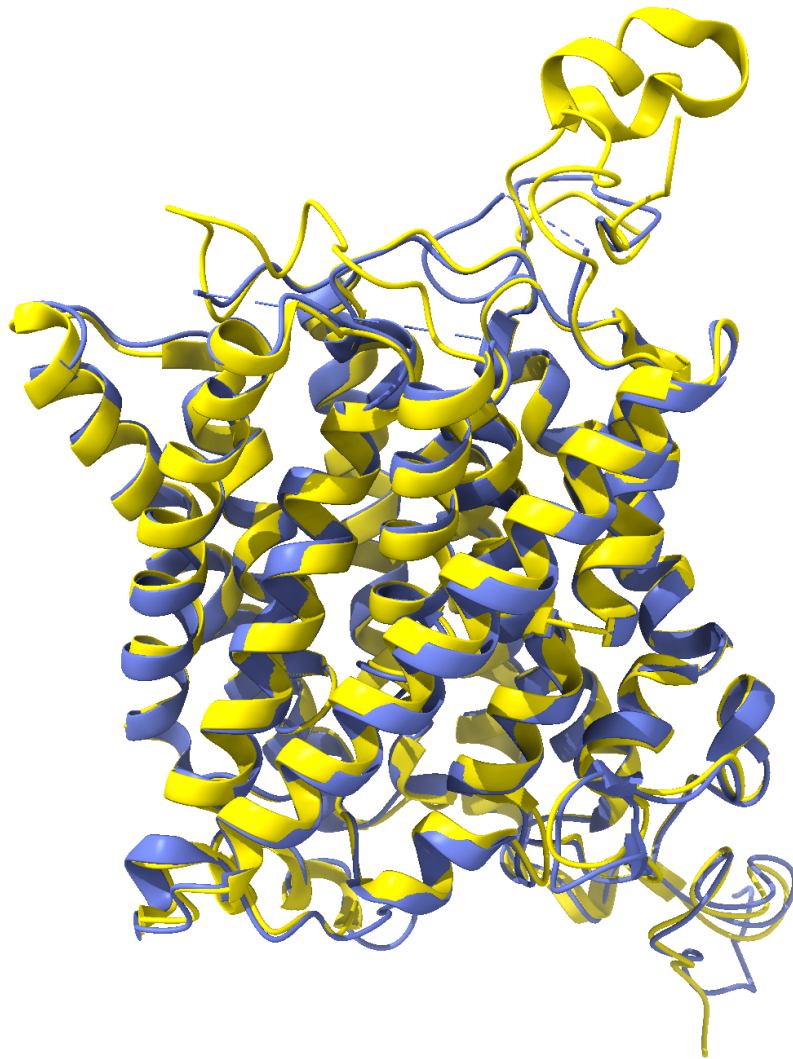


Figure 3.1 **Comparison of inter helical loops between two closely related proteins.** The Mep2 proteins from *S. cerevisiae* (blue) (pdb 5aex) and *C. albicans* (yellow) (pdb 5af1) are superimposed. While the core transmembrane structure is conserved even in two closely related proteins the interhelical loops differ in length and structure.

3.2 Aims and objectives

As outlined above, the evolutionary history of the ammonium transporter (Amt/Mep/Rh) superfamily has been addressed in several previous studies. However, most of these investigations either focused on a single subfamily or included the broader superfamily without making its evolutionary relationships the central objective. Furthermore, while phylogenetic trees have been constructed across all three subfamilies, they often lacked consistent criteria for root placement and did not comprehensively integrate mechanistic or structural data into the evolutionary interpretation.

The central aim of this study was therefore to generate a phylogenetic tree of the ammonium transporter superfamily that is both comprehensive in its taxonomic and subgroup representation and mechanistically informed in its rooting.

A critical objective, often overlooked in previous analyses, is the biologically justified placement of the root of the phylogenetic tree.

To achieve these goals, the following specific objectives were defined:

- To construct a phylogenetic tree of the ammonium transporter superfamily, based exclusively on conserved TM regions, to improve alignment accuracy and ensure structural equivalence across homologous positions.
- To include a taxonomically broad and mechanistically diverse set of sequences, representing all three subfamilies (Amt, Mep, Rh) and all domains of life.
- To assess the robustness of the resulting tree topology using transfer bootstrap expectations, thereby quantifying the reliability of key branches and subgroup relationships.
- To propose the placement of a root using mechanistic and evolutionary evidence and interpreting root positions in light of functional divergence.

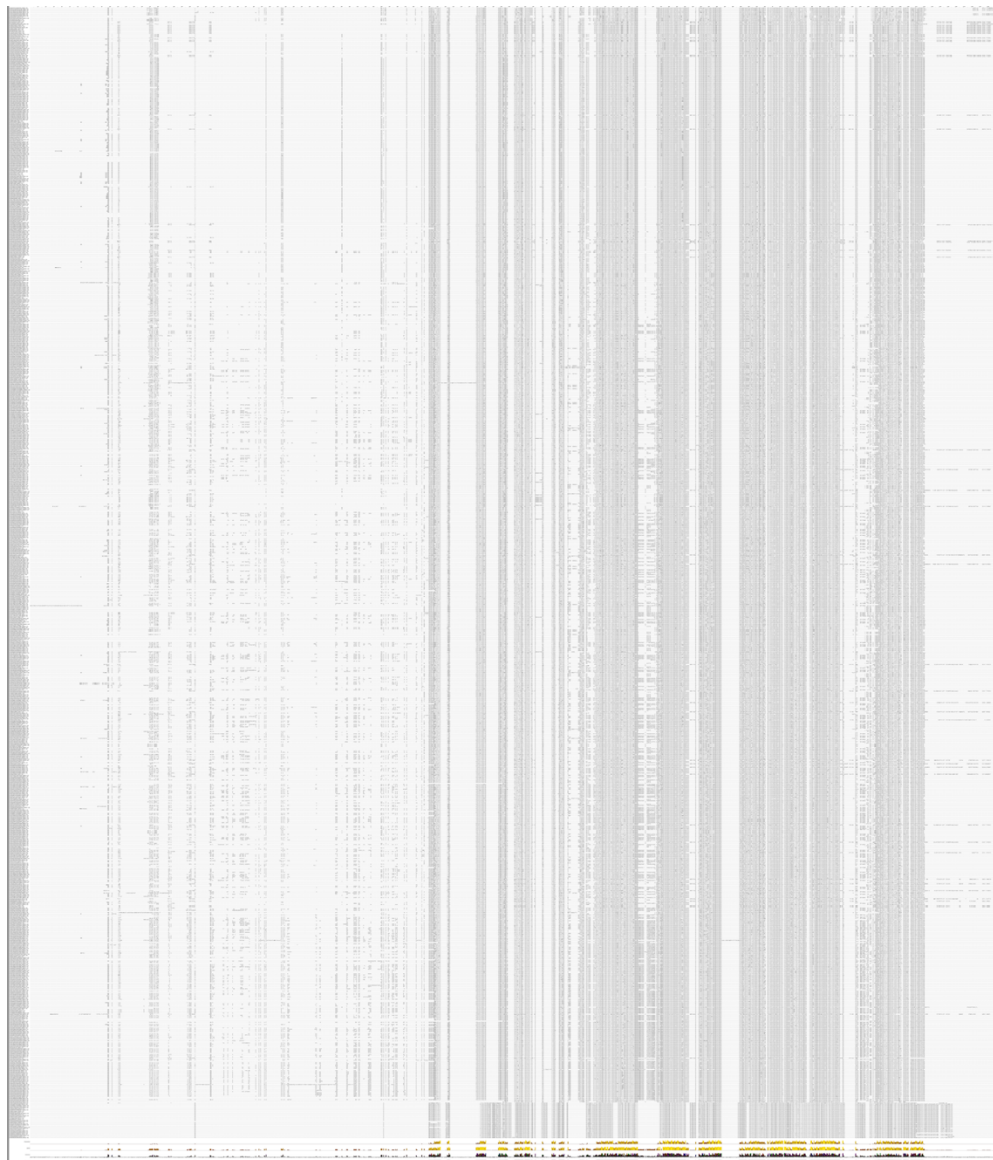


Figure 3.2 Example protein alignment of representative full-length sequences from pfam family PF00909. In an alignment of around 1000 ammonium transporters using default settings in MAFFT, although there seem to be areas of good alignment that represent the TM regions, large gaps form, exaggerated by some proteins being longer than others. This is an example of when using full length sequence alignments of this superfamily leads to poor quality alignments. I aim to reduce this by removing the interhelical loops and only using the more conserved transmembrane regions

3.3 Results

3.3.1 Seven transporters

I validated my approach by using only the TM regions of the seven proteins that had had their structures solved to date (Table 1.1). To investigate the relationships of these seven proteins I took the sequences of each eleven TM helices aligned each helix using MAFFT as described in methods section 2.2.1 (Kato and Daron M Standley, 2013) and concatenated the sequences. These seven sequences were used to make a maximum likelihood phylogenetic tree using RaxML (Figure 3.3A). The topology of the tree closely aligns with what is reported in the literature, with Rhesus and Mep proteins each forming their own monophyletic clusters. Additionally, the clustering of EcAmtB with the Mep proteins has also been observed (Figure 3.3B) (Pitts *et al.*, 2014). Using only the TM regions, I have, on a small scale, replicated results previously seen using the full protein sequences.

3.3.2 Phylogenetic tree based on the Tree of Life

To construct a phylogenetic tree with a diverse selection of organisms, I selected only taxa included in Banfield's Tree of Life (TOL) (Hug *et al.*, 2016) and identified their corresponding ammonium transporter proteins (see paragraph 2.2.2.1 in the Methods section). These sequences were used to generate a maximum likelihood tree using RaxML 100 bootstrap trees and bootstrap values reported as Transfer Bootstrap Expectation (TBE) (Figure 3.4). The tree is not divided into distinct Eukaryotic (yellow), Bacterial (blue), or Archaeal (red) clades (Figure 3.4). This has been observed in previous studies and suggests that the history represented by the tree is complex and suggests that functional/mechanistic divergence occurred prior to the division into Eukaryotic/Bacterial/Archaeal groups. (Matassi *et al.*, 1999; Huang and Peng, 2005; Cherif-Zahar *et al.*, 2007; McDonald, Dietrich and Lutzoni, 2012; Tami R McDonald and Ward, 2016; Matassi, 2017). Since the origin of life is hypothesised to trace back to the last universal common ancestor (LUCA), for a clade that is divided into at least two subclades of Archaea and Bacteria it implies that there could have been a protein present in LUCA which is ancestral to these subclades but not ancestral to any other sister clades that have Archaeal and Bacterial subclades.

If there was one single ancestral protein present in LUCA, then only a single protein would be present at the origin of each of the separate Archaeal and Bacterial clades. Any subsequent divergence or radiation would have occurred independently within the Bacterial or Archaeal clades. This would result in a tree that separates Archaea from Bacteria except when HGT occurs (Figure 3.5A). An HGT event would be observable as the presence of an Archaeal or Eukaryotic subclade within the Bacterial lineage (Figure 3.5B). Multiple events would produce multiple such subclades. I do not see this however, but rather multiple Archaeal/Bacterial splits at the basal points of Archaea and Bacteria. This suggests that there were likely multiple distinct transporters in LUCA (Figure 3.5C).

Therefore, placing a root between Archaea and Bacteria to represent speciation is not feasible, and an alternative approach is necessary.

The properties of the tree can be summarised:

- The tree is not divided into two distinct Archaeal and Bacterial subtrees which does not support the hypothesis that LUCA possessed a single ammonium transporter.

- The tree contains numerous subgroups consisting of bacteria–archaea pairs, some also including eukaryotes, each with its own distinct root. This pattern supports the hypothesis that LUCA may have possessed multiple ammonium transporters, which were subsequently inherited vertically across lineages (Figure 3.5C).
- Rhesus and Mep proteins cluster into their own distinct groups forming separate clades consisting of a single-function/family groups
- Rhesus proteins appear to be among the most ancient members of the ammonium transporter superfamily, as indicated by their position near the root of the phylogenetic tree in this midpoint rooted tree. This is unlikely given that the most parsimonious history is adaptation from pre-existing Bacterial/Archaeal proteins. Therefore, a new approach to rooting this tree is justifiable.

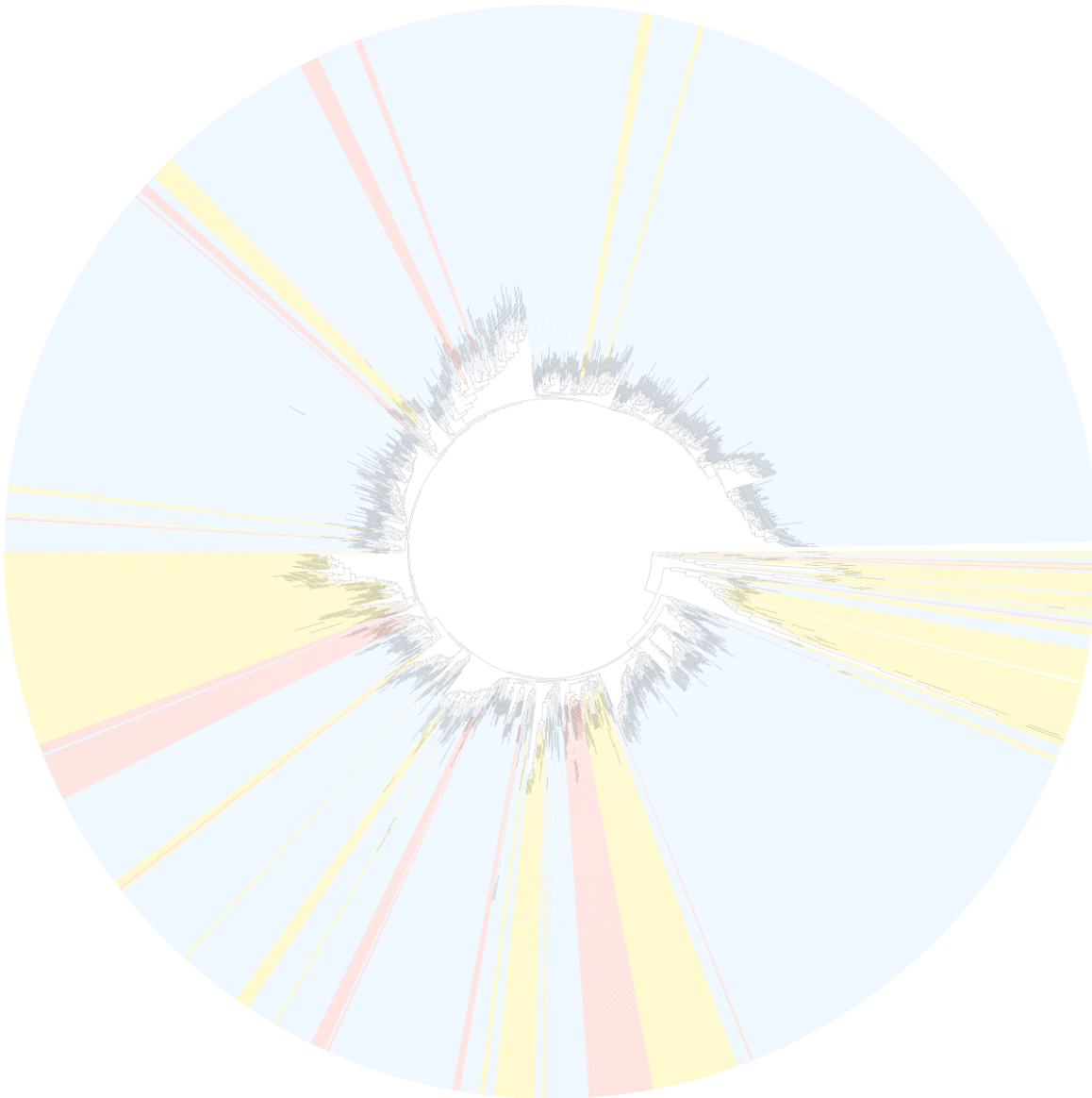


Figure 3.4 **Midpoint rooted phylogenetic tree of ammonium transporters.** Transmembrane sequences of the ammonium transporters of organisms from Banfield's TOL and the proteins from our review were used to generate a maximum likelihood tree of 1375 proteins using RaxML with the evolutionary model TIM1+G4 (methods section 2.2.2.5). The tree calculation was repeated 20 times and 100 bootstrap trees were calculated. The tree is coloured by kingdom with bacteria (blue), archaea (red) and eukarya (yellow) represented.

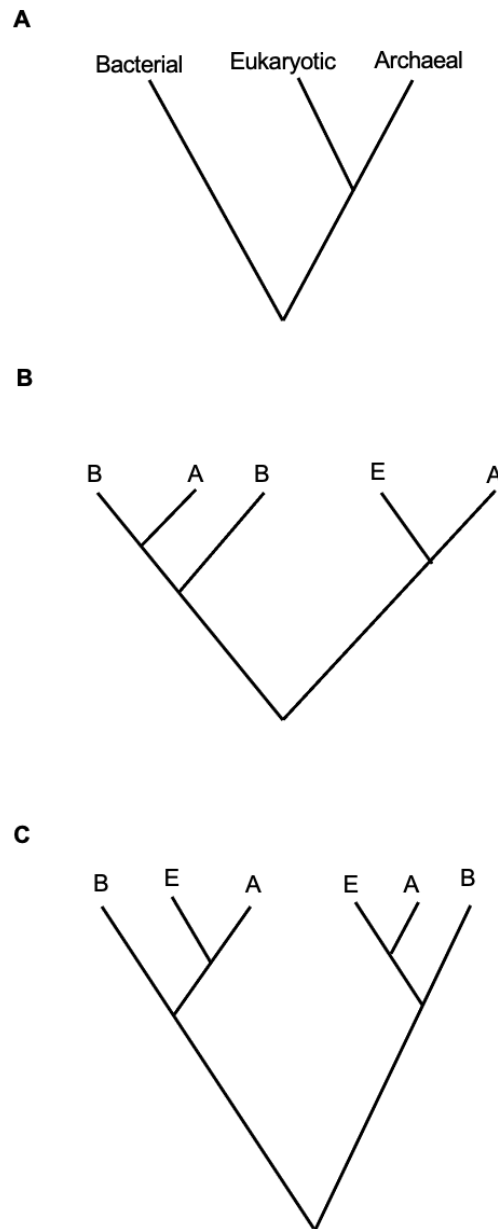


Figure 3.5 **Simple schematic of what a phylogenetic tree would look like depending on proteins present in LUCA.** A) A single protein in LUCA is passed vertically to its daughters leading to three separate clades following the kingdoms of life. This is not consistent with the topology we see in our tree (Figure 3.4). B) An example showing what HGT would look like with an Archaeal group nestled within a bacterial clade. C) In this simple tree two distinct proteins are present in LUCA, they are passed vertically to its offspring resulting in two patterns of bacterial/archaeal/eukaryotic groups. This is more in line with what is seen in my tree.

3.3.3 Editing of the tree

After construction of the tree in Figure 3.4, investigation and reannotation of some proteins indicated that they were likely not ammonium transporters and so these were removed from the analysis and a new tree was generated with 1332 proteins (for detail of this process see paragraphs 2.2.2.7-10 in the Methods section). The tree was midpoint rooted and showed multiple groups, with rhesus and Mep proteins each forming their own clade (Figure 3.6). Some groups within the topology were altered compared to the previous tree (Figure 3.4) which is to be expected in a tree of this size.

The main points can be summarised:

- The broad topology is consistent with the first tree (Figure 3.4), even though single proteins with longer branches have been removed. This suggests that I have removed appropriate sequences.
- Mep and Rhesus proteins each form their own distinct clades.
- The Mep proteins appear to share a common ancestor with an archaeal group and therefore could derive from archaea.
- The placement of the Rhesus proteins in this midpoint rooted tree is still inconsistent with evolution as discussed in section 3.1.1.

Although the largest published analysis of ammonium transporters contained 18,000 sequences, it clustered the sequences without producing a phylogenetic tree (Pflüger *et al.*, 2024). Therefore, my tree is the largest produced concerning ammonium transporters and allows us to address important questions:

- What is the distribution of the Phe-Gate and Twin-His Motif, identified in EcAmtB as functionally essential?
- Can I identify any other functionally important conserved features, thereby opening new avenues for further mechanistic studies?
- What is the most justifiable root for this tree?
- What can this tree reveal about the evolutionary history of this protein family, and can it provide insights into the ammonium transporter complement of LUCA?

I will present further analysis of the first two points in the next chapter and elaborate on the last two in this chapter.

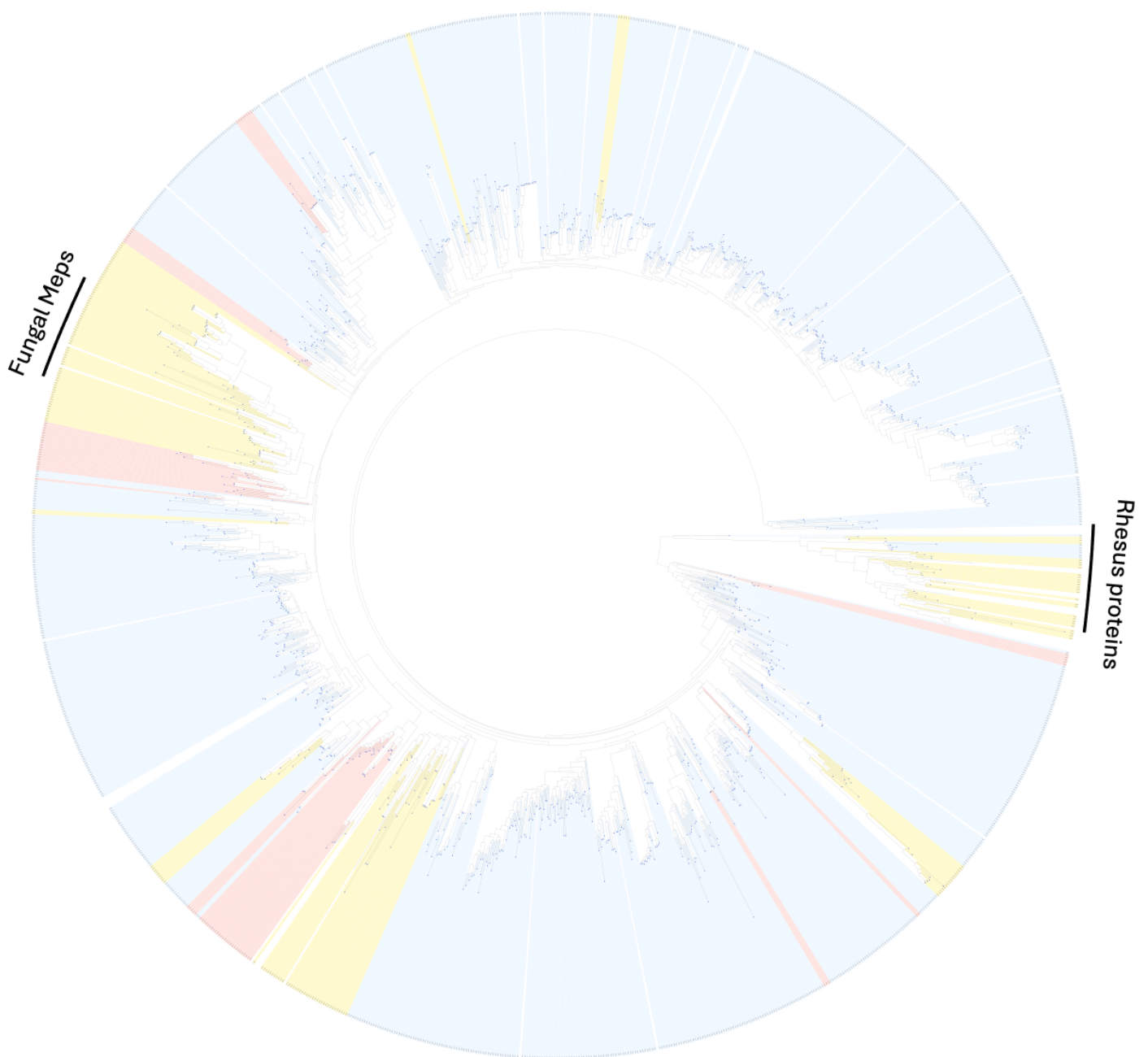


Figure 3.6 **Final midpoint rooted tree with 1332 members.** The largest, most comprehensive ammonium transporter family tree to date. The tree is coloured by kingdom with bacteria (blue), archaea (red) and eukarya (yellow) represented. Also labelled are known groups being rhesus protein and mep proteins. The tree was constructed with RaxML and the evolutionary model TIM1+G4 (methods section 2.2.2.10).

3.3.4 Rooting the ammonium transporter tree

3.3.4.1 Root placement between residues denoting key functions

The structure and function of two notable ammonium transporters, Amt5 in *Candidatus Kuenenia stuttgartiensis* and Amt1 in *Shewanella denitrificans*, have been described in the literature (Pflüger *et al.*, 2018; 2024). Each protein consists of a membrane-integral ammonium transporter domain fused to an additional cytoplasmic domain: a histidine kinase (HK) for KsAmt1 and a diguanylate cyclase (DGC) for SdAmt1. These proteins are examples of systems where an Amt protein acts as a specific ammonium receptor, modulating signal transduction to the HK or DGC domain in response to NH_4^+ concentration. These sensors operate by permitting ammonium to enter the pore, however, rather than translocating it into the cytoplasm, ammonium is redirected into a binding pocket, where it triggers the activation of the extracellular domain. The ammonium-binding pocket in these sensors is stabilised by a Glu residue, which holds ammonium within the pocket (Pflüger *et al.*, 2018; Pflüger *et al.*, 2024). In contrast, in the protein functioning as a transporter, this pocket is blocked by a Tyr residue, facilitating the translocation of ammonium through the pore. In the Rh protein, potentially capable of bidirectional transport (Soupene, Lee and Kustu, 2002; Deschuyteneer *et al.*, 2013), the position is occupied by a Met residue (Figure 3.7). This suggests that the residue at this position may be linked to the protein's fundamental function: **Tyr** for **transporter**, **Glu** for **sensor**, and **Met** for **bidirectionality**. The change in function is sufficiently fundamental that once it has happened in one direction, the protein cannot revert to its original function and therefore sets up a new lineage. Pflüger *et al.*, 2024 proposed a signature of QX₆EX₁₅NX₂DF/TX₅₄₋₋₆₀TX₂S/T to denote sensors (Pflüger *et al.*, 2024). Because I only used the TM domains of these proteins, I was not able to verify this, but I rather based the categorisation of the single Glu residue at the entrance of the binding pocket.

If an annotated phylogenetic tree reveals distinct clustering corresponding to the three variants (i.e., Glu, Tyr, and Met) at this position, it may imply that the core functional role, such as transporter, sensor, or bidirectional activity, was established early in evolutionary history, with subsequent diversification building upon this foundational function. Conversely, a lack of clear separation among these groups would suggest functional plasticity, indicating that transitions between roles (e.g., transporter and

sensor) are relatively facile. In this analysis, I observed a pronounced separation among the Tyr, Glu, and Met clades, supporting the hypothesis of early functional specialisation. This is the first time that these residues have been used to annotate a tree. This distinct grouping pattern was the more interesting because the residue was not present in the alignment used to generate the tree. This is striking because the TM-based tree reveals clear functional splits in the protein, despite excluding the region responsible for these differences from its construction. An intriguing group of eukaryotes have a Cys in place of the Met/Glu/Tyr residues. Although the functional implications of this substitution are currently unclear, its presence across both plants and protozoa suggests it may be associated with a previously unidentified function or transport mechanism. This remarkable result opens entirely new avenues for further characterising the structural-functional relationships within the Amt/Mep/Rh superfamily of transporter/sensors/bidirectional transporters, which will be discussed in greater detail in section 3.4.1.

Defining four groups on the basis of the residue at the pocket position provided the rationale for placing the root between the Glu and Tyr groups (Figure 3.8). The residue changes to flip the function between sensor and transporter appear to be irreversible as once the lineage is established, I only see two cases of divergence in the Glu group. If the sensors and transporters share a common ancestor which I believe they do, the root of the overall tree should lie between their two functions. The clear Tyr/Glu divergence, combined with the presence of representatives from all three kingdoms within each clade (Figure 3.9), suggests that LUCA may have possessed at least two distinct proteins: an ammonium transporter (Tyr) and an ammonium sensor (Glu) (Figure 3.10). In the midpoint rooted tree (Figure 3.6), the root was placed so that a large group of mainly eukaryotes (rhesus proteins) were at the base of the tree. This does not align with the principles of speciation, as eukaryotes emerged over a billion years after the first prokaryotes (Eme *et al.*, 2017). It is therefore more likely that the specific function arose late in evolution and spread in the pattern observed (Figure 3.8), rather than a scenario where all organisms initially had a copy of the Rhesus protein and most subsequently lost it over time.

If the split between Glu sensors and Tyr transporters occurred early in the evolution of these proteins we can see that the residue appears to be essential as there are only two instances, both in the Glu group, where the residue varied.

The basal branch support in this tree configuration (Figure 3.9) is much improved compared to the previous midpoint rooted tree (Figure 3.6). There are still instances of low support for example the TBE is low higher in the sensor group suggesting instability. TBE should account for internal reorganisation so this may not be the most reproducible topology. The support for the branching of the rhesus group is high at 0.978. The branch below the Mep/archaea group is also high at 0.87.

When looking at my newly rooted tree annotated with the pocket residues (Figure 3.8) there are clearly other positions that split the tree by this residue where root may be placed for example where the midpoint root was, at the base of the rhesus proteins. In this scenario, LUCA would have had a bidirectional transporter and a sensor with the Cys proteins and the Tyr transporters emerging from the sensors. I do not have enough information to dismiss this hypothesis.

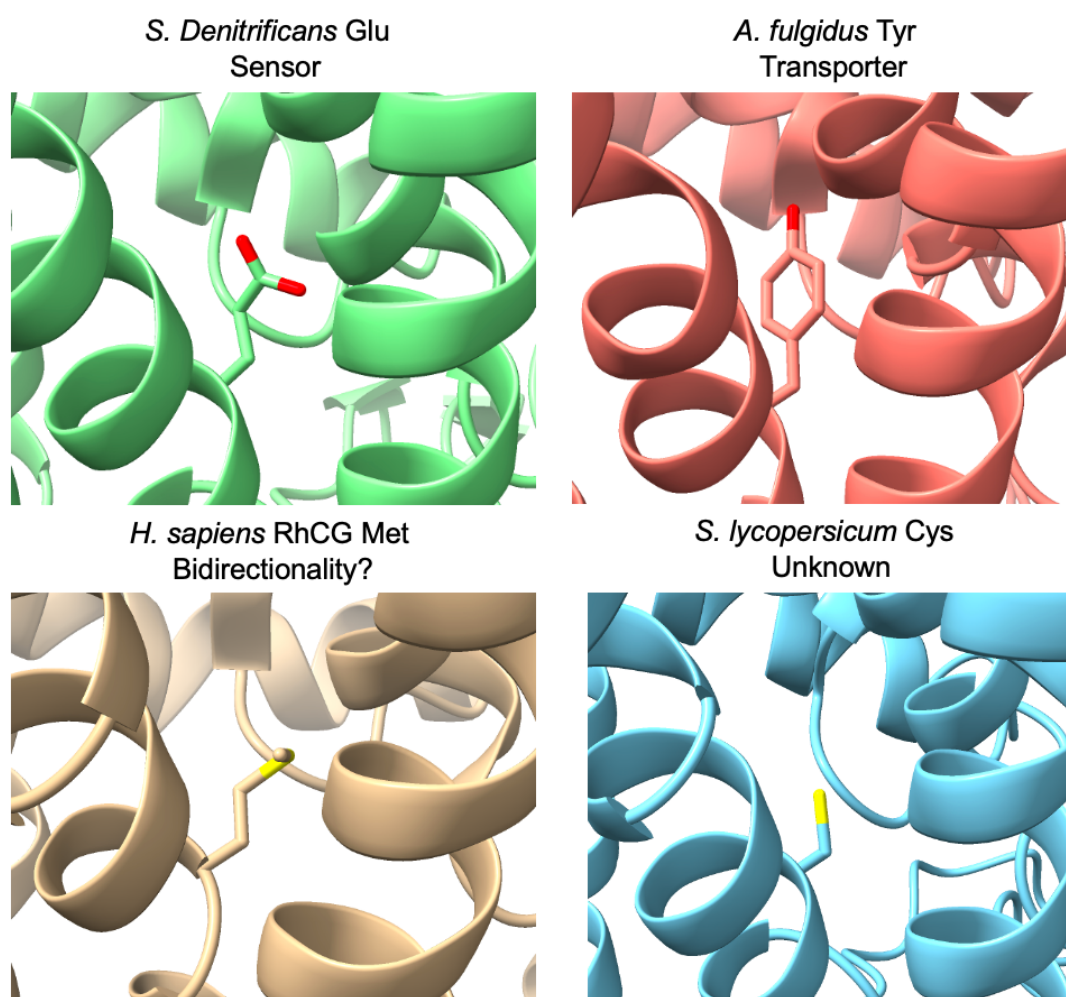


Figure 3.7 **The different residues present at the opening of the binding pocket in the pore.** Glu29 in *S. denitrificans* AmR1 (pdb 8qpf (Pflüger *et al.*, 2024)) opens access to the pocket *allowing* it to function as a sensor, Tyr27 in *A. fulgidus* Amt1 (pdb 2b2i (Andrade *et al.*, 2005)) is hypothesised to block entrance of ammonium to the binding pocket *allowing* it to be. transporter. Met78 from *H. sapiens* RhCG (pdb 3hd6 (Gruswitz *et al.*, 2010)) function is not known but could be involved in bidirectionality. Cys70 from *S. lycopersicum* (pdb AF-O04161-model_v4 (alpha fold structure)) also has an unknown function but its size does not appear to be one that would block a binding pocket if there was one present. The change to the residue highlighted here must have been accompanied by additional adaptations.

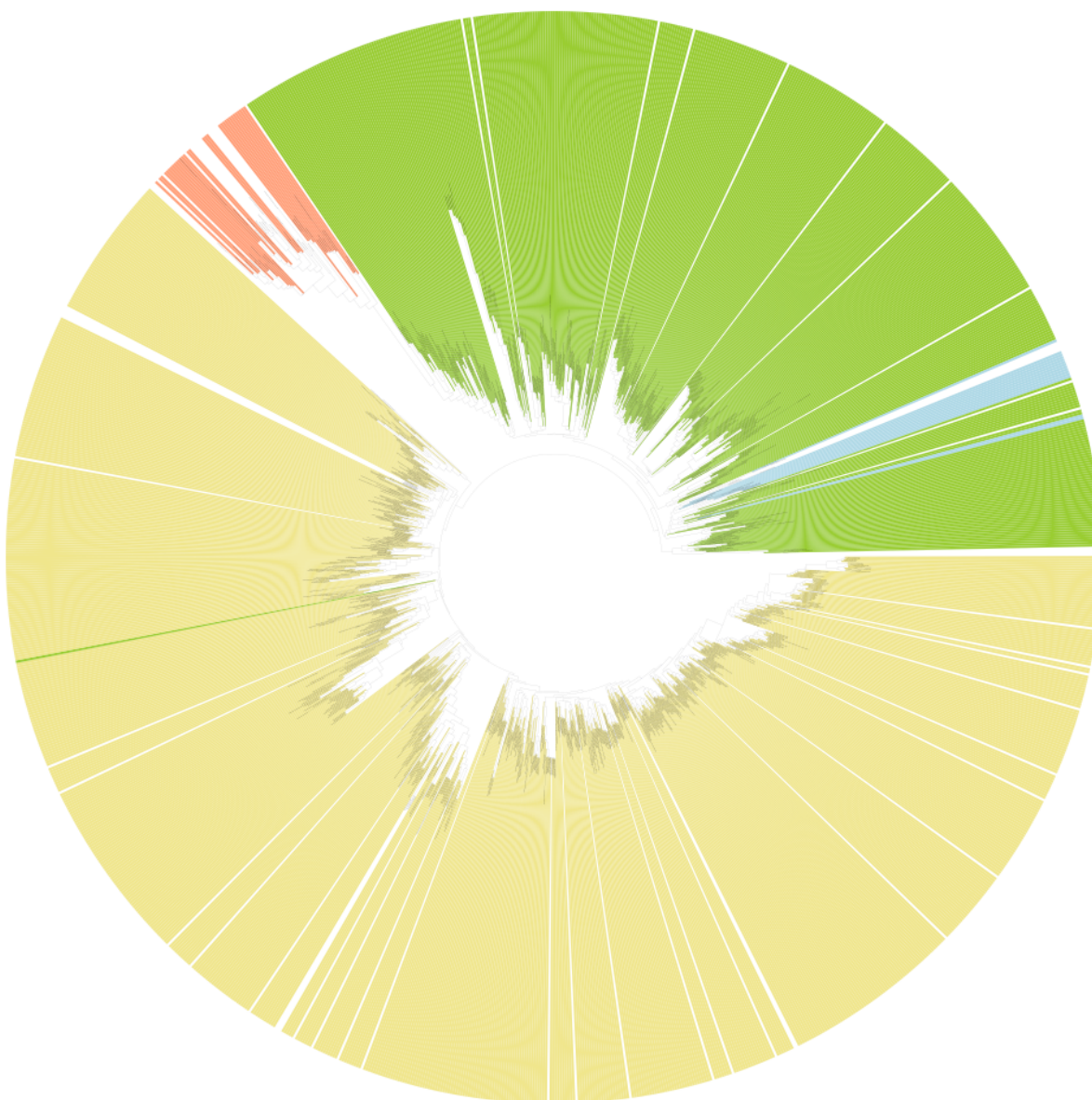


Figure 3.8 **Root of the tree placed between Glu and Tyr groups.** Ammonium transporter tree from Figure 3.6 colours by pocket residue. Glu (green), Tyr (tan), Met (red) and Cys (blue).

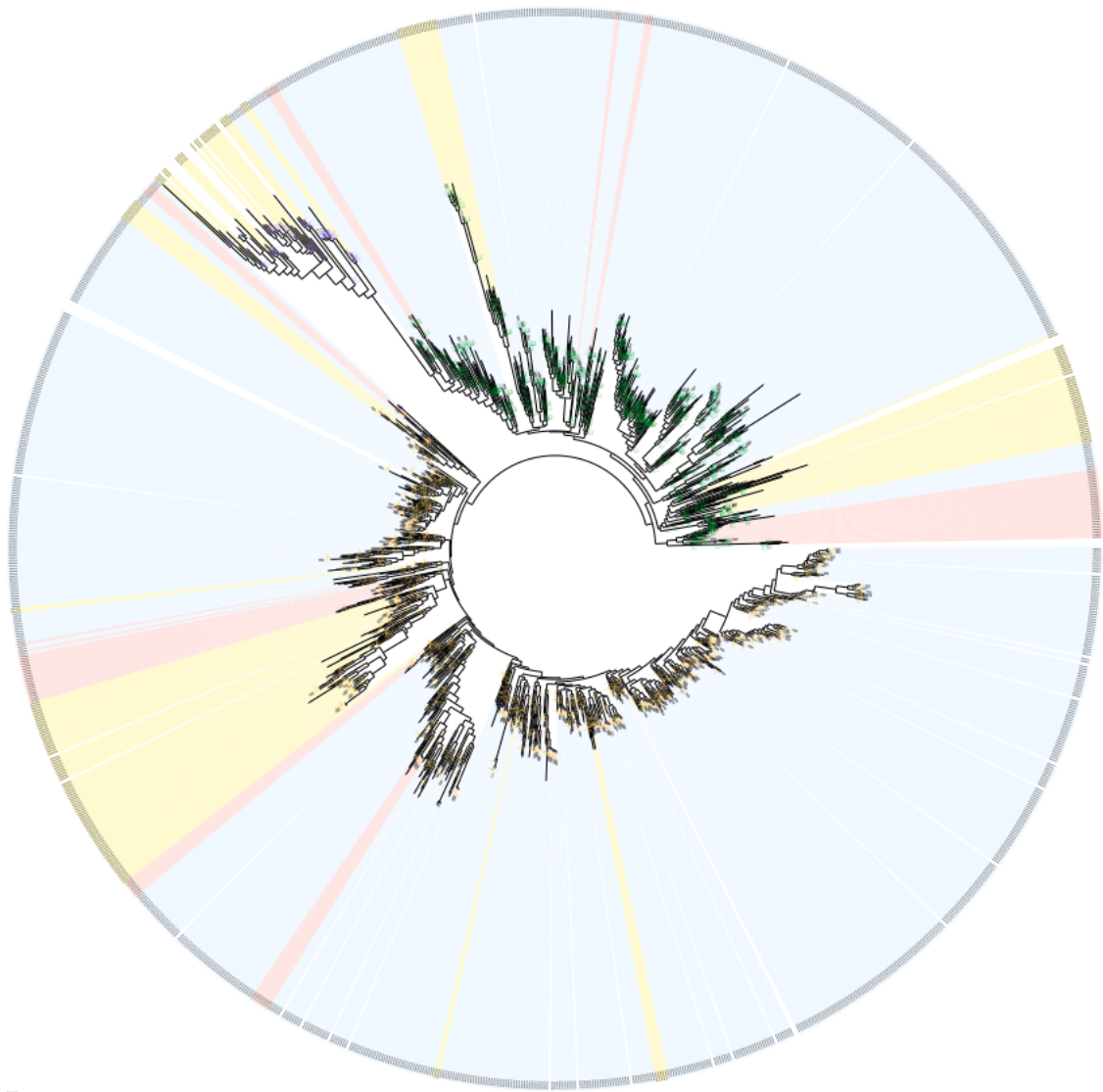


Figure 3.9 **Glu/Tyr rooted tree coloured by kingdom.** Bacteria (blue), archaea (red) and eukaryotes (yellow).

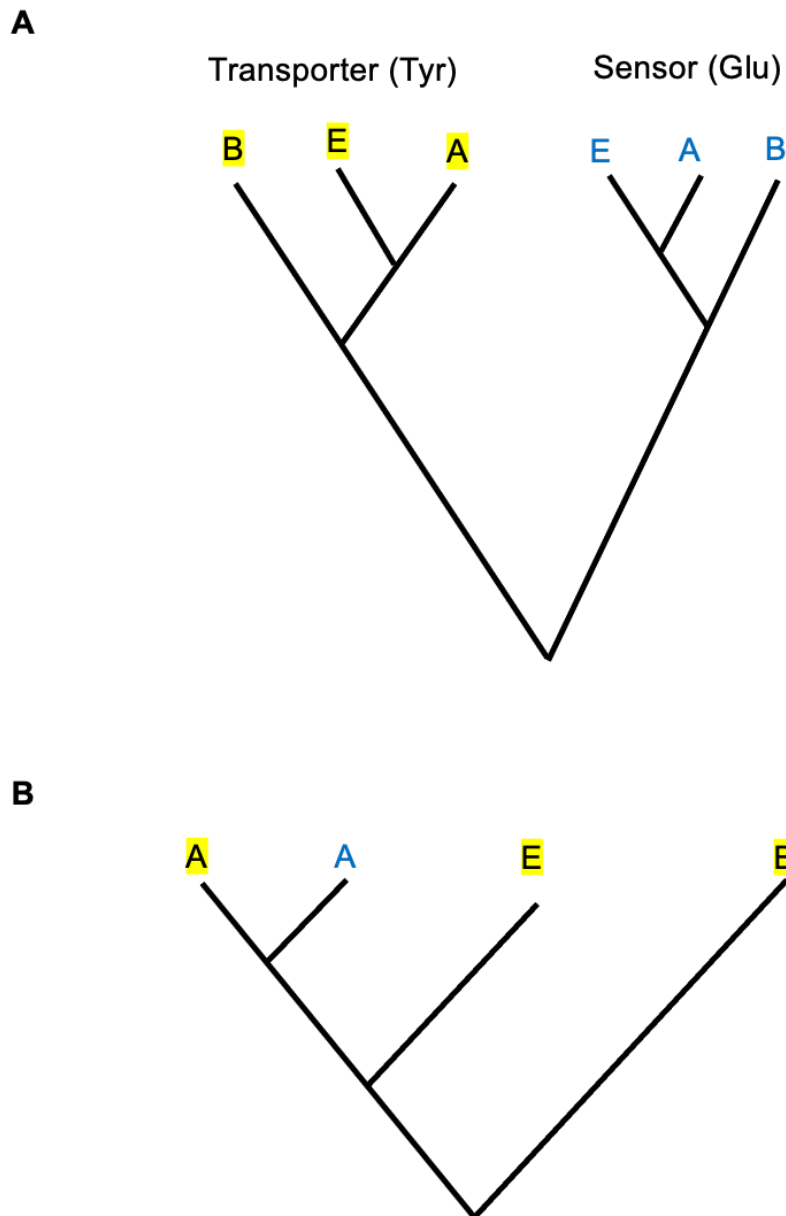


Figure 3.10 **A sketch showing the potential origins of the two protein functions.**

A) In this scenario the protein diverges into a transporter (yellow highlight) and a sensor (blue) in LUCA and the different functions are inherited vertically. We see bacterial, archaeal and eukaryotic proteins on both sides of the root. B) In this scenario, I have chosen the original protein to have been a transporter as an example. The transporter is inherited vertically by LUCA's offspring. In, for example, an archaeon the transporter evolves into a sensor meaning the common ancestor for the two functions comes from within the archaea. We see scenario A represented in our tree.

3.3.4.2 The pore exit residue

3.3.4.2.1 Residue variation at the cytoplasmic pore exit and its evolutionary implications

Another residue that differs between sensors and transporters is found at the cytoplasmic exit of the pore (Pflüger *et al.*, 2024). In the sensor SdAmR1, this residue is Asn269, which blocks the exit, whereas in the transporter AfAmt1, it is Ser251, whose smaller side chain leaves the pore open, allowing full translocation. In some proteins for example a Mep from *Cryptococcus neoformans* this residue is a Thr, the function of which is not known however the size of the side chain is more similar to Ser suggesting it allows the pore to stay open (Figure 3.11). The residues at this position in the alignment were overlayed onto the tree (Figure 3.12). The ratio of different amino acids at this position was found to be Ser 49.34%, Asn 36.46%, Thr 8.82%, Ile 1.80%, Gln 1.48% and other amino acids at less than 1%. It was found that the presence of Ser or Asn broadly follows the Tyr/Glu (transporter/sensor) split with rhesus proteins and Glu sensors appearing to conserve the “blocker” Asn and the Tyr transporters mainly having the Ser “opener”. While the split of exit residues broadly follows the pocket residues, there are some instances where Ser is found with Glu (sensor) and Asn is found with Tyr (transporter). Thr residues appear in both Glu and Tyr groups with instances in archaea, bacteria and eukaryotes. This distribution may be from vertical inheritance from LUCA, convergent evolution or HGT. In this instance I would argue that HGT or convergent evolution in the eukaryotes that have the Thr in the Glu group is more likely than vertical inheritance because of the rarity of this variant in other eukaryotes.

Gln is found only at the base of the Tyr (transporter) group across a group consisting of bacterial and eukaryotic proteins with strong support. Ile is also only found in a group of bacterial proteins in the Tyr group.

The increased variation and inconsistencies of distribution of this residue suggests it is not as critical to protein function as the pocket residue and therefore less subject to conservation. In order to explore alternative theories to the evolution of this family I will, in the following paragraphs, propose and discuss a new root based on the distribution of the pore exit residue.

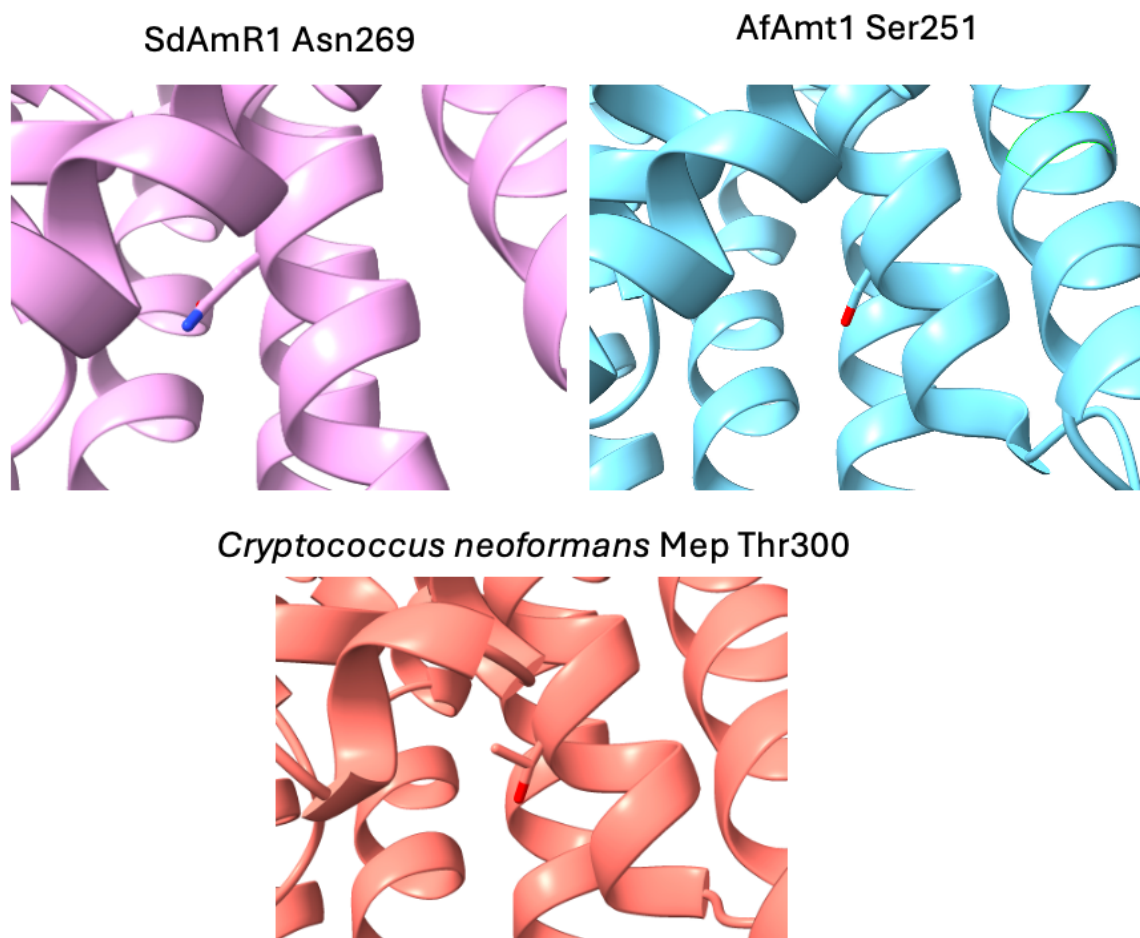


Figure 3.11 **Structural alignment showing the residue at the exit of the pore.** Asn269 in SdAmR1 (pink) (pdb 8qpf (Pflüger *et al.*, 2024)) blocks the exit to the pore meaning ammonium is not transported while Ser251 in AfAmt1 (pdb 6eu6 (Andrade *et al.*, 2005)) (blue) does not allowing transport. Thr300 in *Cryptococcus neoformans* Mep (pdb AF-A0A854QN45-F1-model_v4 (alpha fold structure)) (red) function is not known but it seems to share the lower profile of Ser meaning it may not be blocking the pore.

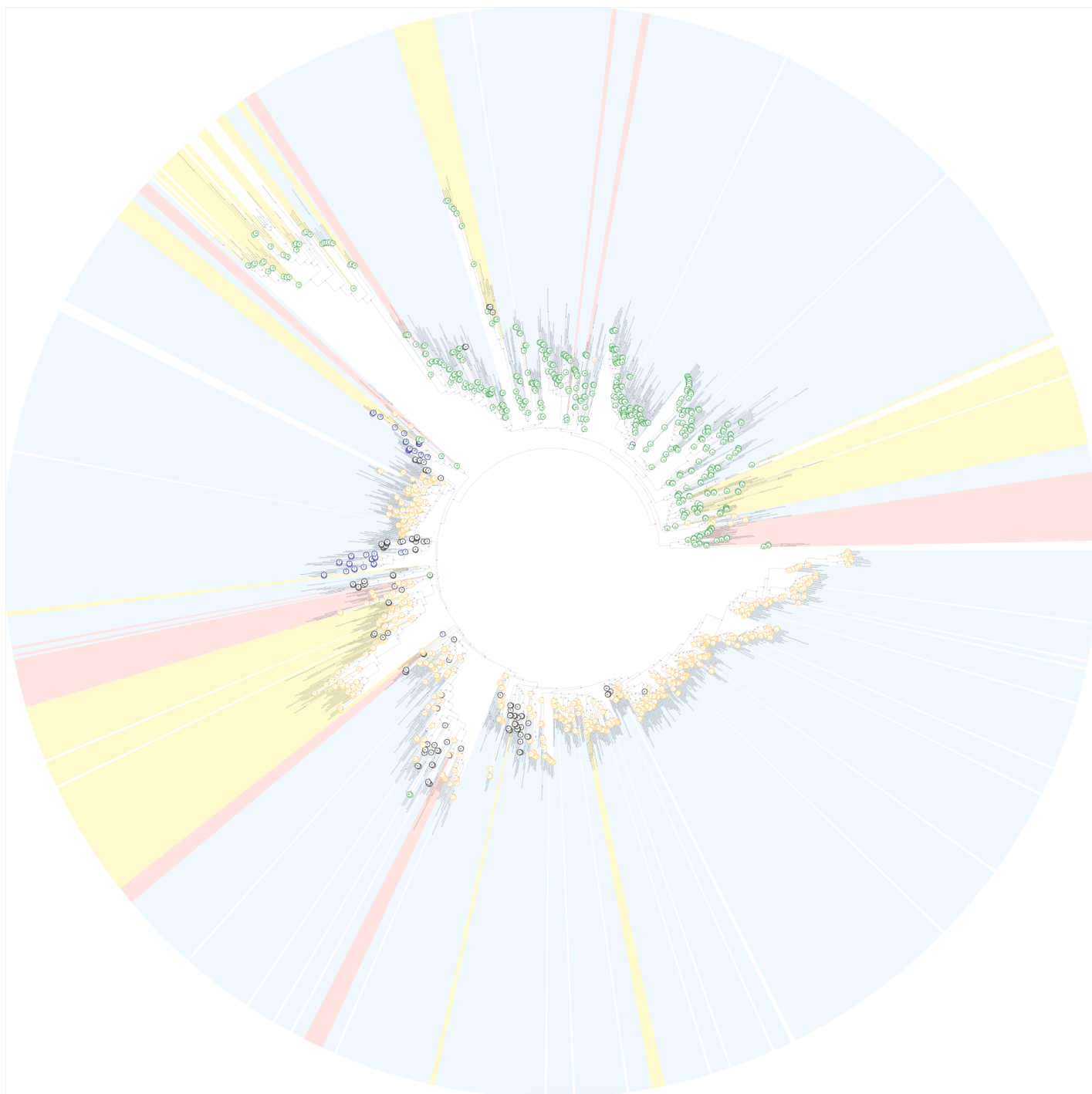


Figure 3.12 **Tree coloured by kingdom and annotated with the pocket residue.** Ser (yellow), Asn (green) Ile (blue) Gln (blue) and Thr (grey). Kingdoms are coloured by bacteria (blue), archaea (red) and eukaryotes (yellow).

3.3.4.2.2 *A different root based on pore exit residues*

To explore potential alternative evolutionary histories from the Tyr/Glu root (transporter/sensor), I propose a new root based on the split between Ser “opener” and Asn “blocker” residues at the exit of the pore. Specifically, I placed the root at the base of the largest clade of Ser-containing proteins (Figure 3.13). This node has strong bootstrap support (0.959) and excludes all Asn-containing sequences. Although it would have been possible to root the tree at the base of the Asn clade to exclude Ser proteins instead, this would have excluded a greater number of Asn sequences than were excluded from the Ser clade at the selected root. Therefore, the chosen root better represents the Ser/Asn split with minimal exclusion. Also supporting the root placement is that when clustering, the “transporter” group exclusively had a Ser/Tyr that leaves the pore open, allowing the full translocation of ammonium.

The newly rooted tree shows a potentially different origin for the super family (Figure 3.14). In this case, it suggests that LUCA may have originally possessed two transporter proteins, both featuring a Tyr residue at the entrance of the binding pocket but differing at the pore exit with either a Ser or Thr, or alternatively an Asn or Gln. The protein with the Tyr/Asn or Gln arrangement then evolved, probably still in LUCA, into the Glu/Asn sensors. Since the change between Tyr/Asn to Glu/Asn requires only one mutation (Figure 3.15A), it is more likely than the possibility that the sensors originated from a Tyr/Gln protein which requires two (Figure 3.15B). In this scenario the original protein to the superfamily would have been a transporter.

The change to the topology of kingdoms is in someways less pronounced (Figure 3.16). To the right of the root at the base of the tree in the small Tyr group (transporter) there are still representatives from each of the kingdoms of life. When we move to the base of the Glu group (sensor) this is also the case. The large group of bacteria branching to the left of the root gave rise to archaea, which subsequently gave rise to eukaryotes.

This root supports a scenario that preserves the distinction between sensors and transporters based on the presence of Glu or Tyr at the binding pocket, while offering an alternative view of the superfamily’s evolution, suggesting that the original proteins were transporters, from which sensors later evolved.

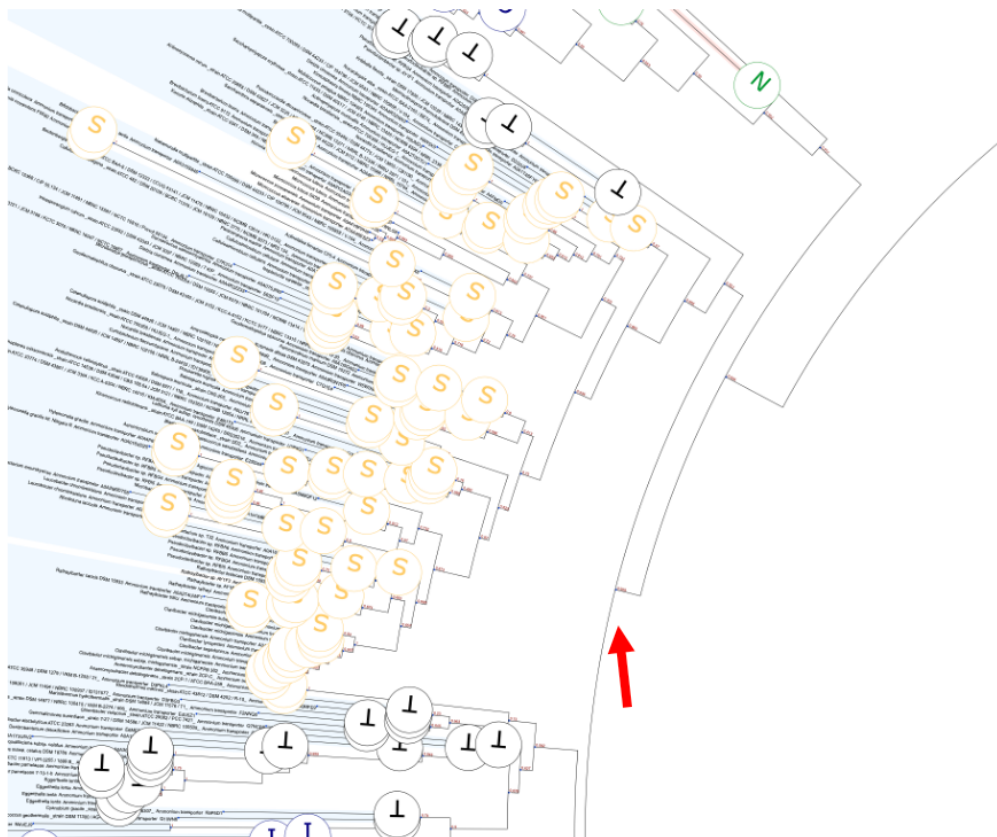


Figure 3.13 **Location of new root between different pore exit residues.** The root was chosen as it appears between the broad Ser/Asn groups.

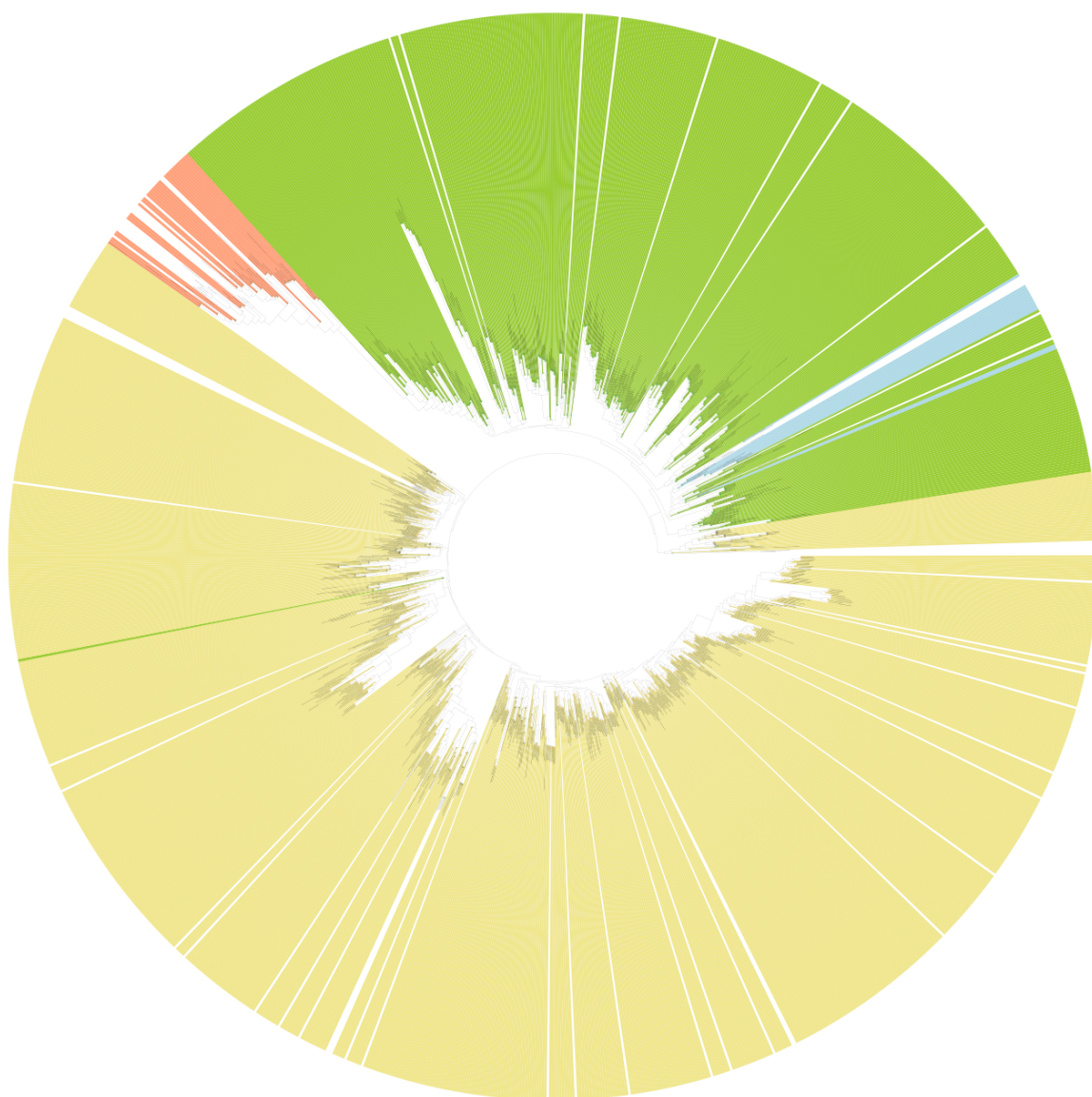
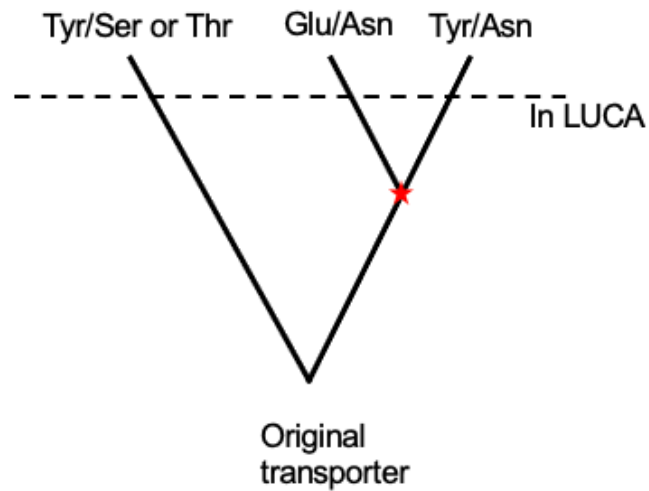


Figure 3.14 **Outcome of new root placement on the topology of the tree.** This tree is coloured by pocket access residue being Tyr (yellow), Glu (green), Cys (blue) and Met (red). The residue found at the exit of the pore is also annotated onto the tree.

A



B

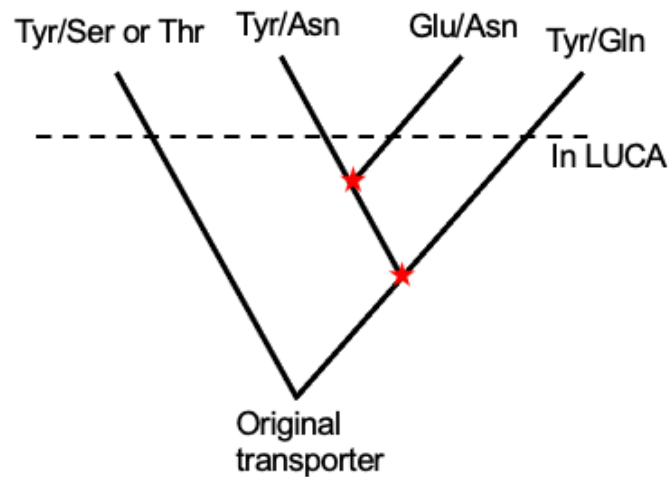


Figure 3.15 **Different scenarios for the origin of the Glu/Asn sensor proteins.**

A) If the original protein had a Tyr/Asn arrangement it only requires one change (red star) to become a Glu/Asn sensor. B) If the protein had a Tyr/Gln arrangement it would have required two changes. Therefore, the simplest explanation with the fewest changes is possibly the correct one.

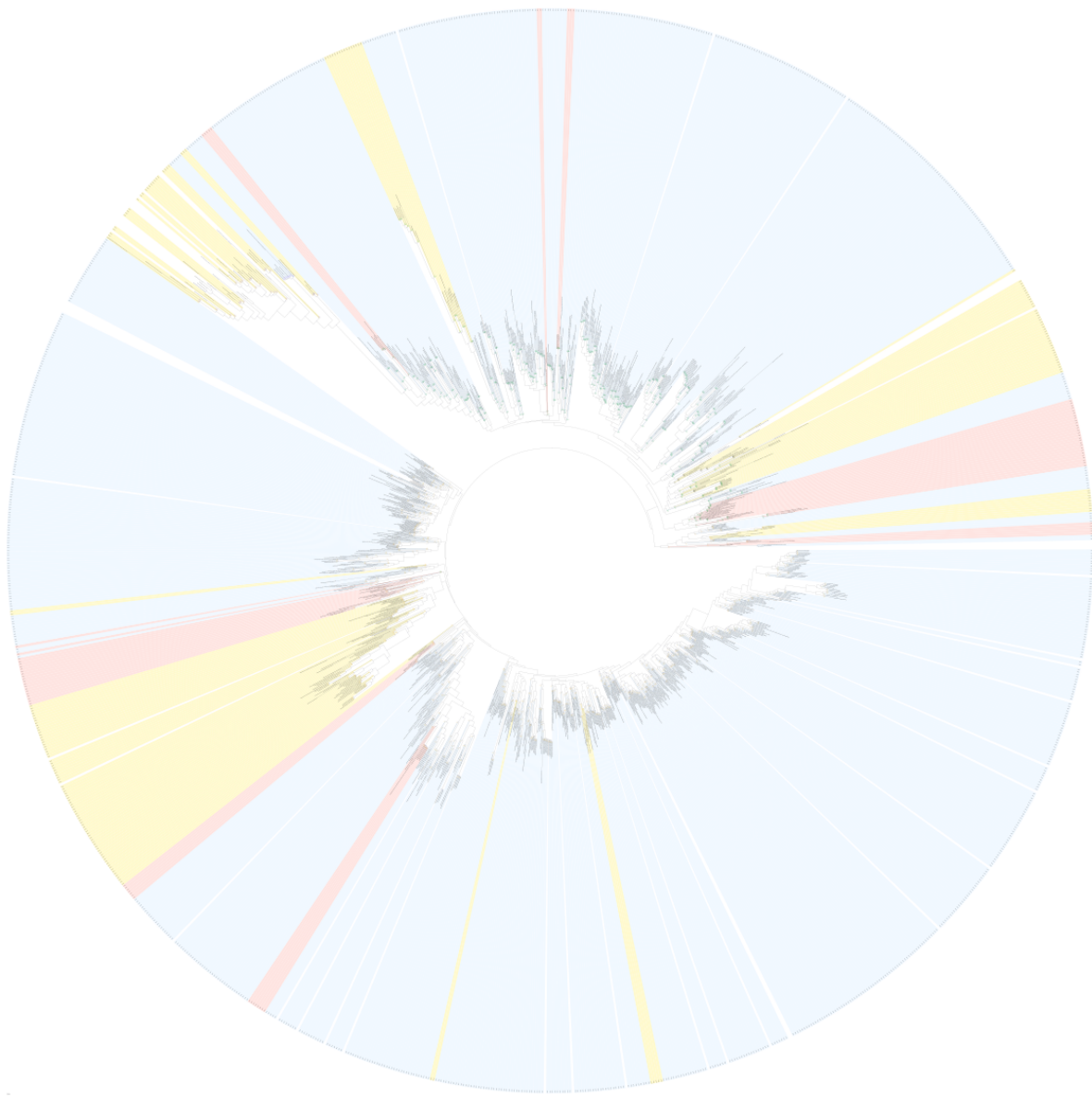


Figure 3.16 **Kingdoms annotated onto tree rooted between Ser and Asn groups.**
Bacteria (blue), archaea (red), eukaryote (yellow).

3.4 Discussion

The work presented in this chapter is a preliminary investigation into the phylogenetic history of the ammonium transporter family that can be iterated upon in future work. This was done in conjunction with investigations into the family's activity using SSME. In order to fully explore a phylogenetic reconstruction of the ammonium transporter family it would require more time than it was possible to give in this PhD. While this analysis uncovers some interesting possibilities concerning their history and grouping, there are some methodological limitations that, if addressed, could improve upon the work performed here.

My analysis shows that these ammonium transporters can be distinctly classified based on the residue found at the opening of the ammonium-binding pocket: either Glu, Tyr, Met, or Cys, or the residue found at the exit of the pore: Ser, Asn, Thr, Ile, and Gln. I hypothesise that proteins with Tyr at the pocket position are transporters, Glu indicates a sensor, and Met is characteristic of rhesus proteins. The fact that this residue was not included in the input sequences to create the tree also implies that the phylogenetic reconstruction is consistent with the divergence of these groups from a common ancestor. Interestingly similar analysis did not identify Rh proteins as having a Met in the pocket opening position but rather a Leu or Phe (Pflüger, Gschell, Zhang, Shnitsar, Zabadné, Zierp, Günther, Einsle and Andrade, 2024). Because I have structurally overlayed these residues rather than aligned sequences, I am confident that the Rh proteins do indeed have a Met.

This section will include a discussion of the tree as it is presented in this chapter and then a discussion of the methodological limitations.

3.4.1 The Asn/Ser root gives an indication of the evolution of the proteins within LUCA
Two roots have been proposed for this tree, one being between Glu and Tyr representing a sensor and a transporter and one being Asn and Ser and splitting the tree based on the pore exit residue. Both offer insights into the history of the protein superfamily.

In the scenario where the root lies between Glu and Tyr, LUCA appears to have had at least one sensor protein and one transporter. However, as noted, this arrangement is not necessarily contradicted by the second root I propose based on the residue at

the exit of the pore. The proteins were vertically inherited from this point, and both seem to have had an Asn at the pore exit since it occurs on both sides of the root. The pocket residues Glu/Tyr/Met/Cys appear stable in that the residues seem to form distinct groups and are not found scattered throughout the tree. The occurrence of the three kingdoms of life at the base of each group makes it seem like it is plausible that LUCA vertically transferred these proteins to its ancestors. The single exception to Glu/Tyr split is *Nitrososphaera viennensis* sitting in the archaeal group at the base of the fungal proteins. The support for this leaf is extremely low, however, at 0.04 so it can be assumed that this possibly does not belong here.

Rooting on the Asn/Ser residue at the exit of the pore shows that LUCA possessed two ancestral Tyr transporters one of which evolved into the Glu sensors. This supports a model in which the ancestral gene underwent duplication eventually giving rise to two distinct functional classes: an Asn/Glu sensor and a Ser/Tyr transporter. The phylogenetic placement of the root suggests that this divergence either took place in LUCA or, alternatively, that sensors emerged after the split between bacteria and archaea and were subsequently acquired through horizontal gene transfer early in the evolution of life, as homologs are found across all kingdoms. Because I am attempting to generate a model of the evolution of this protein family, rather than the evolution of life, it is plausible that this tree extends into the history of the protein's evolution in LUCA and does not begin solely at the point when LUCA diverged into bacteria and archaea. Hence, it appears that one of the original ammonium transporters featured a Tyr at the binding pocket entrance and an Asn at the pore exit, and later evolved into a sensor, with a Glu at the pocket entrance, in LUCA.

A clade of bacteria containing Ser residues at the pore exit shows moderate bootstrap support (0.612) and is positioned within the Asn-containing lineage near the root. This distribution suggests that the protein may have either been introduced into this group through HGT or that the observed mutations arose independently through convergent evolution. The low bootstrap values may also mean that this group should occur at another point in the tree.

The substitution of Ser in transporters may have been selected to increase transport efficiency. This hypothesis could be tested by measuring the activity of a AmtB variant Ser236Asn using SSME. I would predict that transport would not be abolished, but rather reduced.

The placement of the root in a phylogenetic tree is crucial for understanding evolutionary history. The two scenarios I have presented do not seem to be mutually exclusive; however, given that the groups of the pocket residue are more defined and therefore there is less ambiguity about root placement, the Glu/Tyr rooted tree will be carried forward in further research.

3.4.2 The Glu/Tyr rooted tree is more consistent with TOL than the midpoint rhesus rooted

The kingdom distribution pattern observed in the Glu/Tyr-rooted tree aligns more closely with established theories of speciation compared to the patterns seen in the midpoint or in previous studies that present a rhesus-rooted trees (Tami R McDonald, Dietrich and Lutzoni, 2012; Tami R McDonald and Ward, 2016). If the root represents the most ancient ammonium transporter, one would expect both branches emerging from it to reflect evolutionary divergence and include a mix of bacteria, archaea, and eukaryotes. However, in the midpoint or Rhesus-rooted phylogenetic tree, this expectation is not met, as the Rhesus branch is predominantly composed of eukaryotic sequences. This does not reflect speciation as there is not a Bacteria/Archaea/Eukaryote (B/A/E) grouping suggesting either the proteins are not ancient or were lost in vast numbers of organisms. In the Tyr/Glu rooted tree however at the base of the two groups there is a B/A/E arrangement. This means that potentially two or more ancient ammonium transporters/sensors were passed vertically to offspring. Annotation of the tree by phyla reveals more resolution about the taxonomy of the organisms. In some cases, for example the plant and fungal clusters the variety of phyla is low. This would suggest that these proteins either (i) arose in a certain phylum and not in others or (ii) were present in LUCA and were lost in most organisms. The distribution may also be explained by horizontal gene transfer, a theory widely accepted for this superfamily (Huang and Peng, 2005; McDonald, Dietrich and Lutzoni, 2012; McDonald and Ward, 2016; Matassi, 2017).

3.4.3 LUCA likely had multiple ammonium transporters

Assuming that each distinct grouping of bacterial and archaeal ammonium transporters represents a lineage that inherited an ancestral transporter from LUCA,

this phylogenetic tree reveals approximately seven such independent occurrences. These groupings are distributed as three clades on the Glu related side and four on the Tyr related side. This pattern suggests that LUCA may have possessed up to or at least three sensory and four transport-active proteins.

This raises the question: why would a unicellular organism such as LUCA require such a diversity of ammonium transporters? Modern unicellular organisms exhibit considerable variation in the number of Amt they possess, ranging from a single transporter in *E. coli* (AmtB) to as many as seven putative transporters and sensor in *K. stuttgartiensis* (Pflüger, Camila F Hernández, *et al.*, 2018). One plausible explanation could be adaptation to a chemically heterogeneous environment with variable ammonium availability or chemical gradients. Alternatively, it remains possible that not all of these transporter types were present in a single LUCA; instead, some may have emerged post-LUCA, evolving new functions over time and spreading across lineages through HGT. Given the extensive evolutionary timescales involved, both the diversification of function and the widespread distribution via HGT are plausible scenarios.

The two Glu and Tyr groups have members present in the three kingdom, bacteria, archaea and eukaryotes, at the base of their separation. We see another B/A/E group again in the region of the Mep group. Did LUCA then have three proteins, one sensor and two transporters, or even more? There are in total seven archaeal/bacterial pairings, each one could be examples of ancient proteins inherited from LUCA. In these groups just a bacterial and archaeal pairing, rather than bacterial/archaeal/eukaryotic group, may be enough to indicate that it is an ancient protein. It is thought that eukaryotes arose from the endosymbiosis of an alphaproteobacterium and an asgard archaeon (Roger, Muñoz-Gómez and Kamikawa, 2017). This means that other lineages of archaea and bacteria may have shared proteins that are not seen in eukaryotes (Eme *et al.*, 2017).

I propose that, originally, LUCA possessed at least one ammonium transporter, based on its widespread presence across all domains of life. However, previous studies aiming to reconstruct the genome of LUCA or other ancient genomes have not included ammonium transporters in their predictions (Sousa, Nelson-Sathi and Martin, 2016; Weiss *et al.*, 2016). Instead, it has been proposed that LUCA was capable too of nitrogen fixation, reducing atmospheric dinitrogen (N_2) to ammonia (NH_3) using the nitrogenase. If true, this would imply that LUCA may not have required an ammonium

transporter for external NH_4^+ uptake (Weiss *et al.*, 2016). The criteria for inferring the presence of a protein in the LUCA require the identification of monophyletic groupings comprising both archaeal and bacterial sequences. This approach minimises the possibility that shared sequence similarity arose through HGT after LUCA's divergence. Accordingly, ammonium transporters were excluded from LUCA reconstructions, as their phylogeny reveals multiple archaeal–bacterial pairings, resulting in an apparently polyphyletic distribution. This pattern fails to satisfy the criterion of monophyly, which the researchers define as indicative of vertical inheritance from LUCA.

This raises another question of whether proteins present in multiple paralogous copies in the LUCA were excluded from the analysis due to the stringent requirement for strict monophyly. The authors further propose that LUCA regulated nitrogen assimilation through the glutamine synthetase pathway, a mechanism that is highly conserved across prokaryotes (Merrick and Edwards, 1995). In modern organisms this pathway involves both ammonium transporters and a highly conserved family of trimeric proteins, known as PII, the most widely distributed signal transduction proteins in the bacterial world. Many prokaryotic organisms encode two PII proteins, typically designated GlnK and GlnB, with some also encoding a third, GlnJ (Arcondéguy, Jack and Merrick, 2001). Notably, it has been repeatedly reported that PII proteins play also a critical role in regulating post-translationally the nitrogenase activity, a function considered vital for nitrogen acquisition in LUCA (Huergo *et al.*, 2012). Nevertheless, and somewhat unexpectedly, neither ammonium transporters nor the PII protein were inferred to have been present in LUCA, despite their ubiquity in prokaryotic nitrogen metabolism and their essential roles in the functionality of both the glutamine synthetase and nitrogenase enzymes, which are believed to have been present in LUCA. What would a scenario look like where LUCA did not have an ammonium transporter? The most plausible explanation is that a protein present in LUCA was vertically inherited by an early bacterium or archaeon and subsequently adapted to function as ammonium transporters during evolution. This adaptation would then have been disseminated through HGT between kingdoms, ultimately becoming incorporated into a wide range of organisms. This is not seen in the tree however as there are multiple cases of archaeal and bacterial proteins sharing a common ancestor.

This scenario would be supported if archaea emerged from bacteria, with the first ammonium transporter originating in bacteria and then being vertically inherited by archaea. However, this scenario is not consistent with the hypothesis that both bacteria and archaea originated directly from LUCA. The exclusion of ammonium transporters in these studies was likely a mistake, and reasonable elements of biology are altered because of it.

3.4.4 Methodological limitations

3.4.4.1 *Sequence Type and Saturation in Deep Phylogenetic Inference*

A fundamental issue affecting my analysis is the use of nucleotide sequences for inferring relationships across ancient divergences. Phylogenetic inference works best with datasets that are only slightly saturated and owing to their reduced state space (four possible bases), nucleotide sequences saturate more rapidly than protein sequences with twenty possible amino acids (Philippe *et al.*, 2011). No tests for saturation were performed in this analysis and it is possible that it is a significant feature of this tree. When nucleotide sites undergo multiple substitutions over time, the observed sequence divergence becomes lower than the actual divergence that occurred, a phenomenon that both underestimates evolutionary distances and produces homoplasy that can mislead phylogenetic inference toward incorrect topologies through long branch attraction (Delsuc, Brinkmann and Philippe, 2005).

The prevailing consensus regarding the diversification of life posits that our LUCA gave rise to both Bacteria and Archaea domains. Subsequently, a highly rare endosymbiotic event occurred wherein an archaeal host engulfed a bacterial cell, which persisted intracellularly and eventually evolved into the progenitor of eukaryotic cells (Eme *et al.*, 2017). This theory is backed up by the large differences seen in the membrane chemistry and ribosomes of bacteria and archaea and their respective similarity to eukaryotes (Valas and Bourne, 2011; Eme *et al.*, 2017). Some scientists challenge this view however by, instead of comparing ribosomes (Hug *et al.*, 2016), including other conserved proteins into consideration, observing their distribution across the bacterial and archaeal clades and searching for the most parsimonious explanation. They claim that archaea arose from the bacterial clade either from firmicutes or gram-negative mycobacterium (Valas and Bourne, 2011). At a kingdom scale, in the tree presented in this chapter, we often see a split of bacteria and archaea before eukaryotic proteins

branch from bacteria. The origin of eukaryotic proteins being from bacteria could have occurred during the symbiotic event that gave us mitochondria where there were transfers of genetic material. This event is thought to have given eukaryotic cells the same membrane as bacterial cells rather than retaining the archaeal one (Gould, 2018). The split of archaea and bacteria, rather than archaea branching from higher in the bacterial group means that from my tree the origin of archaea still appears to be from LUCA rather than bacteria. If these instances were due to saturation or homoplasy it could be that they would not occur repeatedly in this similar pattern. This interpretation only holds if it is accepted that each bacterial/archaeal grouping is evidence of a separate protein subfamily present in LUCA. If this is not the case, then the topology seen in this tree could be due to saturation of nucleotide sites leading to long branch attraction or many cases of HGT.

The historical signal in nucleotide sequences becomes eroded over time by substitutions occurring repeatedly at the same sites, recognised as one of the primary obstacles to deep-time phylogenetic inference (Rodríguez-Ezpeleta *et al.*, 2007). In protein-coding genes, third codon positions saturate most rapidly due to the degeneracy of the genetic code allowing synonymous substitutions to accumulate without functional constraint. First and second codon positions are more conserved because changes at these positions more frequently result in amino acid replacements subject to purifying selection (Xia, 2018).

The adaptation to high temperature environments favours a GC rich genome owing to the three hydrogen bonds this gives and the improved stability of the double stranded DNA (Verma, Kumar and Satyanarayana, 2022; Steindorff *et al.*, 2024; Takemata, 2024). This means that using nucleotide sequences exposes this analysis to the potential grouping of sequences that have convergently evolved to fill a niche but are distantly related in reality. One could test for this to see if GC rich species are clustered together in this tree. Even when using sophisticated substitution models that account for among-site rate variation, nucleotide data for ancient divergences remain fundamentally problematic because the model cannot distinguish between true phylogenetic signal and noise once saturation has occurred (Philippe *et al.*, 2011; Xia, 2018).

The alternative approach, using amino acid sequences, could be a next step in this analysis and a comparison of the topologies between the nucleotide and amino acid tree could be made. Amino acids are less prone to saturation because convergent

evolution of amino acids is inherently less likely than convergence at nucleotide sites simply due to the larger character state space (Delsuc, Brinkmann and Philippe, 2005). Multiple studies have demonstrated that amino acid sequences retain phylogenetic signal over much longer evolutionary timescales than nucleotide sequences (Delsuc, Brinkmann and Philippe, 2005; Philippe *et al.*, 2011). However, the trade-off is that amino acid sequences lose information from synonymous substitutions, which are valuable for resolving recent divergences (DeFilippis and Moore, 2000). If the topology of an amino acid tree were to match the nucleotide tree presented here, then the scenarios of many distinct proteins in LUCA or that of potential HGT into difference organisms still holds. If the topologies do not match, then nucleotide sequences may not have been an appropriate choice. In this instance nucleotide sequences were chosen because the highly conserved structure of the transmembrane region of the ammonium transporters suggested that this area was under significant selection pressures potentially reducing the rate of mutation and making nucleotide use possible for this deeply branching tree.

An intermediate approach that could have improved my analysis would be to analyse only first and second codon positions, excluding the rapidly saturating third positions. This approach retains nucleotide-level information while reducing the noise contributed by saturated sites. Alternatively, codon-based models that explicitly account for the structure of the genetic code and different evolutionary rates at different codon positions could provide a better treatment of nucleotide data across deep timescales (Delsuc, Brinkmann and Philippe, 2005). The topologies of the trees calculated using these sequences could then be compared to observe if any differences have occurred.

Diverse taxa were chosen to calculate the tree in order to have a spread of more recently diverged organisms. The text based search in UniProt was done to retrieve many sequences with the lowest computational power. There are limitations to a text based search based on functional annotations on UniProt however as it is not guaranteed to find all ammonium transporters. To be sure that these proteins are included, a search through complete genomes would have been better. In conjunction to this, more sequences for the sake of more sequences will not add validity to a tree; a diverse spread of sequences and an accurate evolutionary model would add more insight than many closely related proteins (Philippe *et al.*, 2011).

3.4.4.2 Long branch attraction

A major concern in my analysis is the potential for long branch attraction (LBA), a well-documented phylogenetic artifact in which distantly related lineages that have both undergone extensive sequence evolution erroneously group together (Felsenstein, 1985b; Bergsten, 2005). LBA occurs because the limited character states in molecular sequences mean that convergent evolution is common, and two long branches separated by a short internode may accumulate more convergent changes than the few synapomorphies on the short branch linking one long branch to its true sister group (Hendy and Penny, 1989; Bergsten, 2005).

The deep phylogenetic relationships being reconstructed involve ancient divergences where saturation and homoplasy are expected to be high. For ancient protein families, even amino acid sequences can suffer from LBA when branches are sufficiently long and divergence times sufficiently ancient, and nucleotide sequences are substantially more vulnerable (Delsuc, Brinkmann and Philippe, 2005; Philippe *et al.*, 2011).

As discussed above, nucleotide saturation creates homoplasy that directly fuels LBA. In the case of my tree there is a group of eukaryotic proteins from *Guillardia theta* embedded deeply in a bacterial group with support of 0.67. This may be evidence of LBA in this tree or an example of HGT. Removing this group and recalculating the tree could be a method to check for LBA, if the topology changes a lot then this is evidence that there may have been LBA but if not it could be evidence of HGT. Recalculating this tree using amino acid sequences or just the first and second codon positions and comparing the topologies of the trees could also provide an answer (Bergsten, 2005; Widhelm *et al.*, 2019).

3.4.4.3 ML susceptibility to LBA

While maximum likelihood methods are generally more resistant to LBA than parsimony (Huelsenbeck, Bollback and Levine, 2002), they are not immune to this artifact.

Critically, ML susceptibility to LBA increases substantially under model misspecification. It is well known that if among-site rate variation (asrv) is ignored in tree reconstruction, the ML approach underestimates substitution rates, and these estimates become progressively worse with increasing evolutionary distances, making ML tree inference susceptible to long branch attraction (Susko and Roger, 2021). The

TIM1+G4 model used to calculate the final tree in this chapter is part of the GTR family. It was fitted to the data using modeltest-ng which does not have site specific models available as they are more computationally intensive (Darriba *et al.*, 2020). TIM1+G4 assumes the same substitution rate across all branches, that base frequencies do not change over time, that evolution is not directional and that each site evolves independently. This model is not the most appropriate for deeply branching trees as there may be variations in substitution rates and base frequencies may change over time for example in thermophiles. Future work could include choosing a model that accounts for site specific variation. If a tree calculated using this model were to change topology significantly it would mean that TIM1+G4 is potentially too simple to explain the alignment.

3.4.4.4 Use of TM regions

The TM regions used for the alignments to calculate this tree were retrieved from the information page on UniProt for each protein. Here UniProt displays TM regions as predicted by TMHMM. It is known that some ammonium transporters have signal peptides that are cleaved before insertion into the membrane (Khademi, O'Connell *et al.*, 2004; Lupo, Li, Durand, Tomizaki, Cherif-Zahar, Matassi, Merrick and Fritz K. Winkler, 2007) and some that have an extra helix at the beginning of the chain (Gruswitz, Chaudhary, Joseph D. Ho, *et al.*, 2010a). In order to make sure that signal sequences were not included, proteins were only included if their TM count was appropriate and the alignment was checked.

Another feature of these proteins that was not investigated was whether any of the helices arose through duplication. This is a phenomenon that has been seen in other membrane proteins (Hennerdal *et al.*, 2010; Duran and Meiler, 2013). It is potentially possible that the ammonium transporters underwent such a duplication as the pseudosymmetrical helical bundles are a feature seen in other membrane proteins that have helical duplication in their histories (Duran and Meiler, 2013).

3.4.4.5 Rooting Methods and Their Limitations

An aspect of this work was to find a justifiable root for the ammonium transporter family, as previous studies have not investigated this fully (Tami R. McDonald, Dietrich

and Lutzoni, 2012; Tami R. McDonald and Ward, 2016). The method of manually choosing a root based on observation of the tree was employed. Further work could use computational methods to calculate a root for comparison. Standard ML implementations use time-reversible substitution models (such as GTR), which are computationally efficient but cannot inherently determine root placement. Time-reversible models are incapable of identifying the root because they disregard the direction of evolution, making the phylogenetic inference problem computationally tractable but yielding unrooted trees (Huelsenbeck, Bollback and Levine, 2002; Kinene *et al.*, 2016).

The most common solution is outgroup rooting. The outgroup method is widely used but accurate root indication strongly depends on the availability of a proper outgroup (Mai, Sayyari and Mirarab, 2017). Outgroups can attach incorrectly, however, particularly to long branched ingroup taxa, creating artefactual roots that simultaneously alter ingroup topology (Wheeler, 1990; Holland, Penny and Hendy, 2003). An outgroup has not been proposed for the ammonium transporter family so this was not considered for this work but a future line of enquiry may be to identify one and use this to calculate a tree to compare the root placement.

Alternative rooting methods exist but have their own limitations. Midpoint rooting assumes a molecular clock meaning substitution rates are homogeneous along evolutionary time and that phylogenetic trees follow the strict evolutionary clock model (Mai *et al.*, 2017). This premise is disrupted by variation of evolutionary rates, which has been reported both between and within lineages, negatively impacting midpoint rooting performance (Mai *et al.*, 2017). This variation in evolutionary rate has been observed in the Amt/Mep/Rh family where it was found that the Rh30 antigens likely underwent rapid evolutionary changes following their split from the Rh transporters (Matassi *et al.*, 1999; Matassi, 2017). Fitting a different model to the Rh protein group could produce an improved tree, especially given the group's longer branch. If the topology was found to be the same then it could be argued that the method used here was appropriate. Given that when midpoint rooting was used in this chapter the root was placed between Rh proteins and the rest of the sequences, mid-point rooting is arguably not a reliable method in this instance.

Non-reversible models offer a promising alternative that can estimate root placement within the ML framework. Non-reversible Markov models can be used to compute the likelihood of potential root positions because, when using a non-reversible model, the

direction of time affects the likelihood of the tree (Huelsenbeck, Bollback and Levine, 2002; Squartini and Arndt, 2008). Using this property, the most likely location on the tree for the root can be found, so long as the model has an appropriate fit. Where no reliable outgroups can be found, or where there is reason to question the position of a root inferred using an outgroup, non-reversible models can provide a useful additional line of evidence for the position of the root (Squartini and Arndt, 2008). However, these models are computationally intensive and require substantially more parameters to be estimated than reversible models, making them challenging to apply to large datasets (Squartini and Arndt, 2008). Furthermore, non-reversible models still assume that the substitution process is stationary across the tree, an assumption that may be violated if lineages have experienced shifts in compositional bias or other evolutionary pressures. Nevertheless, this approach could be valuable for future analyses of the Amt/Mep/Rh family.

3.4.4.6 Future work

In order to test the findings of this chapter the tree must be recalculated using protein sequences. This will establish whether homoplasy/saturation/GC bias has had a meaningful effect on the topology of this tree. There could also be reanalysis using nucleotide sequences of just the first and second codons positions to compare to the three-codon position tree and the protein sequence tree. To improve the variety of proteins present and also reduce the chances of failing to include some paralogues present in an organism, ammonium transporters could be searched for and extracted from whole genomes.

An additional line of enquiry would be to refine the large, broad search that has been done here to use as a guide to identify a limited number of high-quality genomes across a reasonably diverse set of clades and repeat the analysis to try to optimise model choice and methodology. This would also establish whether homoplasy is an issue truly affecting this tree.

Chapter 4: Phylogenetic investigations into conserved features of the ammonium transporters

4 Chapter 4: Phylogenetic investigations into conserved features of the ammonium transporters

4.1 Introduction

Proteins of the ammonium transporter (Amt/Mep/Rh) superfamily participate in diverse physiological functions, all supported by a conserved transmembrane helix architecture. In the previous chapter, I proposed that a single residue at the entrance of the ammonium-binding pocket serves as a key marker for distinguishing three functional specialisations: proteins with a glutamate (Glu) at this position act as sensors, those with a tyrosine (Tyr) function as transporters, and those with a methionine (Met) operate as bidirectional Rhesus proteins. This Glu/Tyr-based distinction formed the basis of my proposed model for the evolutionary origin of these functions.

4.1.1 Is there a common mechanism underlying the different physiological roles?

Some organisms possess multiple ammonium transporter genes; for example, the poplar tree, *Populus trichocarpa*, has fourteen *amt* genes (Couturier *et al.*, 2007). Maintaining this genetic diversity points to functional specialisation, with different transporters likely contributing to distinct physiological roles, ranging from ammonium uptake in the roots to its distribution and localisation in the leaves. Organisms with more tissue types are likely to have a greater variety of ammonium transporters variants owing to the differing requirements. Many other organisms also code for multiple ammonium transporters, sometimes from different subfamilies like *Kuenenia stuttgartiensis* which possess one rhesus and four *amt* proteins despite being a prokaryote (Bizior *et al.*, 2023; Williamson *et al.*, 2024). From a functional standpoint, these observations raise the question of whether a universal transport mechanism may underlie the diverse biological roles exhibited by this protein superfamily across different organisms.

In this context, various methods have been employed to determine if the transport mechanism of a single transporter is electroneutral or electrogenic. Yeast and oocyte complementation assays have shown that plant Amt2 proteins operate in an electroneutral manner (Neuhäuser, Dynowski and Ludewig, 2009) and a phylogenetic study aimed at a mechanistic classification of the family was conducted under the

assumption that only certain plant AMTs were electrogenic (McDonald and Ward, 2016). It was recognised at the time that the interpretation of the phylogenetic tree might need to be revised if electrogenic activity were discovered in additional members beyond those plant proteins. In fact, one of the most reliable methods currently available for distinguishing between electroneutral and electrogenic transport mechanisms is solid-supported membrane electrophysiology (SSME), which utilises artificial liposomes containing the reconstituted, purified protein. Notably, all proteins investigated to date using this approach have been identified either as electrogenic transporters (Wacker *et al.*, 2014; Mirandela *et al.*, 2019; Williamson *et al.*, 2020) or non-transporting sensors (Pflüger *et al.*, 2018; Pflüger *et al.*, 2024). These transport mechanisms share a fundamental characteristic: the binding of the substrate NH_4^+ rather than NH_3 . Taken together, these findings indicate that members of this transporter superfamily may differ in their fundamental transport mechanisms, electroneutral versus electrogenic, despite preserving all structural features essential for their activity, including a substrate binding site separated from the entrance of a highly hydrophobic pore by the Phe-gate, into which the twin-His motif protrudes (See paragraph below). This conservation raises an important question: If we can relate functional differences to the presence or absence of specific residues, these can be mapped onto the Glu/Tyr tree from Chapter 3 Figure 3.9 and this can be used to determine if some functions arose once or multiple times. Understanding these distinctions could provide critical insights into the evolution and functional plasticity of this transporter family.

4.1.2 Evolution and functional characterisation of conserved essential structural features.

The ammonium transporters share conserved features like the Phe Gate (Javelle, 2008) and the Twin His motif (Javelle *et al.*, 2006). The EcAmtB Twin His motif has been found to be crucial for stabilising water wires that conduct the proton after NH_4^+ deprotonation therefore allowing the protein to conduct a charge through its hydrophobic pore, ensuring ammonium-specific transport (Williamson *et al.*, 2020) (section 1.4.2). Variations in the region have been found to be rare and as far as we know confined only to the fungal Mep1 and Mep3 proteins which have a Glu/His instead of the His/His arrangement. When the His-to-Glu mutation is introduced into

EcAmtB, it leads to increased transport activity but reduced substrate selectivity, as the mutation allows the pore to become water-filled and conduct hydrated K⁺ ions (Williamson *et al.*, 2020).

Another conserved structural feature that appears to be important in the translocation mechanism is the Phe-Gate. The precise function of this “gate” remains unclear but mutational analyses have shown that deletion of the second Phe residue leads to a loss of transporter activity (Javelle *et al.*, 2008).

Because these features appear to be important mechanistically, I will therefore analyse their distribution through the superfamily.

4.1.3 Evolution of ammonium transporters with extra domains

Proteins often consist of multiple domains with distinct functions, enhancing their efficiency in complex processes. For example, the histidine kinase in a two-component system contains several specialized domains that enable it to sense environmental signals, undergo autophosphorylation, and transfer the phosphate to a response regulator, ensuring an effective and coordinated cellular response (Podgornaia and Laub, 2013). To date, two proteins within the Amt superfamily that are fused to additional enzymatic domains have been functionally characterised: KsAmt5, which is linked to a histidine kinase domain, and SdAmr1, which is fused to a diguanylate cyclase domain (Pflüger *et al.*, 2018; Pflüger *et al.*, 2024). These proteins have been found to be sensors that do not translocate ammonium. The origin of these fused proteins remains unclear. It is uncertain whether the enzymatic domains were acquired independently in response to specific functional needs within different organisms, or if a single fusion event occurred for each domain type, giving rise to all related fused proteins through subsequent diversification.

4.2 Aims

The aim of this chapter is to extend our phylogenetic analysis and investigate the distribution of features of the Amt/Mep/Rh superfamily important for their mechanism and function. This will be done using the Glu/Tyr rooted tree from the previous chapter (Figure 3.9). By examining the distribution of these features, including those established as essential for AmtB and NeRh50 activity, we seek to gain deeper insights

into their functional relevance and evolutionary origins. This approach, combined with the transport assay developed in Chapter 5, will open new avenues for mechanistic studies, advancing our understanding of the translocation mechanisms within this protein superfamily involved in diverse physiological functions.

My specific objectives for this chapter are as follows:

- To create a tree annotated with any extra fused domains present in the proteins.
- To build a tree of ammonium transporters fused to an extra domain.
- To Investigate the distribution of conserved features that have been shown to be critical for the activity of the ammonium transporters.
- Evaluate the origins of subfamilies based on the evidence from the tree.

4.3 Results

4.3.1 Proteins with extra domains

4.3.1.1 *Evolutionary origins of domain-fused ammonium transporters*

In our phylogenetic analysis, the UniProt name used for annotation is not ammonium transporter for several proteins but instead diguanylate cyclase, adenylyate cyclase, or PII nitrogen regulatory protein (Figure 4.1) (methods section 2.2.3.1). This is due to the presence of additional functional domains fused to their C-terminal cytoplasmic regions, downstream of the conserved core translocation domain. This observation raises a key evolutionary question: do these fused proteins originate from a single ancestral fusion event, or did the additional domains arise independently in different lineages as adaptations to specific functional needs?

A potential clue lies in the clustering patterns of these proteins within the phylogenetic tree. A tree was rendered annotated with the presence or absence of a fused domain according to the protein's InterPro page. In this selection of proteins, those with a fused domain were found exclusively in the Glu group and belonged to prokaryotes with one exception (Figure 4.2). This exception is a eukaryotic ammonium transporter from *Chlamydomonas reinhardtii* (accession A0A2K3DQF8), which contains an NPL4 domain, a component involved in endoplasmic reticulum-associated degradation (ERAD), a cellular pathway that removes misfolded proteins from the ER and targets them for proteasomal degradation. According to the Pfam database, as of 16th April 2025, this protein is the only known example with this specific domain architecture, but as it is only a predicted protein it may be genome misannotation. Hence, at first glance,

the restricted presence of fused domains within the Glu-sensor group might suggest a common evolutionary origin. However, deeper analysis of the phylogenetic distribution challenges this hypothesis.

Three plausible evolutionary scenarios must be considered to explain the emergence of these fused proteins:

1- Single ancestral fusion event followed by vertical inheritance: In this scenario, a single fusion event occurred in an ancestral lineage, and the fused gene was subsequently inherited by descendant taxa. If this were the case, we would expect the fused proteins to form a clade that includes representatives from diverse bacterial and archaeal groups.

2- Multiple independent fusion events: Alternatively, the same domain may have been acquired independently in different lineages through separate fusion events. This would result in multiple, taxonomically restricted clades in the phylogenetic tree, each representing an independent origin of the fused form.

3- Mixed scenario – both inheritance and independent acquisition: A combination of the above, where some lineages inherited the fused gene from a common ancestor while others acquired it independently. This would produce a phylogenetic tree with both shared clades and isolated groups across different domains of life.

To evaluate these scenarios, I examined the phyla of the organism these proteins were from. If scenario (1) were correct I would expect to see a clustering of proteins with the same extra domain, all from different phyla, depending how ancient the fusion event was, suggesting that there was a common ancestor from which these organisms inherited the protein. This is indeed what I observed for one group of proteins fused to a diguanylate cyclase domain (Figure 4.1). Interestingly these proteins are also fused to an array of other domains as well as the diguanylate cyclase. It could have been the case that multiple domains were acquired at the same time in a 'pack' or that over time these were fused individually. These include degenerate phosphodiesterase (EAL) and Per-ARNT-Sim (PAS) domains. The combination and order of these domains differ between these proteins so that none are the same suggesting individual acquisition. This is interesting given that these proteins still cluster together on the tree. It may be that, while originating from a common ancestor, recombination has shuffled the order of domains for each individual protein.

In contrast the PII domain fused proteins reflect scenario (2) better. While there are two clusters, one in bacteria and one in archaea, the taxonomic diversity is low. In

Archaea, they are confined to the phyla Methanobacteriota and class *Halobacteria*, and in Bacteria, solely to the phyla Bacillota and class *Clostridia*. This limited representation implies that the single-fusion hypothesis is less likely, as it would require widespread independent loss of the fused gene across all other archaeal and bacterial classes. A more parsimonious and taxonomically consistent explanation is that two independent fusion events occurred, one in *Halobacteria* and one in *Clostridia*. These events likely reflect convergent evolution, where similar selective pressures drove the fusion of functional domains in distinct lineages. It is also possible that there was indeed a single fusion event in *Halobacteria* or *Clostridia* and that this protein was passed through HGT.

Two Amts fused to histidine kinases also cluster next to each other and although some proteins carry the name on UniProt of adenylate cyclase, they are fused to an adenylate and guanylate cyclase domain.

This means that overall, it is likely that scenario (3) is correct and there was a mix of events leading to the distribution of proteins seen in the tree.

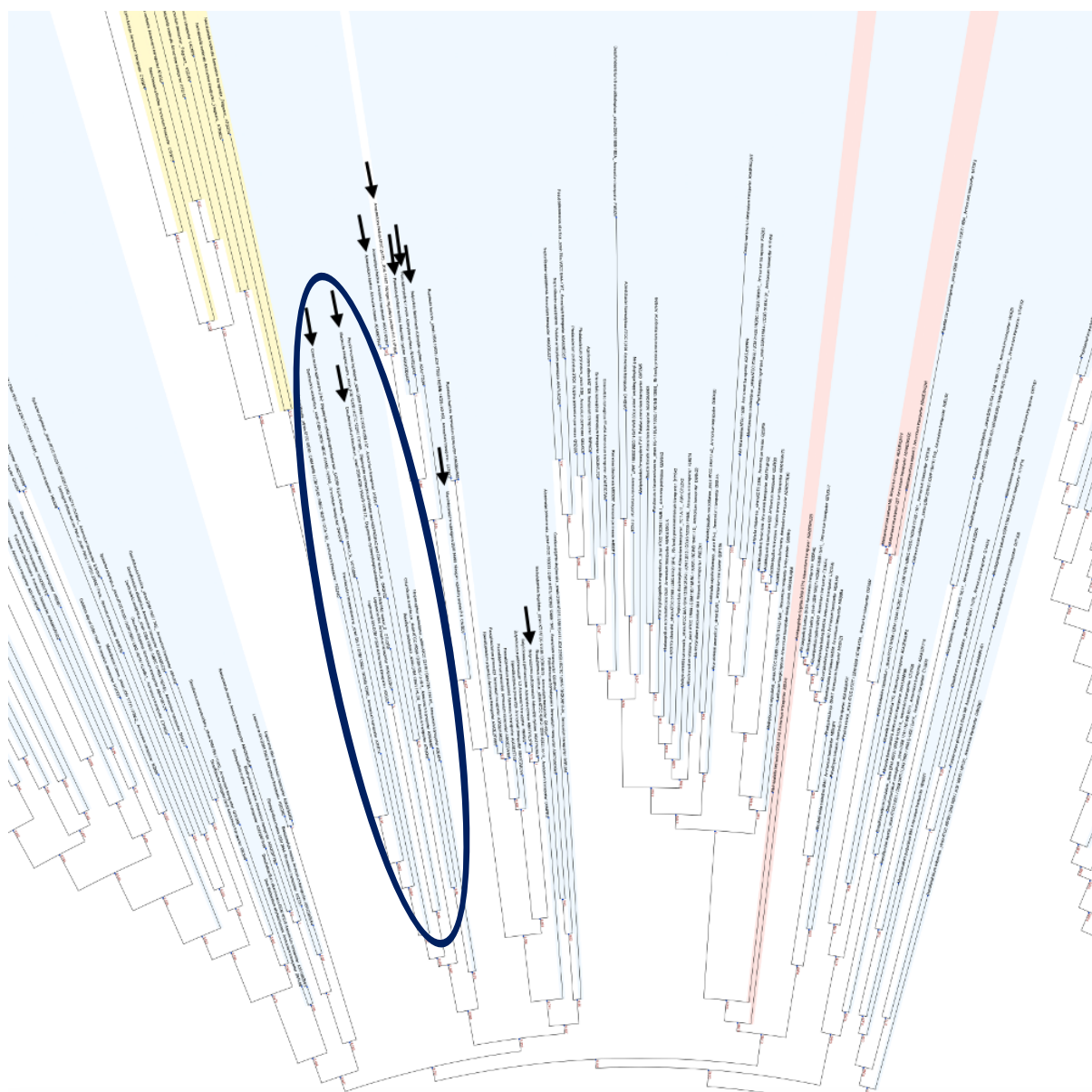


Figure 4.1 **Location of proteins not carrying the name ‘ammonium transporter’ on UniProt which was used to annotate the leaves.** The proteins in this group indicated by the arrows are ammonium transporters fused to an extra enzymatic domain which gives the protein its name. A small cluster of proteins with a fused DCG domain is indicated by the dark blue oval.

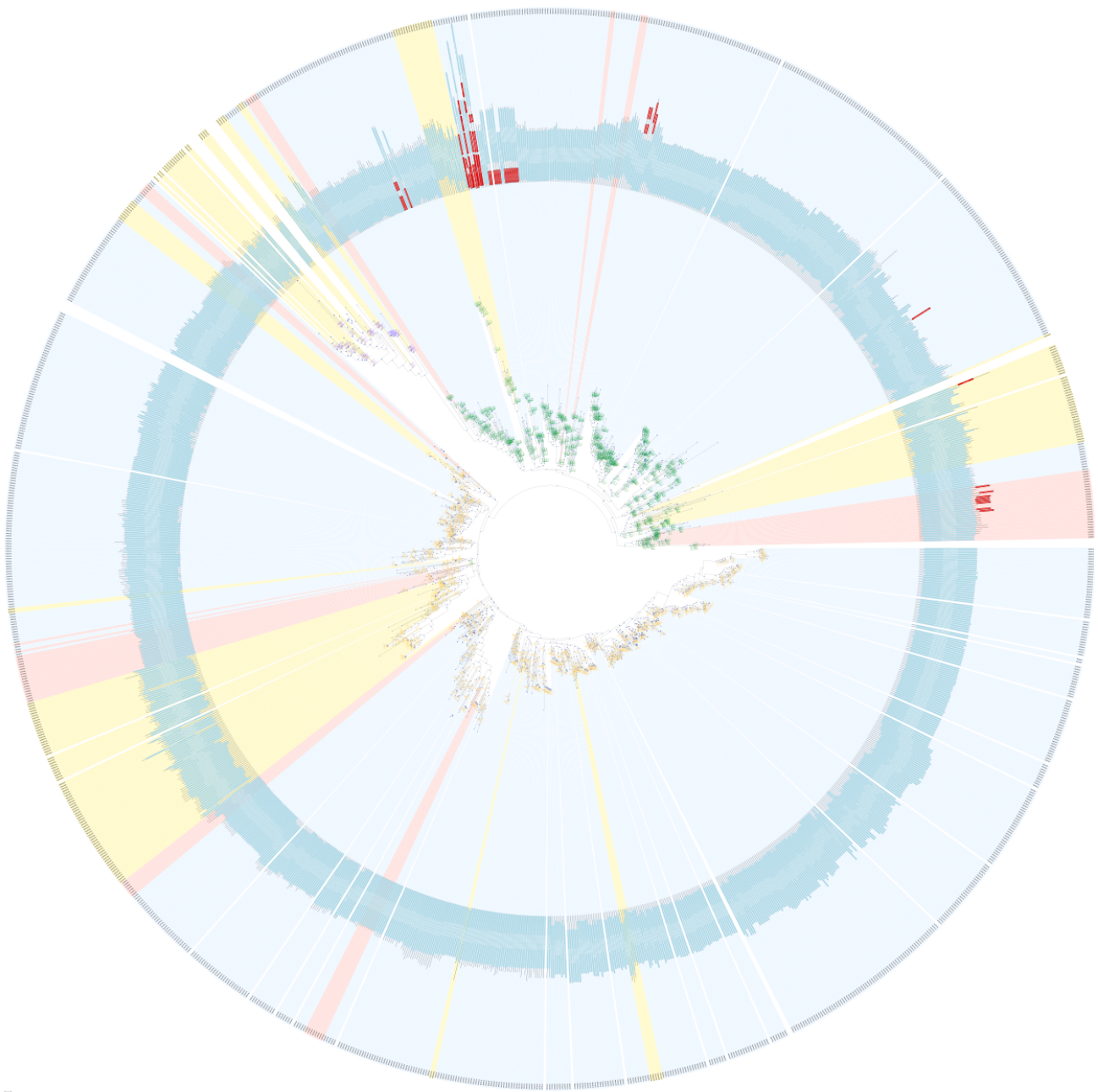


Figure 4.2 **Glu/Tyr rooted tree with addition annotation of proteins with extra domains.** The ammonium transporting domain (blue) can be seen connected to the extra domains (red). The kingdoms are coloured bacteria (blue), archaea (red) and eukaryotes (yellow). The pocket residue is annotated onto the tree in the circles, Tyr (yellow), Glu (green) and Met (pink)

4.3.1.2 Evolution of Amt-PII domain fusions suggests modular adaptation in Amts

4.3.1.2.1 Phylogenetic analysis of Amt and PII domain fusions

To explore the evolutionary relationship between the Amt domain and the domain to which it is fused, two phylogenetic trees were constructed: one based on the transmembrane (TM) regions of the Amt domain, and the other on the full sequence of the fused partner domain. Following a fusion event, the evolutionary trajectory of the resulting chimeric protein is expected to mirror the speciation pattern of the host organisms. Consequently, if a single fusion event occurred, the phylogenies derived from each domain should be broadly congruent. Conversely, substantial discrepancies between the two trees would suggest multiple independent fusion events or subsequent domain shuffling, thus providing evidence against a single ancestral fusion. Based on the previous tree (Figure 4.2) I hypothesise that the Amt-PII fused group appeared to have derived from more than one fusion event and because of the fact that they are the largest group of Amts fused to an extra domain, according to interpro (04/03/25), it was decided that these proteins should be investigated further in an attempt to prove or disprove this hypothesis. Depending on the ammonium concentration, PII proteins (e.g., GlnK and GlnB in *E. coli*) regulate Amt transporters in most prokaryotes and some plants by: (i) directly binding and physically block the pore of AmtB, it has not yet been studied where this mechanism differs if the PII domain is fused to the ammonium transporter, and (ii) modulating the transcription of genes encoding AmtB and glutamine synthetase (GS) through two-component regulatory systems. PII proteins represent one of the most widely distributed families of signal transduction proteins across diverse organisms (Arcondéguy, Jack and Merrick, 2001; Huergo *et al.*, 2012). The relationship of ammonium transporters to their signal transducer proteins GlnK has been hypothesised to be more ancient than the divergence of archaea and bacteria (Thomas, Coutts and Merrick, 2000). The PII protein GlnK is ubiquitous in prokaryotes for the regulation of Amts and forms an operon with the *amt* gene (Thomas, Coutts and Merrick, 2000; Javelle *et al.*, 2004). The PF00909 (ammonium transporter)-PF00543 (PII) family on interpro had 1,400 members (04/03/2025), in these proteins the Amt and GlnK domains are fused. Sequences were extracted from UniProt and there was a reduction in proteins from 1400 to 600 proteins as many did not have eleven or twelve predicted TM regions and

therefore failed the filtering step. The two trees created looked superficially similar (Figure 4.3).

The unrooted amt and PII trees were compared using Robinson-Foulds (RF) distance in ete3 (Robinson and Foulds, 1980; Huerta-Cepas, Serra and Bork, 2016) (2.2.4). This gave an RF Distance of 836 and a Max RF of 1216 meaning 69% of the possible splits in the trees are different to each other. This suggests that while the trees differ significantly, they are not entirely dissimilar to one another. The bootstrap values in the Amt tree were high for the split between bacteria and archaea with some poorer values on more basal nodes. The bootstrap values for the PII tree were generally high at basal nodes especially at the split between archaea and bacteria. The low RF values may be caused by internal reorganisation of leaves higher up in the topology and this is an issue of resolving deep branches in large trees. To the eye, the unrooted trees look similar, archaeal and bacterial protein cluster distinctly. The two distinct groups do not confirm or deny the idea that the fusion of an Amt and PII domain may have happened independently in archaea and bacteria. To investigate this question further I looked deeper at the taxonomy.

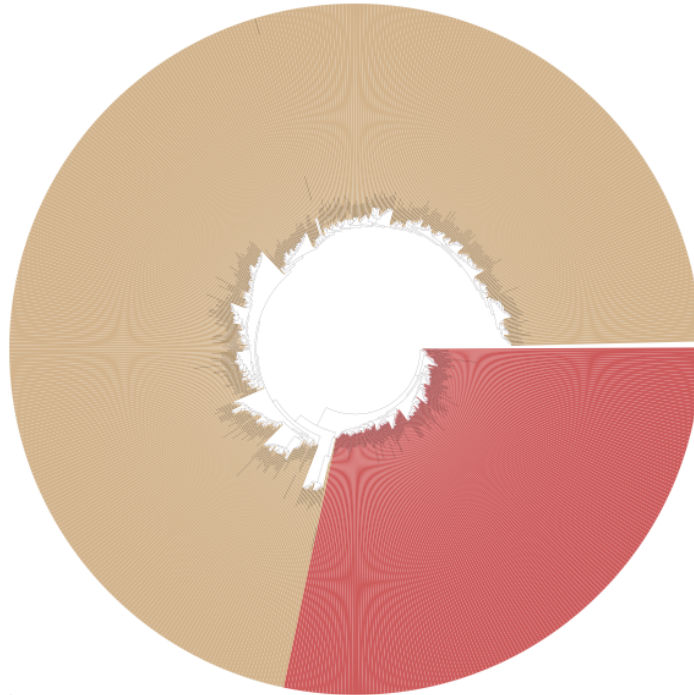
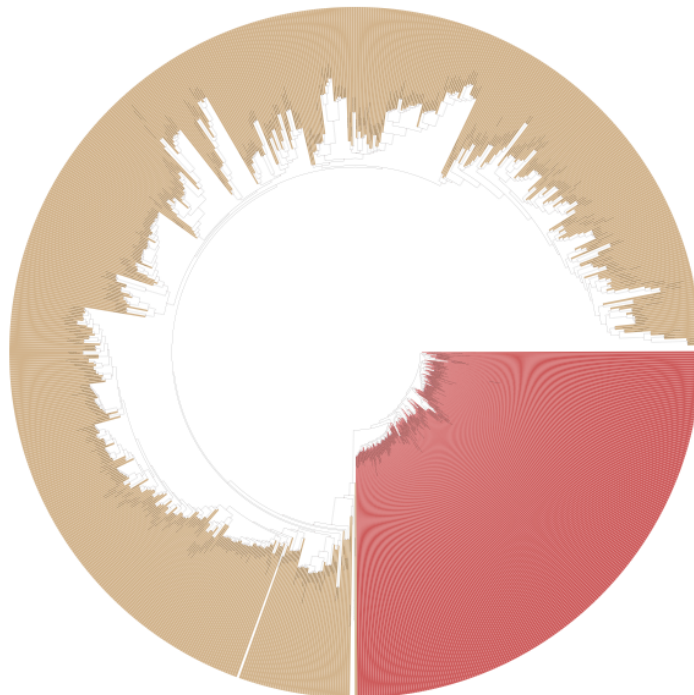
A**B**

Figure 4.3 **Comparison trees between the ammonium domains and PII domains of transporters that are fused.** Ammonium transporters fused to a PII domain were investigated. A) The nucleotide sequences for the TM regions of the ammonium transporter domain were used to calculate a tree us ML. B) The nucleotide sequences of the PII domain were used to calculate a second tree. The trees are unrooted and ladderised and coloured to show bacterial (tan) and archaeal (red) sequences.

4.3.1.2.2 Independent origins of Amt-PII Fusion Proteins in Archaea and Bacteria?

To investigate the evolution of these two groups, the trees were coloured by phyla (Figure 4.4). This has the benefit of visualising if the organisation of groups reflects speciation. The phyla present are Actinomycetota (bacteria), Bacillota (bacteria), Elusimicrobiota (bacteria), Spirochaetota (bacteria) and Methanobacteriota (archaea). The reduction in sequence numbers as described in methods section 2.2.4.3 may have led to a decrease in diversity. The two phyla with greatest numbers of proteins are bacillota and methanobacteriota.

The clear separation between archaeal and bacterial proteins observed in the phylogenetic tree is consistent with known patterns of speciation. This pattern suggests that there has not been extensive HGT events between these two domains of life, at least with respect to the genes in question. If the fusion had originated in LUCA, I would have expected to observe a broader distribution of taxa that either retained the fusion or exhibit signs of its loss because otherwise the retention in only two main phyla means that many other organisms would have had to lose the protein. However, the apparent absence of such taxa makes this scenario less likely though not impossible. The fusions provide evidence for the modular nature of ammonium transport proteins, as they appear capable of acquiring additional domains while maintaining their conserved structural core.

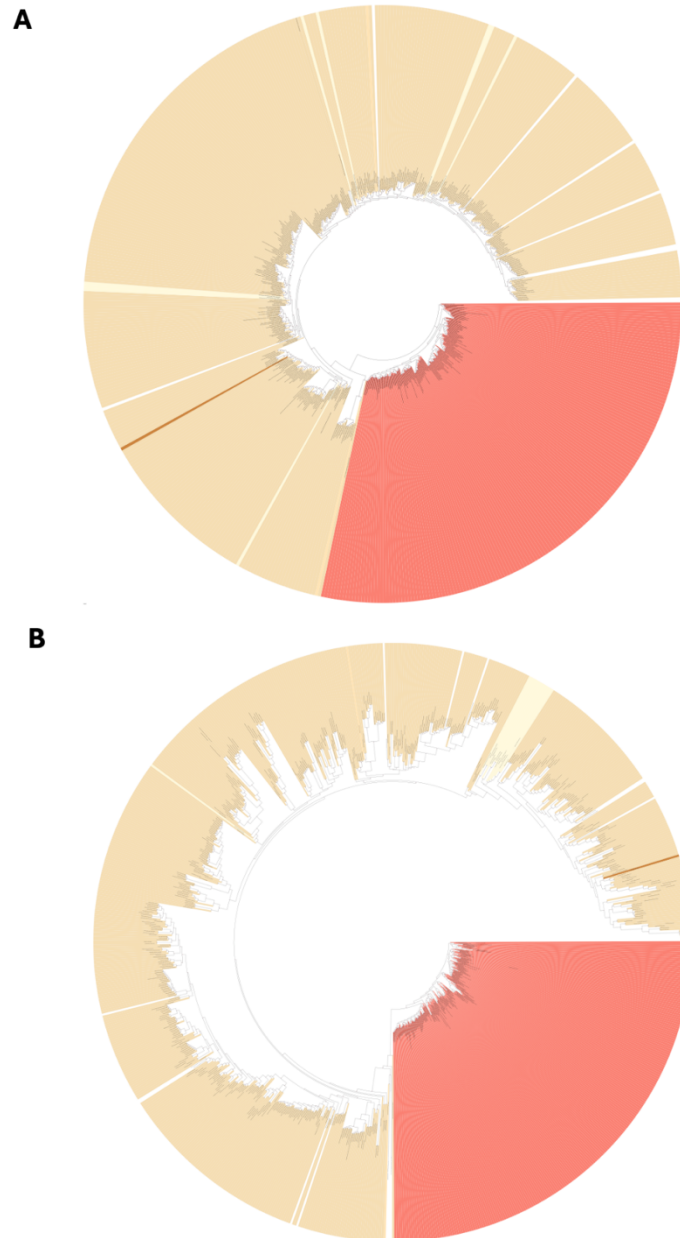


Figure 4.4 **Comparison trees between the ammonium domains and PII domains of transporters that are fused.** Ammonium transporters fused to a PII domain were investigated. A) The nucleotide sequences for the TM regions of the ammonium transporter domain were used to calculate a tree using ML. B) The nucleotide sequences of the PII domain were used to calculate a second tree. The trees are unrooted, and ladderised and coloured by phyla two phyla, methanobacteriota (red) and bacillota (light brown). Other phyla present are actinomycetota (orange) and spirochaetota (light brown).

4.3.2 Known ammonium transporter functions

To investigate whether distinct functional patterns correspond to specific clusters within the phylogenetic tree, I incorporated proteins with experimentally validated functions. These proteins were given a broad classification depending on the outcome of the publication behind their analysis, specifically, sensors (do not transport or expression influences cell activity), detoxifiers (bidirectional import/exporters), and transporters (import only), as described in our recent review concerning the evolution and diversification of ammonium transporters (Williamson *et al.*, 2024) (see 2.2.2.8 in the *Methods section* for which proteins made it into the tree). By mapping these well-characterised proteins onto the phylogenetic tree, my aim was to examine whether proteins with similar biological roles cluster together, potentially revealing shared evolutionary origins or evidence of functional convergence within the broader protein family. Out of the ninety-six functionally annotated proteins initially considered, only nineteen met the inclusion criteria and were retained in the final tree (Figure 4.5, Table 2.12).

This analysis revealed a general pattern of correspondence between functional categories and conserved residues, with two notable exceptions. In most cases, Met-containing proteins were associated with detoxification, Glu-containing proteins with sensing functions, and Tyr-containing proteins with transport.

The first exception involves the presence of sensor-annotated proteins in the Tyr group, specifically the fungal Mep2 proteins. These are recognised as ammonium *transceptors*, i.e. proteins that combine transport and signalling functions, rather than sensors in the strict physiological sense (Michael C Lorenz and Heitman, 1998). In Mep2, signal transduction is thought to be triggered by a specific ammonium transport mechanism distinct from that of Mep1 and Mep3, rather than by activation of a cytoplasmic domain following substrate binding and a transduction cascade involving cytoplasmic proteins (Brito *et al.*, 2020; Williamson *et al.*, 2022). This suggests that Mep2 evolved from a transporter into a transducer-like protein, rather than having originated as a sensor.

The second exception is found in the Glu group, which includes the *Aedes aegypti* protein AeAmt1, annotated as a detoxifier (Chasiotis *et al.*, 2016; Durant *et al.*, 2017; Durant and Donini, 2018, 2019, 2020). In their 2020 study, Durant and Donini used RNA interference (RNAi) to show that AeAmt1-knockdown males exhibited reduced

fertility, with fewer eggs laid and lower egg viability. Based on these results, the authors proposed that AeAmt1 protects spermatozoa from ammonia toxicity. However, no direct functional studies have yet demonstrated how AeAmt1 performs this role, making its designation as a detoxifier provisional and subject to further validation.

In conclusion, the clustering of proteins by function and conserved residues supports the hypothesis that evolutionary constraints shape these protein families in ways that reflect their roles. While most cases align with this pattern, the exceptions highlight the complexity of functional evolution and underscore the need for careful interpretation of protein annotations.

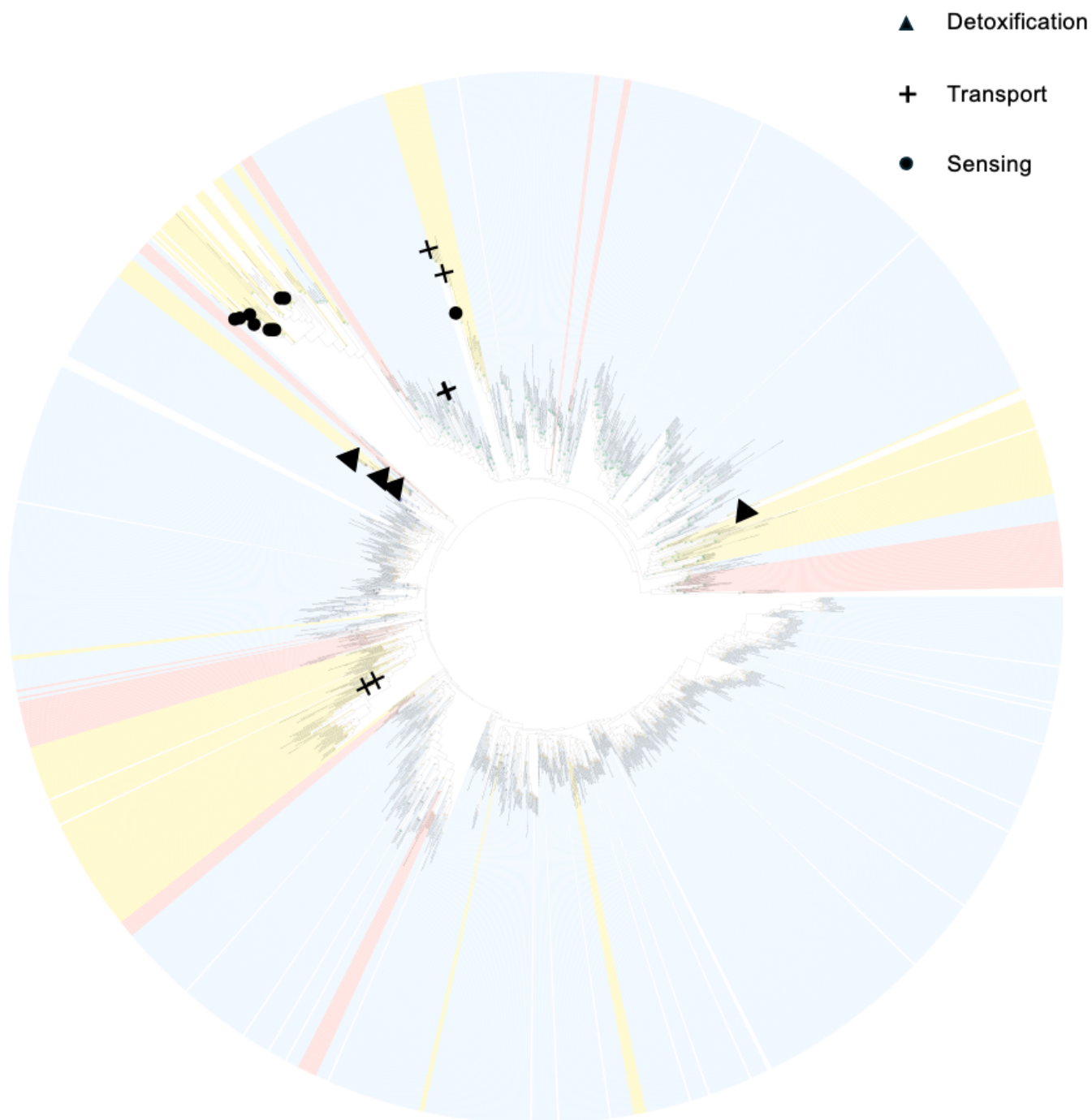


Figure 4.5 **Tree annotated with the pocket residue and the function.** The residue that blocks or opens the pore being Glu (green), Cys (grey), Met (purple) and Tyr (yellow) are shown along with the function of the protein if known. The tree is also coloured by kingdom bacteria (blue), archaea (red) and eukaryotes (yellow)

4.3.3 Conserved features

4.3.3.1 *Twin-His motif*

The Twin-Histidine (HH) motif is a conserved structural feature that has been shown to be critical for the function of ammonium transporters in bacteria and plants (Bizior *et al.*, 2023). This motif consists of two His residues positioned within the translocation pathway and is present in nearly all characterised members of the Amt/Mep/Rh family. Notably, an exception to this conservation exists in fungal transporters, Mep1 and Mep3, in which the first His is substituted with a Glu, resulting in an EH motif. In contrast, fungal Mep2-type transporters maintain the canonical HH motif (Marini *et al.*, 1997; Boeckstaens, André and Marini, 2008). These structural differences correspond to distinct functional characteristics. Mep1 and Mep3 are associated with higher rates of ammonium transport but exhibit lower substrate affinity. By contrast, Mep2 displays higher affinity for ammonium and is optimally active under acidic conditions (pH 4), whereas Mep1 and Mep3 function best at a more neutral pH (pH 6) (Marini *et al.*, 1997; Boeckstaens, André and Marini, 2008). Importantly, Mep2 serves a dual function in fungi, acting not only as an ammonium transporter but also as a transceptor, a protein that combines transport and signalling functions. Mep2 is specifically essential for inducing pseudohyphal growth under nitrogen-limiting conditions, a dimorphic transition that promotes nutrient foraging and plays a role in the virulence of certain fungal species, like the human pathogen *Candida albicans*. In contrast, although Mep1 and Mep3 are fully functional in ammonium transport, they do not trigger pseudohyphal differentiation (Lorenz and Heitman, 1998; Boeckstaens, André and Marini, 2008). Substitution of the conserved HH to EH motif in Mep2-type from *S. cerevisiae* and *C. albicans* has critical functional consequences. This alteration abolishes the protein's ability to mediate the transition from yeast growth to pseudohyphal development in response to nitrogen starvation, underscoring the critical role of the Twin-His motif in fungal pathogenicity (Williamson *et al.*, 2020). To explore the evolutionary origins of this variation, the amino acid residues at the twin-histidine positions were mapped across our broad phylogenetic tree of Amt/Mep/Rh family members (Figure 4.6). Intriguingly, in addition to the known EH-containing Mep1 and Mep3 proteins, several bacterial and archaeal sequences were also found to possess the EH motif, a previously unreported observation. This raises important questions about their inheritance. One possibility is that fungi inherited a single EH-type ammonium

transporter gene from archaea, which subsequently underwent two gene duplication events, giving rise to Mep1, Mep2, and Mep3. In this scenario, Mep1 and Mep3 retained the EH motif, while Mep2 reverted to the ancestral HH motif. Alternatively, fungi may have inherited two or three distinct ammonium transporter genes from archaea, some carrying the EH motif and others the HH motif. To investigate these possibilities, the tree was coloured according to fungal taxonomic phyla (Figure 4.7) and compared to the established phylogeny of fungal evolution, particularly the divergence between Ascomycota and Basidiomycota (Figure 4.8; Spatafora *et al.*, 2017). The results revealed that fungal ammonium transporters consistently group into three distinct and well-supported clades of repeating taxa. These clades correspond to three divergent types of ammonium transporters, Mep1/Mep2/Mep3, found across both Ascomycota and Basidiomycota, the two major fungal phyla. This indicates that diversification into these three transporter types occurred early in fungal evolution, before the divergence of these lineages. Generating a subtree of the Mep proteins with a suitable outgroup would possibly be further work to show this evolution better. The presence of EH domains in both archaea and fungi challenges the long-standing notion that the EH motif is a fungal-specific innovation and highlights an important area for further evolutionary and functional investigation. It is not clear if the EH domain was acquired by the fungi from archaea or if it appeared in the fungi and archaea independently.

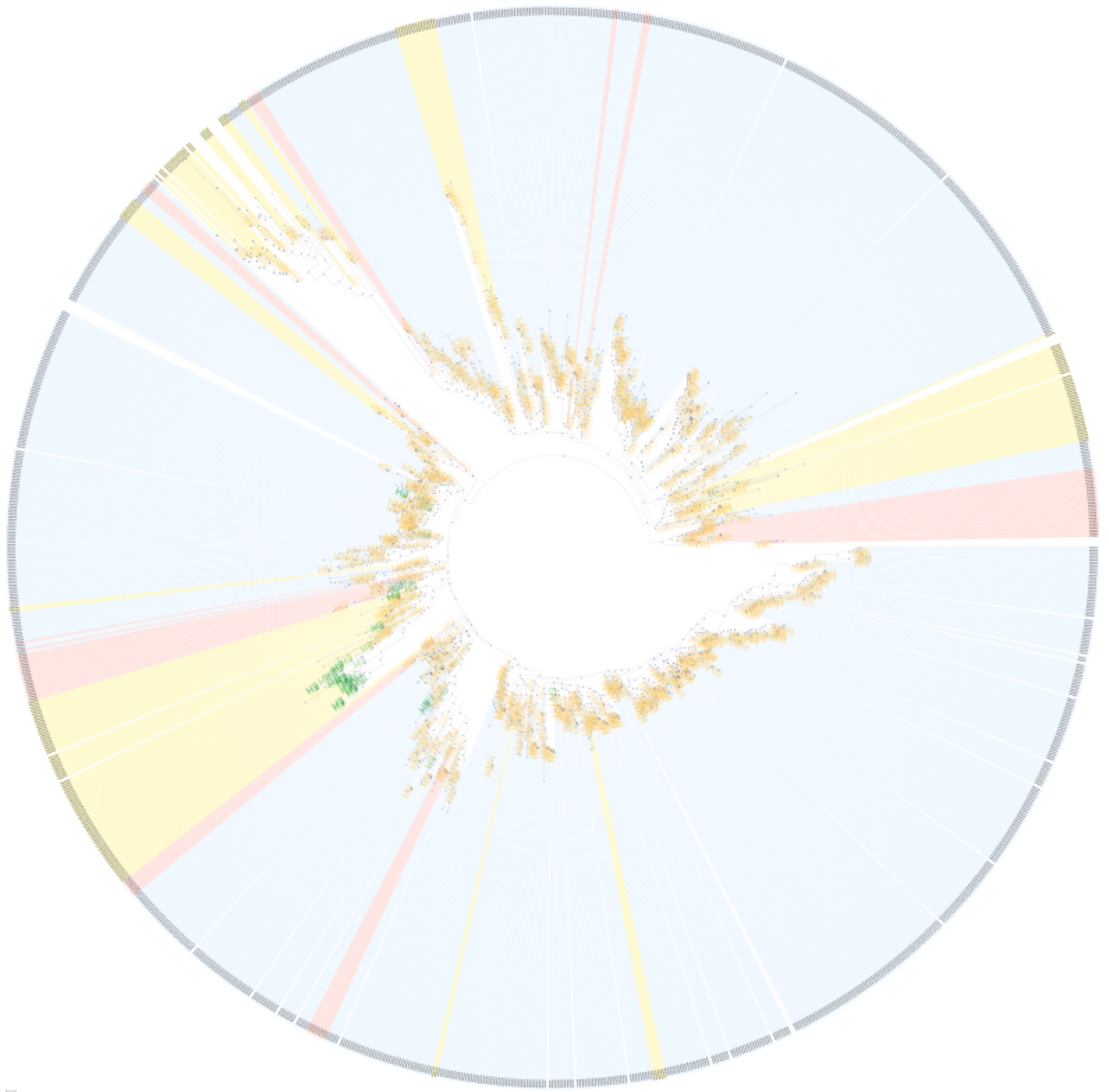


Figure 4.6 **Twin-His motif annotated onto tree.** Proteins with the typical HH motif have a yellow annotation while proteins with the EH motif seen in Mep1 and Mep3 proteins have a green annotation. The tree is also coloured by kingdom with Bacteria (blue), Archaea (red) and Eukarya (yellow).

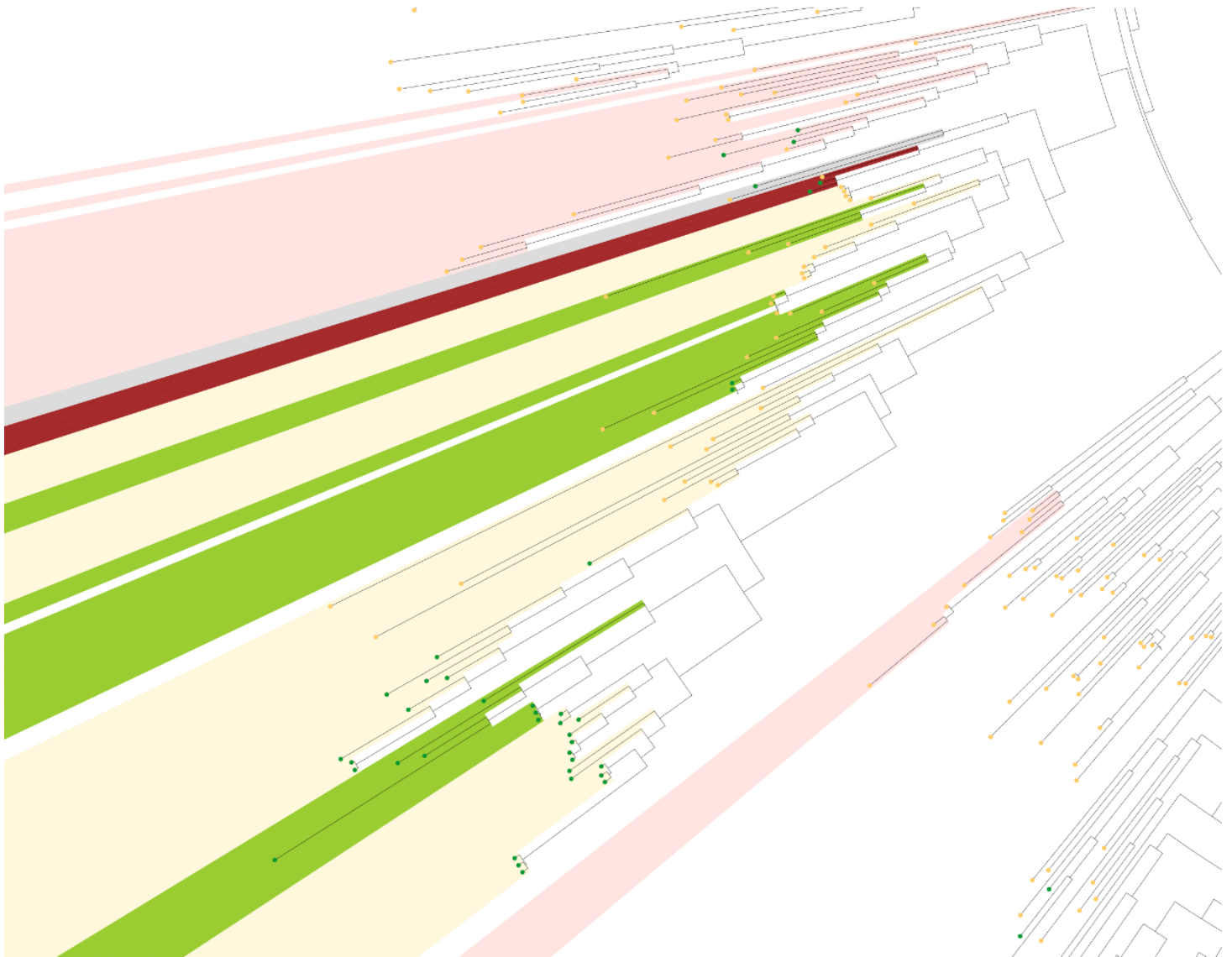


Figure 4.7 **Evolution of the fungal Mep group.** The tree was coloured by fungal phyla Ascomycota (cream), Basidiomycota (green), Blastocladiomycota (dark red), Chytridiomycota (grey) to explore the history of the group. Archaea are coloured light red. The presence of the HH motif is denoted by a green dot and the EH by a yellow dot.

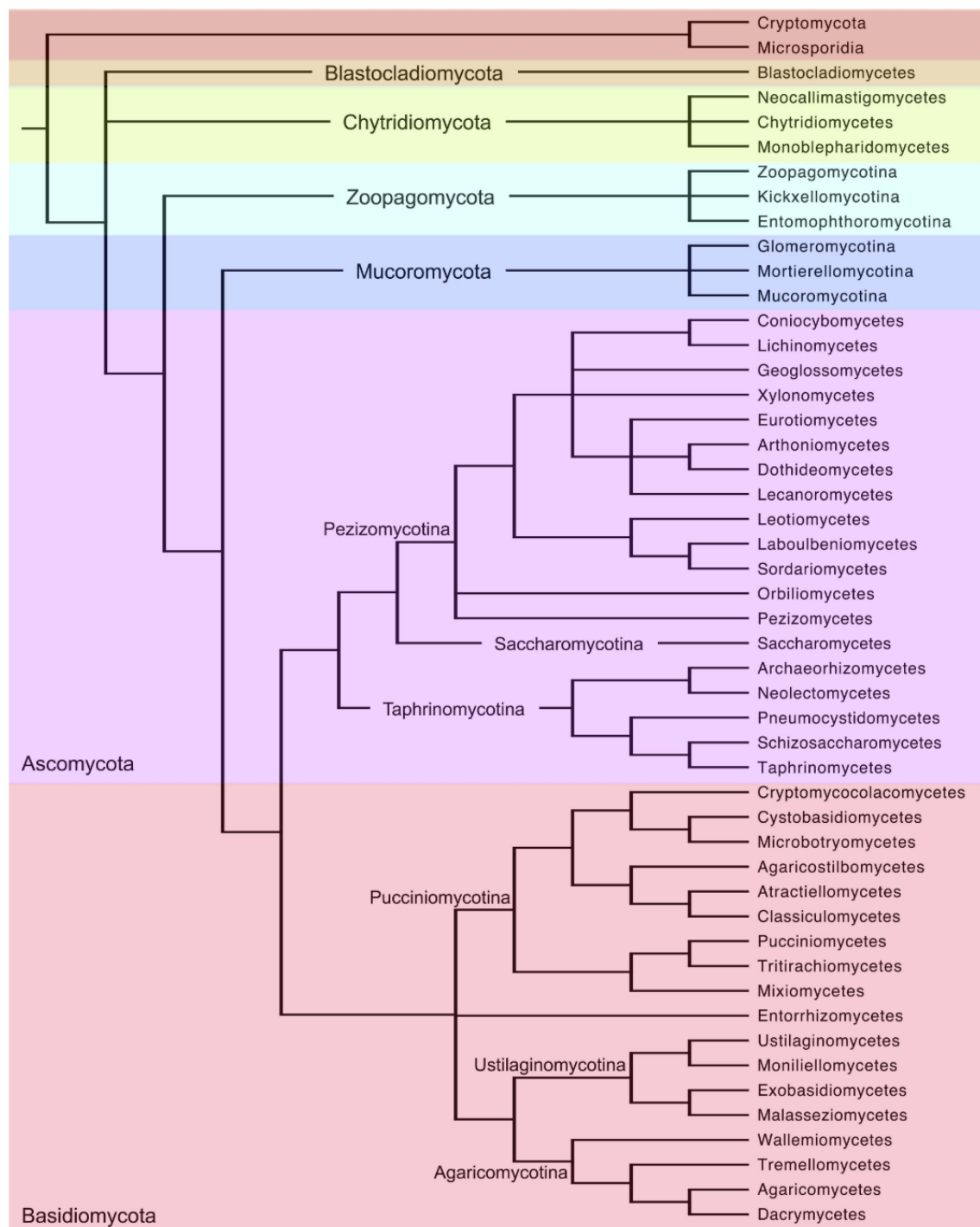


Figure 4.8 **Cladogram of the evolution of fungi.** Figure adapted from Spatafora *et al*, (2017).

4.3.3.2 Phe Gate conservation

The Phe gate is another important conserved motif for ammonium transporters. Mutational analyses have shown that deletion of the second Phe residue leads to a loss of transporter activity (Javelle *et al.*, 2008). However, these studies were conducted using methylammonium (MeA) as a substrate, which is known to be an imperfect analogue of ammonium and may yield results that are not entirely consistent with assays employing ammonium (Williamson *et al.*, 2023). The crystal structures of *E. coli* AmtB and *N. europaea* NeRh50 reveal structural differences in the Phe-gate. Specifically, in NeRh50, the ring of the first phenylalanine (Phe-86) is oriented perpendicular to that of the second (Phe-194) widening the pore (Lupo *et al.*, 2007), in contrast to the parallel ring orientation observed in Amt protein structures that completely obstructs the conduction pathway (Khademi *et al.*, 2004). The structure of the Phe-gate in RhCG also reveals that the outer phenylalanine (Phe130) does not obstruct the pore, resulting in an “open” conformation (Gruswitz *et al.*, 2010). Despite these differences, all Amt/Mep/Rh structures solved to date show that the Phe gate, even when not fully occlusive, forms a constriction along the translocation pathway (Khademi *et al.*, 2004; Andrade *et al.*, 2005; Lupo *et al.*, 2007; Gruswitz, *et al.*, 2010; Berg *et al.*, 2016; Pflüger, *et al.*, 2018; Pflüger *et al.*, 2024). This recurring structural feature suggests that the phenylalanine rings must undergo dynamic movements to permit ammonium translocation from the cytoplasmic binding site into the hydrophobic pore. Consistently, molecular dynamics simulations have observed frequent 180-degree flips of the Phe gate (Nygaard *et al.*, 2006). Thus, although the central core of Amt/Mep/Rh proteins is highly conserved, the Phe gate emerges as a rare site of structural variation. This highlights the need to (i) determine the extent of its conservation across the superfamily and (ii) experimentally define its specific role in the ammonium conduction mechanism. I will now analyse the distribution of this motif in the superfamily and, in Chapter 6, evaluate its functional importance by measuring the activity of EcAmtB and NeRh50 Phe-gate variants. To analyse the distribution of the motif in the superfamily, an alignment of the protein sequences of all the members of the tree was generated using MAFFT, and the columns corresponding to the Phe gate residues were examined. To visualise this, each leaf in the tree was annotated with the amino acids present at the relevant columns. The Phe gate was completely

conserved across the tree with eight exceptions (Table 4.1). Further investigations into these exceptions were carried out using an AlphaFold structural alignment (Figure 4.9).

- In *Debaryomyces hansenii*, the first phenylalanine residue is uniquely substituted by a methionine, while the second phenylalanine remains conserved. Notably, the orientation of the second phenylalanine closely mirrors that observed in the crystal structures of bacterial *E. coli* AmtB (Figure 4.9). Structural studies of the AmtB Ala-Phe variant have shown that the positioning of the second phenylalanine residue is dependent on the presence of the first (Javelle *et al.*, 2008), implying that substitution of the first phenylalanine may disrupt the proper conformation of the Phe gate. In this context, the observed Met-Phe configuration in *D. hansenii* appears unusual and may represent a sequencing artifact rather than a *bona fide* variant (Figure 4.9). Further validation of the sequence is therefore necessary.
- The distribution and substitution patterns of the Phe gate variants do not exhibit any clear phylogenetic or functional trends, with the notable exception of two leucine/leucine (Leu/Leu) variants identified in *Leifsonia xyli*, which cluster closely together. As these proteins originate from two subspecies of *L. xyli*, it is plausible that they represent the same sequence from two different samples. Structural modelling using AlphaFold indicates that, in this case, the pore beyond the S1 binding site is widened, suggesting the possibility of a transport mechanism distinct from that of *Escherichia coli* AmtB (Figure 4.9). A similar pattern is observed in the ammonium transporters from *Nematostella vectensis* and *Rhodospirillum rubrum*, where only the second phenylalanine residue is substituted by leucine (Figure 4.9). If confirmed as genuine, functional characterisation of this ammonium transporter, using SSME or the assay I have developed and detailed in Chapter 5, would provide valuable insights into its unique transport properties. However, given that the codons encoding phenylalanine (UUU/UUC) and several leucine codons (UUA, UUG, CUC) differ by only a single nucleotide, these substitutions may represent sequencing errors rather than authentic evolutionary divergence.
- In *Dictyostelium purpureum*, the motif at the position of the Phe gate is composed of two tyrosine residues (Tyr/Tyr), which can be structurally compared to the canonical Phe gate as it similarly contributes to constricting

the translocation pathway (Figure 4.9). Interestingly, the conformation of this Tyr/Tyr gate closely resembles that observed in Rhesus proteins, with the upper tyrosine oriented perpendicularly to the lower one, mirroring the spatial arrangement typically formed by the Phe gate motif in Rh proteins. A comparable scenario is observed in the ammonium transporter AeAmt1 from *A. aegypti*, in which the first phenylalanine is substituted by a tyrosine residue. Interestingly it is also the detoxifier exception to the Glu sensor group in section 4.3.2. However, considering that the codons encoding Phe (UUU/UUC) and Tyr (UAU/UAC) differ by only a single nucleotide, such substitutions may also result from sequencing errors rather than true evolutionary divergence.

This analysis demonstrates that, although the identified variants may present promising opportunities for structural and functional characterisation, rigorous sequence verification is essential to confirm their authenticity prior to further study. Nonetheless, the high conservation of the Phe gate across species highlights its crucial role in ammonium transporter function. Accordingly, I will assess its functional significance in Chapter 6 by measuring the activity of EcAmtB and NeRh50 Phe-gate variants.

Table 4.1 List of the Phe Gate exceptions.

Organism name	Phe Gate variation
<i>Leifsonia xylii</i>	Leu/Leu
<i>Leifsonia xylii</i>	Leu/Leu
<i>Aedes aegypti</i>	Tyr/Phe
<i>Nematostella vectensis</i>	Phe/Leu
<i>Dictyostelium purpureum</i>	Tyr/Tyr
<i>Oikopleura dioica</i>	Phe/Met
<i>Rhodospirillum rubrum</i>	Phe/Leu
<i>Debaryomyces hansenii</i>	Met/Phe

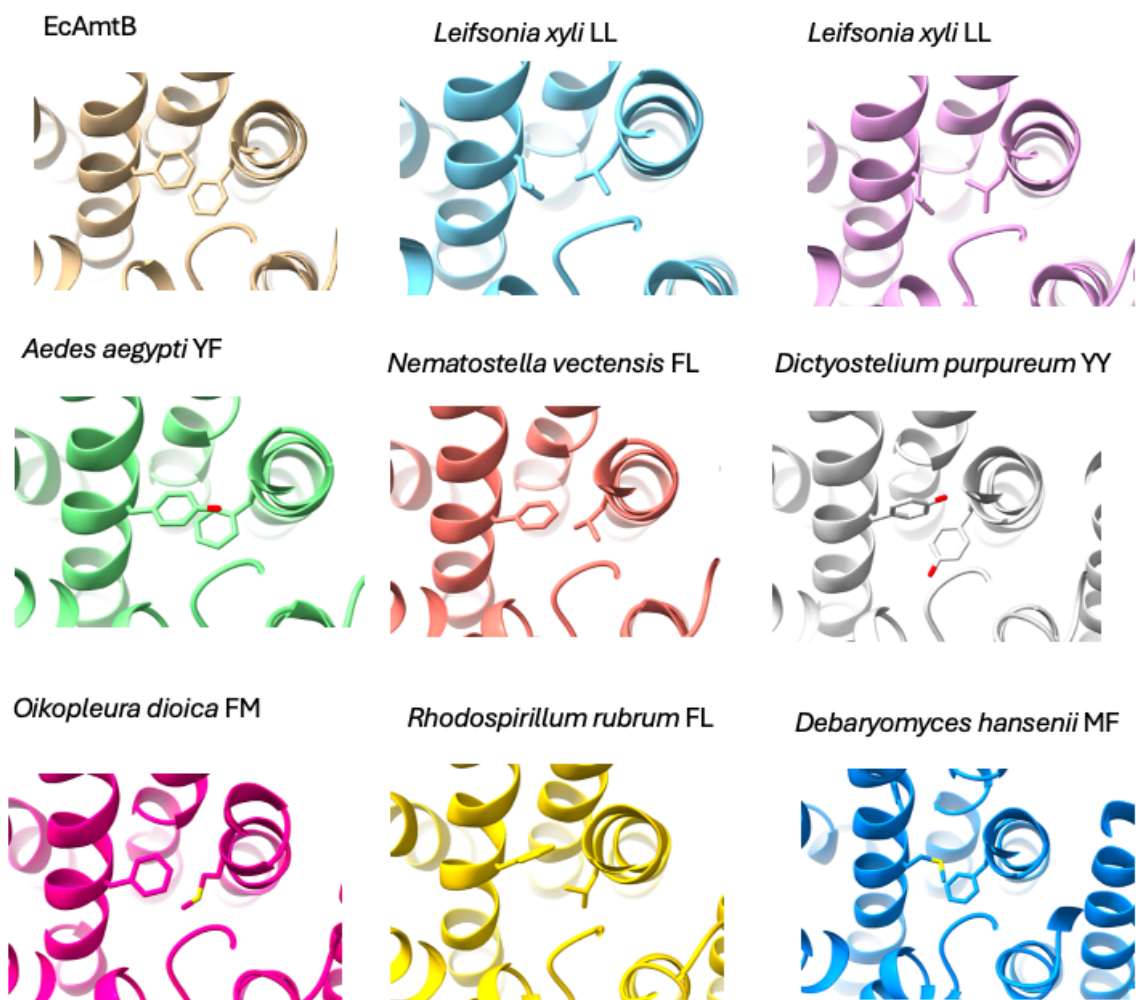


Figure 4.9 **Different arrangements of the Phe Gate.** These proteins are part of nine that do not appear to have the otherwise fully conserved Phe Gate. Aside from EcAmtB, these structures were obtained from AlphaFold. The structure of one protein from *Apis mellifera* was not available, but the rest are shown here with EcAmtB as a reference. Notably the Tyr/Tyr arrangement in *D. purpureum* with the first residue being perpendicular to the other is in the same orientation as that found in rhesus proteins.

4.4 Discussion

In this chapter, I have explored the evolutionary history of ammonium transporters and sought to uncover mechanistic patterns underlying their diversification. Excitingly, these findings open new avenues for structural and functional studies aimed at understanding how these proteins have acquired novel roles in diverse biological processes.

4.4.1 Origins of protein groups

4.4.1.1 *Origins of proteins with extra domains*

In my tree all ammonium transporters with the extra domains were to be found in the “sensor” protein group. This makes sense as a sensing signal is often part of a transduction chain leading to a physiological regulation for an organism, so being fused to a domain with an enzymatic activity allows for more efficient transfer of a signal rather than relying on diffusion through a crowded cell. A transporter would only need to move ammonium into the cell. In section 4.3.1.1 the single eukaryotic protein that had an extra domain fused in *C. reinhardtii* is an interestingly unique case. According to pfam it is not just the only eukaryotic protein in our tree to be fused to an extra domain but also the only protein known to date to have this specific fusion. A search for homologues of this protein may find more but the fact that this protein clusters with another *C. reinhardtii* amt and not with another organism may mean it is indeed unique to this organism and the result of a duplication then fusion.

The Amt-PIL protein relationship is old as mentioned before. This means the two are found together in both Glu sensor and Tyr transporter organisms. The fact that we have found only fused PIL domains to be present in the Glu sensors is interesting and suggest that (i) their function in sensors is slightly different than in transporters meaning it is more likely they will fuse (ii) fusion is very rare and happened to have occurred once (followed by vertical inheritance) or twice (independent fusion) in the sensor group (iii) transporters perform worse when fused which would select against fusion.

An analysis of 18,000 ammonium transporters that also managed to cluster the proteins based on their “sensor” or “transporter” capabilities found that fused domains are not exclusive to sensor proteins (Pflüger *et al.*, 2024). This may mean that the

diversity of my tree is not extensive enough to pick up these proteins or that there is further clustering within the groups than what I have proposed here.

4.4.1.2 Eukaryotic proteins

The origin of eukaryotic ammonium transporters seems to differ in both the Tyr and the Glu group. In the Glu group, eukaryotic proteins appear to have been inherited from bacteria. This corroborates with previous findings about the rhesus group (Huang and Peng, 2005). In the Tyr group it appears that the Mep proteins originated from archaea. This does not match what has been seen as the Mep origin was thought to be bacterial (McDonald, Dietrich and Lutzoni, 2012). The development of the ammonium transporters in the domains of plant, fungal, and other eukaryotic proteins is noteworthy as they are not arranged in a manner that reflects speciation. An explanation could be that plants and fungi, while being complex organisms with organs, are still stationary and can only explore an area by growing roots or hyphae. Their stationary nature means they are still reliant on ammonium scavenging for their nitrogen sources but also their complex structures mean they are also reliant on ammonium localising and detoxification. It may be that they acquired their ammonium scavengers through vertical inheritance, and these were lost in other eukaryotes that evolved the ability to move and digest food meaning they no longer needed to scavenge ammonium from the environment. It could also be that they obtained them from either archaea or bacteria through HGT.

4.4.1.3 Rhesus proteins

The topology of the rhesus group is consistent with published literature (Huang and Peng, 2005). It follows this pattern: fish proteins branching to a group containing both invertebrate and fish proteins, which then leads to RhAG, RhBG, and RhCG proteins in mammals and other animals. Out of almost 4000 prokaryote genomes analysed by Matassi (2017) only 34 coded for a Rhesus protein leading to their hypothesis that Rhesus proteins were introduced into prokaryotes via a single HGT event. However, the subsequent distribution and evolutionary spread of Rhesus proteins across prokaryotic lineages following this initial transfer remains unresolved though there is evidence for more instances of HGT (Matassi, 2017). In contrast to previous studies,

our analysis includes a rooted phylogenetic tree, allowing for the identification of a basal eukaryotic clade with strong statistical support. This clade comprises proteins from *Chlamydomonas reinhardtii*, a unicellular green alga, a bacterial group then appears higher in the tree topology, which could be evidence of HGT. This is different to previously seen rhesus trees which placed *Nitrosomonas europaea* and *Nitrospira* in a separate clade (Cherif-Zahar *et al.*, 2007). This means that including more proteins into the tree changes the inferences that can be made about the evolution of the group. This is important, because of the work of Matassi (2017) we know that, despite using a methodology that should incorporate a broad taxonomical diversity, representatives in the rhesus group are missing. For instance, no archaeal Rhesus proteins were included in our tree, despite evidence of their existence (Matassi, 2017). Notably, the archaea *Methanosalsum zhilinae* has been shown to encode both an Amt and an Rh homologue (Matassi, 2017). In this context, I aligned the AlphaFold-predicted structure of the *M. zhilinae* rhesus protein with the published structure of AfAmt1 (Andrade *et al.*, 2005), an archaeal Amt protein, confirming the presence of the characteristic methionine residue that I have proposed as a hallmark of Rh proteins (Figure 4.10). Further Increasing the diversity in the tree may offer better insights into the protein's history.

The origin of the rhesus proteins in our tree appears to have been from a bacterial source as there is the ammonium transporter G7WDB5 in *Desulfosporosinus orientis*, sulphate-reducing bacterium, with high support at the base of the group. This implies that this protein shares a common ancestor with the Rhesus group. However, just one level below in the topology, there is a clade that includes both archaeal and bacterial sequences. The support for the split of the clade leading to the rhesus proteins and the clade containing the bacterial and archaeal groups is reasonable with a TBE value of 0.768. This means that the rhesus proteins could have originated from a distinct sensor group vertically passed from LUCA.

We can identify the transition of rhesus proteins to 'sensors' through the change of Met to Glu if it is accepted that the Met residue is a characteristic of the rhesus proteins. The rhesus group is mainly composed of eukaryotes and is not as diverse in terms of kingdoms as other parts of the tree. The most parsimonious explanation for this rarity of rhesus proteins in prokaryotes in this tree is that they were acquired through HGT from eukaryotes, rather than being ancestrally present in all organisms and subsequently lost in lineages. The number of times the protein would have to have

been lost outweighs the single HGT event into prokaryotes that this tree suggests. The prokaryotes coding for the rhesus protein seen in this tree, *Nitrosomonas europaea* and *Nitrosospira multiformis*, are both ammonia oxidising bacteria (AOB). These organisms must regulate a specific internal ammonium concentration so perhaps their acquisition and retention of the rhesus protein allows them to control this through import and export. The idea that prokaryotes obtained the Rh protein through HGT suggests that the origin of Rhesus proteins could have been a result of the duplication and adaption of an ammonium transporter, likely a Glu sensor. As discussed above, this may have happened in a eukaryote in response to changing selection pressure. If this was the case, we would expect to see some eukaryotes retaining a sensor which we do. It is likely that the evolution of a bidirectional transporter in eukaryotes was a necessary adaptation for maintaining ammonium homeostasis within a multicellular organism, rather than solely an adaptation between the cell and its external environment. The duplication origin theory is supported by previous research, which also claims the origin of rhesus proteins was in eukaryotes with subsequent HGT into prokaryotes (Huang and Peng, 2005; Matassi, 2017).

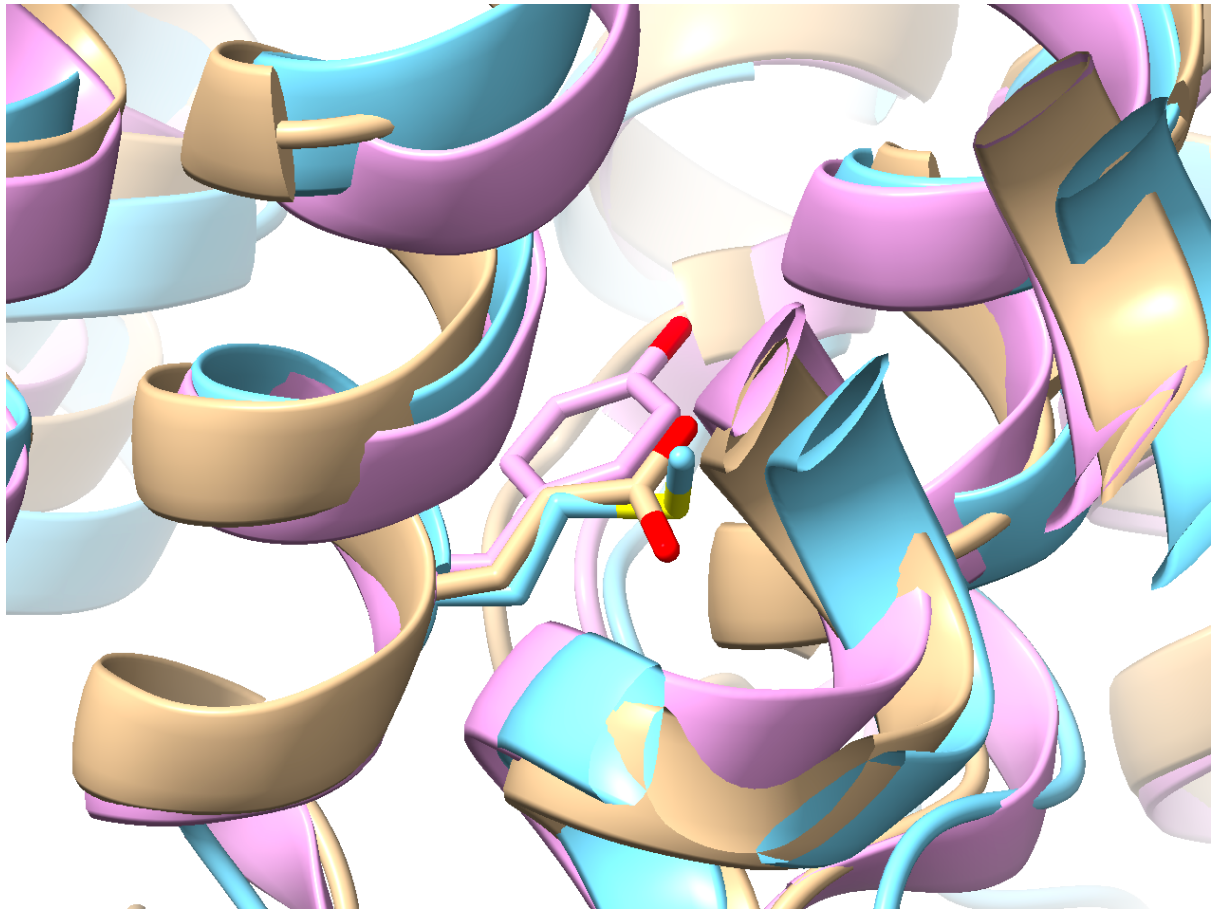


Figure 4.10 **Superimposition of the pocket residue, confirming the archaeal rhesus protein.** The archaeal rhesus protein from *Methanosalsum zhilinae* (blue) with the rhesus protein's characteristic Met residue is shown aligned with the same organism's other ammonium transporter (sand) which has a Glu. AfAmt1 (pink) is shown as a reference with its Tyr.

4.4.2 Distribution of functions

In the previous chapter, we stated that Glu-type proteins may function exclusively as sensors. An exception to this is the fungal Mep2 proteins, which have been identified as ammonium transducers (Biswas and Morschhäuser, 2005; Berg *et al.*, 2016). These proteins appear to have evolved a distinct transport mechanism that is tightly linked to their transceptor function (section 1.4.4) meaning the Tyr transport hypothesis still holds as the signal comes from Mep2's distinct mechanism of transport, different to that of Mep1/3, and not from a canonical sensor followed by a signal transduction cascade (Brito *et al.*, 2020; Williamson *et al.*, 2022). This suggests the protein started as a transporter and was adapted to be a transducer. The fungal Mep proteins are also unconventional as Mep1 and Mep3 are among the few that have exchanged the first His in the Twin-His motif for a Glu. Reasons for this are unclear but when this substitution is made in EcAmtB, the activity increases compared to the WT (Williamson *et al.*, 2022). In *S. cerevisiae* the three Mep proteins display differing K_m and V_{max} values with Mep2 having the lowest K_m of 1-2 μ M, Mep1 5-10 μ M and Mep3 1.4-2.1 mM (Marini *et al.*, 1997). Mep2 has the lowest V_{max} of the three however, suggesting that while it is the most sensitive it is also the most precise, which would align with its role as a transceptor.

In bacteria and archaea, the gene encoding the ammonium transporter (*amtB*) is consistently co-localised with *glnK*, which encodes a member of the PII family of signal transduction proteins that regulate key aspects of carbon and nitrogen metabolism. Experimental evidence shows that in *E. coli*, AmtB is functionally inactivated through the rapid and reversible formation of a membrane-bound complex with GlnK in response to micromolar changes in extracellular ammonium concentrations (Javelle *et al.*, 2004). This regulatory interaction is modulated by the uridylation state of GlnK, which in turn reflects fluctuations in the intracellular glutamine pool. Notably, under physiological conditions, AmtB activity is required to trigger GlnK deuridylation. These findings demonstrate that AmtB functions not only as a transporter but also as an integral component of the ammonium sensing system, effectively coupling substrate transport with signal transduction, and could thus be classified as an ammonium sensor (Javelle *et al.*, 2004). These observations underscore the blurred boundary between transporters and sensors, highlighting that certain membrane proteins can simultaneously fulfil both roles. It may be that my categorisation of these proteins as

detoxifier, sensor or transporter is too narrow, and their functions are either more varied or less definable for a single protein. The two sensors SdAmR1 and KsAmt5 both showed no transport through the pore when measured using SSME but merely binding of ammonium to a pocket inside the pore (Pflüger *et al.*, 2018; 2024). Building on the transport assay based on vesicles developed in Chapter 5, one could potentially measure the activity of a diverse array of ammonium transporters, selected based on the phylogenetic tree, to explore their functional dynamics in unprecedented detail. Proteins to measure could be ones where the organism has ammonium transporters in both the sensor and the transporter group, proteins where the organism just has a member in one group, and proteins where the organism has multiple members in one group but not the other. This approach could be further extended by preparing vesicles directly from yeast or human cells, enabling the testing of nearly all non-canonical Amt/Mep/Rh proteins identified in my work. This opens exciting new perspectives for exploring this fascinating family of proteins in a way that has never been done before. The extra domains fused to the ammonium transporters must convey some functionality. The extra domain combinations vary with some proteins having more than others. Among the extra domains there are diguanylate cyclase domains. These are recognised second messenger proteins and are key in bacterial biofilm formation (Whiteley and Lee, 2015). Often coupled to the diguanylate cyclases are found degenerate phosphodiesterase (EAL) domains and Per-ARNT-Sim (PAS) folds. EAL domains are often found in the same proteins as diguanylate cyclases domains and are also involved in the signalling to form biofilms (Kimbrough and McCarter, 2021). PAS domains are common signal transducers with some proteins capable of binding small ligands while others merely interact with proteins to activate them (Stuffle, Johnson and Watts, 2021). It suggests itself that the proteins would possibly contribute to biofilm formation in times of low or high ammonium availability.

There are also two proteins fused to histidine kinase and DNA gyrase B-, and HSP90-like ATPase domains. This combination is very similar to the GHKL conserved domain containing a histidine kinase, HSP90, DNA gyrase B and MutL (Dutta and Inouye, 2000). A histidine kinase domain has already been found to be fused to an ammonium transporter in the bacterial protein KsAmt5 (Pflüger *et al.*, 2018). Together these extra domains suggest that this protein interacts with another protein and DNA potentially meaning it influences protein transcription.

One protein in this tree has a direct chemotaxis protein fused to its ammonium transporter. This suggests that in response to low ammonium the bacterium will increase movement or perhaps in response to ammonium sensing the bacterium will move towards the source.

4.4.3 Measuring on SURFE²R

The mechanism of transport has only been elucidated using SSME for a handful of proteins in this superfamily (Wacker *et al.*, 2014; Pflüger *et al.*, 2018; Mirandela *et al.*, 2019; Williamson *et al.*, 2020; Pflüger *et al.*, 2024). Due to limitations in yield and protein stability, we have been unable to assess the activity of eukaryotic proteins in our laboratory, which has hindered efforts to determine whether a universal mechanism operates across the superfamily. However, there are several reasons to hypothesise such a conserved mechanism. Notably, key structural features such as the Twin-Histidine motif and the phenylalanine gate are retained even in members of the family that do not transport ammonium through the pore (Pflüger *et al.*, 2018; 2024). In terms of a functional categorisation, the conservation of these features makes it more difficult to distinguish between proteins of different groups. Furthermore, the overall structural conservation across diverse homologues supports the possibility of a shared mechanistic framework.

In this and the previous chapter we have found that certain specific residues at the opening of the binding pocket or the exit of the pore may have a role in defining the groups and functions of these proteins. For this reason, to investigate the mechanism of the superfamily further I propose that the following proteins could be measured using SSME (Table 4.2). These proteins were chosen based on their location in the tree and not on any other characteristic of organism habitat or function.

If the question of evolution were to be asked, I would propose a different line of investigation. This would involve trying to see if we can group proteins based on activity and whether this grouping matches what is seen in the tree. For example, each archaeal/bacterial pairing could be evidence of an ancient protein. If representatives from each kingdom in a pairing were measured and found to function in the same way and if this function was distinct from other archaeal/bacterial pairings it would be consistent with sharing a common mechanism, distinct from other members of the superfamily, and that this was a protein that was represented in LUCA.

Table 4.2 Details of proteins to investigate further using electrophysiology

Group	Reason and what to measure	Organism	Protein UniProt ID
Whole tree	Test for universal mechanism by taking proteins from the extremes of the Glu and Tyr groups	<i>Methanococcus maripaludis</i> (Glu)	G0H0Z6
		<i>Methanosarcina barkeri</i> (Glu)	A0A0E3SHQ9
		<i>Acidithiobacillus ferrivorans</i> (Tyr)	A0A257T8W6
		<i>Hafnia alvei</i> (Tyr)	A0A377PLP5
Cys proteins	Function of Cys, function of protein with fused domain	<i>C. reinhardtii</i>	Q8RUT6
			A0A2K3DQF8.
His/His Glu/His Mep and archaeal	Do the HH and EH proteins from archaea and fungi share mechanisms	His/His	
		<i>Methanofollis liminatans</i>	J1ASR8
		<i>Cryptococcus neoformans</i>	J9VXX9
		<i>S. cerevisiae</i>	P41948
		Glu/His	
		<i>Methanosphaerula palustris</i>	B8GGC3
		<i>Methanofollis liminatans</i>	J1L5M8
		<i>Cryptococcus neoformans</i>	A0A854QN45
		<i>S. cerevisiae</i>	P40260
		His/His	

His/His Glu/His bacterial	Is the mechanism different in these proteins to the ones around them in the tree?	<i>Amycolatopsis mediterranei</i>	A0A9R0NTP8
		Glu/His	
		<i>Nocardia brasilliensis</i>	KOER80
		<i>Nocardia brasilliensis</i>	A0A6G9XJV9
		<i>Catenulispora acidiphila</i>	C7Q1E4
Pocket entrance and pore exit	What is the purpose of different pore exit residues in combination with different pocket residues	Tyr/Asn	
		<i>Metallosphaera sedula</i>	A0A088E6Q7
		Tyr/Ser	
		<i>Ferroplasma acidarmanus</i>	S0APH6
		Tyr/Gln	
		<i>Ricinus communis</i>	B9SA70
		Tyr/Thr	
		<i>Dehalococcoides maccartyi</i>	D2BIB2
		Tyr/Ile	
		<i>Myxococcus xanthus</i>	A0AA42Q839
		Glu/Ans	
		<i>Haloferax mediterrani</i>	B8ZYW3
		<i>Rubrobacter xylanophilus</i>	Q1AUG3
		Glu/Thr	
		<i>Nematostella vectensis</i>	A7S731
		Glu/Ser	

		<i>Lutibaculum baratangense</i>	V4RK08
		Met/Asn	
		<i>Chlamydomonas reinhardtii</i>	Q94CJ2
		<i>Homo sapiens</i>	Q9UBD6
		Cys/Asn	
		<i>Micromonas pusilla</i>	C1N6X9
Rhesus proteins	Is bidirectionality shared across the rhesus proteins? What is the transported substrate - NH ₃ or NH ₄ ⁺ or both?	<i>Nitrosospira multiformis</i>	A0A1I0AQM4
		<i>Dictyostemilum discoideum</i>	Q9NIV0
		<i>Metacarcinus magister</i>	F2YIC4
		<i>Homo sapiens</i>	Q9UBD6
Phe Gate	What is the activity of the proteins with an altered Phe Gate? If different, is normal function restored upon mutation back to normal Phe Gate?	<i>Leifsonia xyli</i>	A0A1E2SKM1
		<i>Leifsonia xyli</i>	U3PCH5
		<i>Aedes aegypti</i>	Q1I727
		<i>Nematostella vectensis</i>	A7SGD4
		<i>Rhodospirillum rubrum</i>	Q2RWY7
		<i>Oikopleura dioica</i>	E4YA63
		<i>Dictyostelium purpureum</i>	F0ZPL3
		<i>Debaryomyces hansenii</i>	Q8BK68
Proteins with fused domains	Are all proteins with fused domains non-transporters like the	<i>Psychromonas ingrahamii</i>	A1SY01
		<i>Leptonema illini</i>	H2CJI0

	two previously investigated?	<i>Pseudobutyrvibrio ruminis</i>	A0A2G3DSU6
		<i>Delsulfosporosinus acidiphilus</i>	I4D6Z9
		<i>Marichromatium purpuratum</i>	W0E077
		<i>Chlamydomonas reinhardtii</i>	A0A2K3DQF8
		<i>Halorubrum lacusprofundi</i>	B9LU00

The proteins proposed for future study are from archaea, bacteria and eukaryotes. Eukaryotic and archaeal proteins are more difficult to work with because of poor yield and protein stability once removed from the membrane using detergent. Therefore, the next step of this PhD was to develop an assay to circumvent this by creating vesicles directly from cells used to overexpress our protein. This would be first attempted in *E. coli* then expanded to yeast or other eukaryotic cells in order to be able to investigate eukaryotic proteins.

Mutational studies and SSME measurement regarding the pore exit and binding pocket residues can also be carried out in the EcAmtB and NeRh50.

4.5 Future phylogenetic work

In summary I propose the following future work could help us understand this superfamily better:

- Build a more comprehensive tree using the proteins in the PF00909 pfam entry and using their TM regions
- Carry out the same annotations as with this tree to see if the Glu/Tyr/Cys/Met split holds
- Make subtrees from this larger one with the full-length sequences. Only using more closely related proteins should in theory reduce the gaps in the alignment and the full-length sequence will allow for better resolution and may reveal more about their functions. However as seen in Figure 3.1 more closely related proteins do not always share the same extra helical segment length. Subtrees

that could be explored are the fungal/archaeal group, the rhesus proteins and any other group that is well supported.

- Investigate the origins of all the extra domain proteins by building trees that compare the amt domain to the extra one. This could also be done using proteins that are not fused to give context to the fusion.
- Potentially identify other residues that could be implicated in the function of these proteins and explore other rooting options
- Search for evidence of recombination in the EH/HH archaeal and fungal proteins.

As has happened with this chapter, many more questions will likely arise from these investigations.

Chapter 5: Creation of vesicles from cells used to overexpress protein

5.1 Introduction

To date, the ammonium transporters that have been measured using Solid-Supported Membrane Electrophysiology (SSME) include the archaeal AfAmt1, the bacterial transporters EcAmtB and NeRh50 and the bacterial ammonium sensors KsAmt5 and SdAmt1 (Wacker *et al.*, 2014; Pflüger *et al.*, 2018; Mirandela *et al.*, 2019; Williamson *et al.*, 2020, 2022; Pflüger *et al.*, 2024). As detailed in Chapter 1 section 1.4, essentially all other functional studies have been undertaken using intact cells or cell-derived vesicles. However, in all these systems, the parameters used to measure and analyse substrate translocation (pH change, electric current, uptake of labelled analogue) are likely to be affected by other physiological phenomena for example the methods by which *X. laevis* oocytes regulate their internal ammonium conditions (see section 1.4.5), requiring careful controls and often lead to contradictory results (Khademi *et al.*, 2004; Javelle *et al.*, 2006; Williamson *et al.*, 2023). Using SSME based measurements in our lab allowed us to investigate conditions that would not be possible *in vivo*, for example D₂O measurements which experimentally have shown the importance of the water wires to EcAmtB and NeRh50 transport (Williamson *et al.*, 2020). However, there is a limitation in the need to express, purify, and reconstitute the protein into liposomes. Attempts in our lab to express and purify eukaryotic ammonium transporters from yeast has not proved successful owing to poor yield and stability (Javelle A., personal communication). This has meant that we have had to rely on electrophysiological measurements of the bacterial models EcAmtB and NeRh50 and infer the activity and mechanism by using yeast complementation (Williamson *et al.*, 2022). The purification-reconstitution process also uses detergents to stabilise the protein when they are removed from the membrane until they are reconstituted. The lipid composition of the proteoliposome membrane, although optimised for our study, are not the native lipids. In our lab we have found that EcAmtB has specific binding sites on its outer surface for 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) and if this is not present both the activity and stability of the protein are compromised (Mirandela *et al.*, 2019). Another drawback is that the protein insertion orientation is also not controllable and previous investigations using trypsin to cleave the His-Tag of the C-terminal tail of EcAmtB or NeRh50 found that the proteins are inserted both

inside out (ISO) and right side out (RSO). For these reasons the first aim of my PhD was to develop a new method for measuring Amt/Mep/Rh protein activity without the need to purify and reconstitute them into artificial liposomes. This *in vitro* approach involves creating vesicles containing our protein of interest directly from the membrane of the cells used for overexpression by disrupting them with a hypotonic solution. This *in vitro* assay enables precise control over experimental parameters, both inside and outside the vesicles, such as substrate concentration, pH, and the presence of potential inhibitors. This would bypass the purification-reconstitution steps, remove the need for detergent and ideally allow us to increase the diversity of the proteins we are able to investigate to include eukaryotic proteins. Another benefit is that the French press or osmotic shock can be used to seal the vesicle in ISO or RSO orientation, respectively (Kaback, 1970). This may allow for direct comparisons between transport activity in different directions, particularly useful for the bidirectional rhesus proteins (Deschuyteneer, Boeckstaens, Mees, *et al.*, 2013).

In this chapter, "proteoliposomes" will refer to instances where we have purified our protein and reconstituted it into artificial liposomes. "Vesicles" will refer to spheroplasts created directly from a cell's membrane, while "empty vesicles" will describe vesicles generated from cells that do not express any ammonium transporter. Unlike proteoliposomes, however, since the vesicles are created from the cell membrane, they will naturally contain other native proteins present and the concentration of our protein of interest will not be known.

Eukaryotic ammonium transporters have been studied in the literature, but only by using non-direct measurements like pH change as discussed in Chapter 1 section 1.4.5. Rhesus proteins RhAG, expressed in red blood cells (RBCs) have been previously studied using the technique of creating spheroplasts straight from the red blood cell membrane (Genetet *et al.*, 2012; Ripoche *et al.*, 2004). In this instance RBC 'ghosts' were created in the presence of the fluorescent pH sensitive dye pyranine. Using stopped flow spectrofluorometry these two studies were able to measure the pH change within the ghosts following an ammonium and methylammonium pulse. In this way the transport of ammonium could indirectly be measured. Since the NH_4^+ ion is acidic and NH_3 is basic, using a pH dye should theoretically allow researchers to discriminate between the transport of the two molecules. They found that following an ammonium pulse, the pH inside the ghosts increased suggesting that RhAG translocates NH_3 and that the F65S mutation found in people suffering from

overhydrated stomatocytosis impairs the activity of the transporters (Genetet *et al.*, 2012).

Other labs have had success in measuring the activity of their target proteins in membrane vesicles using SSME (Schulz *et al.*, 2010; Niessen *et al.*, 2016; Gerbeth-Kreul *et al.*, 2021; Pommereau, Licher and Bärenz, 2023). In these instances, the membrane fragments were isolated and not further purified using, for example, sucrose gradient centrifugation to remove organelles. Proteins also transported both large and small substrates for example, human B0AT1 (SLC6A19) amino acid transporter (Gerbeth-Kreul *et al.*, 2021), and rat CLC-7 transporting Cl⁻/H⁺ (Schulz *et al.*, 2010), suggesting that it is possible to do this analysis on a range of substrate sizes. For this reason, we hypothesised that this method would also work for ammonium transporters.

5.2 Aims and objectives

In the previous chapter I built a phylogenetic tree and used it to identify potentially important proteins that could be measured using SSME. In this chapter I intend to develop a method to measure these proteins. In order to develop a system in which we are able to directly measure the activity of eukaryotic proteins we decided to use first a method to prepare vesicles directly from the cells used for overexpression (Kaback, 1970). We would also begin by using the model ammonium transporter AmtB from *E. coli*.

Our aims were as follows:

- Identify the strain and culture conditions for AmtB overexpression.
- Optimise the system used for AmtB overexpression.
- Characterise the vesicle size and AmtB content.
- Develop the assay conditions.
- Optimise the assay further.

5.3 Results

5.3.1 Vesicles made from *E. coli* Strain C43 over expressing EcAmtB

5.3.1.1 *Expression induced by IPTG*

In order to find the best strain for overexpressing AmtB and making the vesicles we began by using *E. coli* C43. When expressing EcAmtB in C43 cells for purification and reconstitution, autoinduction media is used. Autoinduction media has the disadvantage of lacking control over protein expression, as it does not allow for the culture to be grown to a desired optical density (OD) before induction. Not all the lactose present in the media is used to induce expression of the lac promoter as some is metabolised for biosynthetic purposes. For this reason, Isopropyl β -D-1-thiogalactopyranoside (IPTG) which serves as a potent inducer of the lac operon system within the pET22b expression vector was used. C43 cells were first transformed with the pET22b vector harbouring the *EcamtB* gene. A single transformed colony was used to inoculate 100ml LB. This was allowed to grow in 37°C until it reached an OD of 0.3. At this point it was left to cool for 30mins then 1 mM IPTG was added, and the culture was incubated at 30°C. Samples were collected at 3 hours, 6 hours, and after overnight culture. However, western blot analysis revealed minimal overexpression, even after overnight induction (Figure 5.1). This outcome was unexpected, given that IPTG typically acts as a robust inducer of recombinant protein expression. As a control a transformed colony was grown in the same manner but without the addition of IPTG.

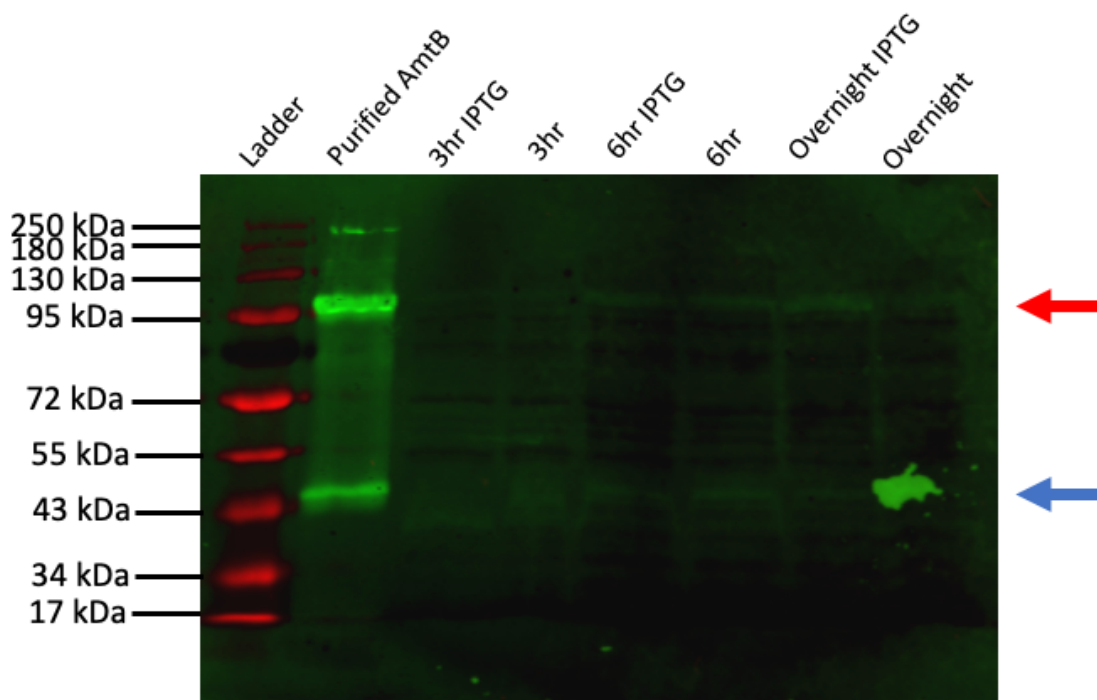


Figure 5.1 **IPTG-induced expression of AmtB from a pET vector in C43(DE3) cells.** His-tagged AmtB was assessed by Western blotting of lysed C43(DE3) cells. Single colonies were used to inoculate 100 mL LB cultures grown with or without 1 mM IPTG. Samples were collected after 3 h, 6 h, and overnight (~16 h). Cell lysates (15 μ L) were resolved by SDS–PAGE, transferred to a membrane, and probed with a mouse anti-His primary antibody followed by a fluorescent goat anti-mouse secondary antibody. Membranes were imaged on a Li-Cor Odyssey system using automatic exposure. The red arrow marks the expected position of the AmtB trimer, and the blue arrow marks the monomer.

5.3.1.2 Autoinduction media

Since IPTG did not work in inducing protein expression we returned to using autoinduction media. 1L flasks inoculated with transformed and untransformed C43 cells were grown overnight and vesicles were made through lysis of the cells in a hypotonic buffer. The protein concentration of these vesicles was measured using a nanodrop and adjusted to be 5mg/ml. This ensured continuity between batches and comparable protein levels between vesicles and proteoliposomes even if the concentration of AmtB was not known. The result was visualised with a 10% SDS gel and western blot (Figure 5.2). The analysis revealed a protein of the same size as purified AmtB in both vesicles from transformed and, to a lesser extent, untransformed C43 cells. To assess whether the protein remained detectable at higher dilutions, relevant for SSME testing, the gel samples were subjected to serial dilutions. The results demonstrated that AmtB was still visible at up to a 50x dilution in vesicles from transformed C43 cells, and up to a 2x dilution in vesicles from untransformed C43 cells. Using an anti-His antibody it was found that while AmtB was seen up to a 50x dilution in vesicles from transformed cells, it was also present in the untransformed sample to a dilution of 2x. This is unexpected as there should have been no His-tagged protein in these samples.

We overexpressed EcAmtB carrying an His6-tag fused to its C-terminal tail and western blot analysis using an anti-His antibody confirmed the presence of His-tagged AmtB vesicles from transformed cells, suggesting the potential expression of native AmtB in untransformed cells. These findings emphasise the necessity of establishing conditions to repress native AmtB expression in untransformed cells.

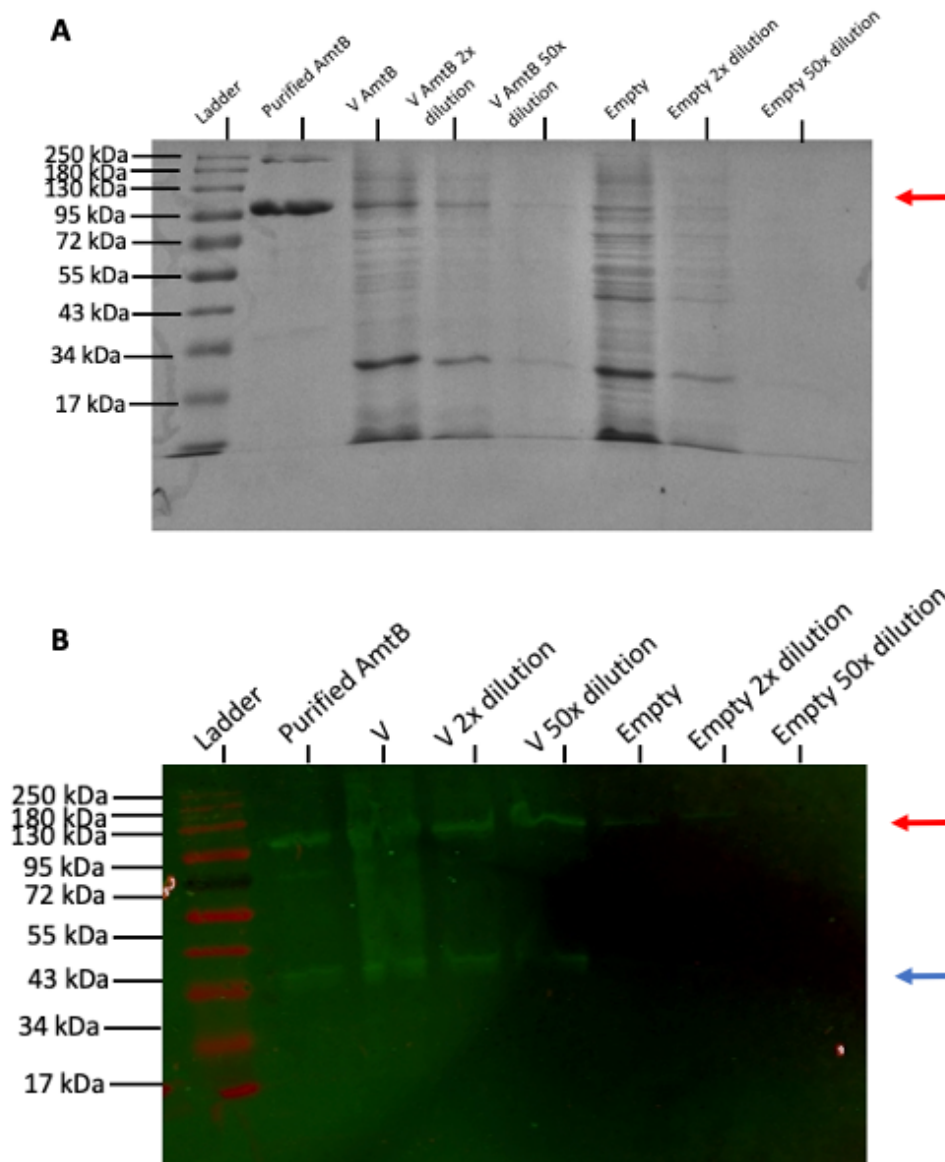


Figure 5.2 **SDS–PAGE and Western blot analysis of vesicles prepared from C43(DE3) *E. coli* cells.** Cells expressing AmtB (V) and untransformed cells (Empty) were lysed under hypotonic conditions, and resealed vesicles were collected by ultracentrifugation. Vesicle samples (20 μ L) were analysed by SDS–PAGE (A) and immunoblotting (15 μ L) (B) to assess the presence of AmtB in undiluted (V, Empty), 2x diluted and 50x diluted samples. Purified His-tagged AmtB reconstituted into liposomes was included as a positive control. Red arrows indicate the expected position of trimeric AmtB, and blue arrows indicate monomeric AmtB.

5.3.1.3 SSME Measurements of vesicles

To discover whether the activity of AmtB could be measured using these vesicles, sensors were prepared. The vesicle samples were not diluted before addition to the sensor, and these were compared to AmtB activity measured in proteoliposomes and empty liposomes as controls.

SSME measurements following a 200 mM ammonium pulse of these samples revealed a peak of comparable height to the proteoliposomes containing purified AmtB in both vesicles derived from transformed and untransformed C43 cells and representative traces aligning with the average maximum amplitude were chosen to display this (Figure 5.3). Here and throughout, each SSME measurement has been repeated three times per sensor and at least three sensors were measured for each condition from the same biological sample, for a representation of these data see appendix Figure 0.1. In the next sections, the appendix figure displaying these data will come after the main text figure cross reference. The peak observed in vesicles prepared from untransformed cells may be due to the presence of native AmtB, confirming the importance of using culture conditions that effectively repress AmtB expression.

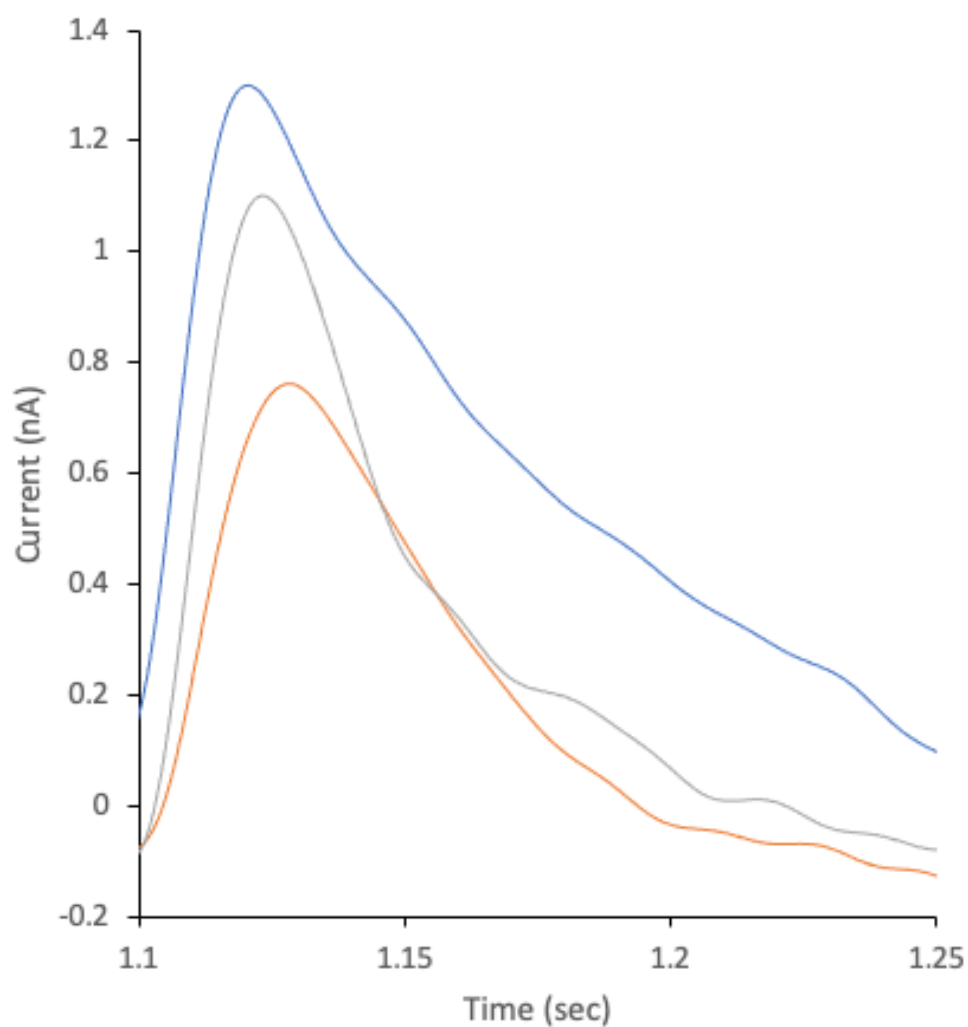


Figure 5.3 **SSME analysis of vesicles created from C43 cells.** Representative traces are shown generated from SSME analysis on vesicles containing AmtB made from C43 cells (orange), empty vesicles (grey) or purified AmtB reconstituted into an artificial liposome (blue). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.1.4 *Dilutions of vesicles*

Since there were peaks after an ammonium pulse using empty vesicles, sensors with 2x, 5x, 10x and 50x dilutions of the samples were prepared. The results from this were similar to the previous assay as peaks of a comparable size were recorded for empty and AmtB vesicles and representative traces close to the average maximum amplitude was chosen for display (Figure 5.4, Figure 9.2 for full trace, Figure 9.3 for expanded view).

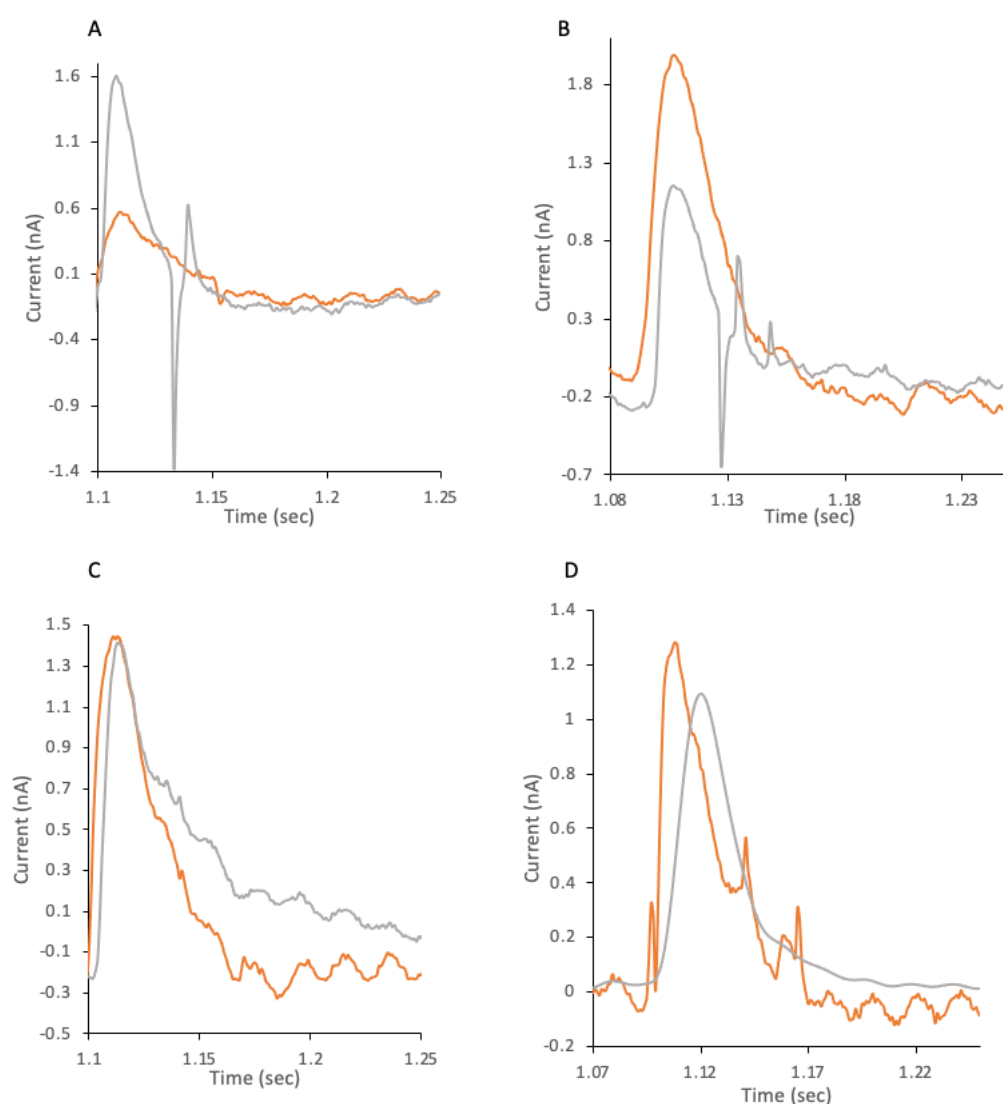


Figure 5.4 SSME analysis on different dilutions of vesicle samples. Figure showing representative traces from SSME measurements on diluted AmtB containing vesicles (orange) and empty vesicles (grey). Sensors with A) 2x diluted vesicles, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles were pulsed with 200mM ammonium. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.1.5 Repression of expression of native AmtB

AmtB is expressed as part of an operon with the PII protein GlnK, under the regulation of a promoter repressed in the presence of high ammonium concentrations in the media. The expression of the AmtB-GlnK operon is tightly regulated and occurs only under conditions of low ammonium concentration (Javelle *et al.*, 2004). To obtain vesicles devoid of native AmtB, I suppressed its endogenous expression by adding 20 mM ammonium to the culture, as previously demonstrated (Javelle *et al.*, 2004). C43 cells transformed with *amtB* in the pET22b vector and untransformed C43 cells were cultured under previously described conditions, with the addition of 20 mM ammonium to the growth medium. Analysis via 10% SDS-PAGE revealed a distinct band for transformed and a faint band corresponding to the size of EcAmtB in the untransformed C43 samples, indicating successful suppression of most of the native expression (Figure 5.5A). Western blot analysis confirmed the presence of AmtB in the transformed C43 cells, while no detectable AmtB was observed in the empty vesicles derived from untransformed C43 cells (Figure 5.5B). These results confirm the successful generation of comparable AmtB-containing and relatively more empty vesicle preparations.

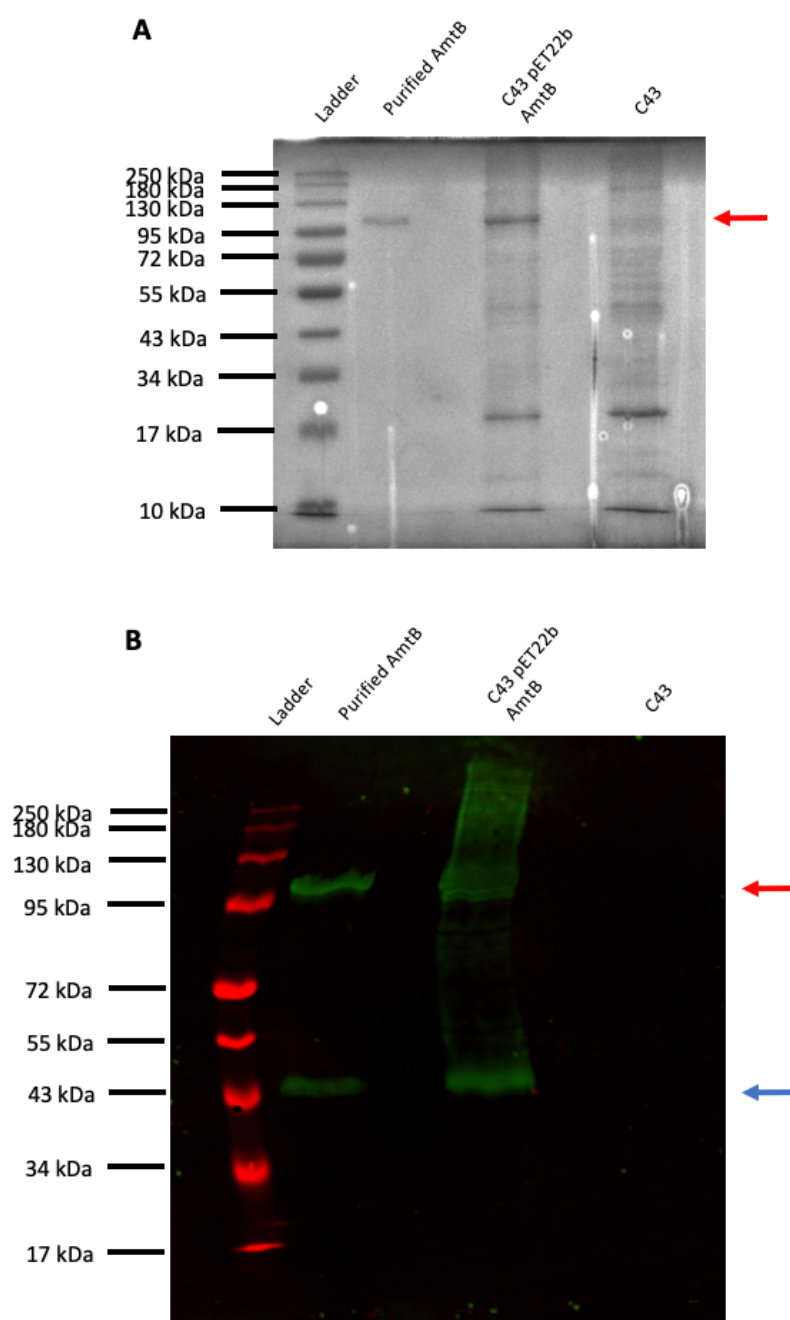


Figure 5.5 **SDS-PAGE and western blot analysis of the suppression of native AmtB production in C43(DE3) *E. coli* cells grown in the presence of 20 mM NH_4^+ .** Cells expressing AmtB (V) and untransformed cells (Empty) were grown in the presence of 20 mM NH_4^+ then lysed under hypotonic conditions, and resealed vesicles were collected by ultracentrifugation. Vesicle samples were analysed by SDS-PAGE (20 μL) (A) and Western blotting (15 μL) (B) to assess whether native AmtB production had been suppressed. Purified AmtB reconstituted into liposomes was used as a positive control. Red arrows indicate the expected position of trimeric AmtB, and blue arrows indicate monomeric AmtB.

5.3.1.6 SSME measurements

Native AmtB is not expressed under the experimental conditions used in this study, consistent with previous findings reported by Javelle *et al.* (2004). With AmtB-containing proteoliposomes and control vesicles lacking AmtB in hand, we proceeded to SSME measurements. Sensors were prepared as described previously and pulsed with an activating solution containing 200 mM ammonium. The traces obtained still showed peaks in empty vesicles and a representative trace close to the average maximum amplitude and decay value was chosen for display for each condition (Figure 5.6, Figure 9.4). Decay values of 31.57 ± 8.33 for empty and 14.71 ± 0.13 for AmtB vesicle did not correlate with transport either.

We hypothesised that variations in vesicle size and quality could account for these results. Indeed, studies using electron microscopy on *E. coli* C43 cells have shown that these cells adapt to membrane protein overexpression by increasing membrane production, which leads to the formation of abnormal membrane structures. These structures include invaginations and proliferate internal membranes, which may contribute to changes in vesicle size and quality (Wagner *et al.*, 2007).

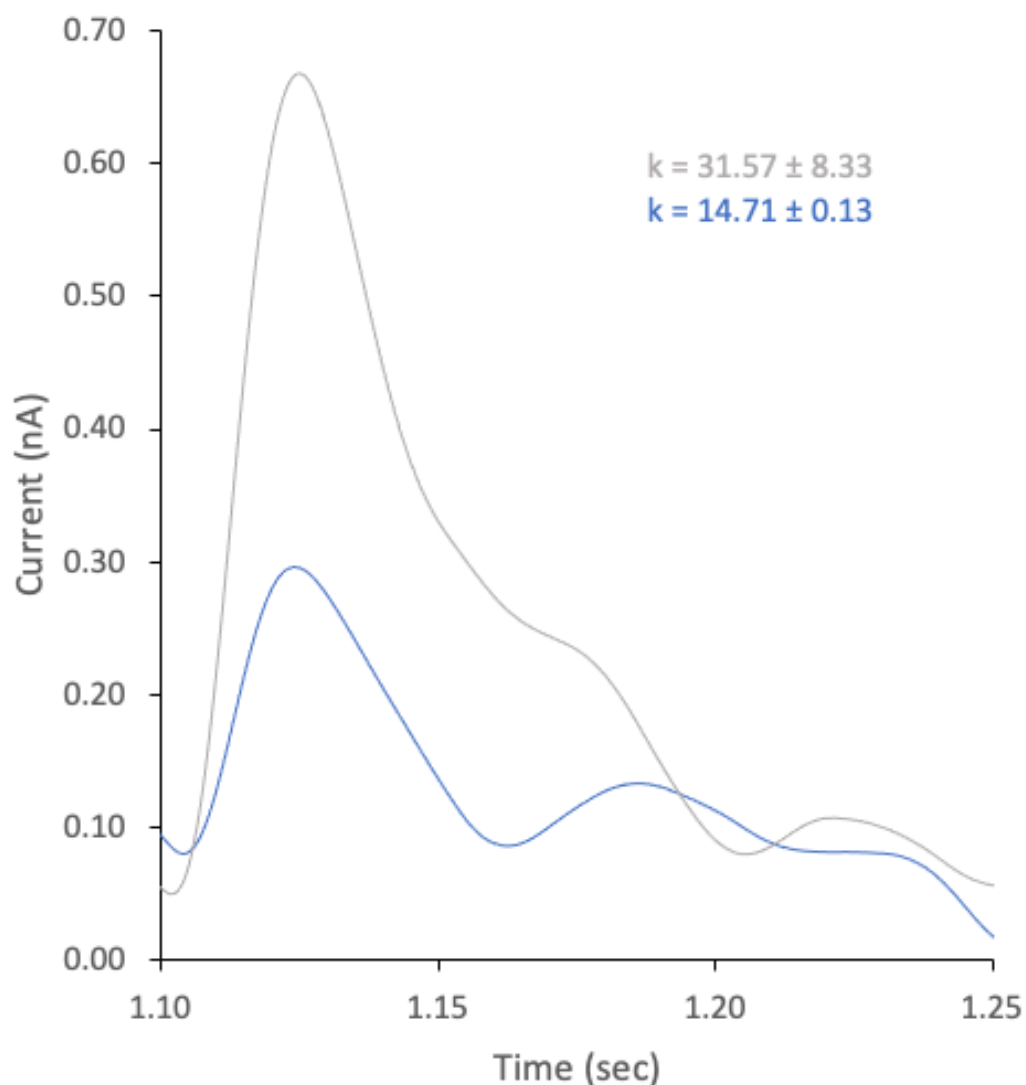


Figure 5.6 **SSME analysis of vesicles produced in the presence of 20 mM NH_4^+ in the culture medium.** SSME analysis of empty vesicles (grey) and AmtB containing vesicles (blue) following a 200 mM NH_4^+ pulse. Both measurements were conducted with no dilution of the vesicles. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.1.7 Quality of vesicles

Vesicle size is an essential parameter in electrophysiological measurements, as larger vesicles accumulate charge at a different rate compared to smaller vesicles. First, we measured the sizes of the vesicles containing AmtB and the empty vesicles using dynamic light scattering (DLS) to ensure that their sizes were similar. DLS measures the Brownian motion of liposomes in solution by analysing fluctuations in light scattering intensity at defined detection angles. An autocorrelation function is applied to the resulting signal to extract the particle size distribution (pdi). This is a crucial factor for accurately comparing ammonium flux in these vesicles. The sizes of the vesicles containing AmtB differed to empty vesicles, and both were significantly larger than the liposomes used in reconstitution, (~440 nm, ~ 530 nm and ~100 nm respectively) (Figure 5.7). The pdi value was also high for these samples. The pdi sits on a scale of 0-1 and refers to the range of sizes present in a given sample, 0 being the most uniformly sized and 1 being the least. AmtB containing vesicles had a pdi of 0.42 and Empty 0.34. These values show that along with the size being large, the range of sizes present are also large making the samples even less reliable. Artificial liposomes are extruded through a 100nm filter to give a uniform size, so an attempt was made to extrude the vesicles. It proved impossible to force the sample through the 100nm filter potentially owing to cellular debris in the sample compared to the artificial liposomes. This was not improved with dilution of the sample. As previously said, when C43 cells overexpress membrane proteins they can become invaginated and the membrane loses integrity possibly explaining the larger size of the vesicles (Wagner *et al.*, 2007).

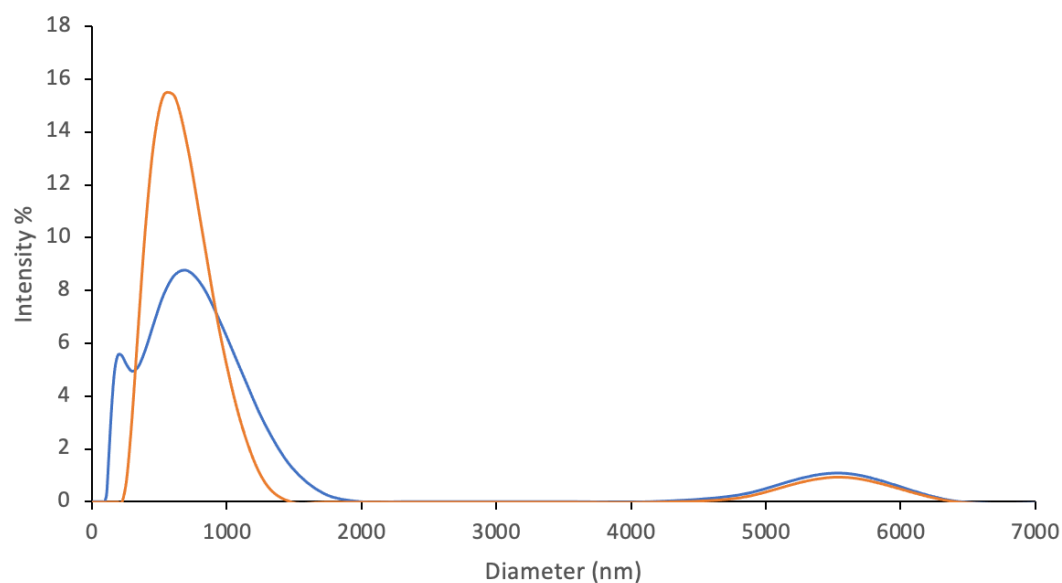


Figure 5.7 **Size of C43 vesicles as measured by DLS.** AmtB (blue) and empty (orange) vesicles were diluted 50x and subjected to dynamic light scattering analysis to find their size.

5.3.2 AmtB Expression in GT1000 strain

The issues encountered thus far appear to be less likely due to heterologous protein expression itself and may instead be related to possible expression of native AmtB in the negative control even after suppression. Given the unsuitability of the *E. coli* C43 strain, it was decided to switch to the GT1000 strain. GT1000 is an *E. coli* $\Delta amtB$ *glnK* strain, developed in the early 2000s to study ammonium transporters (Coutts and Thomas, 2002). Critically, this strain does not express endogenous *amtB* and carries a $\Delta glnK$ background, thereby preventing GlnK-mediated inhibition of AmtB activity (Javelle *et al.*, 2004).

To express AmtB in GT1000, we utilised the plasmids pJT5 and pJT6E, both of which contain the *amtB* and *glnK* genes on the native promoter. AmtB on pJT6E is a WT variant with a 6-histidine tag fused to its C-terminal tail (Javelle *et al.*, 2004). AmtB-6 His tag protein has been shown to be fully active *in vivo* but the non-His-tag version was included for comparison (Javelle *et al.*, 2005). The promoter is induced in minimal media containing only 0.2 mg/mL of glutamine as the sole nitrogen source. These conditions address two key issues: 1) the absence of native AmtB in the GT1000 strain, and 2) the ability to express the protein under its native promoter, thereby circumventing the complications associated with excessive overexpression in C43 cells using a T7 promoter (Figure 5.8). The pACYC184 vector that pJT is based on is a low copy number vector compared to the high copy number pET22b vector meaning expression will be lower.

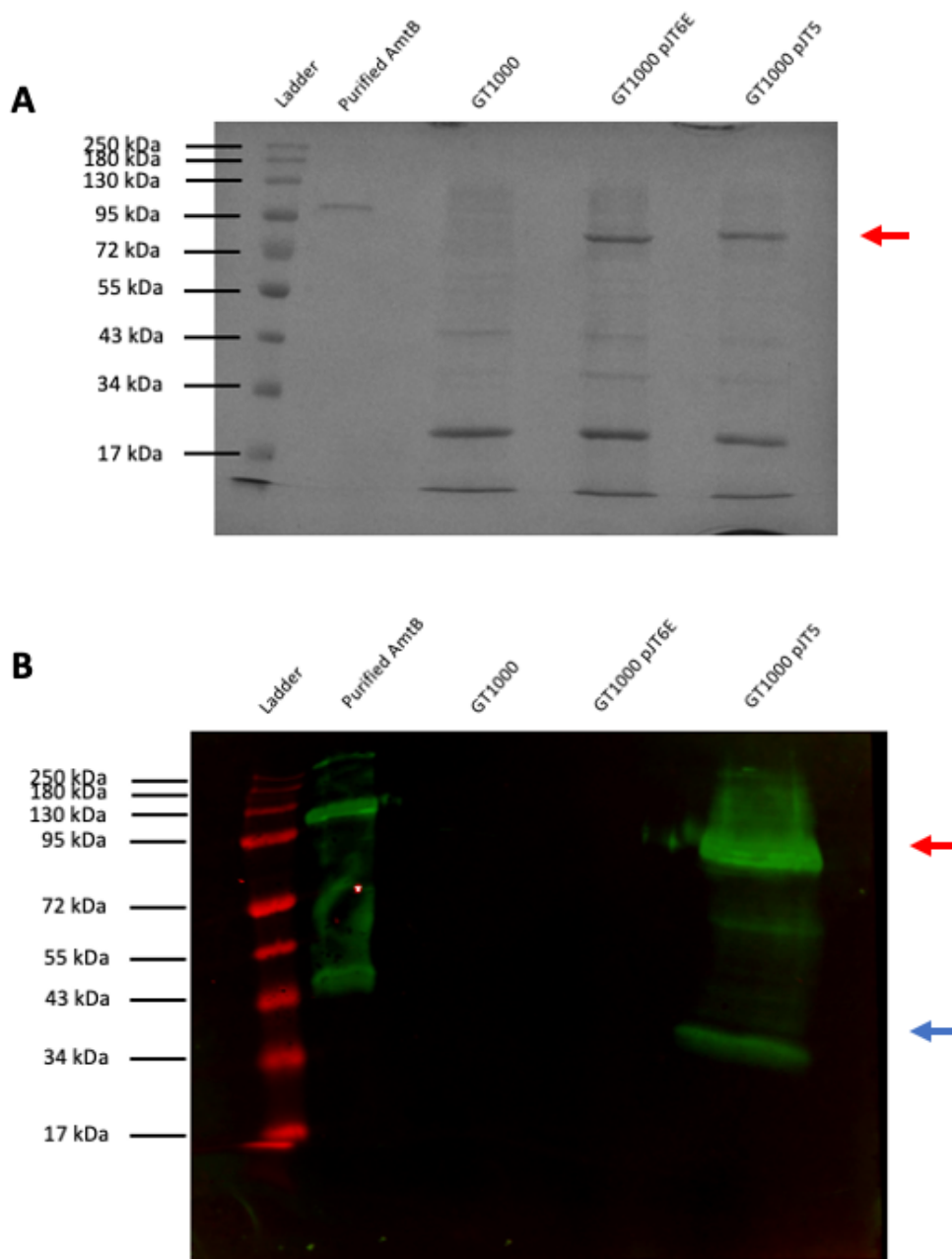


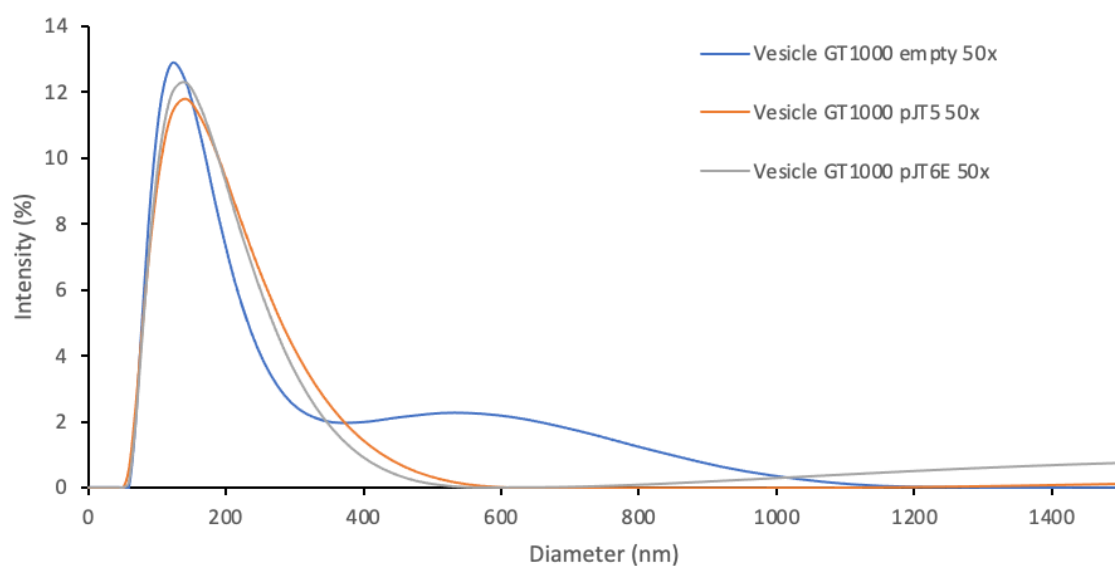
Figure 5.8 **SDS-PAGE and Western blot analysis of expression of AmtB using a pJT vector in GT1000 *E. coli* cells.** Cells expressing AmtB (GT1000 pJT6E, GT1000 pJT5) and untransformed cells (GT1000) were lysed under hypotonic conditions, and resealed vesicles were collected by ultracentrifugation. Vesicle samples were analysed by SDS–PAGE (20 μ L) (A) and immunoblotting (15 μ L) (B) to assess the presence of AmtB. Purified His-tagged AmtB reconstituted into liposomes was included as a positive control. Red arrows indicate the expected position of trimeric AmtB, and blue arrows indicate monomeric AmtB.

5.3.2.1 Size of GT1000 vesicles

In order to check the quality before measurements, the sizes of these vesicles were again measured using DLS with a Zetasizer machine. The diameter was found to be both more uniform and much smaller than C43 vesicles (Figure 5.9A). With empty being 169.1nm (0.36pdi), pJT5 being 148nm (0.23 pdi) and pJT6E being 157nm (0.23 pdi). The relatively large pdi values may be explained by the presence of cellular debris in the sample. An attempt was made to extrude the vesicles to achieve more uniformity between the empty and AmtB containing samples but again it proved impossible to force the sample through the filter. This problem continued even when the sample was diluted. However, the sizes between samples were close enough, therefore suitable for SSME measurements.

Firstly, since vesicle fusion and/or lysis frequently occur after freeze/thaw cycles, leading to the formation of multilamellar or enlarged vesicles, those that had been previously thawed once or twice were thawed again, sonicated, and their size was measured at a 50x dilution using DLS (Figure 5.9B). The vesicle sizes were broadly comparable to those of freshly prepared vesicles. For pJT5 vesicles, the mean diameter was 148 nm after the first thaw, 157 nm after the second, and 156 nm after the third. Empty vesicles showed mean diameters of 169 nm after the first thaw and 184 nm after the second. Although the average size of the empty vesicles did not differ substantially between thaw cycles, their size distribution changed markedly, with a broad population extending from 200 to 800 nm (Figure 5.9B). This may reflect variability in sonication, resulting in a fraction of vesicles that were not fully unilamellar. Nevertheless, efforts were made to maintain consistent conditions across batches, with each sample subjected to two 10 s rounds of sonication separated by a cooling period on ice to prevent overheating.

A



B

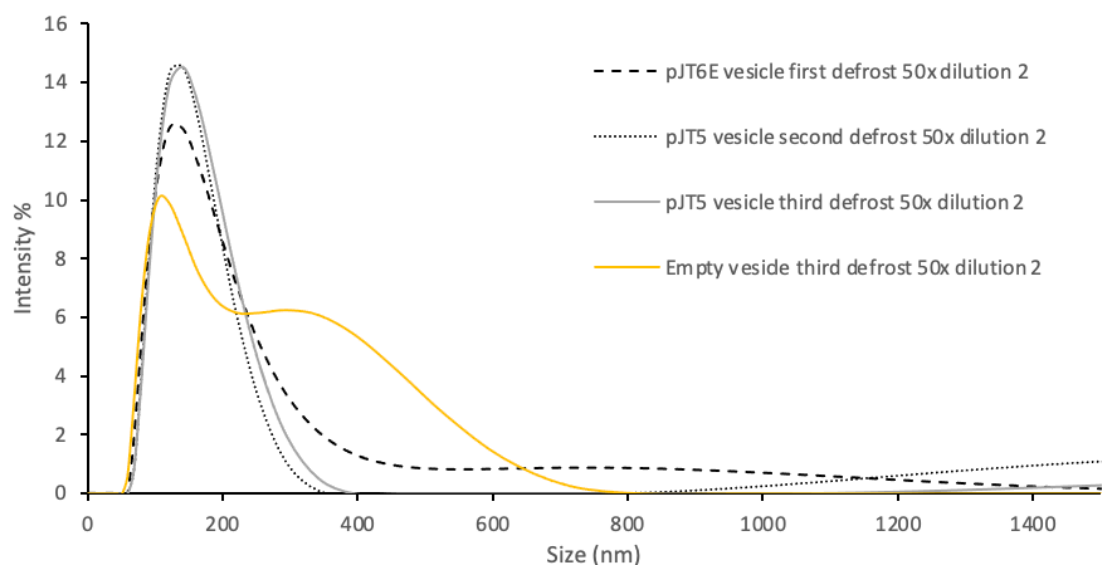


Figure 5.9 Sizes of vesicles produced from GT1000. A) GT1000 vesicles from cells expressing (pJT5 and pJT6E) and not expressing (empty) AmtB were sonicated, diluted 50x and had their size measured using DLS. Samples chosen had not been defrosted or sonicated before. B) Samples of GT1000 vesicles from cells that expressed (pJT5 and pJT6E) or did not express (empty) AmtB were defrosted and sonicated then had their sizes measured using DLS. These samples had been defrosted and sonicated on previous occasions as noted on the figure.

5.3.2.2 *Quality of vesicles*

The parental *E. coli* K-12 strain from which GT1000 is derived contains four potassium transport systems: TrkH, TrkG, KdpA, and Kup. Trk-mediated transport depends on the proton motive force, KdpA requires ATP hydrolysis, and Kup is induced under osmotic or acidic stress (Epstein, 2003). As no such stress conditions were present during *E. coli* growth, and neither ATP nor a membrane potential was present during the SSME measurements, potassium transport activity would not be expected. Therefore, provided the vesicles were properly sealed, potassium leakage should not be observed. Therefore, to check that the vesicles did not leak, their potassium permeability was used. Activating (A) and non-activating (NA) solutions were made using sodium phosphate salts rather than potassium phosphate with 200 mM K⁺ used as the substrate in activating solution (Table 2.8). Sensors were prepared using NA solution and thus were equilibrated to these conditions. Following a pulse of A solution containing 200 mM K⁺ there was no peak recorded for both pJT5 and empty vesicles suggesting that there is both no translocation of K⁺ into the vesicles or leak through gaps, representative traces that are close to the average were chosen for display (Figure 5.10, Figure 9.5). This suggests that the vesicles are sealed correctly and not leaking.

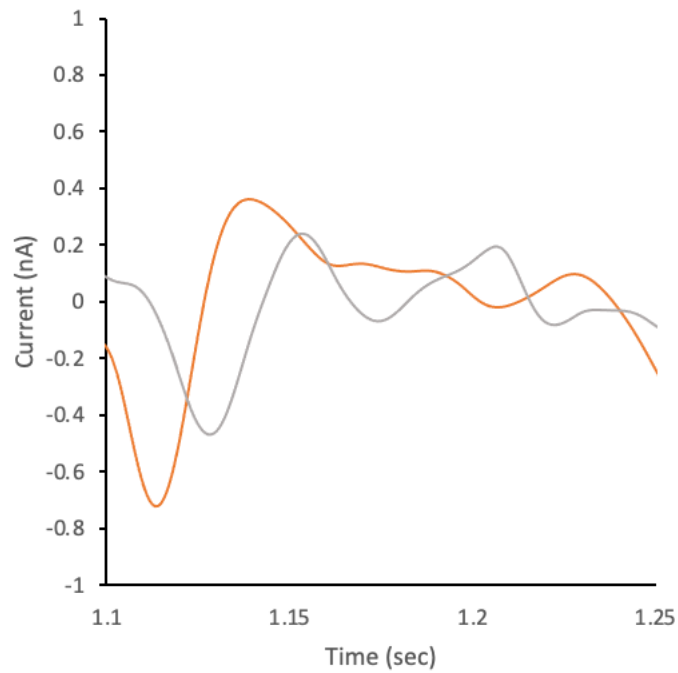


Figure 5.10 **SSME analysis to determine potassium leakage in vesicles.** Vesicles made from GT1000 cells expressing AmtB (orange) and empty (grey) were diluted 2x and measured using SSME. The sensors were pulse with 200 mM K^+ . Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.2.3 Initial measurement of GT1000 vesicles

Since these vesicles were found to likely not leak K^+ , sensors were prepared with a vesicle dilution of 2x. A pulse of 200 mM NH_4^+ or MeA triggered a transient current of 1.1 and 1.2 nA respectively, whereas a smaller current of 0.4 was measured in empty vesicles, traces that were close to the average maximum amplitude were chosen as representatives (Figure 5.11, Figure 9.6 for NH_4^+ , Figure 9.7 for MeA).

To further characterise our assay, we determined the transport kinetics. The transient currents were measured in vesicles contain AmtB after ammonium pulses ranging from 3.125 to 100 mM (Figure 5.12A). The peak currents started to saturate between ammonium pulses of 50-100 mM. The data were then fitted according to the Michaelis-Menten equation, and a K_m of 6.5 ± 4.5 mM and a V_{max} of 1.6 ± 0.3 nA was calculated. This compares to the kinetics of empty vesicles which did not display a pattern (Figure 5.12B)

All these results are promising; however, two issues need to be addressed: 1) the empty GT1000 vesicles still produced a peak, and 2) the calculated K_m is eight times higher than the value obtained from artificial liposomes containing purified AmtB (Mirandela *et al.*, 2019), suggesting that further optimisation is required.

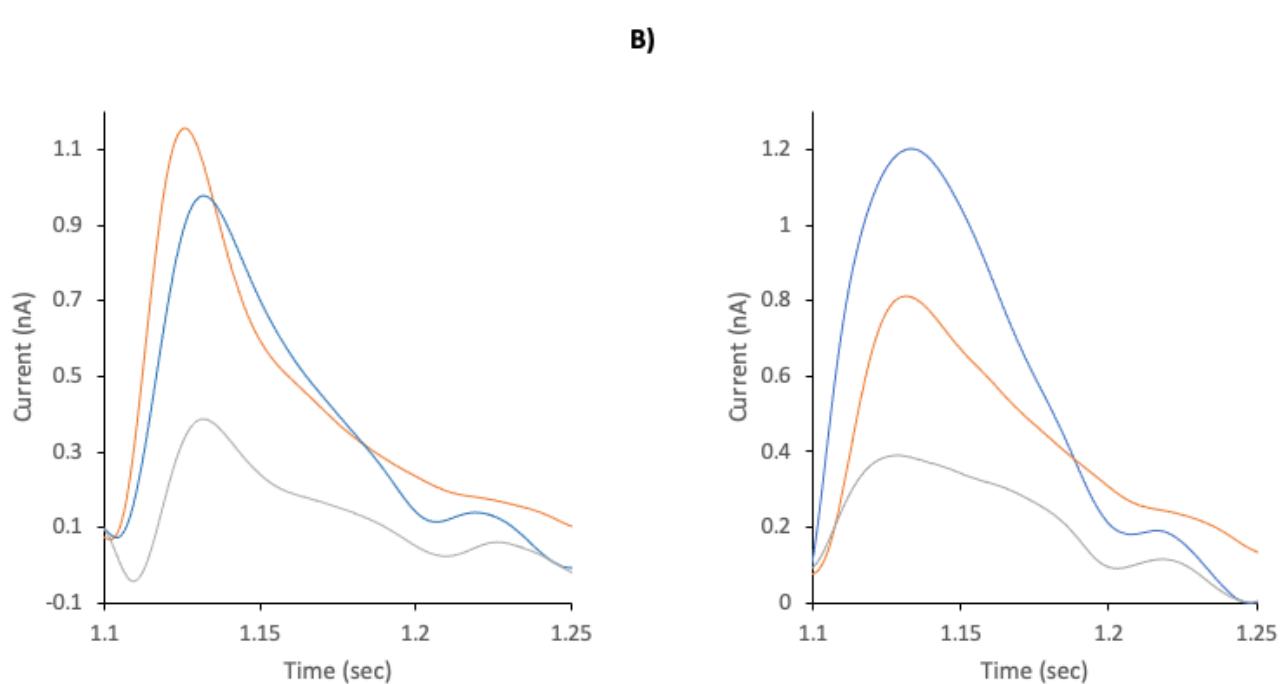


Figure 5.11 **SSME analysis of vesicles created from GT1000 cells.** A) Vesicles containing AmtB (pJT5 (orange), pJT6E (blue)) and empty (grey) were diluted 2x, affixed to sensors and pulsed with 200 mM NH_4^+ . B) The same sensors were pulsed with 200 mM MeA. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

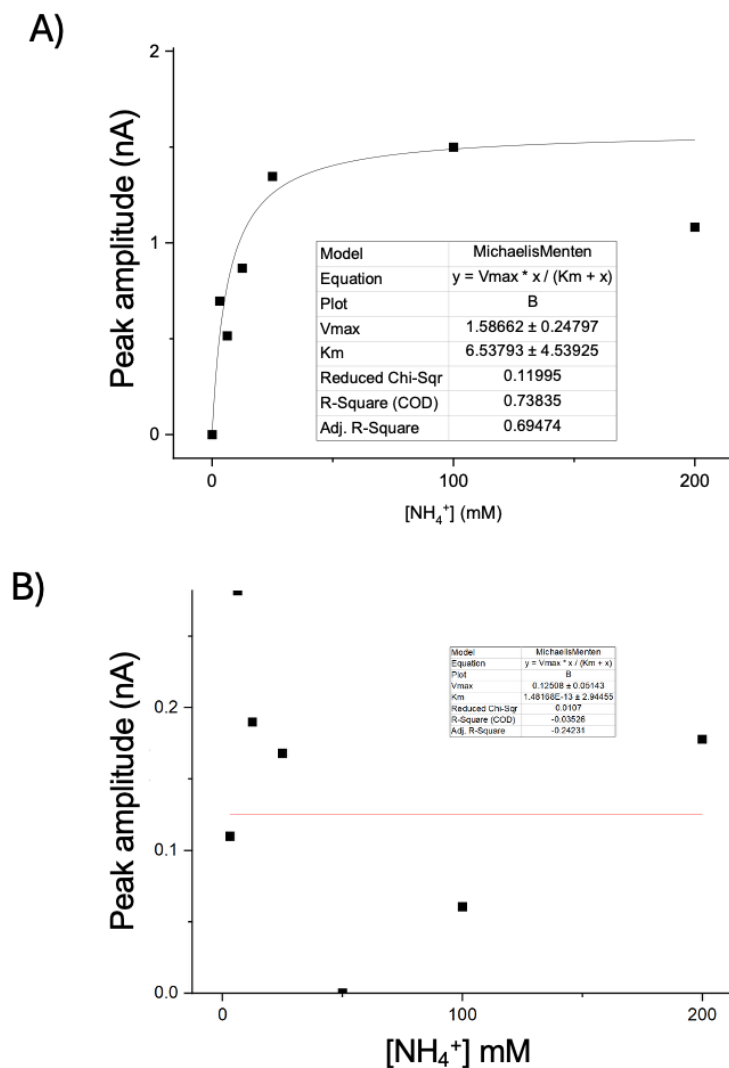


Figure 5.12 **Ammonium transport kinetics measured in vesicles.** The maximum amplitude recorded following an ammonium pulse in a range of 3.125 to 200 mM were measured and the data were fitted according to the Michaelis-Menten equation using Origin software. A) vesicles containing AmtB from pJT5 plasmid. B) Empty vesicles.

5.3.3 Optimisation

5.3.3.1 *Dilution of vesicles*

To try to reduce the size of the peak produced by empty vesicle following an ammonium or MeA pulse, the vesicles were diluted further. Dilution may help to decrease the background noise further until only the signal from AmtB is detected. The dilutions chosen were 10, 50, 100 and 1000, to replicate the dilutions we used in proteoliposome measurements. After pulsing with 200 mM NH_4^+ (Figure 5.13, Figure 9.8 for full trace, Figure 9.9 for expanded view) and 200 mM MeA (Figure 5.14, Figure 9.10 for full trace, Figure 9.11 for expanded view) the traces obtained did not show a correlation with dilution or were not found to be reproducible with empty vesicles generating the same sized peaks as those from AmtB vesicles. This could mean that the buffer rather than the vesicle sample needs to be optimised. A concentration of 200 mM NH_4^+ may be too high for the pulse, so that other transporters/channels translocates ammonium unspecifically.

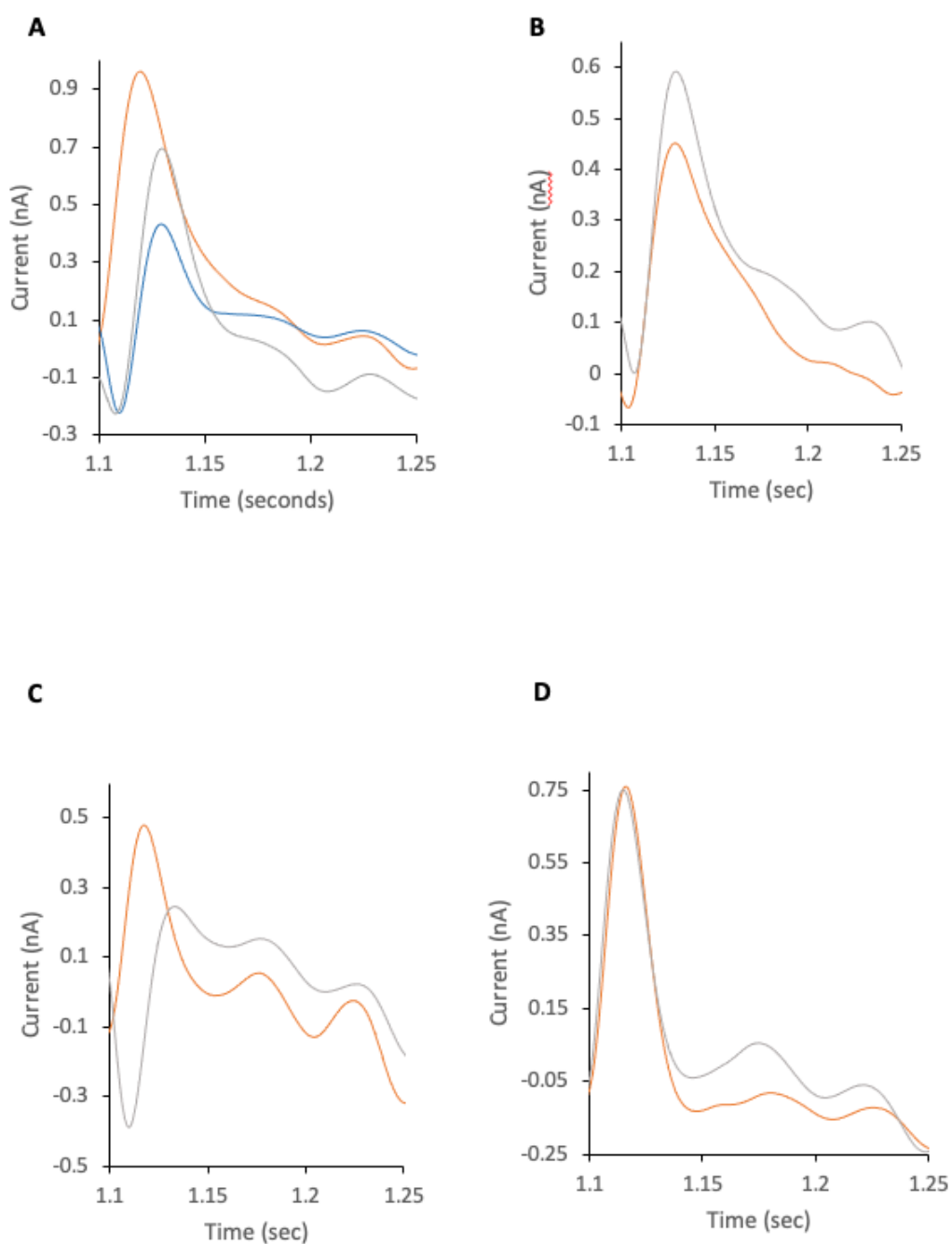


Figure 5.13 **Transport of NH_4^+ in diluted vesicle samples.** Samples of vesicles containing AmtB (pJT5 (orange) and pJT6E (blue)) and empty (grey) were diluted 10x (A), 50x (B), 100x (C) and 1000x (D) and pulsed with 200 mM NH_4^+ . Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

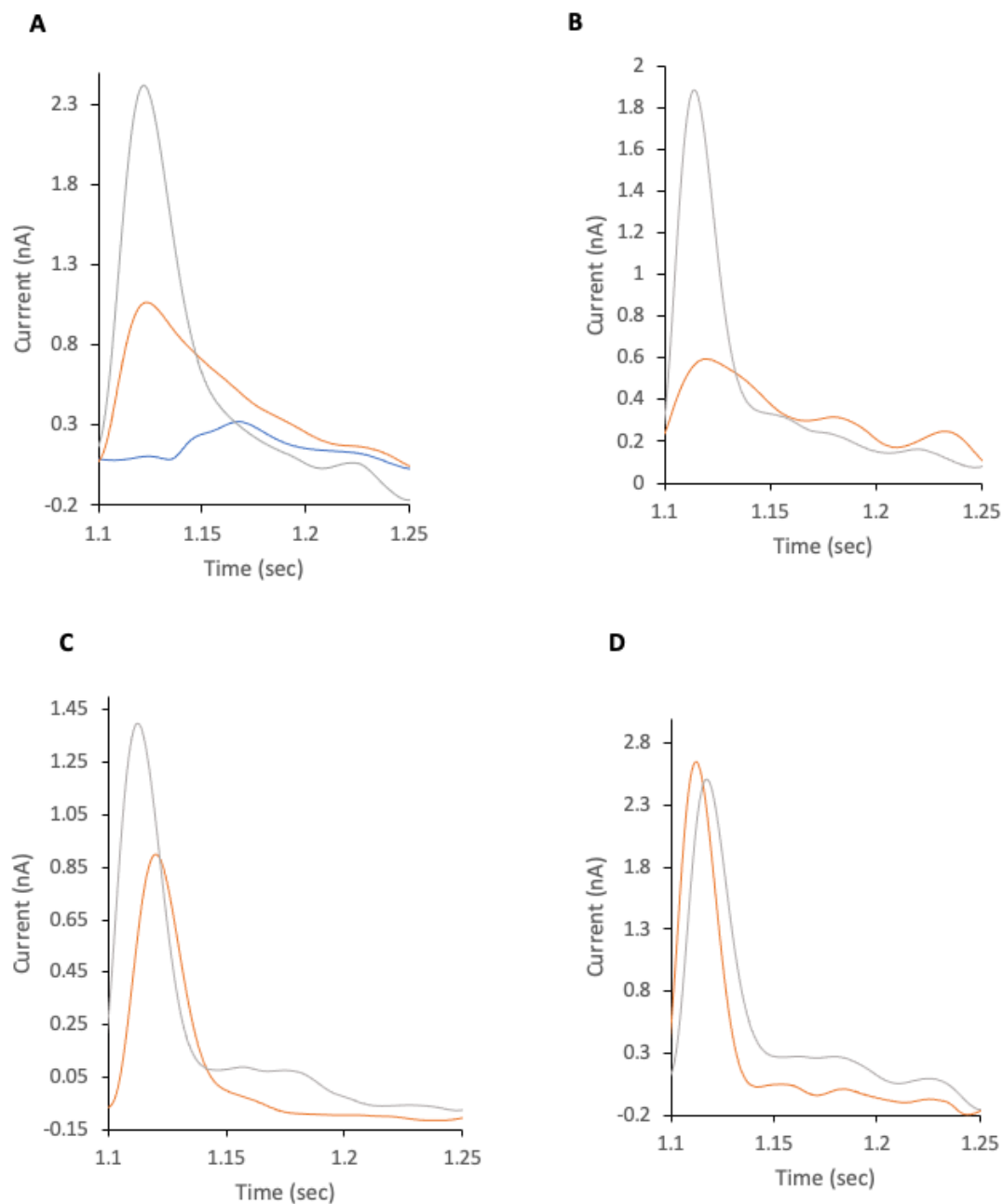


Figure 5.14 **Transport of MeA in diluted vesicle samples.** Samples of vesicles containing AmtB (pJT5 (orange) and pJT6E (blue)) and empty (grey) were diluted 10x (A), 50x (B), 100x (C) and 1000x (D) and pulsed with 200 mM MeA. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.3.2 NH_4^+ in a Sodium Phosphate buffer background

The ions used in the buffer to ensure osmotic equivalence between the NA and A solutions have a crucial influence on the measurements (Bazzone *et al.*, 2013; Bazzone, Barthmes and Fendler, 2017). Therefore, we tested whether substituting K^+ with Na^+ would improve the assay. The A and NA solutions were prepared with a NaPO instead of KPO buffer. Traces from this experiment showed a reaction to a 200 mM ammonium pulse in empty and pJT5 vesicles but not one corresponding to transport (Figure 5.15, Figure 9.12). The reaction may have been due to interaction of ammonium with the membrane, but the shape of the peaks makes it impossible to determine a decay value to investigate this further. This shows that the presence of a sodium phosphate buffer in native vesicles impacts the AmtB activity.

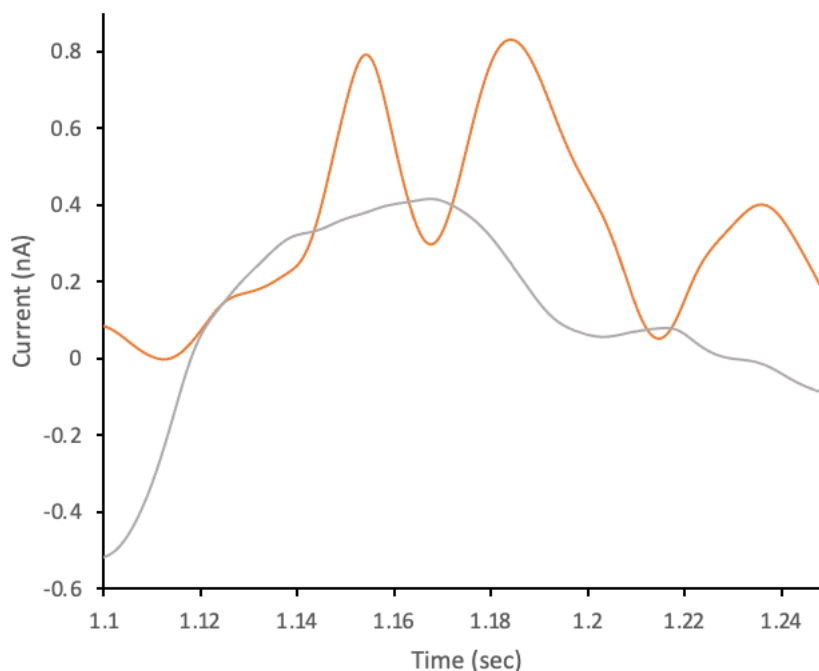


Figure 5.15 **SSME analysis of NH_4^+ transport in vesicles using a sodium phosphate buffer.** Representative traces showing sensors pulsed with 200 mM NH_4^+ in a sodium phosphate buffer background as opposed to potassium phosphate for samples of AmtB containing vesicles (orange) and empty (grey). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.3.3 *Non-activating + substrate*

In standard SSME measurements, it is crucial to balance the ionic concentrations of both the activating and non-activating solutions. This ensures that when the sensors are pulsed with the activating solution, the ion concentration remains constant, with the only variable being the presence or absence of the substrate. As a result, the measured activity can be confidently correlated with substrate presence rather than an increase in positive ion concentration. However, due to the unexpected activity observed in the empty vesicles when using conventional buffers, a new strategy was implemented. In this approach, ammonium was directly added to the NA solution to serve as the A solution. This meant that rather than having a balanced ion composition as before, an imbalance was deliberately generated. A 200 mM ammonium pulse triggered peaks with similar amplitude for both empty and pJT5 vesicles (Figure 5.16, Figure 9.13). The average decay values were higher for empty vesicles but the standard deviation for each condition made this insignificant. This suggests that ammonium is interacting with the membrane or with proteins in the membrane of both vesicle conditions.

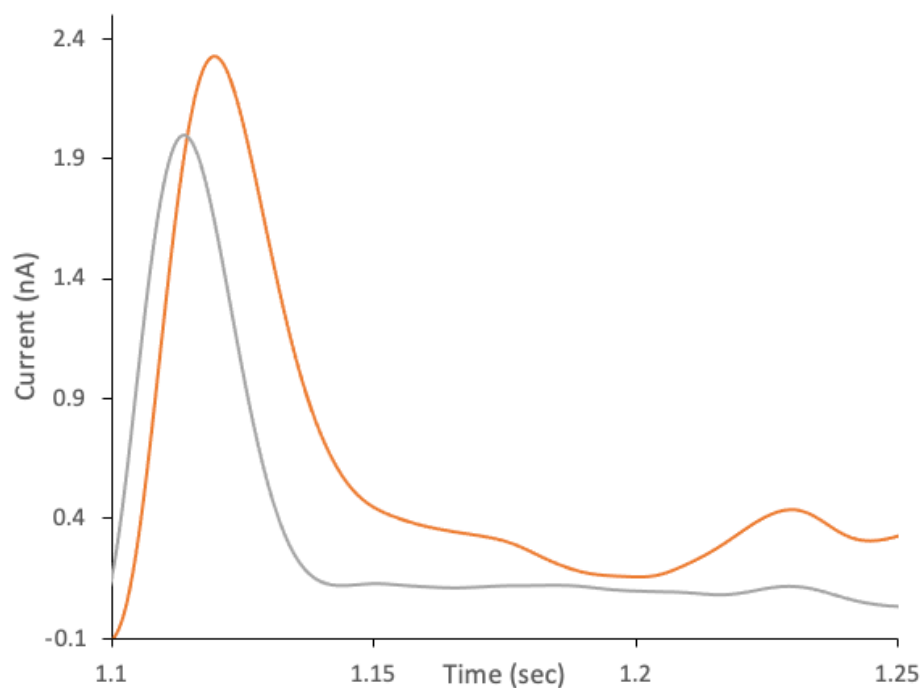


Figure 5.16 **Representative traces of GT1000 vesicles following a pulse of imbalanced A and NA solutions.** pJT5 (orange) and empty (grey) vesicles were diluted 2x and affixed to sensors. These were pulsed with non-activating solution +200 mM ammonium which served as the activating solution. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.3.4 *Tris Buffer*

In order to avoid potassium buffers which, produce a peak after an ammonium pulse in empty vesicles and sodium buffers which reduce the amplitude of the peak it was decided to use Tris salts to replace the phosphate buffer. In this instance vesicles were pulsed with an activating solution of non-activating tris buffer pH 7 with an added 200 mM or 100 mM ammonium as in the previous experiment. Unfortunately, peaks of similar amplitude were observed after ammonium pulses of 100 mM and 200 mM in both empty and AmtB-containing vesicles (Figure 5.17, Figure 9.14 for full trace, Figure 9.15 for expanded trace). The decay values were significantly lower in both AmtB and empty vesicles pulsed with 100 mM ammonium compared to those pulsed with 200 mM ammonium.

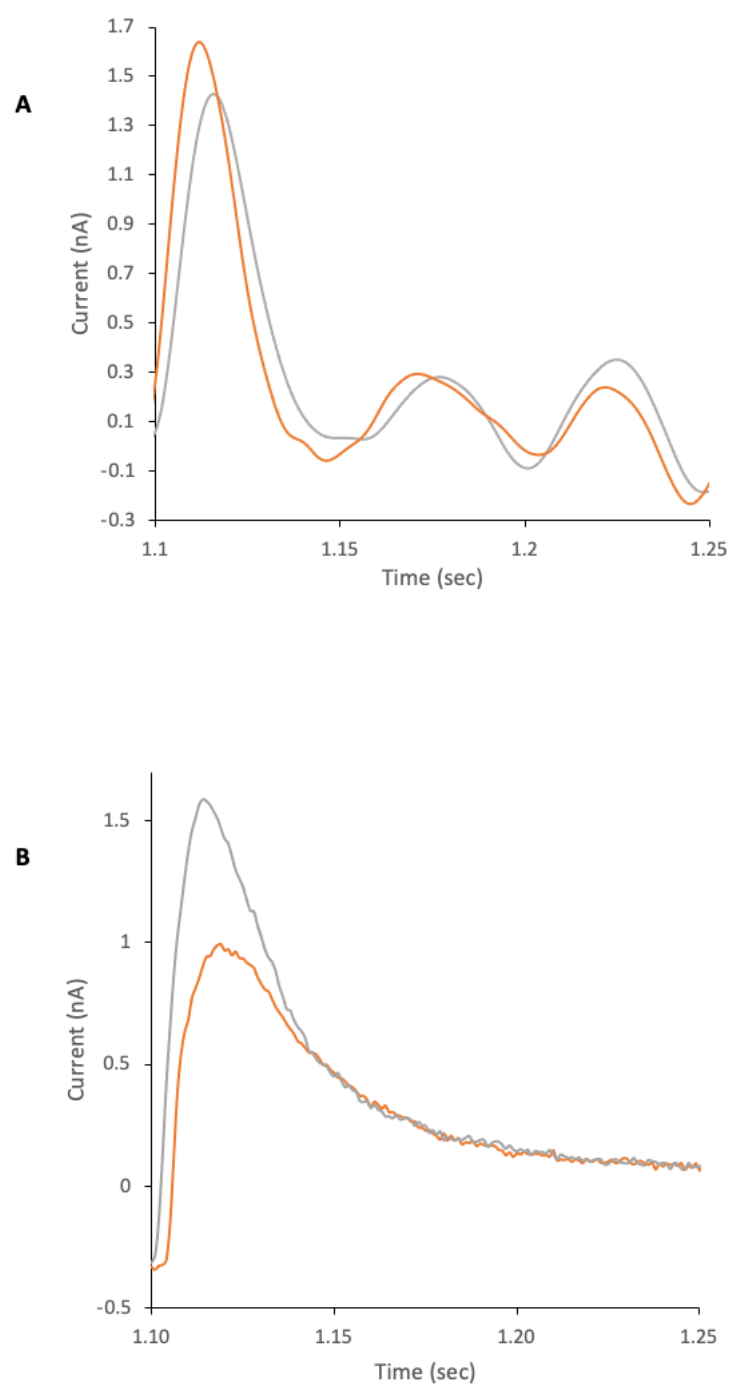


Figure 5.17 **GT1000 vesicle measurements using tris buffer background.** pJT5 (orange) and empty (grey) vesicles were diluted 2x with a NA tris buffer, affixed to sensors and left to equilibrate. These were pulsed with A) 200 mM NH_4^+ in a tris buffer and B) 100 mM NH_4^+ in a tris buffer. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.3.3.5 Wacker solutions

In 2014, Wacker *et al* published the first electrophysiological measurements of the archaeal Amt1 protein from *A. fulgidus* using the SSME method (Wacker *et al.*, 2014). The composition of the buffer in their measurement was 100 mM Tris, 500 mM KCl and either 300 mM NaCl for non-activating or 300 mM ammonium for activating at pH 8. This uses the same concentration of buffer as we do but much higher salt concentrations. There was no evidence of a peak indicative of transport following a transient pulse of 300 mM ammonium in both the empty and AmtB-containing vesicles (Figure 5.18, Figure 9.16).

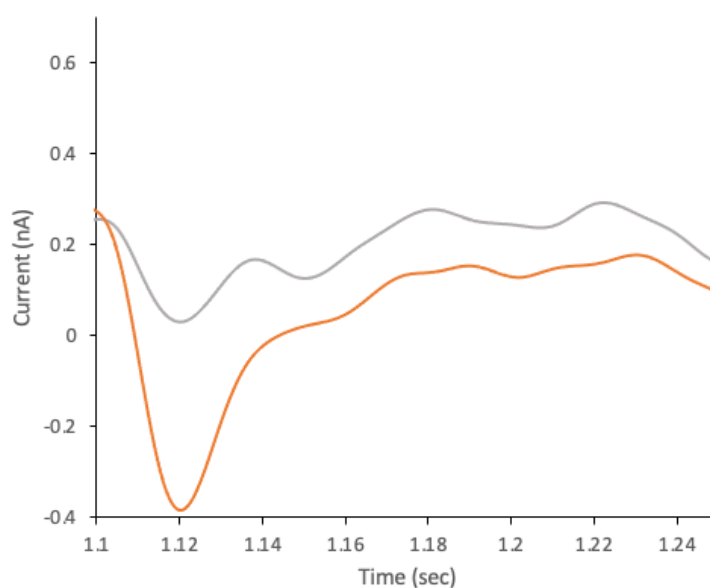


Figure 5.18 **GT1000 vesicle measurements using the NA - A buffers from Wacker *et al* 2014.** Sensors were prepared with either pJT5 vesicles (orange) or empty vesicles (grey) using NA buffer composed of 100 mM Tris, 500 mM KCl and 300 mM NaCl. These were pulsed with the activating buffer which differed in that the 300 mM NaCl swapped with 300 mM ammonium. These buffers were both at pH 8. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

5.4 Discussion

This work is not the first time vesicles have been prepared from GT1000 cells to investigate ammonium transporters. In the original study by Khademi *et al.*, (2004), the authors proposed that AmtB functions as an NH_3 channel, based on the observed alkalinisation inside proteoliposomes containing purified EcAmtB following an ammonium pulse (Khademi *et al.*, 2004). In a subsequent study, Javelle and colleagues attempted to reproduce the results of Khademi *et al.*, (2004), but used vesicles derived from the GT1000 strain instead of proteoliposomes containing purified AmtB, as in the original study (Javelle *et al.*, 2007). Despite extensive troubleshooting, Javelle and colleagues were unable to measure internal pH changes following a 25 mM ammonium pulse using stopped-flow spectrofluorometry, raising concerns about the validity of the results obtained by Khademi *et al.* Furthermore, their work did not report any acidification, which would have indicated NH_4^+ transport. In this chapter, I present data showing there is the potential following further optimisation to create an assay to measure the activity of AmtB in vesicles of this nature (Figure 5.11). The ammonium transport kinetics in AmtB-containing vesicles exhibit a saturable transport system, with a K_m value within the range observed for proteoliposomes containing purified AmtB (Williamson *et al.*, 2020). However, the assay requires further optimisation, as a residual signal was also detected in empty vesicles following ammonium pulses. Further dilution of the vesicles did not yield a good result and contrary to expectations the peaks of the traces did not decrease in the highest dilutions. This may be due to the following reasons:

- The concentration of AmtB in the vesicle samples is lower than that in the liposomes used previously (Mirandela *et al.*, 2019), as we adjusted the total protein concentration to 5 mg/mL, which included other native membrane proteins. However, it is important to note that this concentration is higher compared to similar studies aimed at characterising other transporters in vesicles (Schulz *et al.*, 2010; Bazzone *et al.*, 2022; Körner *et al.*, 2024). Increased dilution could therefore have led to too low transport to be measured, and the traces seen are background noise affecting both empty and AmtB containing vesicles. I did not control for *amtB* expression in this chapter and each biological replicate may have had different levels of the protein in the membrane.

- The peaks measured in the empty vesicles increased with dilution, suggesting a potential issue with the vesicles themselves. Investigations into the stability of spheroplast membranes have shown that the buffer in which they are stored can lead to increased leakage of proteins, RNA, and DNA (Bodmani and Welker, 1969). Given that larger molecules such as proteins, DNA, and RNA are affected by the buffer, it is likely that smaller ions like NH_4^+ could also be influenced. Therefore, optimising the buffer for vesicle storage is crucial.
- This investigation lacks a critical control in that the GT1000 cells forming the empty vesicles were not transformed with any empty vector or inactive transporter, for example the H318A mutant (Javelle *et al.*, 2007). This means that there was no control to see whether the antibiotic used during expression or the insertion of the protein may have affected the permeability of the membrane. This should be taken forward with any future work.
- Although other studies characterising bacterial Amt transporters using SSME have employed ammonium pulses up to 300 mM (Pflüger *et al.*, 2018; Pflüger *et al.*, 2024; Wacker *et al.*, 2014), the 200 mM pulse used in our study may be too high, generating an electrochemical gradient strong enough to drive the unspecific translocation of ammonium by other membrane proteins. Given that the K_m value is measured in the mM range, lower concentrations of ammonium or alternative substrate analogues (such as methylamine or hydrazine) should be tested.

5.4.1 Other proteins in the membrane

Since the vesicles are being created directly from the cells used for overexpression, there will be other native proteins present alongside AmtB. This is different to preparations of artificial liposomes where the only protein present is the purified proteins that is inserted. For this reason, the effects these native membrane proteins may have on the measurements in this chapter have been considered.

5.4.1.1 Aquaporins

Aquaporins are a family of proteins found in the membrane and are responsible for the selective passive transport of water when there is a need for rapid transfer, for example in tear production. *E. coli* expresses two aquaporins, GlpF and AqpZ. Of these the narrower pore in AqpZ makes it more selective for water while the wider channel in

GlpF allows it to facilitate the passage of glycerol and other molecules (Borgnia *et al.*, 1999; Savage *et al.*, 2003). In mammals there are thirteen aquaporin families and while they are highly selective for water AQP3, 7, 9, 10 also transport other small molecules like glycerol, urea and ammonia (Wang, Solenov and Yang, 2023). Ammonia and water have a very similar molecular volume of 24.9cm³/mol and 18cm³/mol respectively meaning selectivity against one or the other is difficult (Nakhoul *et al.*, 2001). The transport of ammonia through AQP1 and AQP4 has been experimentally proven through observation of an alkalinisation in oocytes expressing aquaporins when pulsed with 20mM NH₄Cl (Nakhoul *et al.*, 2001; Assentoft *et al.*, 2016). Importantly it was found that aquaporins are not permeable to the ammonium ion with no acidification being detected in the oocytes following pulses. In opposition to this, experiments on ghosts made from red blood cells expressing RhAG and AQP1 from *H. sapiens* and *M. musculus* found that it is unlikely that the aquaporins are contributing to NH₃ transport since blocking their action did not decrease transport rates (Ripoche *et al.*, 2006). Only 20mM ammonium was used for the experiment however, 10x less than our working concentration. The 200mM ammonium we use for SSME may be too high and the ability of the aquaporin to be selective against NH₄⁺ may be decreased, thus forcing ammonium to pass through the pore of the *E. coli* aquaporin GlpF.

A possible way to counteract aquaporin interference is to make a $\Delta amtB glnK aqpZ glpF$ *E. coli* strain or to use an aquaporin inhibitor. There are three classes of aquaporin inhibitors: metal related, ammonium salts and small molecules.

- Metal inhibitors for example HgCl₂ and AgNO₃ may not be selective enough for aquaporins and therefore interfere with AmtB as it has been found in previous studies on human RhAG (Ripoche *et al.*, 2004; Wang, Solenov and Yang, 2023).
- Tetraethylammonium (TEA) has been shown to selectively inhibit the activity of aquaporins that are more permeable to water, while having no effect on those responsible for glycerol transport (Brooks *et al.*, 1992; Detmers *et al.*, 2006). The question arises as to whether Amt proteins capable of transporting methylammonium could also facilitate the translocation of TEA, thereby rendering this inhibitor ineffective for these experiments. Studies have demonstrated that in the euryarchaeon *Archaeoglobus fulgidus*, the activity

measured using SSME on proteoliposomes containing purified Af-Amt1 significantly decreases by 35% and 90% when pulsed with methylamine or dimethylamine, respectively, compared to ammonium. This reduction in activity is likely attributed to steric hindrance imposed by the larger size of the cargo molecules. Therefore, it is highly unlikely that TEA is translocated by Amt proteins and could be suitable for these experiments (Wacker *et al.*, 2014).

- Small molecules are likely the most specific inhibitors of aquaporins. Many of these inhibitors seem to have been developed primarily to target aquaporins with high water selectivity, rather than those responsible for glycerol transport (Wang, Solenov and Yang, 2023).

5.4.1.2 Potassium channels

As mentioned in section 5.3.2.2 it is unlikely that any potassium channel could translocate ammonium under our experimental conditions; however, this needs further clarification. *E. coli* expresses three potassium channels, the low affinity Trk and Kup proteins and the high affinity KdpFABC protein (Schramke *et al.*, 2017). Some evidence has been found that NH_4^+ is able to travel through potassium channels in plant roots when the ratio of ammonium to potassium was extremely high (Hoopen *et al.*, 2010). If the potassium channels in *E. coli* were transporting ammonium efficiently enough to have an impact on our traces, we would have presumably seen a peak resulting from potassium transport in section 5.3.2.2. The fact that the channels are also highly selective for K^+ (Thompson *et al.*, 2009), it is unlikely that ammonium can be getting through these channels. With this in mind, it has been shown that a single mutation, H168E, in EcAmtB significantly alters the conduction mechanism and AmtB selectivity, converting it from a highly selective ammonium transporter into a non-selective potassium channel (Williamson *et al.*, 2020). Taken together, these observations highlight the strong selectivity of Amt proteins and potassium channels. However, we cannot rule out the possibility that, under our experimental conditions, using a 200 mM gradient ammonium pulse, a potassium channel might be compelled to conduct ammonium.

5.4.1.3 Mechanosensitive proteins

In response to stress *E. coli*'s, MscS and MscL proteins open to dissipate osmotic pressures (Haswell *et al.*, 2011). These have been found to transport Na⁺ and other ions (Cui, Smith and Adler, 1995). Could the pressure induced by the physical pulse of non-activating and activating solutions be causing these proteins to open? It is unlikely for two reasons

- The data: No transport is recorded when the vesicles are pulsed with potassium
- Our experimental design: We ensured that the NA and A solutions were isosmotic to prevent osmotic stress and, consequently, the activation of mechanosensitive channels.

5.4.2 Substrate and buffer

It could be interesting in the context of our assay to use other ammonium analogues that could 1) be exclusively translocated through Amt proteins and 2) are membrane impermeable, such as hydrazine. The transport rates of MeA and hydrazine through RhAG and aquaporins have been studied in red blood cell ghosts (Ripoche *et al.*, 2006). This study found that MeA and hydrazine exhibit very low permeability across membrane lipids and are predominantly translocated via the RhAG for two reasons:

- HgCl₂ treatment, a well-known inhibitor of ion channels and transporters, applied to Rhnull cells (Rh/RhAG⁻, AQP1⁺) that express AQP1 but lack Rh protein, does not reduce the residual hydrazine transport observed in these cells prior to HgCl₂ treatment. In COnull cells (Rh/RhAG⁺, AQP1⁻) expressing Rh but not aquaporin, HgCl₂ treatment reduces hydrazine transport rates to levels comparable to those observed in treated control or Rhnull cells. This suggests that hydrazine transport is primarily mediated by lipids and RhAG, rather than aquaporins.
- Structural considerations regarding the narrow constriction of the AQP1 water channel make it unlikely that molecules such as hydrazine or MeA could be efficiently translocated, as they would (Murata *et al.*, 2000; Sui *et al.*, 2001)(Murata *et al.*, 2000; Sui *et al.*, 2001).

Hence, hydrazine could be the substrate of choice to measure Amt/Rh/Mep activity in cell vesicles.

Unfortunately, altering the constituents of the buffers in our experiment did not lead to improved results. Although no potassium transport was detected (Figure 5.10) when using a sodium phosphate buffer background, no ammonium translocation activity was observed, suggesting that sodium phosphate or NaCl may either reduce AmtB activity or interfere with the transport measurements. Previous studies of the K⁺ transport of AmtB and ScMep2 has found it is only transported by mutants (Williamson *et al.*, 2022). A clear transient current was observed when using a tris buffer; however, the current amplitude in vesicles and empty vesicles was comparable. No transport activity was recorded with the buffers used by Wacker *et al.*, (2014), which also contained NaCl, indicating that NaCl could be limiting the activity (Khademi *et al.*, 2004).

5.4.3 AmtB and GlnK

In *E. coli*, AmtB is inhibited through interaction with its PII protein GlnK, which physically blocks the pore following an ammonium pulse (Javelle *et al.*, 2004). The GT1000 strain is $\Delta amtB \Delta glnK$, and the plasmids used in this study express both AmtB and GlnK. This raises the question of whether culture conditions used to prepare our vesicles could cause GlnK to bind AmtB and thereby inactivate it. The standard procedure we followed, cultivating GT1000 cells expressing both AmtB and GlnK in media supplemented with 20 mg/ml glutamine, is the same as previously used to measure AmtB activity in *E. coli* cells via *in vivo* [¹⁴C]-MeA transport assays (Javelle *et al.*, 2005; Javelle *et al.*, 2006). Therefore, it is highly unlikely that GlnK would inhibit AmtB in our vesicles. However, as an alternative, it may be beneficial to use a plasmid expressing only AmtB under its natural promoter in GT1000 cells to prepare our vesicles.

5.5 Additional future work

To improve the quality of the vesicles, further purification using a sucrose gradient could be employed (Pommereau *et al.*, 2023). This would help eliminate any internal membrane structures that were co-extracted during ultracentrifugation and enhance the uniformity of vesicle size. Additionally, the protein concentration could be adjusted to a higher value.

Styrene Maleic Acid Lipid Particles (SMALPS) may be a further avenue of research. Using styrene and maleic acid which together form a polymer, parts of the membrane can be hole punched out (Knowles *et al.*, 2009). Anything in the membrane of this section is isolated and can be purified further if required. These SMALPs are then able to be reconstituted into liposomes (Dörr *et al.*, 2014; Krajewska and Koprowski, 2021; Catania *et al.*, 2022). This has the benefits as no detergents will be necessary to extract the protein from the native membrane while also being surrounded by its native lipids. Previous work has shown that reconstitution of SMALPs into either planar or liposomal membranes results in a mostly RSO orientation (Li *et al.*, 2015; Mazurenko, Hatzakis and Jeuken, 2019; Catania *et al.*, 2022).

In order to measure the activity of eukaryotic protein it should also be tried to make vesicles expressing eukaryotic proteins from yeast or human embryonic kidney (HEK) cells.

Owing to time constraints of my other chapters, I was not able to fully explore the options available for future work.

Chapter 6: Investigations into the functional importance of the Phe Gate in EcAmtB and NeRh50

6 Chapter 6: Investigations into the functional importance of the Phe Gate in EcAmtB and NeRh50

6.1 introduction

In Chapter 4 section 4.3.3.2, I determined that of the conserved regions of the Amt/Mep/Rh superfamily, the Phe Gate is the only one to be totally conserved in every protein of the superfamily. The Phe Gate is found at the entrance to the pore and is composed of two Phenylalanine residues arranged in a stacked formation that constricts the pathway of any substate trying to transverse the pore (Figure 6.1A). Most crystal structures of the Amt/Mep proteins show the same conformation of Phe residues with the two rings stacked in parallel to each other, blocking the pore (Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005; Javelle *et al.*, 2008; Berg *et al.*, 2016; Pflüger *et al.*, 2018, 2024). The Rhesus proteins have a different conformation of the first Phe in that it is turned 90° so that it lies perpendicular to the second (Figure 6.1B) (Lupo *et al.*, 2007; Gruswitz *et al.*, 2010). Based on the fact that the pore is blocked by these rings and that there is space around the gate, it has been postulated that the Phe Gate is dynamic and moves during translocation to allow NH₃ or NH₄⁺ into the pore. Movement of the gate has been seen in MD simulations (Nygaard *et al.*, 2006; Yang *et al.*, 2007; Akgun and Khademi., 2011) and in only one crystal structure where in EcAmtB the F107A variant caused the second F215 to be perpendicular to its usual conformation giving a hint at what an 'open' gate may look like (Figure 6.2) (Javelle *et al.*, 2008).

The different Phe Gate conformation seen in the Rhesus proteins may be due to the fact that the protein must accommodate bidirectional transport while as far as we know, the other proteins in the superfamily are either unidirectional or do not transport. If this is the case, the fact that the rhesus proteins adapted the conformation of the Phe Gate rather than substituted the amino acid seems to be evidence of its integral role.

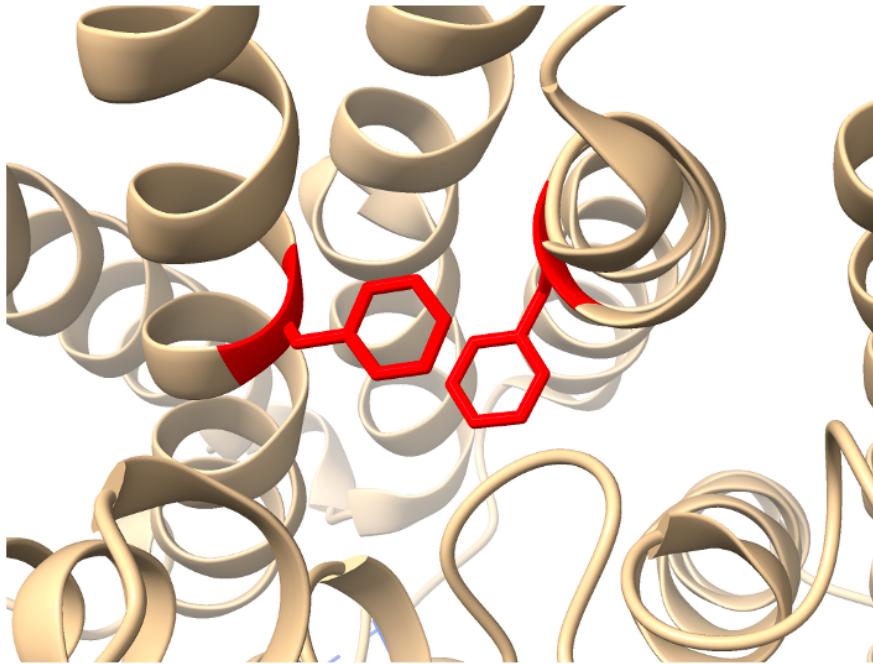
6.2 Aims

To briefly summarise, the Phe gate is highly conserved across the Amt/Mep/Rh superfamily, yet it represents a rare site of structural divergence between Amt/Mep and Rh proteins. This highlights the need for experimental investigation into its specific role in the ammonium conduction mechanism in both protein families to assess whether it fulfils a conserved function throughout the superfamily. For these reasons,

the function of the Phe Gate in EcAmtB and NeRh50 will be explored in this chapter using SSME. The objectives are as follows:

- Create Phe Gate mutants
- Express, purify and reconstitute them into liposomes
- Measure their response to NH_4^+ and K^+ using SSME

A



B

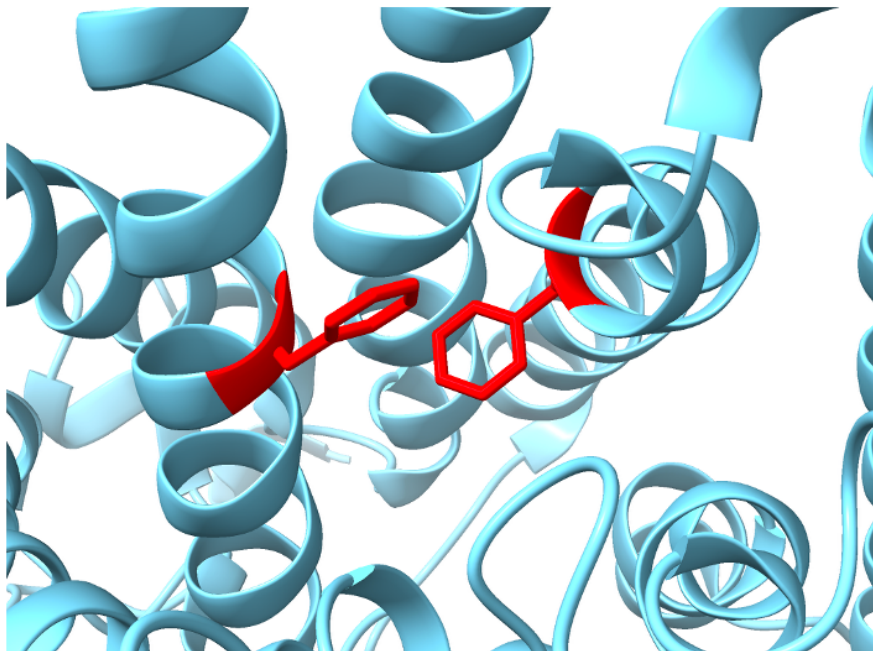


Figure 6.1 **The different arrangements of the Phe gate.** In the crystal structure of EcAmtB (pdb 3c1g (Javelle *et al.*, 2008)) (A) and most ammonium transporters known about, both Phe residues are parallel to each other while in the crystal structure of NeRh50 (pdb 3b9w (Lupo *et al.*, 2007)) (B) and other rhesus proteins the first Phe is rotated so that it sits perpendicular to the second which opens the pore more than in EcAmtB. The Phe Gate is coloured red.

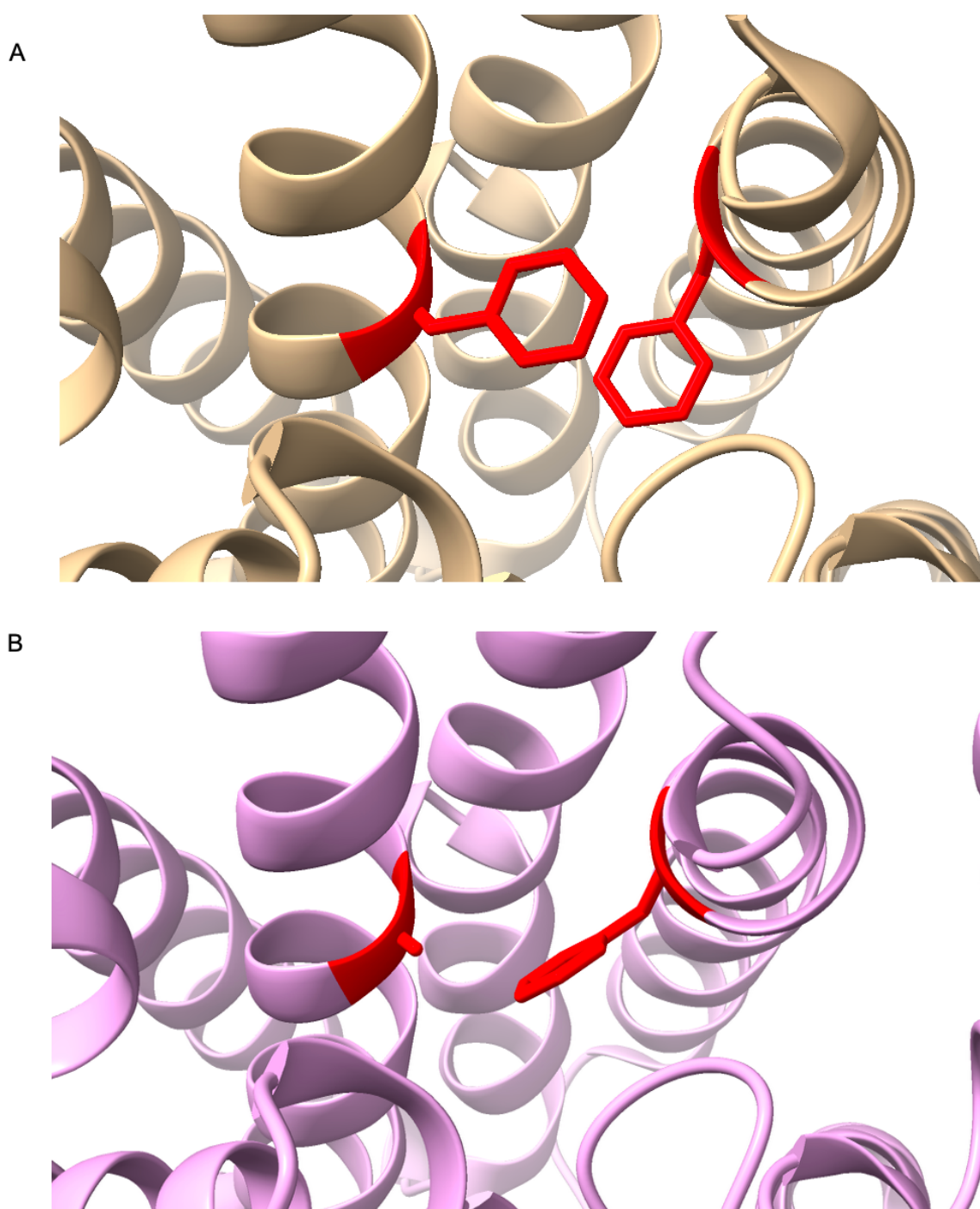


Figure 6.2 **Comparison of WT EcAmtB Phe gate conformation to the F107A mutant in crystal structures.** The WT (A) (pdb 3c1g (Javelle *et al.*, 2008)) has two parallel stacked Phe rings. In the F107A mutant (pdb 3c1h (Javelle *et al.*, 2008)) the second Phe is rotated potentially giving a hint at what an 'open' pore conformation would look like. The Phe Gate is coloured red.

6.3 Results

6.3.1 Instrumentation Issues Affecting Experimental Progress

The objective of this chapter was to investigate the role of the Phe Gate in two ammonium transporters using SSME on the Nanion SURFE²R N1 system. However, the SURFE²R system was not functioning reliably for a significant portion of the experimental period. Sensor statistics were consistently poor, baseline signals from empty liposomes were abnormal, and the recorded traces exhibited irregular shapes. As a result, considerable time was devoted to troubleshooting the underlying issues. Efforts to resolve the problem included purchasing new sensors, which unexpectedly showed lower durability and no improvement in data quality, remaking empty liposomes (with and without detergent), remeasuring previously validated frozen proteoliposomes and controls, verifying vesicle size, thorough cleaning of the SURFE²R system, and remaking buffer solutions to ensure consistency apart from the substrate or KCl additions. Additionally, I consulted Dr. Adriana Bizer, who suspected contamination in the tubing and provided a cleaning protocol for the ionjet.

Despite repeated attempts to contact Nanion earlier without success, a renewed inquiry yielded a prompt response. Replacement of the tubing, ionjet, and conductor ultimately led to improved data quality. However, due to the extended troubleshooting period, there was insufficient time to complete the full set of experiments, including the generation of all mutant plasmids, protein overexpression, purification, reconstitution, and functional measurements. This work is now being continued by Pete, Arnaud's next PhD student.

6.3.2 Overexpression and purification of proteins

6.3.2.1 *WT EcAmtB*

6.3.2.1.1 *Immobilised Metal Ion Chromatography*

EcAmtB was overexpressed in *E. coli* C43 (DE3) strain in autoinduction media. The cells were broken and the membrane fraction containing AmtB was solubilised using 2% DDM. Following this the sample was ultracentrifuged to separate the membrane fraction and the supernatant was loaded onto a 1mL HisTrap IMAC column containing Co^{2+} charged Sepharose beads. The column was washed using 10 ml IMAC solution with 40 mM imidazole (for more detail see methods section 2.1.7.2). The protein was eluted using an increasing gradient of imidazole to a maximum of 500 mM over 20 mL. AmtB eluted at around 200 mM imidazole (Figure 6.3A). This does not give any indication of the size/purity of the protein/sample so further analysis was performed using a 10% SDS-PAGE gel loaded with the elution fractions and showed that the sample is pure and AmtB forms a single band at around 130kDa (Figure 6.3B).

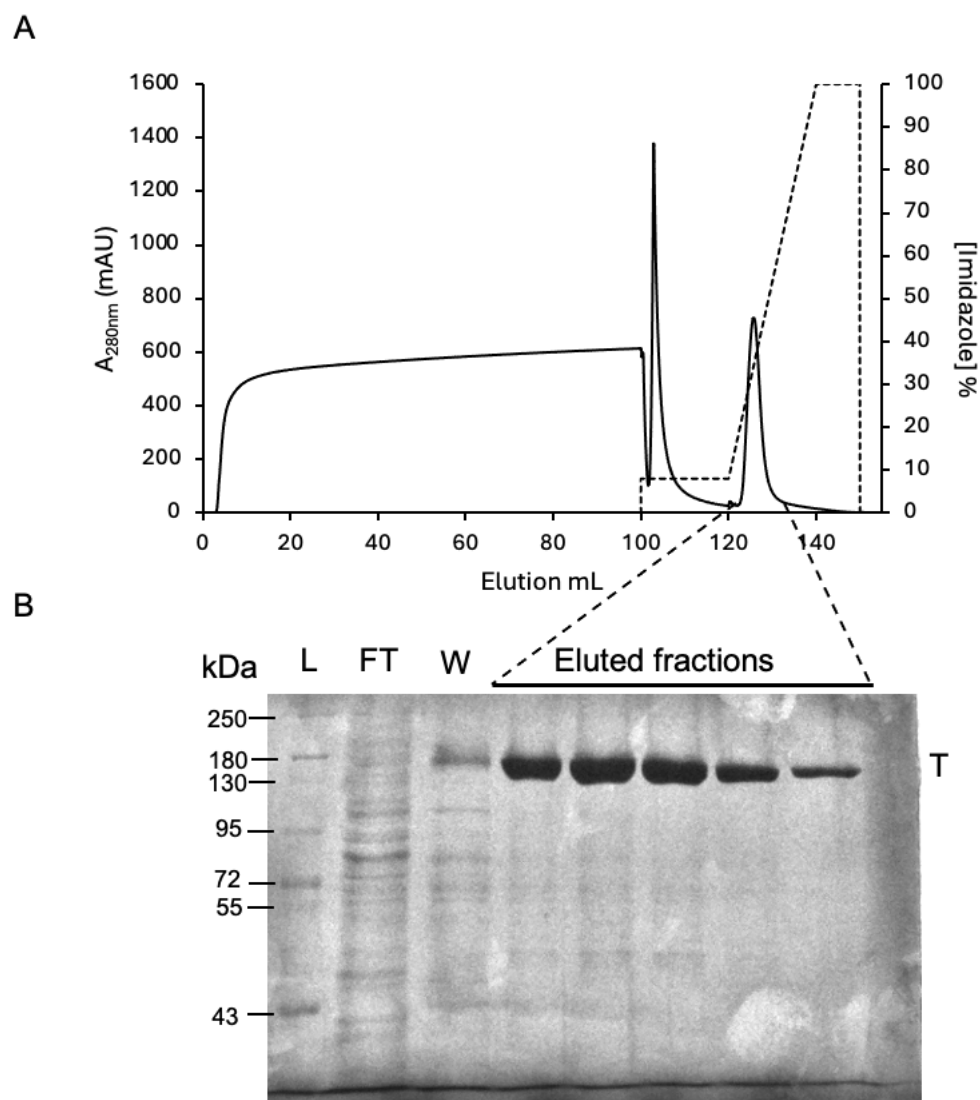


Figure 6.3 **IMAC purification of WT EcAmtB**. A) Profile of IMAC wash and elution of EcAmtB. The dotted line represents the concentration of imidazole where 100% = 500 mM. The solid line represents A_{280nm} . B) A 10% SDS-PAGE gel loaded with the ladder (L), flow through (FT), wash (W) and elution fractions. T on the figure indicates the location of trimeric AmtB

6.3.2.1.2 Size exclusion chromatography

In order to determine the hydrodynamic properties of WT AmtB the sample was subjected to Size Exclusion Chromatography analysis (SEC). The fractions from the IMAC purification were combined and concentrated to ~5mg/ml using a 100kDa filter, and 350µl of the concentrated sample was then injected onto a Superdex S200 10/300 SEC column. The protein eluted at ~11mL with a small shoulder before a distinct peak (Figure 6.4). The hump may be due to the protein unfolding slightly or changing conformation or due to protein aggregation. The fractions were analysed with a 10% SDS-PAGE gel and showed that the AmtB sample is pure (Figure 6.4 insert).

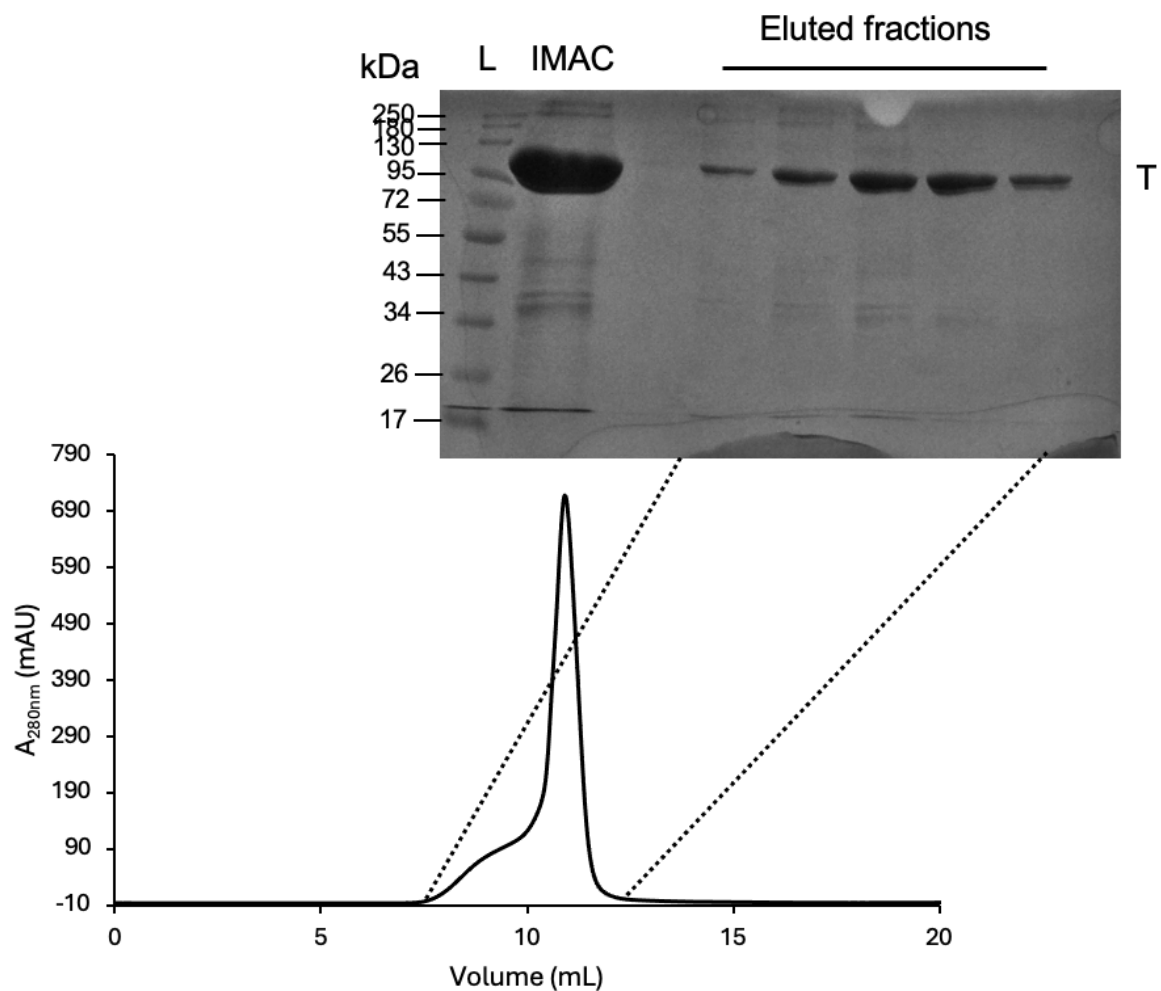


Figure 6.4 **Size exclusion chromatography of WT AmtB.** EcAmtB at a concentration of 5 mg/mL solubilised in 0.033% DDM was injected into a Superdex 200 increase (10/300) column. The sample was left to run through the column for 1 column volume. Insert: 10% SDS-PAGE gel loaded with the ladder (L), a sample from the IMAC purification as a positive control (IMAC) and the SEC elution fractions. The trimer is indicated with T.

6.3.2.1.3 *AmtB* Insertion into liposomes

Reconstitution into liposomes was performed using the sample directly following IMAC purification. Liposomes with a composition of *E. coli* polar lipids and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) at a ratio of 2:1 were destabilised with Triton X-100 detergent to allow for protein insertion at lipid to protein ratios (LPR) of 5, 10 and 50. Residual detergent was removed using overnight incubation in BioBeads and the proteoliposomes were washed by ultracentrifugation three times. The absence of detectable protein in the supernatant by SDS-PAGE analysis suggests that little or no soluble, non-incorporated protein remains; this is consistent with efficient incorporation into the liposomes (Figure 6.5A), although the possibility that non-incorporated protein became insoluble or aggregated and therefore co-pelleted cannot be excluded. The different LPR samples were then run on an SDS-PAGE gel to visualise their different protein concentrations (Figure 6.5B). AmtB was detected exclusively in the proteoliposome fraction, and its amount correlated with the different LPR conditions, indicating successful reconstitution of AmtB into the proteoliposomes

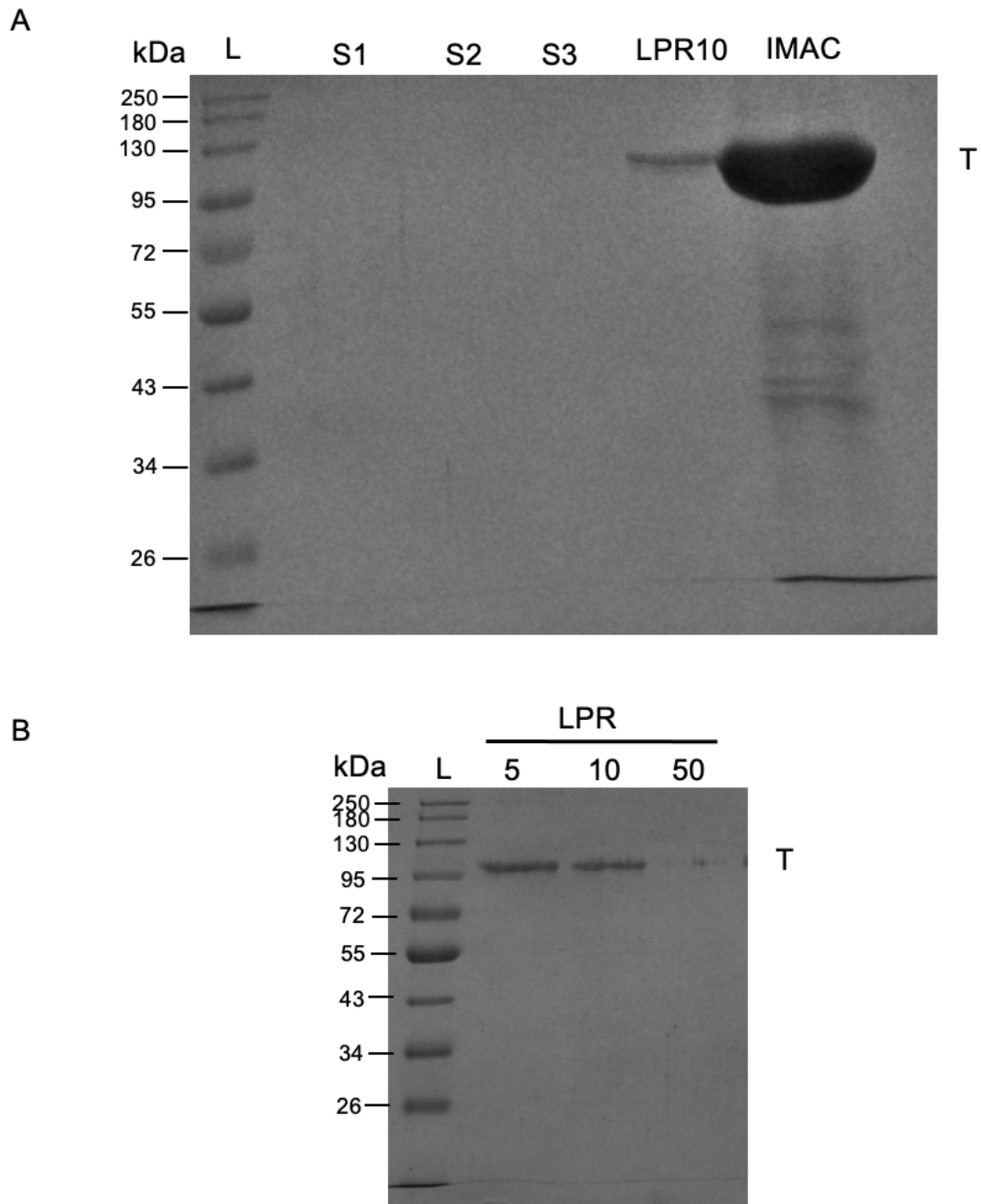


Figure 6.5 **Insertion of AmtB into liposomes.** A) 10% SDS-PAGE was loaded with the ladder (L), S1-3 refers to each sample of supernatant following ultracentrifugation to pellet the proteoliposomes. LPR10 was used to confirm presence in the proteoliposomes and a sample of the concentrated protein from IMAC purification was used as a positive control. B) 10% SDS-PAGE was loaded with the ladder (L) and LPR5, 10 and 50. The trimer is indicated with T.

6.3.2.2 WT NeRh50

Attempts to express NeRh50 using a pET-derived plasmid were unsuccessful, likely due to toxicity caused by the high-level overexpression driven by the T7 promoter in *E. coli*. In a second attempt, NeRh50 was inserted into the pAD7 plasmid with the native AmtB promoter and overexpressed in *E. coli* GT1000 ($\Delta amtB$, *glnK*) using minimal M9 media. The reason to use this plasmid is to have the Rh protein overexpress under the control of the AmtB native promoter and the GT1000 strain was used to avoid the expression of the native AmtB becoming part of the Rh50 trimer and interfering with results. M9 media was used and supplemented with glutamine to activate the promoter. The membrane fraction of *E. coli* GT1000 cells expressing NeRh50 was solubilised using 2% lauryldimethylamine-N-oxide (LDAO), instead of DDM as used for AmtB purification. The insoluble constituents of the sample including the membrane was separated using ultracentrifugation and the supernatant was loaded onto a 1mL HisTrap IMAC column containing Co^{2+} ions. The column was washed using 10mL of buffer containing 40 mM imidazole before the protein was eluted with an increasing gradient of imidazole concentration where the maximum was 500 mM (Figure 6.6A). Again, the peak measured by the absorbance at 280nm gave no indication of the size and purity of Rh50, so a 12.5% SDS-PAGE gel loaded with the elution fractions was used. This showed that, unlike AmtB, Rh50 appeared as multiple bands corresponding to different oligomeric states of the protein (Figure 6.6B). The presence of monomeric Rh50 suggests that the protein is less stable as a trimer under SDS-PAGE conditions than AmtB. The higher-molecular-weight band may be due to contamination by other proteins or, more likely, to an insufficient amount of reducing agent in the loading buffer, resulting in incomplete reduction of disulfide bonds and the persistence of higher-order protein complexes. It has previously been verified with western blots that Rh50 often runs as multiple bands on SDS-PAGE though (Cherif-Zahar *et al.*, 2007).

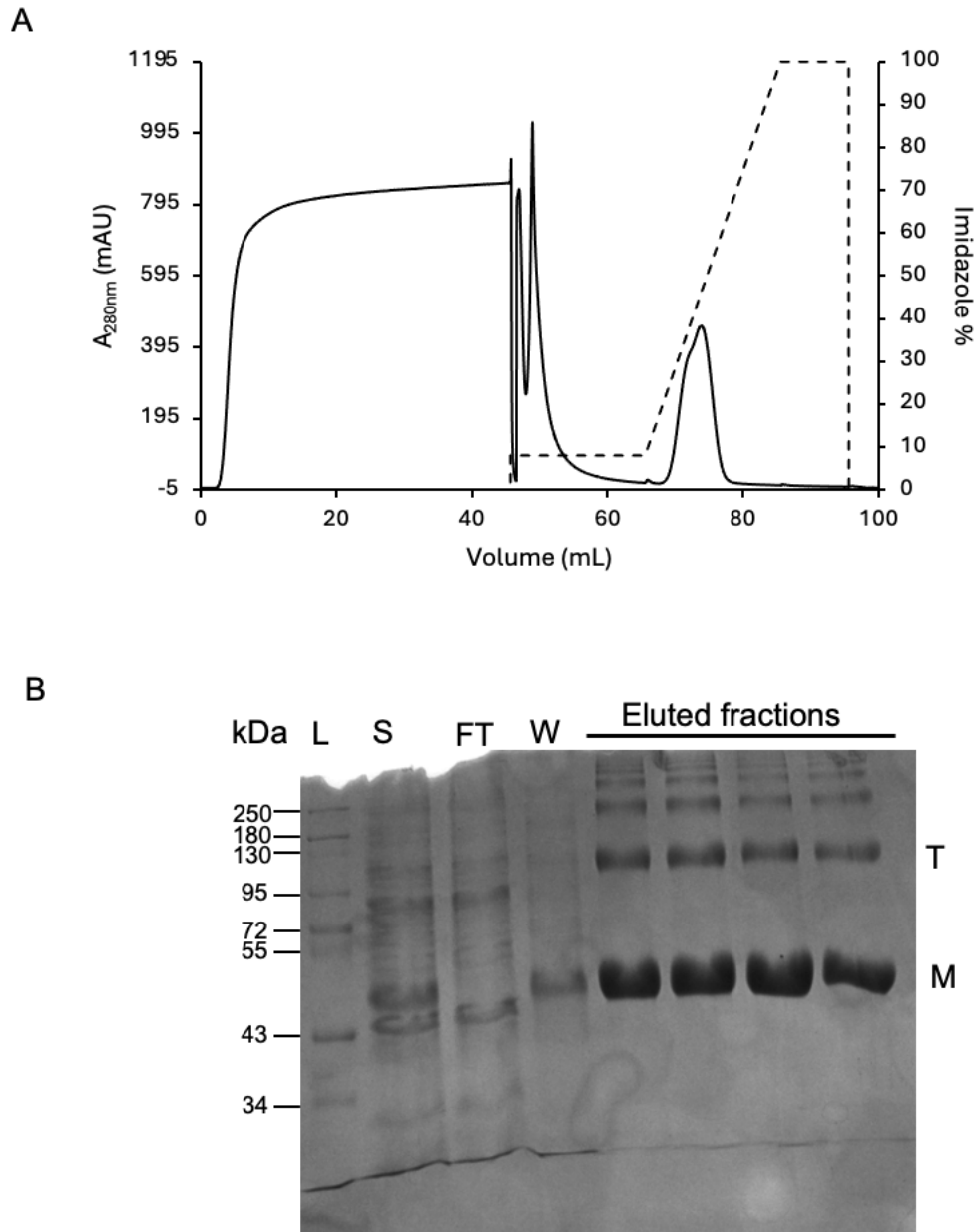


Figure 6.6 IMAC purification of WT NeRh50. A) The IMAC profile of NeRh50 purification. The solid line represents $A_{280\text{nm}}$ while the dotted line shows the concentration of imidazole where 100% = 500 mM. B) 12.5% SDS-PAGE gel loaded with the ladder (L), the supernatant following ultracentrifugation (S), the flow through (FT), wash with 8% imidazole solution (W) and the eluted fractions. The locations of monomers (M) and trimers (T) are shown.

6.3.2.2.1 Size exclusion chromatography

The pooled fractions from the IMAC step were concentrated to ~5mg/ml and 300µL was injected onto a Superdex S200 10/300 SEC column in order to investigate the hydrodynamic properties of Rh50. The absorbance was measured at 280nm and showed that Rh50 eluted as a peak with a small initial shoulder after about 12ml (Figure 6.7). A 12.5% SDS gel loaded with the wells corresponding to the eluted peak showed a band of protein at ~130kDa corresponding to the trimer and another at ~43kDa corresponding to the monomer (Figure 6.7). The higher-molecular-weight band may be due to contamination by other proteins or, more likely, to an insufficient amount of reducing agent in the loading buffer, resulting in incomplete reduction of disulfide bonds and the persistence of higher-order protein complexes.

6.3.2.2.2 Insertion

Again, as with WT EcAmtB, the protein was reconstituted into liposomes following IMAC purification. The liposomes were prepared in the same manner as described and SDS-PAGE analysis of the supernatant following the wash steps showed there was no soluble or non-incorporated Rh50 present (Figure 6.8A), this does not confirm that all protein was incorporated, however, as insoluble protein would have been collected in the pellet *along* with the proteoliposomes. The different LPR samples were also run on a 12.5% SDS-PAGE gel to confirm their differing proteins concentrations (Figure 6.8B). NeRh50 was detected exclusively in the proteoliposome fraction, and its amount correlated with the different LPR conditions, indicating successful reconstitution of NeRh50 into the proteoliposomes. Again, there were bands a higher kDa than what would be expected for the trimer. This may be aggregates of Rh50.

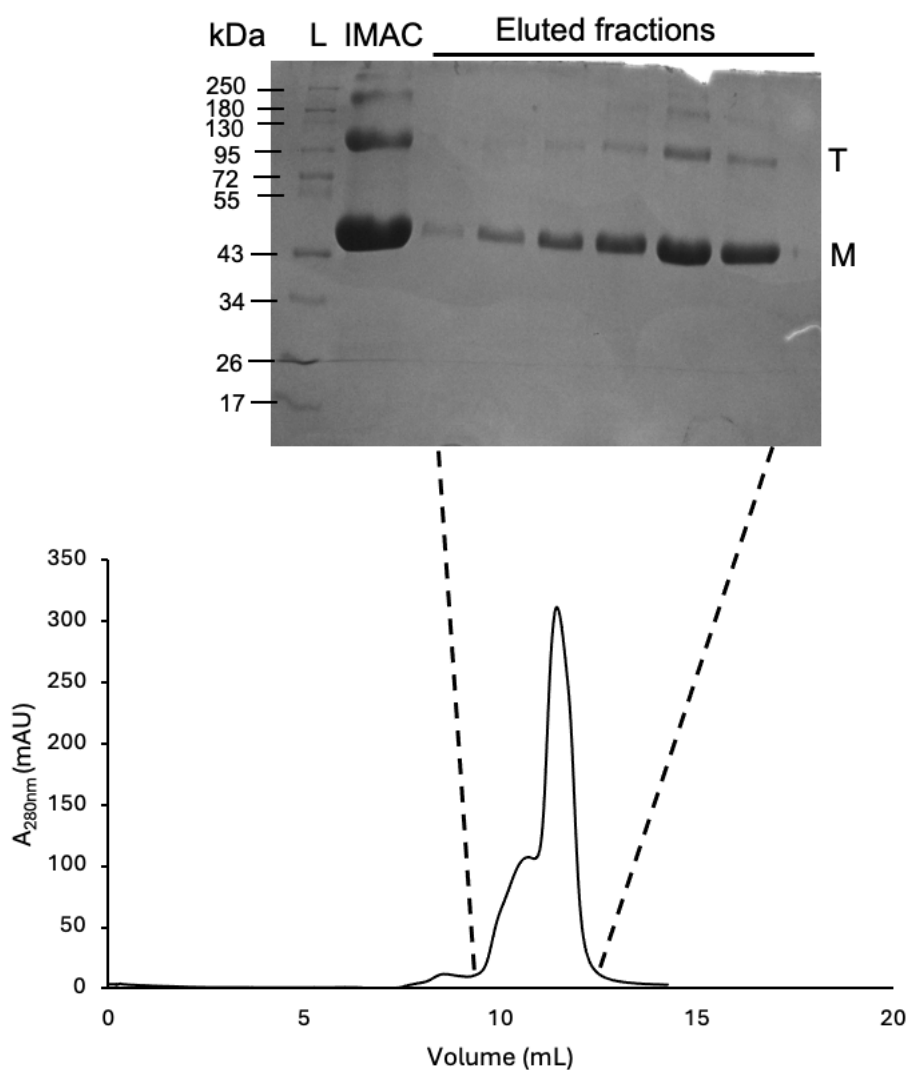


Figure 6.7 **Size exclusion chromatography of WT Rh50.** Rh50 solubilised in 0.09% LDAO was injected into a Superdex 200 increase (10/300) column. Insert: 12.5% SDS-PAGE gel loaded with the ladder (L), SEC fractions and a sample from the IMAC purification step. The trimer and monomers are indicated with T and M.

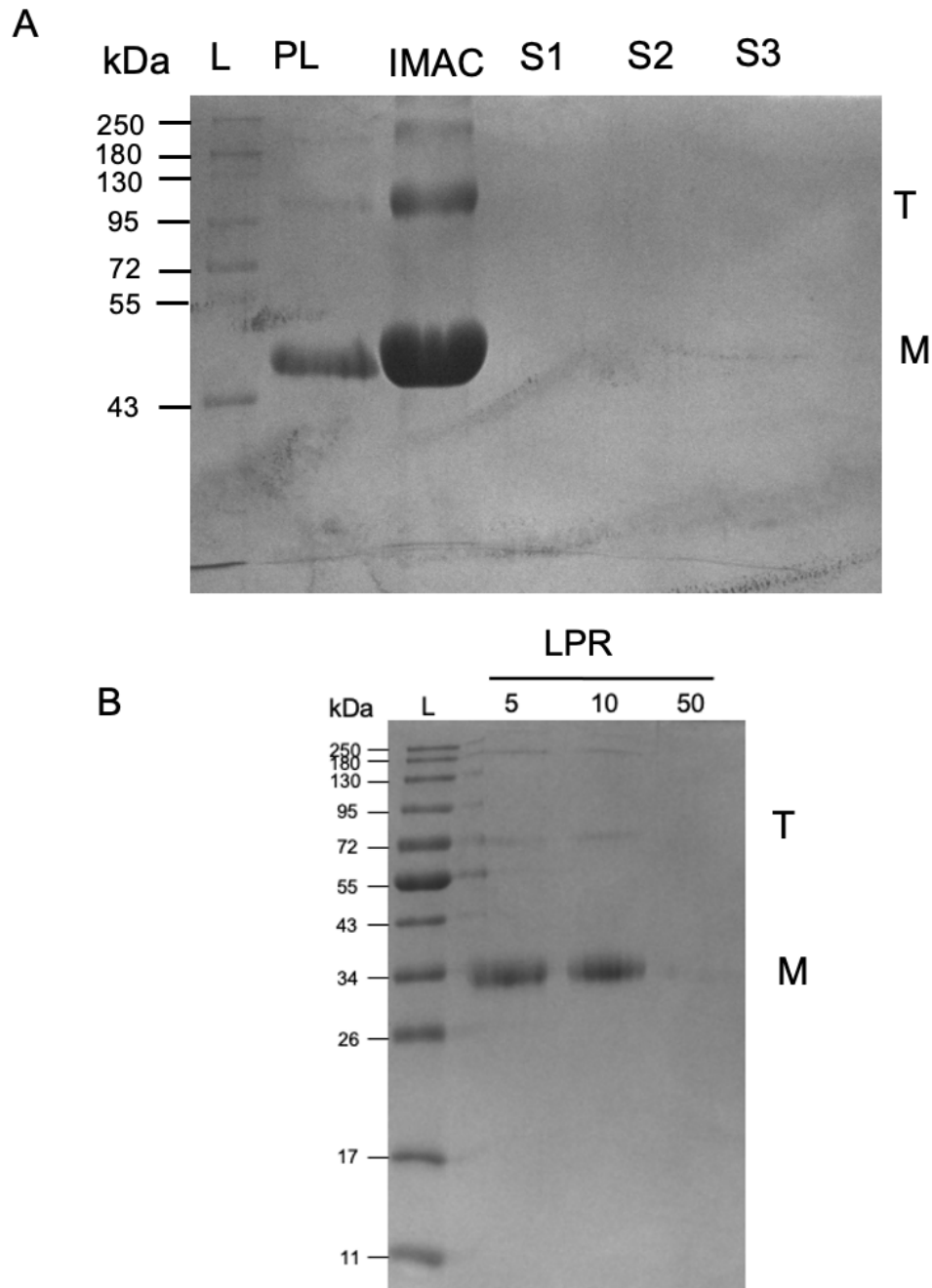


Figure 6.8 **Insertion of WT NeR50 into proteoliposomes.** A) 12.5% SDS-PAGE gel was loaded with the ladder (L), a sample of LPR10 proteoliposome (PL), a sample from the IMAC purification (IMAC) and the supernatant from the proteoliposome wash steps (S1-3). B) 12.5% SDS-PAGE loaded with the ladder (L) and samples of each LPR concentration. The location of the trimer (T) and monomers (M) are indicated.

6.3.2.3 *Proteoliposome diameter*

The transient current measured by SSME arises from the accumulation of charge within the liposome lumen and is therefore dependent on liposome volume. To obtain a uniform size distribution, liposomes were extruded through a 100 nm filter prior to protein reconstitution. This step was important for two reasons: first, to enable direct comparison of transient currents between empty liposomes and proteoliposomes; and second, to ensure that each preparation exhibited a monodisperse size distribution, that is, a narrow and homogeneous liposome population.

The size distribution of each preparation was routinely assessed by dynamic light scattering (DLS). DLS analysis confirmed that both empty liposomes and proteoliposomes displayed a unimodal size distribution (Figure 6.9). Proteoliposomes containing Rh50 had an average diameter of 256 nm, whereas AmtB proteoliposomes and empty liposomes had average diameters of 178 nm and 149 nm, respectively.

The variation in mean liposome diameter may reflect differences in sample preparation, including sonication, extrusion, and protein reconstitution, as well as the sensitivity of DLS to larger particles. In particular, the larger apparent diameter of Rh50 proteoliposomes may indicate that protein incorporation influenced vesicle size or hydrodynamic behaviour, rather than this difference arising solely from variation in sonication (Danaei *et al.*, 2018; Duskocz *et al.*, 2021; Szathmáry *et al.*, 2025).

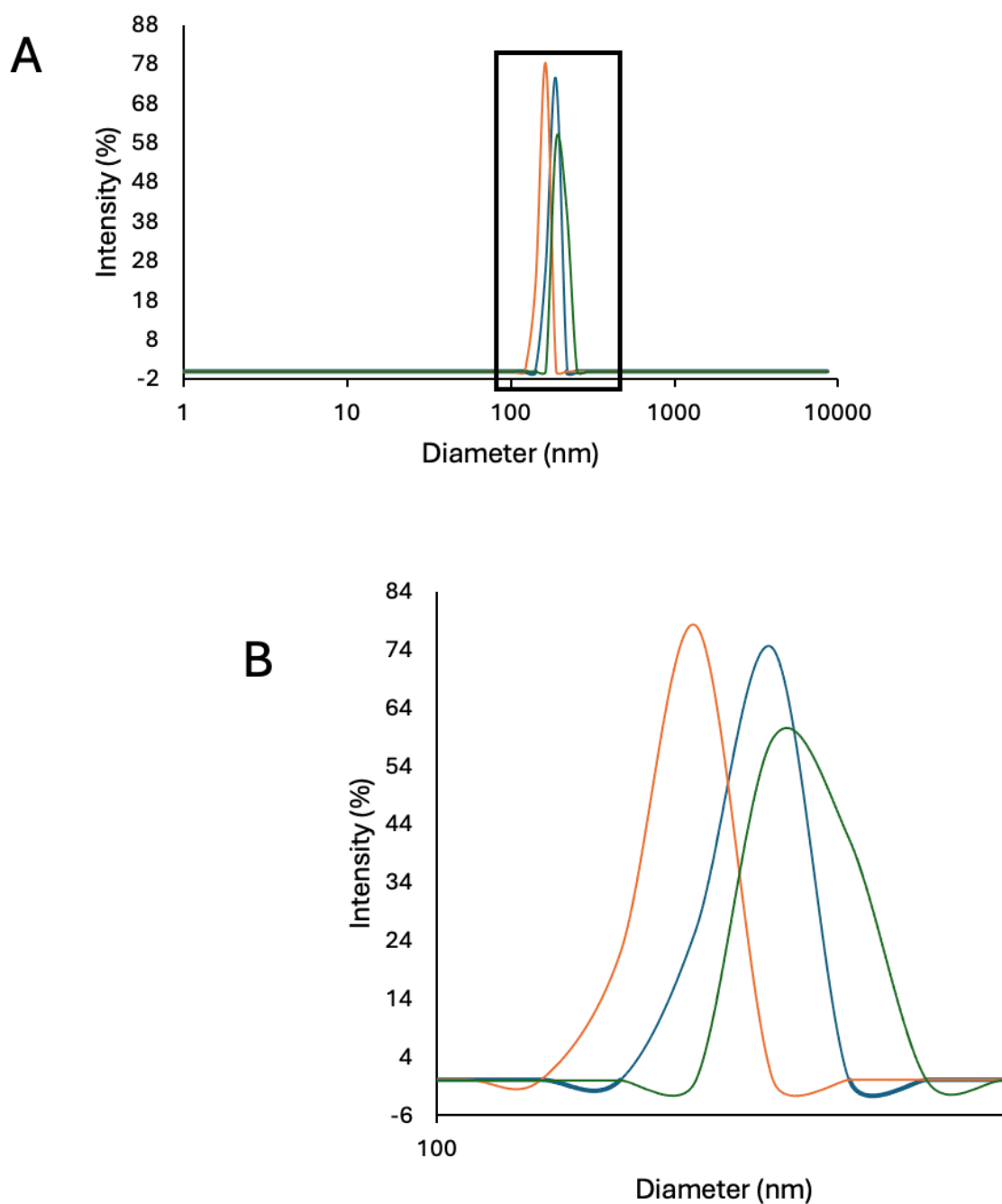


Figure 6.9 **Size distribution of liposomes.** Comparison of diameters of WT Rh50 (green), WT AmtB (blue) and empty liposomes (orange) was made with DLS. LPR5 was used and the samples diluted 50x. A) The full range of diameters as measured by the DLS machine. The black box indicates the enlarged area in (B). B) An enlarged view of the size distribution of the liposome and proteoliposomes. Each measurement was repeated three times, shown here is a measurement representative of the average size and distribution.

6.3.2.4 Overexpression and purification of the mutant Phe Gate proteins

The initial plan when starting the investigations into the Phe Gate was to create all possible combinations of Phe-Ala mutations in both AmtB and Rh50. The Phe residues are replaced with a smaller alanine to physically remove their rings from the pore. Owing to time constraints it was only possible to create Rh50 F110A, F218A and F110A/F218A and AmtB F107A/F215A. The single Phe AmtB mutants have subsequently been made, purified and are currently being analysed by Peter Henderson, a PhD student in the laboratory.

As with the wild type proteins the *amtB* variant with a 6-His tag at its C-terminus was expressed in C43 *E. coli* strain while the Rh50 mutants also with a 6-His tag at their C-termini were expressed in the $\Delta amtB/glnK$ GT1000 strain.

The variants were all purified in the same manner as described for the wild types. First the solubilised protein was loaded onto a 1mL HisTrap IMAC column charged with Co^{2+} ions. The column was washed for 10mL with buffer containing 40 mM imidazole and the proteins were eluted with an increasing gradient of imidazole over 20mL to a maximum concentration of 500 mM. AmtB F107A/215A had the highest yield while the Rh50 mutant yields were consistently poor (Figure 6.10). In the case of F218A and F110A/F218A the expression needed to be repeated as the first attempt provided no protein. SDS-PAGE analysis of the elution fractions showed that AmtB ran as a single band at ~95kDa corresponding to the trimer (Figure 6.11A). Rh50 mutants all ran mainly as a band at ~34kDa (Figure 6.11B-D) corresponding to the monomer.

Pure protein, concentrated to ~5mg/ml, was injected into a Superdex S200 10/300 increase column to assess their hydrodynamic properties (Figure 6.12). For the variants F218A and F110A, the protein yield was low meaning only 100 μ l could be injected into the SEC column rather than 350 μ L as for the other samples, for this reason the peaks are significantly lower than the other proteins. Rh50 F110A/F218A yield was so low that no protein could be spared to perform SEC analysis. AmtB FAFA eluted as a single symmetrical peak, but at a lower elution volume than the WT AmtB protein. The Rh50 mutants also eluted at lower volumes than the WT protein, with F110A eluting as two distinct peaks. One of these peaks appeared at approximately the same elution volume as WT Rh50, suggesting the presence of two different conformations or reduced protein stability due to the mutations in the Phe-Gate.

Protein incorporation was confirmed using SDS-PAGE (Figure 6.13). The Rh50 variants showed a tendency to dissociate into monomers, giving the strongest bands at approximately 34 kDa, whereas the AmtB variant gave the strongest band at around 95 kDa. The gel confirmed the absence of soluble, non-incorporated protein in the supernatant following the wash steps; however, it cannot be excluded that insoluble, non-incorporated protein was also collected in the pellet.

The differences in protein concentration between LPRs was also visualised by running the different LPR proteoliposomes on an SDS-PAGE gel (Figure 6.14). This proved that the most protein was present in LPR5 and the least in LPR50 with bands barely being visible. AmtB and its mutant ran at a single band at ~95kDa while for Rh50 and its mutants the largest band occurred at 34kDa. Rh50 F110A/F218A seems to have smeared through the gel making a distinct band more faint. Unexpected migration of membrane proteins on SDS-PAGE gel is not rare and can be explained by the partial unfolding of hydrophobic domains in the presence of SDS (Rath *et al.*, 2009) and these results are in keeping with previous SDS-PAGE gels loaded with AmtB or Rh50 in our lab.

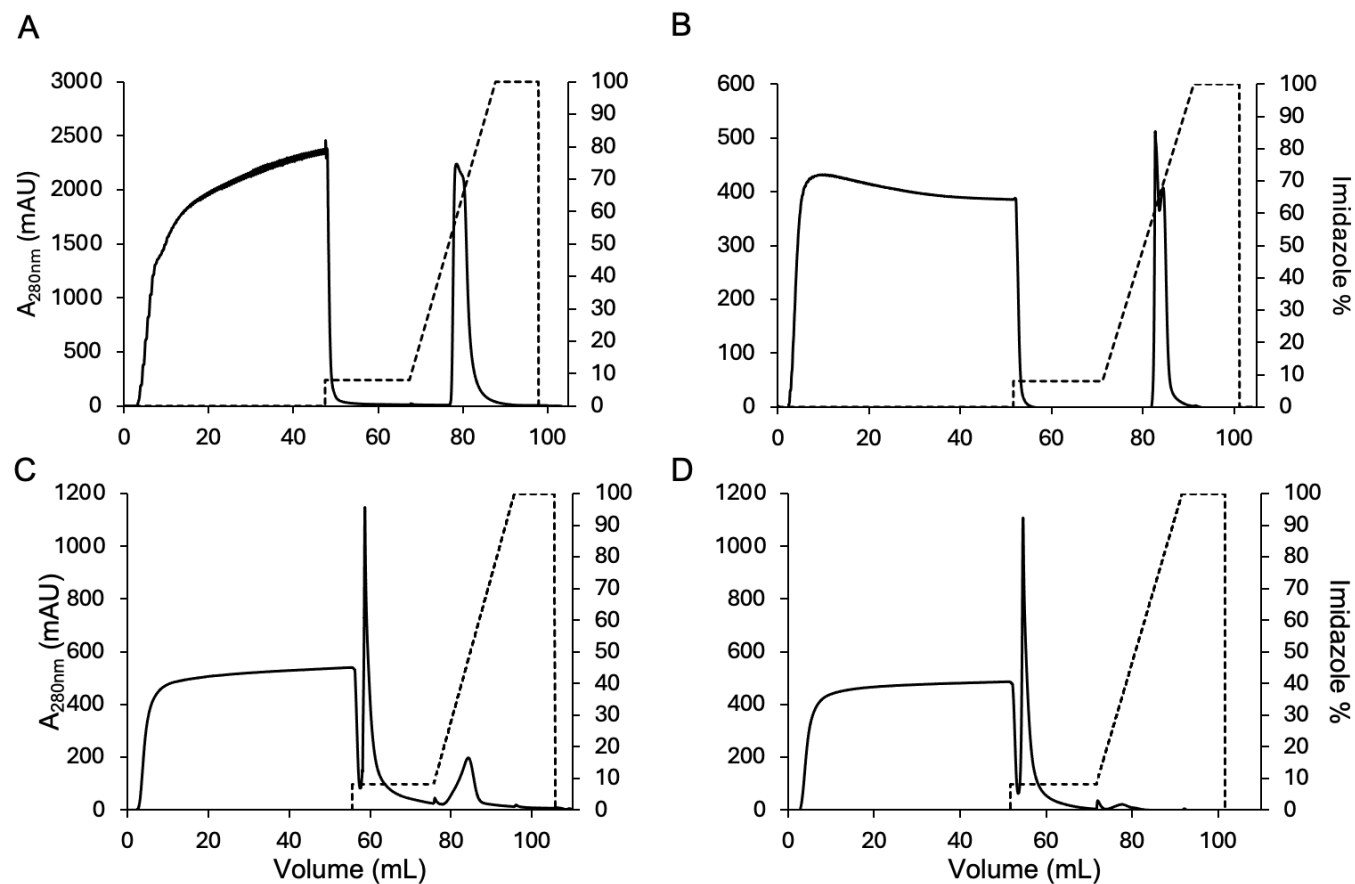


Figure 6.10 **IMAC purification profiles of AmtB and Rh50 mutants.** In each case the solid black line represents A_{280nm} , and the dashed line represents the concentration of imidazole where 100% = 500 mM. A) AmtB F107A/F215A. B) Rh50 F110A. C) F218A. D) F110A/F218A.

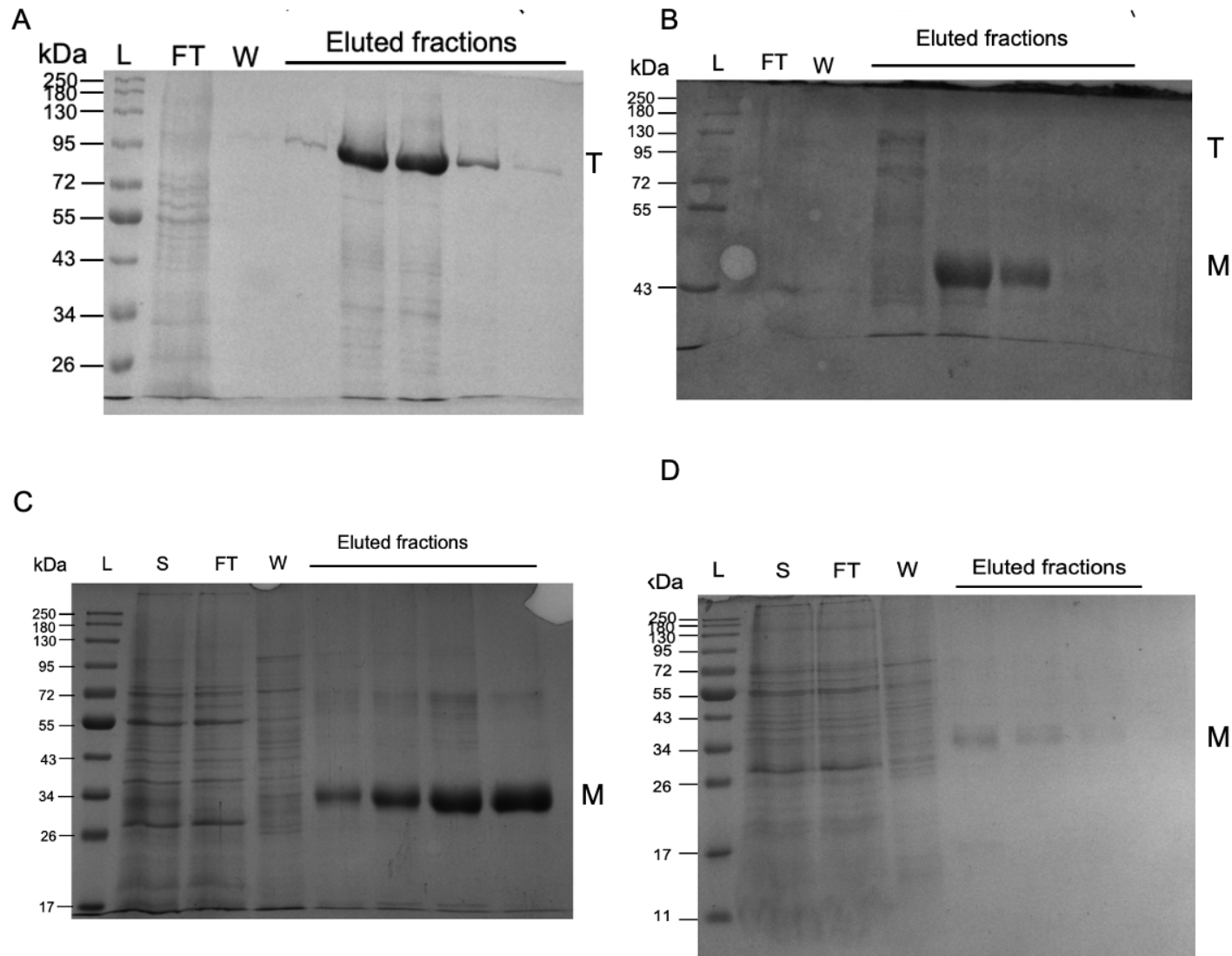


Figure 6.11 **SDS-PAGE analysis of the IMAC elution fractions.** Sample (S), Ladder (L), Flow through (FT), Wash (W) and eluted fractions for each mutant A) 10% SDS-PAGE AmtB F107A/F215A, B) 12.5% SDS-PAGE Rh50 F110A, C) 12.5% SDS-PAGE Rh50 F218A and D) 12.5% SDS-PAGE Rh50 F110A/F218A. Trimers and monomers are indicated with T and M

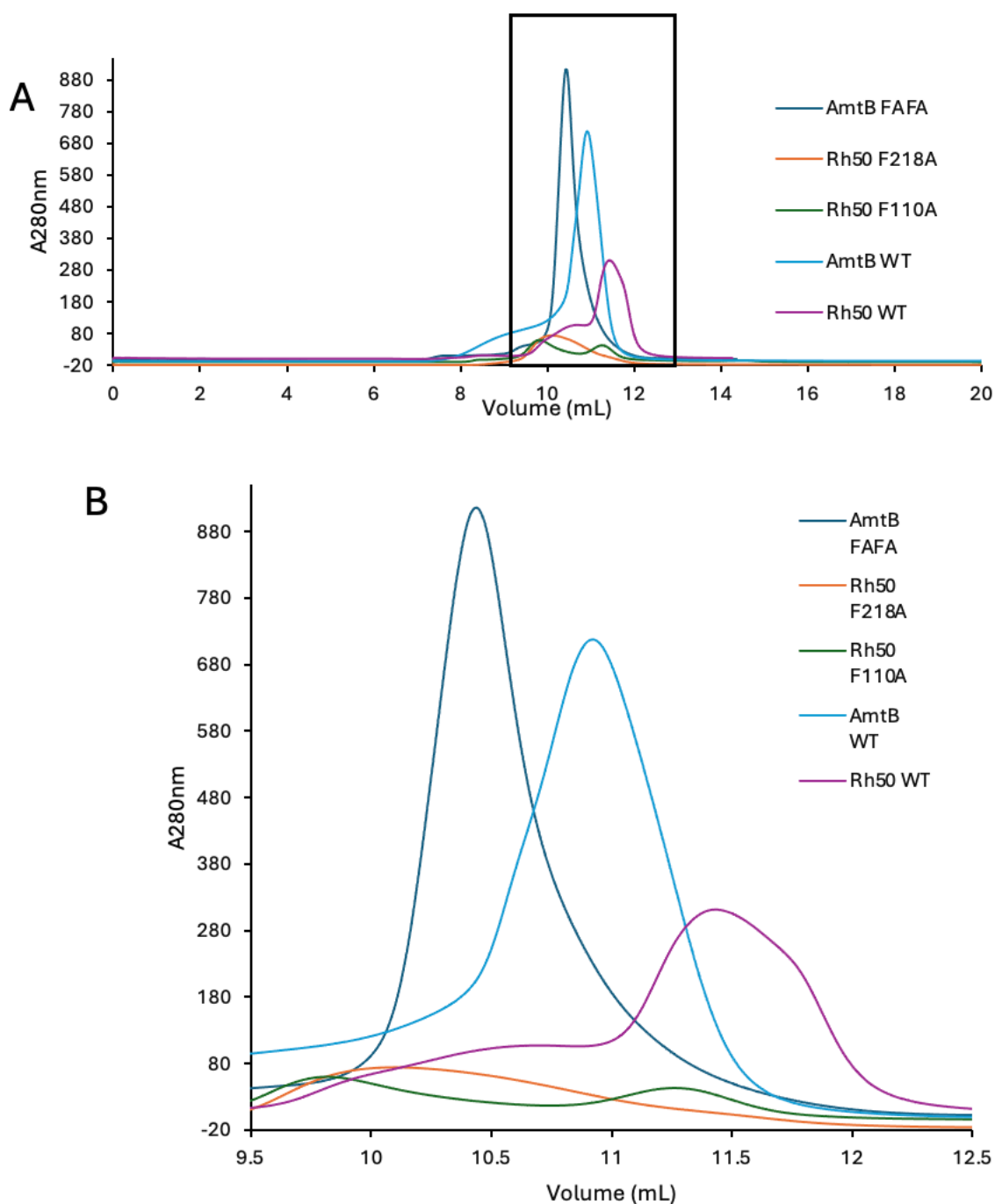


Figure 6.12 **Size exclusion chromatography of WT and mutant proteins.** A) ~5mg/mL protein was injected into a Superdex 200 increase (10/300) column. Either 350 μ L in the case of AmtB WT and FAFA and Rh50 WT or 100 μ L in the case of Rh50 F110A and F218A was used. The region shown in (B) is indicated by the boxed area. B) Expanded view of the elution peaks of the Phe Gate mutants and WT proteins.

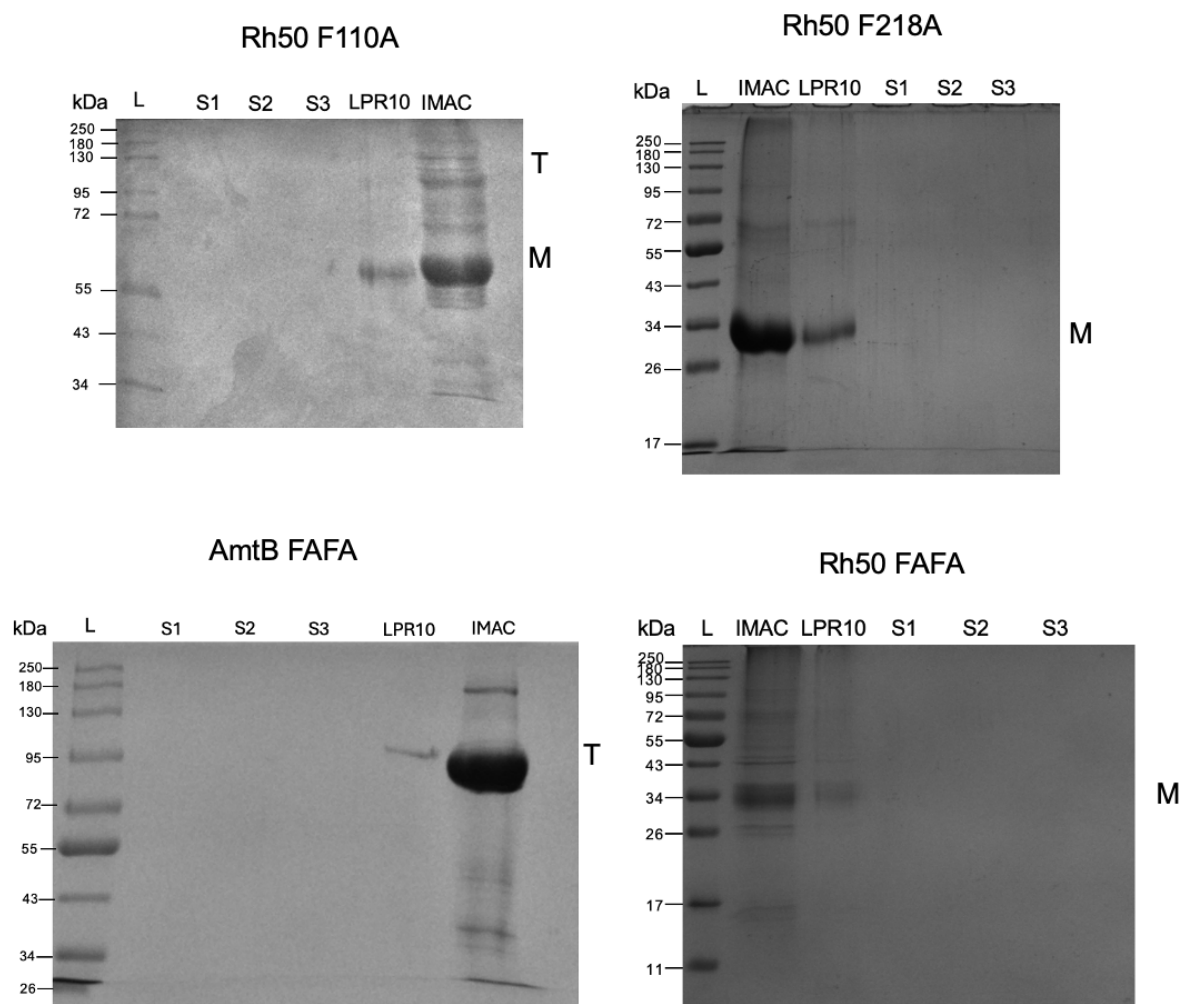


Figure 6.13 **SDS-PAGE analysis of mutant protein insertion into liposomes gels.** SDS-PAGE gels were loaded with the ladder (L) and samples from the supernatant collected during liposome washing (S1-3). Purified protein (IMAC) and samples of LPR10 for each mutant were used as positive control. AmtB FAFA analysis was performed with a 10% SDS-PAGE gel while 12.5% SDS-PAGE was used for Rh50 mutants. Trimers and monomers are indicated with T and M.

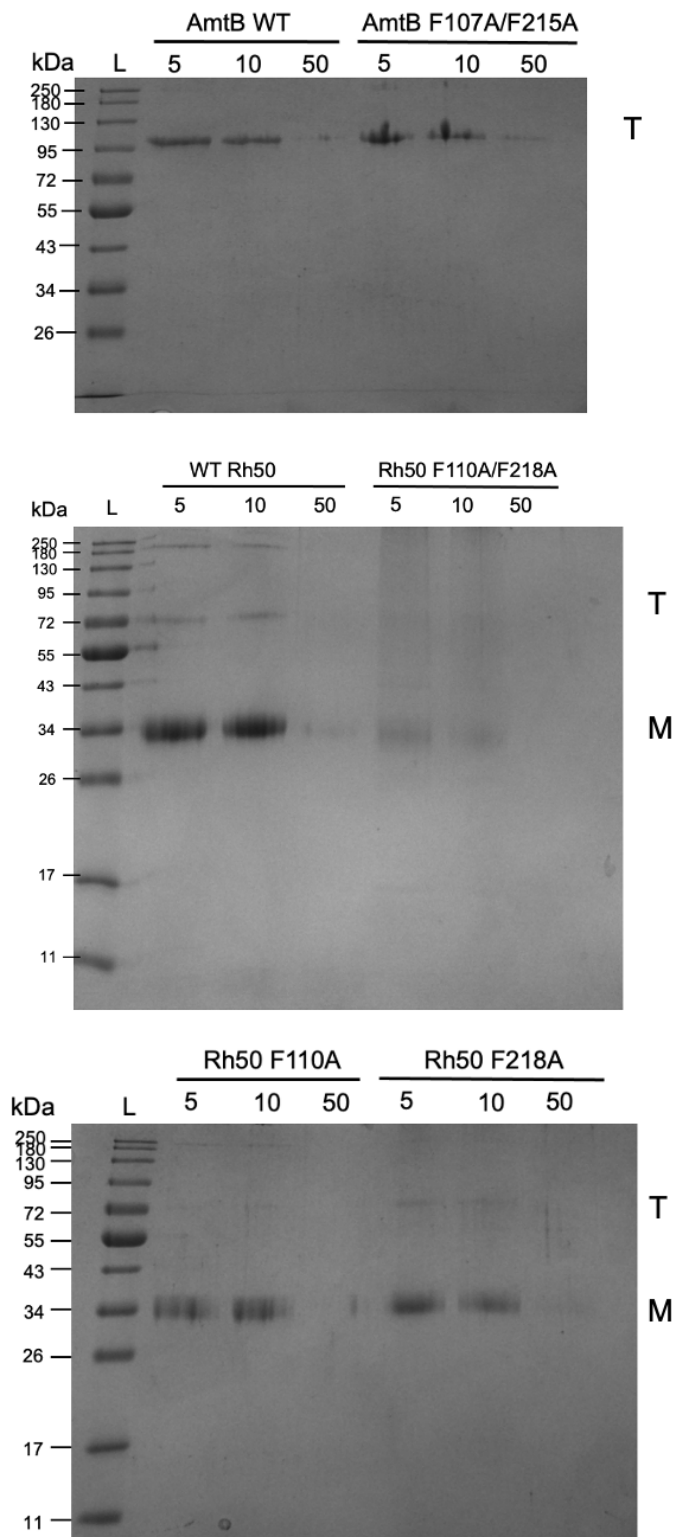


Figure 6.14 **SDS-PAGE analysis of protein concentration in LPR samples.** The SDS-PAGE gels are loaded with the ladder (L) and each LPR sample. AmtB analysis was performed with a 10% SDS-PAGE gel while 12.5% SDS-PAGE was used for Rh50. Trimers and monomers are indicated with T and M

6.3.3 SSME analysis

6.3.3.1 WT proteins

Empty liposomes and proteoliposomes containing either WT AmtB or Rh50 were immobilised on gold-plated sensors for SSME analysis.

One-second pulses of 200 mM NH₄⁺ still elicited measurable peaks in the empty liposomes, most likely reflecting a solution-exchange artefact caused by the rapid change in external ionic composition rather than transporter-mediated charge movement. Such artefacts are well recognised in SSME experiments and are typically controlled for using empty liposomes (Mager *et al.*, 2011; Thomas, Feng and Henzler-Wildman, 2021).

However, these peaks were consistently smaller than those observed for the LPR5 proteoliposomes, suggesting that electrogenic transport was being measured (Figure 6.15). Here and throughout, SSME figures will show a representative trace chosen as it was close to the average maximum amplitude and decay value. Each sensor was measured three times, and every condition was measured in at least three separate sensors. For a figure of the raw data showing the distribution of results see appendix (Figure 9.17 for AmtB, Figure 9.18 for Rh50). The figure showing the raw data in the appendix will be included following each figure.

In order to confirm that the trace seen when pulsing LPR5 proteoliposome with ammonium was due to the presence of protein, sensors with samples of LPR10 and 50 were prepared. The more protein present in the membrane of the proteoliposomes the greater the rate of transport following a pulse of ammonium and therefore the peak amplitude should be greater. When LPR10 and 50 were measured these appeared to show consistency with decreasing transport rates as their amplitudes decreased compared to LPR5 (Figure 6.16, Figure 9.19 for AmtB, Figure 9.20 for Rh50). This proves that our samples were useable to compare the mutants against.

The specificity of both proteins for ammonium was assessed by applying a 200 mM potassium pulse to liposomes and proteoliposomes. This K⁺ pulse did not elicit a positive transient current, indicating the absence of inward potassium transport through either AmtB or Rh50 (Figure 6.17, Figure 9.21 for AmtB, Figure 9.22 for Rh50). Instead, a negative current of comparable amplitude was consistently observed in both liposome and proteoliposome samples. This signal can be attributed to an artifact arising from differences in ionic composition between the non-activating and activating

solutions rather than reflecting genuine protein-mediated transport activity (Bazzone, Barthmes and Fendler, 2017). In other words, when the activating solution was applied, the change in buffer composition generated an apparent charge displacement across the sensor, producing a negative peak detected by the measuring electrode.

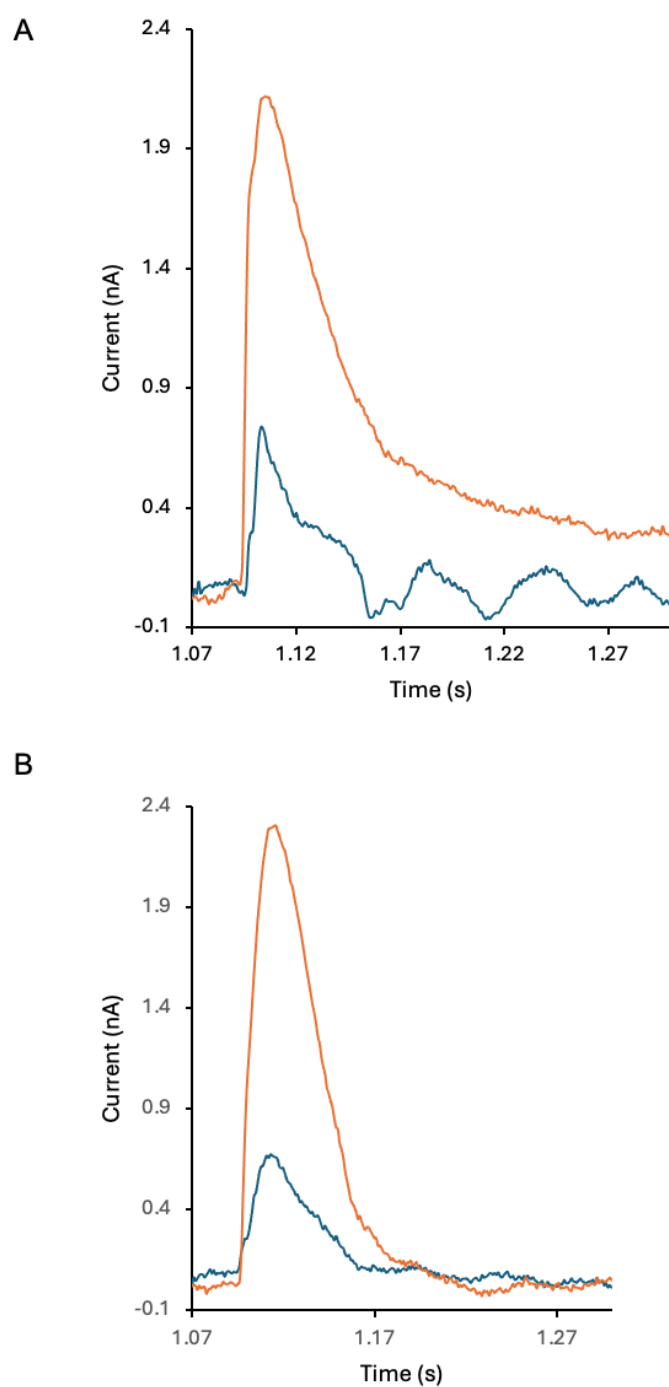


Figure 6.15 **Characterisation of WT protein activity using SSME.** A) Representative traces showing response to pulse of 200 mM NH_4^+ in sample of WT AmtB LPR5 proteoliposomes (orange) or empty liposomes (blue). B) Representative traces showing response to pulse of 200 mM NH_4^+ in sample of WT Rh50 LPR5 proteoliposomes (orange) or empty liposomes (blue). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

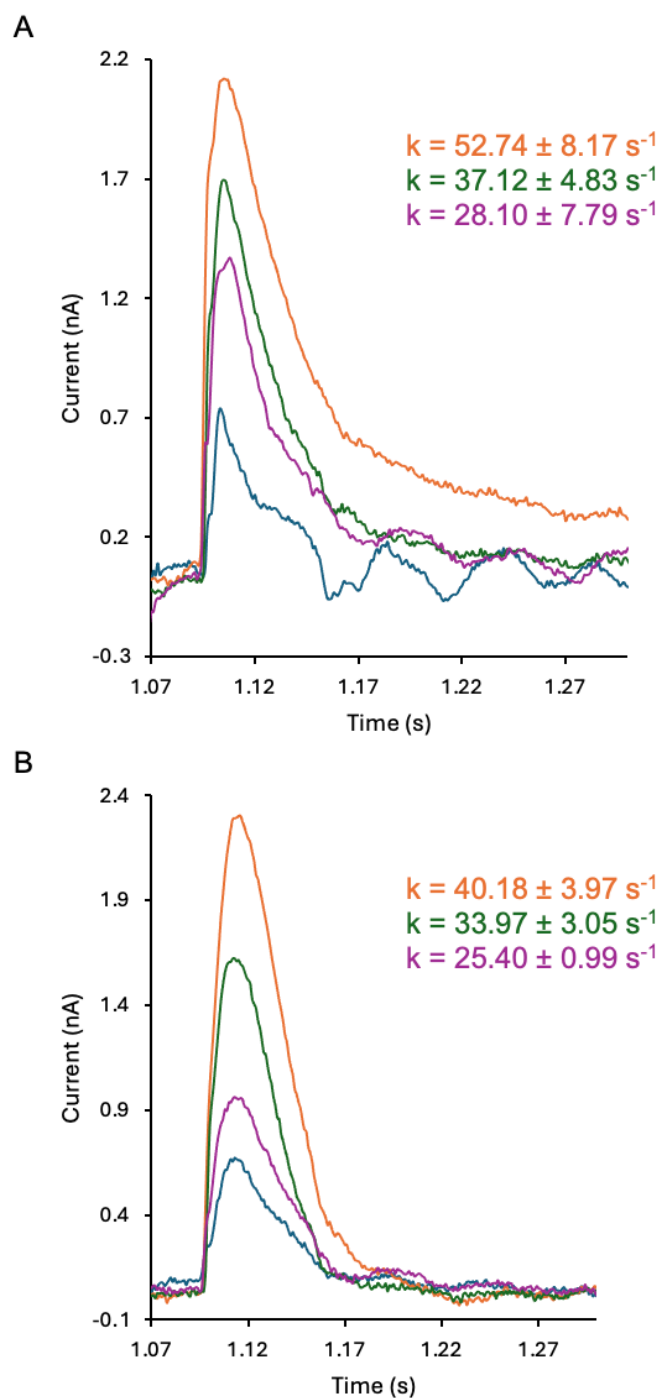


Figure 6.16 **SSME analysis of different proteoliposome LPRs following pulse of 200 mM NH_4^+** . Representative traces close to the average from WT AmtB (A) and WT Rh50 (B). LPR5 (orange), LPR10 (green), LPR50 (purple) and empty (blue). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

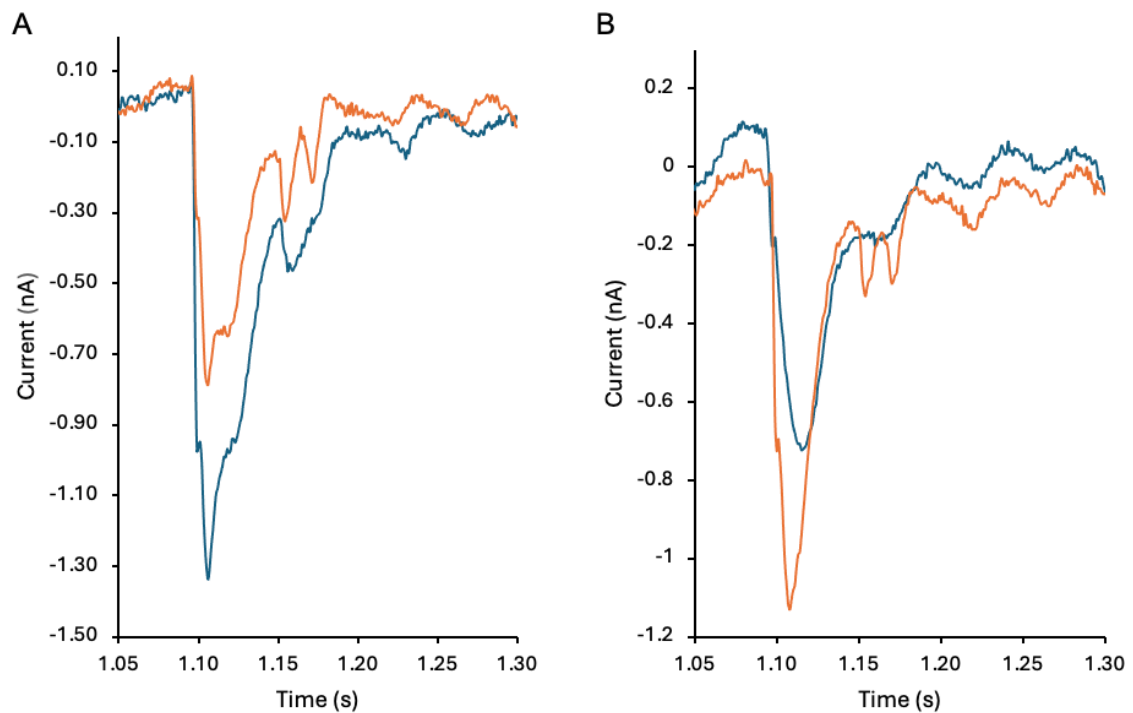


Figure 6.17 **SSME analysis of WT AmtB and Rh50 response to 200 mM K⁺ pulse.** A) WT AmtB LPR5 (orange) and empty liposomes (blue). B) WT Rh50 LPR5 (orange) and empty liposome (blue). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

6.3.3.2 *Phe mutations show reduced activity*

The removal of the pore constriction formed by the Phe-gate is theoretically expected to enhance accessibility of NH_4^+ to the translocation pathway, potentially increasing transport flux. However, all Phe-gate mutants exhibited reduced activity compared to the wild-type protein when subjected to a 200 mM NH_4^+ pulse (Figure 6.18, Figure 9.23 for full trace, Figure 9.24 for expanded view), indicating that the Phe-gate plays a more critical role in transport than merely acting as a physical barrier.

To determine whether the measured transient currents in proteoliposomes containing AmtB or Rh50 variants reflect actual ammonium translocation or merely substrate binding, we investigated the effect of varying the number of transporters per proteoliposome on the current kinetics. If the current results from a complete transport cycle, the decay time of the slower steady-state second phase, would be expected to increase with higher transporter density, i.e. lower lipid-to-protein ratio (LPR) due to enhanced charge accumulation within the vesicle. In contrast, if the current arises solely from substrate binding, the decay kinetics should remain independent of the LPR.

For all variants tested, the decay time of the transient current was independent of the LPR, clearly indicating that the Phe-gate mutants of both AmtB and Rh50 retain the ability to interact with NH_4^+ but have lost the capacity to complete the full translocation cycle (Figure 6.19). This suggests that while substrate binding is preserved, the conformational or structural changes required for substrate translocation are impaired in these variants. An example of normalised currents comparing LPRs in WT proteins can be seen in the appendix (Figure 9.25).

To evaluate whether the Phe-gate mutants retained their selectivity toward ammonium, Rh50 F218A and AmtB F107A/F215A were subjected to a 200 mM K^+ pulse. Both mutants exhibited an inverse current response, similar to that previously observed in empty liposomes and wild-type proteins (Figure 6.20, Figure 9.26 for AmtB FAFA, Figure 9.27 for Rh50 F218A). This indicates that potassium was not bound nor transported by either mutant, suggesting that the mutations did not compromise substrate selectivity. These findings imply that the Phe-gate is likely not directly involved in maintaining ion selectivity at the pore entrance.

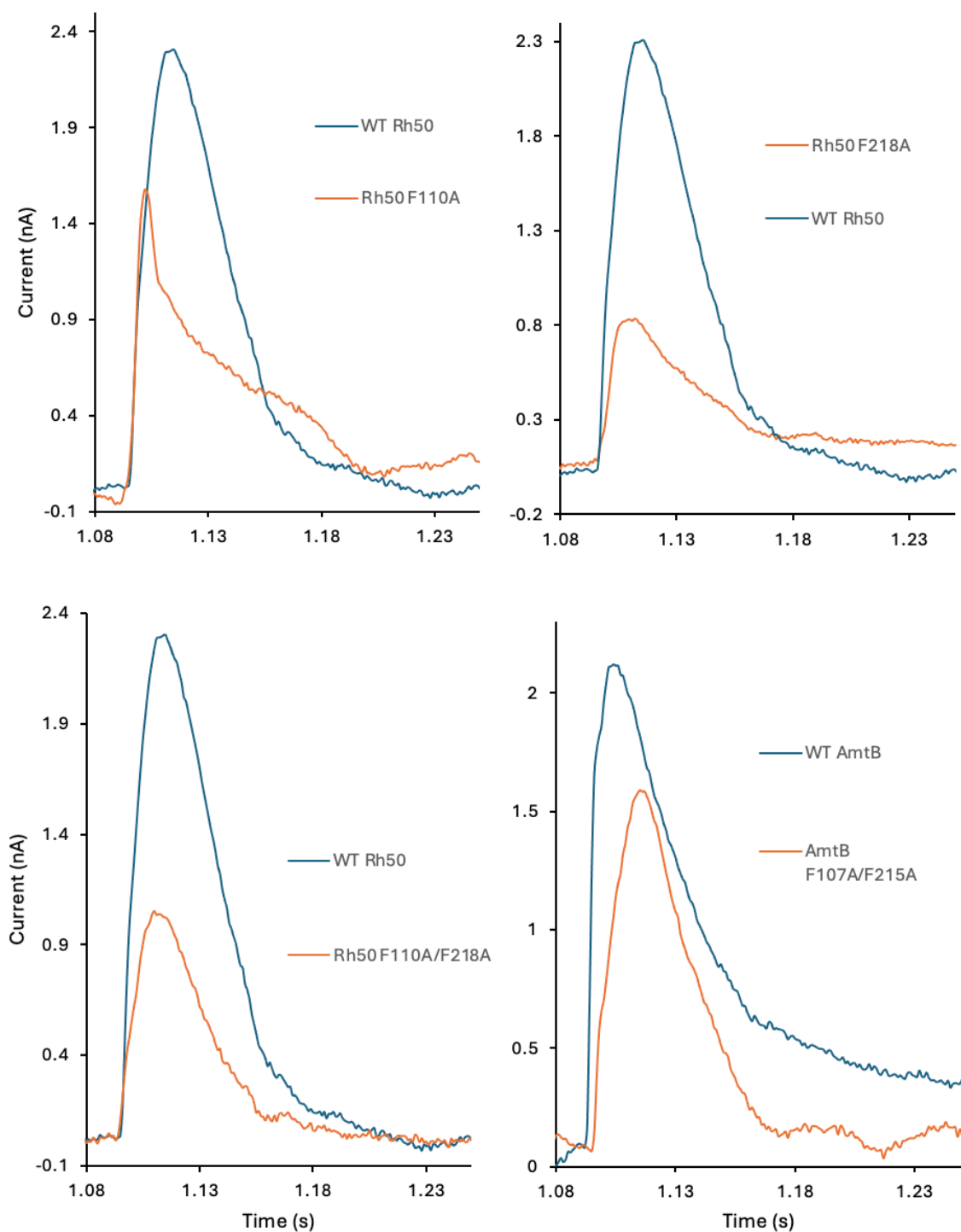


Figure 6.18 **SSME analysis of Phe Gate mutant response to ammonium pulse.** Purified and reconstituted mutant proteins were pulsed with 200 mM NH_4^+ . Representative traces are shown for the mutant proteins (orange) and WT (blue). Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

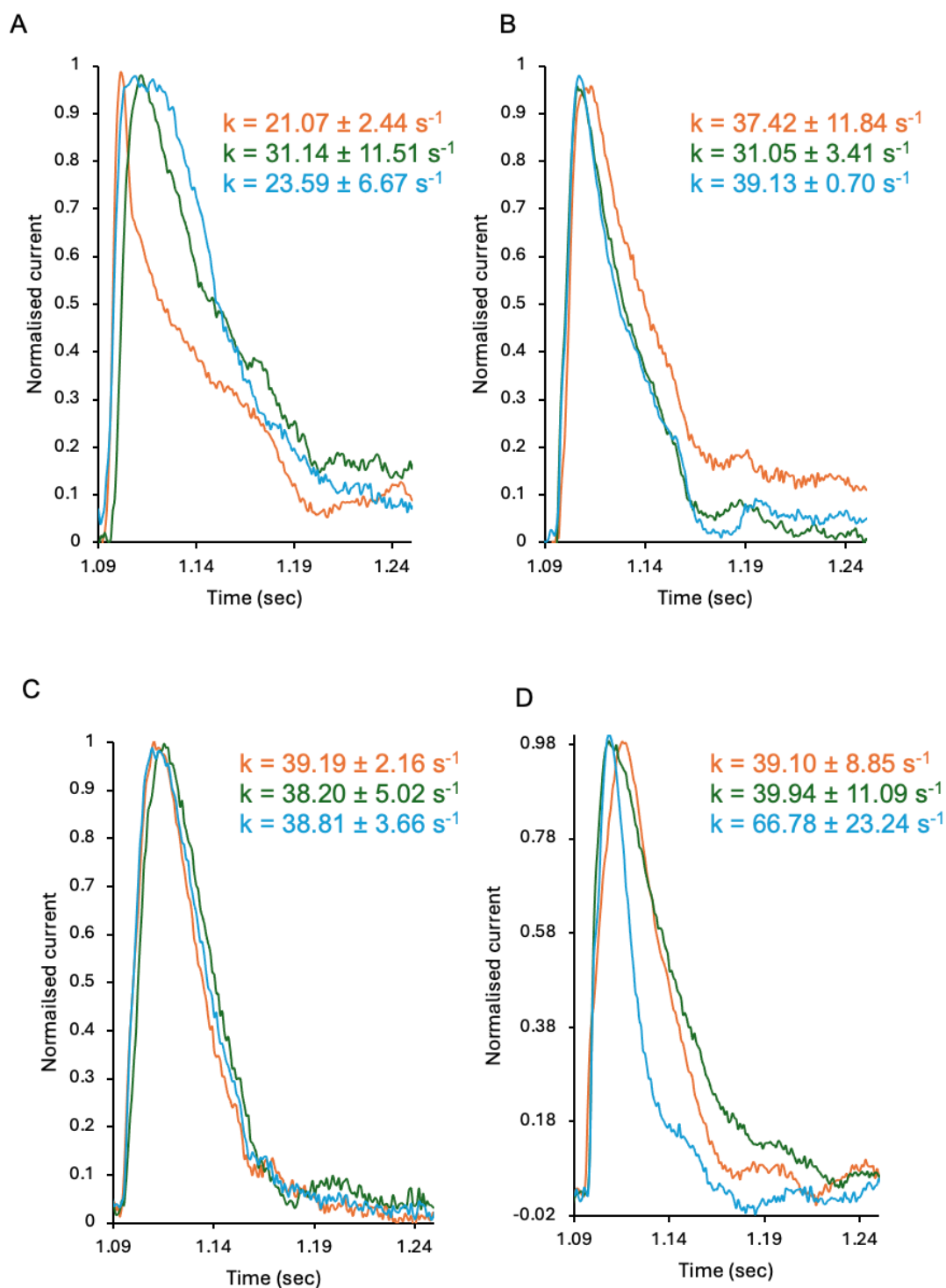


Figure 6.19 **Normalised traces from differed LPR samples in response to ammonium pulse.** Samples of purified and reconstituted proteins at LPR5 (orange), LPR10 (green), LPR50 (blue) in A) Rh50 F110A, B) Rh50 F218A, C) Rh50 F110A/F218A and D) AmtB F107A/F215A. Inset into each graph are the decay values for each trace. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

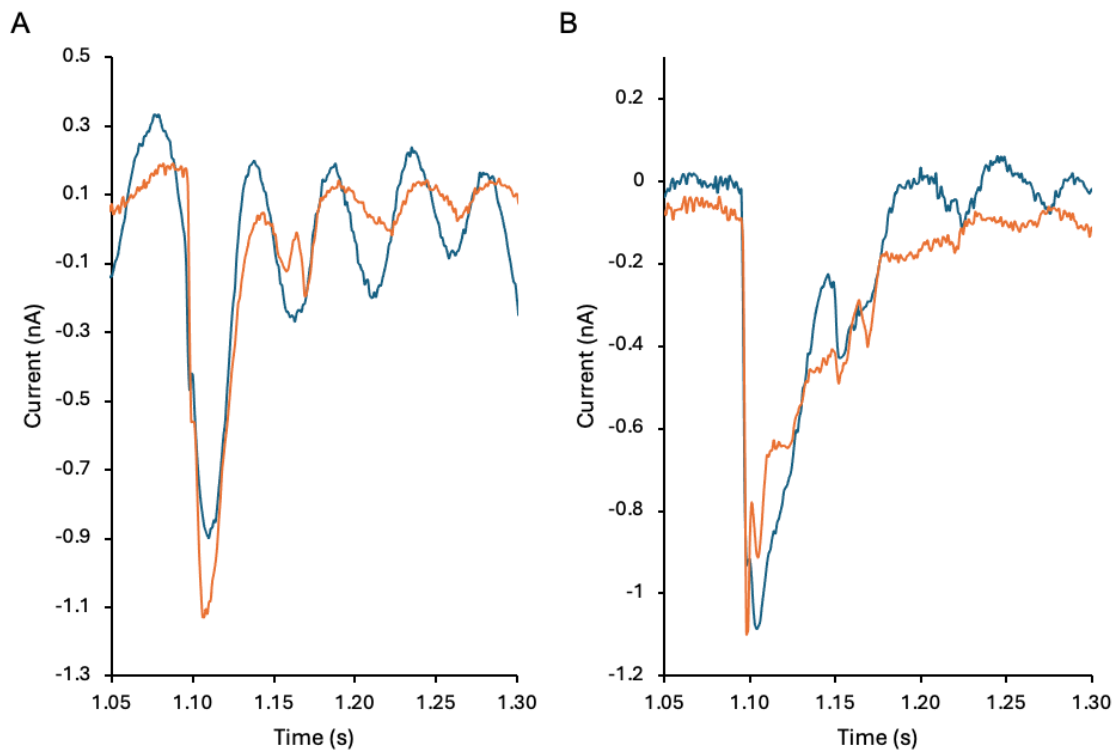


Figure 6.20 **SSME analysis of Phe Gate mutant response to potassium pulse.** Purified and reconstituted A) AmtB F107A/F215A (orange) and B) Rh50 F218A (orange) were pulse with 200 mM K^+ . Empty liposomes (blue) were included as a control. Each condition was measured three times in at least three independent sensors, and one representative trace resembling the average is shown.

6.4 Discussion

6.4.1 Differences in SEC elution peaks

The observed differences in size-exclusion chromatography (SEC) elution peaks for the Phe-gate mutants indicate altered hydrodynamic properties compared to the wild-type proteins. Similar shifts have previously been reported for mutations in the Twin-His motif of Rh50 proteins, suggesting a reduction in structural stability or conformational changes relative to the wild type (Bizior A., unpublished data¹). In the case of *E. coli* AmtB, such changes in elution profile have not been previously observed; all previously characterised mutants have eluted at the same volume as the wild type (Williamson, 2020). Given that the Phe gate is thought to be a dynamic structural element, it may also contribute to the overall stability and conformation of the transporter. Disruption of this gate could therefore alter the protein fold and/or conformation, which may account for the different elution volume observed for AmtB FAFA compared with the WT protein. Reduced stability may likewise have contributed to the low or absent purification yields observed for some of the Rh50 mutants. Weak expression of Phe-gate mutants has also been reported in other members of the family, such as human RhCG, where the F130M mutant showed poor expression (Zidi-Yahiaoui *et al.*, 2009). However, despite these differences in expression and SEC profiles, no major structural differences were observed in the crystal structure of the AmtB F107A, F215A or the double F107A/F215A variant compared to the wild-type protein (Figure 6.21) (Javelle *et al.*, 2008).

¹ A. Bizior thesis "Mechanistic characterisation of the Rh50 ammonium transporter protein from *Nitrosomonas europaea*. (2022).

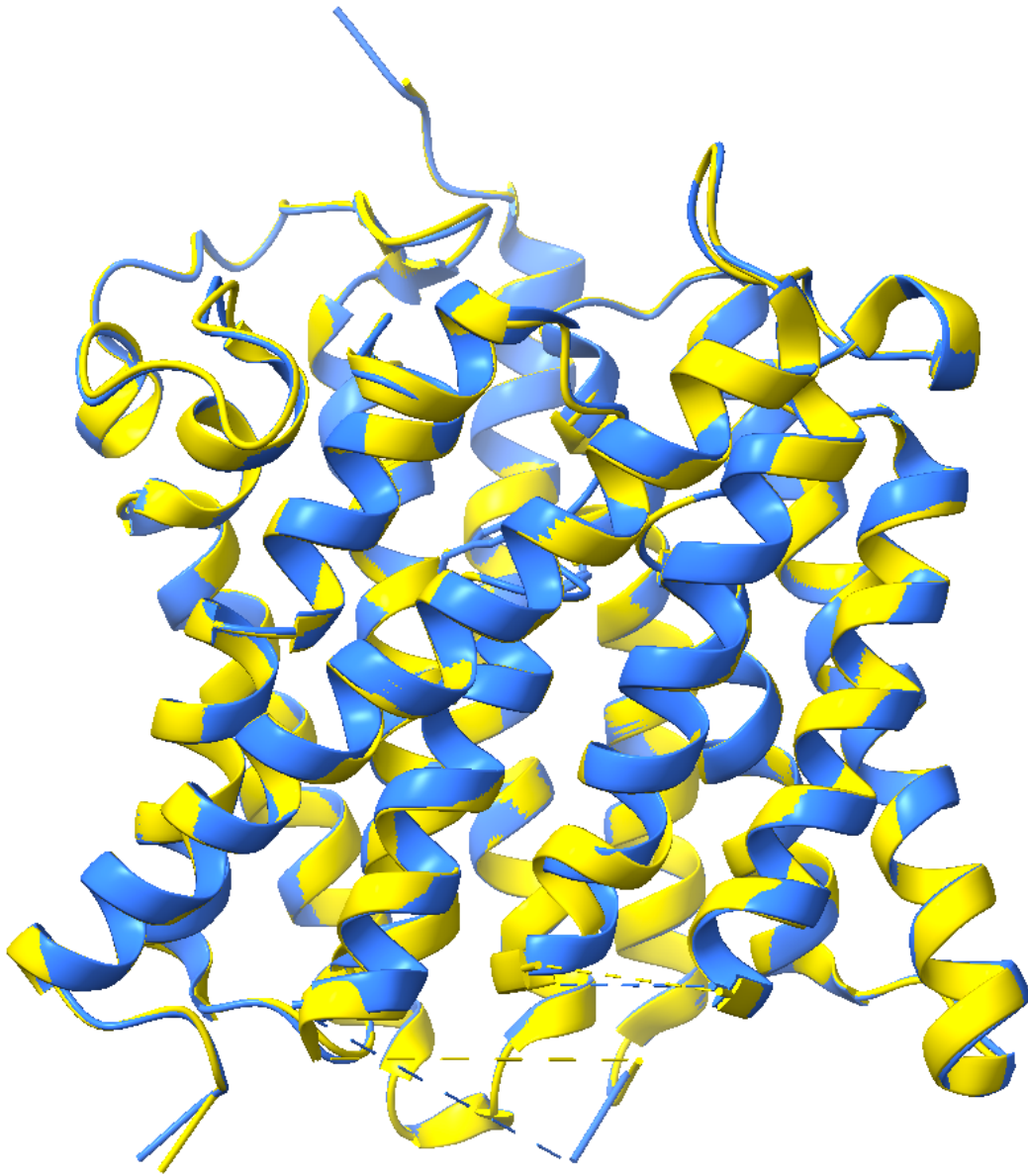


Figure 6.21 Comparison of WT and Phe Gate mutant structures. The variant AmtB F107A/F215A (yellow pdb 3c1j (Javelle *et al.*, 2008)) is structurally identical to the WT AmtB (blue pdb 3c1g (Javelle *et al.*, 2008)).

6.4.2 Mutants' activity

This chapter demonstrated that the Phe-gate mutants of both AmtB and Rh50 initially exhibited reduced activity compared to the wild type (WT). However, when samples with varying LPR were analysed, the decay rate constants did not scale with decreasing LPR, suggesting that transport was potentially impaired and the proteins were unable to complete the full translocation cycle. These findings indicate that mutations in the Phe gate likely result in proteins that retain the ability to bind ammonium but are deficient in its translocation. The function of the Phe Gate has been studied by measuring pH change inside proteoliposomes or HEK cells (Javelle *et al.*, 2008; Zidi-Yahiaoui *et al.*, 2009), *E. coli* growth assay (Hall and Kustu, 2011), yeast complementation (Ganz *et al.*, 2019) and MeA uptake assays (Tremblay and Hallenbeck, 2008; Hall and Kustu, 2011). Numerous MD and Quantum Mechanics/Molecular Mechanics (QM/MM) simulations have also been performed with a focus on the Phe Gate (Cao, Mo and Thiel, 2007; David L Bostick and Brooks, 2007; David L. Bostick and Brooks, 2007; Nygaard *et al.*, 2010). All mutants studied to date with their methods and results can be seen in Table 7.1.

Table 7.1 All Phe Gate mutants studied to date*.

Organism and Protein name	Method	Mutation	Result relative to WT	Reference
<i>E. coli</i> AmtB	MeA transport assay	F107A (1)	Increased activity	(Javelle <i>et al.</i> , 2008)
		F215H (2)	Abolished	
		F215L (2)	Abolished	
		F215Q (2)	Abolished	
		F215S (2)	Abolished	
		F215W (2)	Abolished	

		F107A/F215A	Abolished	
		F215A (2)	Abolished	
<i>Rhodobacter capsulatus</i> AmtB	MeA transport assay	F131A (1)	Reduced	(Tremblay and Hallenbeck, 2008)
	Nitrogenase switch off		Reduced	
<i>H. sapiens</i> RhCG	Internal pH changes in HEK cells, measuring both influx and efflux in response to MeA	F130A (1)	Reduced influx and efflux	(Zidi-Yahiaoui <i>et al.</i> , 2009)
		F235V (2)	Reduced influx and efflux	
		F130A/F235V	Mildly reduced influx and efflux	
<i>E. coli</i> AmtB	<i>E. coli</i> growth assay doubling time	F107L (1)	Slightly increased	(Hall and Kustu, 2011)
		F107A (1)	Slightly increased	
		F215L (2)	increased	
		F215A (2)	abolished	
	MeA uptake assay	F107L (1)	Halved	
		F107A (1)	Trebled	
		F215L (2)	Abolished	
		F215A (2)	Abolished	
<i>Arabidopsis thaliana</i> Amt1;2	Yeast growth assay NH ₄ ⁺ uptake	F147A (1)	Same as WT	(Ganz <i>et al.</i> , 2019)
		F271A (2)	Same as WT	
		F147A/F271A	Abolished	
		F147A (1)	Same as WT	

	Yeast growth assay MeA uptake	F271A (2)	Same as WT	
		F147A/F271A	Abolished	

* (1) denotes the first Phe and (2) the lower in the Phe-gate.

In the case of single mutants affecting the first phenylalanine residue of the Phe-gate, studies have revealed inconsistent results depending on the assay system used. For example, in *E. coli* AmtB, the F107A mutant exhibited increased uptake of methylamine (MeA) relative to the wild type (WT) (Javelle *et al.*, 2011). However, in the same studies, *E. coli* expressing AmtB F107A showed a prolonged doubling time in minimal medium with low concentration of ammonium as the sole nitrogen source, indicating impaired transporter function with NH_4^+ despite enhanced MeA uptake. This discrepancy underscores the limitations of using MeA as a substrate analogue in mechanistic studies of ammonium transporters (Williamson *et al.*, 2023). MeA differs from NH_4^+ not only in size, due to the additional methyl group, but also in physicochemical properties, including a higher pKa (~10.6 versus 9.25), which affects its protonation state and interaction with the transporter. Consequently, MeA may alter pore entry kinetics and translocation efficiency, leading to misleading interpretations of activity, particularly in mutant variants.

Additionally, differences in assay methodology further complicate interpretation. Transport assays provide high-resolution, real-time measurements on the scale of seconds, directly capturing transporter activity. In contrast, growth-based assays, spanning hours to days, integrate multiple physiological processes and can obscure specific transporter-related effects. This likely explains the discrepancy between my results and those of Ganz *et al.* (2019), who used yeast growth assays and concluded that the *A. thaliana* Amt1;2 F147A (equivalent of Rh50 F110A) variant remained functionally active (Ganz *et al.*, 2019). In contrast, studies using more direct methods, including ammonium transport assays in human RhCG and nitrogenase switch-off assays in *Rhodobacter capsulatus* after an ammonium pulse, have consistently demonstrated impaired transport when the first phenylalanine residue is mutated (Tremblay and Hallenbeck, 2008; Zidi-Yahiaoui *et al.*, 2009). These findings, in agreement with the data presented in this thesis, strongly support the conclusion that mutation of the first phenylalanine residue compromises transporter function.

With respect to the second phenylalanine residue of the Phe-gate, most studies, excluding *A. thaliana* Amt1;2 (Ganz *et al.*, 2019), consistently report that single mutations at this site abolish or significantly reduce transport activity (Javelle *et al.*, 2008; Zidi-Yahiaoui *et al.*, 2009; Hall and Kustu, 2011; Ganz *et al.*, 2019). Whether the anomalous activity observed in *A. thaliana* Amt1;2 represents a genuine functional divergence or results from limitations in the assay approach remains unresolved and merits further investigation.

Finally, in the case of double mutants targeting both phenylalanine residues of the gate, all published studies to date, including those reported here, converge on the conclusion that these variants are inactive (Table 7.1).

In conclusion, both experimental data and the broader literature strongly support a model in which the conserved phenylalanine residues of the Phe-gate are critical for ammonium transporter function. Mutation of either residue impairs activity, and disruption of both leads to complete inactivation, highlighting their essential role in substrate translocation. The question is what is the functional role of the Phe-Gate?

6.4.3 Phe Gate role in selectivity

One hypothesis concerning the function of the Phe-gate in the translocation cycle could be that the Phe-gate contributes to substrate specificity by restricting the passage of hydrated ions. In this context, removal of the Phe-gate would increase water accessibility to the pore, enhancing local hydration and potentially allowing the permeation of hydrated ions of similar size as NH_4^+ , such as K^+ . In addition to this functional consideration, structural differences in Phe-gate architecture across the superfamily raise the possibility of divergent roles in different proteins. Specifically, in Rh proteins, the aromatic side chains within the Phe-gate adopt a perpendicular configuration, forming an L-shaped constriction, whereas in bacterial AmtBs such as AmtB, the phenyl rings are arranged in a parallel orientation. This structural divergence suggests that the functional contribution of the Phe-gate may not be conserved across the Amt/Mep/Rh family.

The experimental findings presented in this chapter show that Phe-gate variants of both AmtB and Rh50 do not bind or translocate potassium ions, indicating that the loss of the Phe-gate does not compromise substrate selectivity. These results are

consistent with previous studies employing stopped-flow spectrofluorometry, which demonstrated that deletion of the Phe-gate in AmtB does not significantly alter the overall protein structure, nor does it facilitate water conduction across the membrane (Javelle *et al.*, 2008). In contrast, the situation in Rh proteins has remained less clear. Molecular dynamics simulations have suggested that unlike in AmtB, water molecules in Rh50 can form a continuous water bridge from the periplasmic vestibule to the central pore, potentially enabling water permeation (Hoyhtya *et al.*, 2020). However, my experimental data indicate that removal of the Phe-gate has a similar functional impact in both AmtB and Rh50, specifically inhibiting the proteins' ability to complete the full ammonium translocation cycle. These findings imply that although the Phe-gate does not directly define ion selectivity at the pore entrance, it may play a critical role in facilitating efficient transport in both Amt and Rh proteins.

6.4.4 The Phe Gate's role in the deprotonation of ammonium

The precise location at which ammonium undergoes deprotonation within Amt/Mep/Rh transporters has not yet been conclusively established. The first crystal structures of *E. coli* AmtB revealed several potential $\text{NH}_4^+/\text{NH}_3$ binding sites, designated Am1 through Am4 (Figure 6.22) (Khademi *et al.*, 2004). MD and QM/MM simulations have suggested that the Am1 and Am2 sites are plausible candidates for the deprotonation site (Lin, Cao and Mo, 2006, 2009; Nygaard *et al.*, 2006, 2010; Bostick and Brooks, 2007; Bostick and Brooks, 2007; Ishikita and Knapp, 2007). Am1 is located in the periplasmic recruitment region, while Am2 lies deeper within the pore, just after the second conserved phenylalanine of the Phe-gate (Figure 6.22).

Am2 is situated in a highly hydrophobic region of the pore, where the energetic cost of maintaining a charged species is high. As a result, this environment is thought to promote the deprotonation of NH_4^+ to NH_3 , facilitating its passage through the channel. This has led to the hypothesis that the Phe-gate plays a critical role in the deprotonation process. The results presented in this thesis are consistent with this model: all single or double phenylalanine mutants within the gate exhibit a complete loss of transport activity, supporting the essential functional role of the Phe residues. Computational studies using QM/MM and MD simulations have demonstrated that NH_4^+ can penetrate the pore and pass beyond the Phe-gate (Lin, Cao and Mo, 2006, 2009; Nygaard *et al.*, 2006, 2010; Bostick and Brooks, 2007; Bostick and Brooks,

2007; Ishikita and Knapp, 2007). Moreover, MD simulations have shown that the pKa of the $\text{NH}_4^+/\text{NH}_3$ equilibrium decreases from 9.25 to approximately 6.5 within the vicinity of the Am2 site (Figure 6.22) (Akgun, Khademi and Stroud, 2011), further supporting the notion that the hydrophobic environment at the Am2 site, below the Phe-gate, favours NH_4^+ deprotonation. Thus, it is plausible that the Phe-gate functions as a molecular trap, stabilising NH_4^+ within a hydrophobic pocket that facilitates its deprotonation prior to NH_3 translocation. However, the exact mechanism of deprotonation if it occurs in proximity to the Phe-gate, remains to be elucidated.

6.4.5 Two transport mechanisms

Unlike *E. coli* AmtB, which exhibits complete loss of activity when measured in deuterated water (D_2O) (Williamson *et al.*, 2020), NeRh50 have been found to retain partial activity under the same conditions (A. Bizior, unpublished²). The loss of AmtB activity in D_2O is attributed to the critical role of proton transfer in its translocation mechanism. Following deprotonation of NH_4^+ , the resulting proton is believed to be transferred through the pore via a network of water molecules, often referred to as "water wires." These water wires enable rapid proton hopping through the Grotthuss mechanism, which is highly sensitive to the physicochemical properties of the medium. In D_2O , proton mobility is significantly reduced due to the greater mass of deuterons and the stronger hydrogen–deuterium bond, leading to an approximately 30% reduction in proton mobility per D_2O molecule compared to H_2O (Wiechert and Beitz, 2017). Given that the water network within the AmtB pore involves more than three ordered water molecules, the cumulative effect of this reduced mobility is sufficient to disrupt proton translocation, rendering AmtB inactive in D_2O . This observation underscores the reliance of AmtB on an efficient proton relay mechanism, which may differ in structure or dynamics in Rh50 proteins, thereby accounting for their residual activity in deuterated conditions. This residual 40% activity measured in D_2O implies that in Rh50, although the dominant mechanism of transport is deprotonation followed by transport of the proton via the water wires while NH_3 diffuses through the pore, the same as AmtB, there is also a second mechanism whereby NH_4^+ can move straight through the pore without deprotonation. MD simulations of NeRh50 show that because

² A. Bizior thesis "Mechanistic characterisation of the Rh50 ammonium transporter protein from *Nitrosomonas europaea*. (2022).

of the different orientation of the first Phe, water molecules can penetrate 2Å deeper into the pore than in AmtB (Hoyhtya *et al.*, 2020). This is also coupled with a shorter hydrophobic pore (21Å and 11 Å for AmtB and NeRh50 respectively) and, unlike AmtB, a lack of Phe residues guarding the cytoplasmic exit (Lupo *et al.*, 2007). It is therefore plausible that NeRh50 employs two distinct transport mechanisms: one involving the deprotonation of NH_4^+ to NH_3 prior to translocation, and another in which hydrated NH_4^+ is directly translocated across the membrane. The latter may represent the mechanism utilised by Rh proteins to support bidirectional transport. In agreement with our hypothesis, yeast complementation assays investigating *A. thaliana* Amt1;2 found that variants bearing the F271A mutation resulted in an accumulation of ^{15}N nitrogen in the yeast cells (Ganz *et al.*, 2019). Nitrogen exists as two isotopes ^{14}N and ^{15}N which is more difficult to deprotonate (Ariz *et al.*, 2018). For this reason, a build-up of ^{15}N suggests NH_4^+ is being transported without deprotonation which is similar to what has been seen in the Rh50 protein. This observation strongly suggests that the Phe gate is involved in the deprotonation process, although the precise mechanism remains to be elucidated

6.5 Conclusion

In this chapter, four Phe gate variants of AmtB and Rh50 were generated, expressed, purified, and reconstituted. Their activities were assessed using SSME, which showed that the Phe mutants did not transport ammonium. However, the mutants also failed to transport other cations such as K^+ , suggesting that the Phe gate is not the primary determinant of substrate selectivity. Instead, these findings indicate that the Phe gate plays an essential role in the transport mechanism itself, which may explain its evolutionary conservation. This interpretation is supported by previous studies using growth assays (Ganz *et al.*, 2019) and computational modelling (Lin, Cao and Mo, 2006, 2009; Nygaard *et al.*, 2006, 2010; Bostick and Brooks, 2007; Ishikita and Knapp, 2007), which suggest that the Phe gate may contribute to the deprotonation of ammonium, thereby enabling its transport.

6.6 Future work

Owing to a lack of time, I was not able to fully investigate the Phe Gate in this chapter. Further work should include the development of the single F107A and F215A AmtB mutants along with substituting the residues with other aromatic rings. Some studies have identified a conserved Trp residue that is found near the Phe Gate and appears to have an impact on its function, with mutants either rescuing lost activity or abolishing activity depending on the combination of Phe gate mutants they were paired with (Hall and Kustu, 2011; Ganz *et al.*, 2019). This residue is also a candidate for further research. The ammonium recruitment site in Rh50 and AmtB differs also. In AmtB the recruitment site consists of the residues F107, W148, D160 and S219. In Rh50 and other Rhesus proteins, the Trp and Ser are missing and are replaced with a conserved Glu (E146 in Rh50) sitting at the top of the pore entrance. Because of its negative charge it is thought that this residue is involved in ammonium recruitment (Hoyhtya *et al.*, 2020). Although I managed to create, express, purify and reconstitute E146D and E146Q mutants, I was not able to measure their activity using SSME again due to a lack of time.

Investigating the potential activity of A162 in EcAmtB would also be interesting. MD simulations suggest it may guide ammonium through the Phe Gate meaning it would be integral to the function of the protein and mutation may disable activity (Nygaard *et al.*, 2010). If it does disable activity it could point to ammonium indeed being deprotonated at the Am2 site as if deprotonation were to occur at the Am1 site, ammonium would not need guiding in its uncharged NH_3 form.

More SSME analysis into the ability of the other mutants to transport K^+ would allow for confirmation of the retention of selectivity.

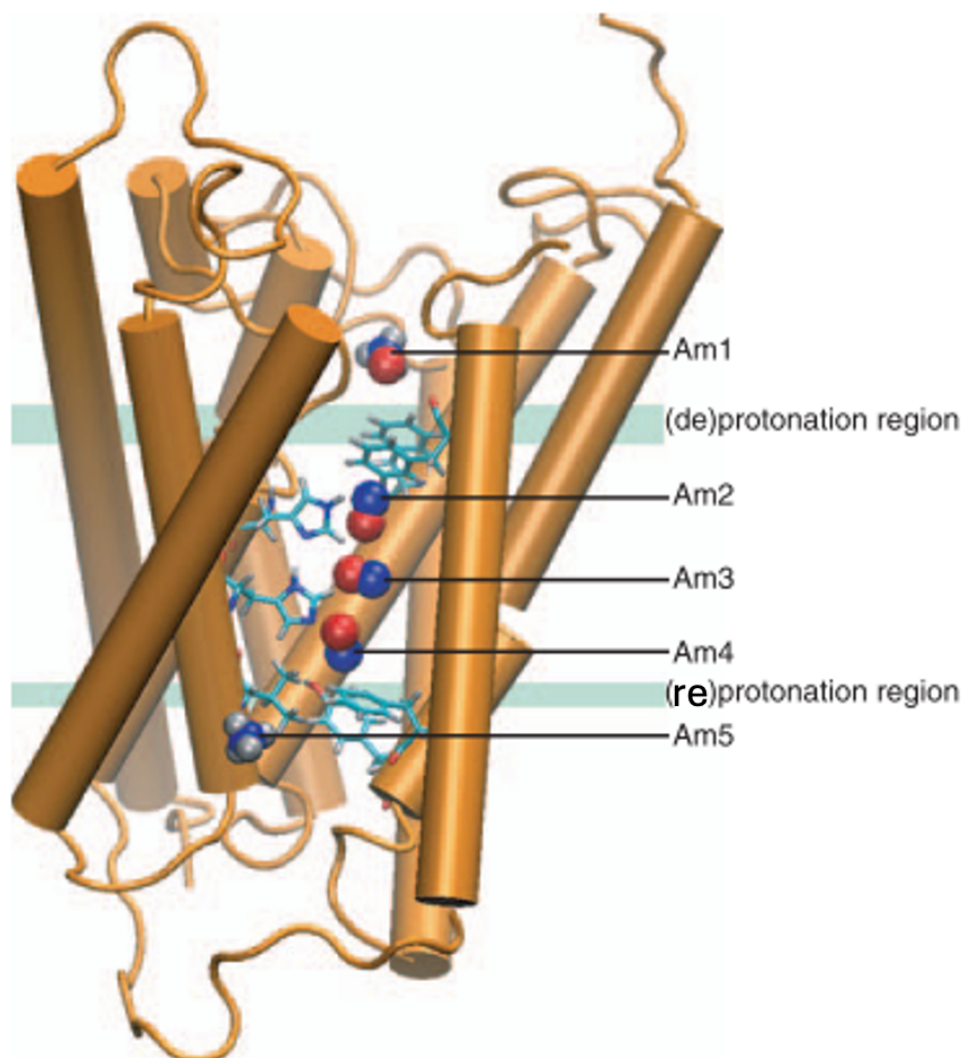


Figure 6.22 Location of Am sites. Deprotonation is thought to occur between the Am1 and Am2 sites. Figure adapted from (Bostick and Brooks, 2007)

Chapter 7: General Discussion

7.1 Overall Aim of the PhD Thesis

The overarching objective of this PhD was to deepen our understanding of the Amt/Mep/Rh superfamily of ammonium transporters by integrating evolutionary, functional, and mechanistic perspectives. This was achieved by combining phylogenetic analysis to explore evolutionary relationships, the development and optimisation of experimental approaches to enable functional characterisation across a wider diversity of family members, and targeted investigation of conserved structural features, specifically the phenylalanine gate (Phe-gate), to elucidate their role in transport mechanisms in representative prokaryotic and eukaryotic homologues.

7.2 General summary

By selecting a diverse set of organisms from across the Tree of Life (Hug *et al.*, 2016), I constructed a large and comprehensive phylogenetic tree tailored to investigate the origins and evolution of a key transporter superfamily. This analysis led to the discovery of a potentially novel classification system, grouping these proteins into three functional categories: transporters, sensors, and bidirectional import/exporters. For the first time, I also proposed a well-supported root to the tree, positioned between the sensor and transporter clades, offering new insights into their evolutionary divergence (Chapter 3).

Further investigation revealed that ammonium transporters fused to enzymatic domains are exclusively found within the sensor group, suggesting a functional and evolutionary link. Interestingly, despite functional diversity across the tree, the Phe Gate motif, a critical element in substrate transport, is strictly conserved in all ammonium transporters (Chapter 4). These findings also suggest that the Last Universal Common Ancestor (LUCA) may have expressed multiple distinct ammonium transporters, highlighting their ancient and fundamental role.

To experimentally probe the functional properties of key proteins identified through phylogenetics, I adopted a novel approach for Solid Supported Membrane Electrophysiology (SSME). By generating vesicles directly from overexpressing cells, I eliminated the need for traditional protein purification and reconstitution, significantly streamlining the workflow (Chapter 5).

Finally, I characterised several Phe Gate mutants in EcAmtB and NeRh50. Despite structural differences in the gate conformation between Amt and Rh proteins, mutations in this region consistently abolished transport activity, reinforcing the gate's essential functional role (Chapter 6).

Overall, this work not only advances our understanding of the evolutionary history and classification of this important superfamily but also opens multiple new avenues for mechanistic exploration, with implications for understanding transport across all domains of life.

7.3 General Discussion

7.3.1 A New Root for Ammonium Transporters: Rethinking Evolutionary Origins and Classification

Previous phylogenetic studies of the Amt/Mep/Rh family have either left the tree unrooted (Couturier *et al.*, 2007; Huang and Peng, 2005; Matassi, 2017; Matassi *et al.*, 1999; Pitts *et al.*, 2014) or used Rhesus (Rh) proteins as an outgroup to root the tree (Cherif-Zahar *et al.*, 2007; Kitano *et al.*, 1999; Kitano and Saitou, 2000; McDonald *et al.*, 2012; McDonald and Ward, 2016). The choice of root fundamentally influences the interpretation of evolutionary relationships, as it imposes assumptions about the order in which subgroups diverged.

7.3.1.1 A Novel Rooting Strategy Using Pore-Entrance Residues

In Chapter 3, I present an alternative rooting strategy based not on outgroups, branch lengths, or global sequence similarity, but on a specific amino acid residue located at the entrance of the ammonium binding pocket within the pore: Glu-containing proteins as sensors, Tyr-containing proteins as transporters, and Met-containing proteins as bidirectional transporters/Rh proteins. Despite omitting traditional rooting criteria, this method successfully partitioned the tree into biologically and functionally plausible groups. Interestingly, this key residue was not included in the multiple sequence alignment used to generate the phylogenetic tree. This is because, although it resides in the conserved helical core, it lies outside the predicted transmembrane (TM) regions based on UniProt annotations. Similarly, inter-TM regions, which may also influence protein function, were excluded from the alignment. Despite these omissions, the

resulting tree still resolved into the three functional categories, sensors, transporters, and bidirectional transporters, supporting the robustness of this classification.

7.3.1.2 *Disrupting the Amt/Mep/Rh Paradigm: Phylogenetic Insights from a Novel Rooting Strategy*

The inclusion of a broad range of ammonium transporters and a justifiable root placement allowed for novel insights into the family as a whole. In my tree the Rh proteins appear to have arisen from the Glu-sensor group. Although it has been hypothesised that Rh proteins arose from an Amt duplication event it was not clear what sort of ammonium transporter this was (Cherif-Zahar *et al.*, 2007; Matassi, 2017). In this study the Rh proteins were defined as those with a Met residue at the ammonium binding pocket, this differs from other studies in that it uses a direct comparison rather than branch length or organism but is still comparable.

The Mep proteins clustered as a distinct sub-group of the AmtS, this has been previously seen in published research (McDonald *et al.*, 2012; McDonald and Ward, 2016; Pitts *et al.*, 2014). While McDonald *et al.*, (2012) extended the Mep name to include proteins that clustered around the fungal proteins (McDonald *et al.*, 2012), in this research I identified archaeal proteins that share the mixture of EH and HH Twin-His motif arrangement sitting at the base of the fungal protein group. Although it is not clear if the fungal Meps arose from these archaeal proteins, their proximity and similarity in terms of the EH/HH mixture suggest this is the case. This may mean that the conventional Amt/Mep/Rh classification of these proteins is too narrow and misses nuance in function that has evolved over time.

It remains an open question whether these phylogenetically defined groups truly reflect functional and physiological differences *in vivo*. To address this, my next line of research focused on developing a method to functionally characterise these proteins using SSME. Crucially, this method eliminates the need for protein purification and reconstitution, allowing for direct measurement of activity in native vesicle preparations.

7.3.1.3 *A New Root, a New Ancestry: Evidence That LUCA Possessed Multiple Ammonium Transporters*

Our novel phylogenetic analysis, based on a newly proposed root placement, reveals a striking pattern: each side of the root contains representatives from all three domains of life, bacteria, archaea, and eukaryotes, indicating that these transporter lineages predate the divergence of modern domains. This strongly suggests that the LUCA already possessed more than one distinct ammonium transporter, which were subsequently passed vertically through evolutionary lineages. Such a conclusion challenges existing assumptions, as previous reconstructions of LUCA's proteome did not include ammonium transporters, likely due to their methodological focus on monophyletic protein families, which excludes the highly divergent ammonium transporter superfamily (Sousa, Nelson-Sathi and Martin, 2016; Weiss *et al.*, 2016).

7.3.1.4 *What's Left Out Matters: Dataset Gaps and Their Role in Interpreting Evolutionary History*

Although attempts were made to acquire proteins for as diverse a group of organisms possible to make the phylogenetic tree in Chapter 3, we know that some have been left out. These include archaea with Rhesus proteins (Matassi, 2017). Although inclusion of extra sequences would possibly not influence the general tree structure of sensor/transporter split, it may offer better insights into the evolution of certain subfamilies. The reasons for the lack of inclusion come from a few angles. Using the organism names from the Tree of Life (Hug *et al.*, 2016) provided a number of challenges as many were not in the same format or came from unnamed organisms and were therefore not included in the list retrieved. Also, when pulling the TM domains, if a protein had fewer than 11 predicted regions it was not included. As was shown in Materials and methods section 2.2.2.9 this was sometimes a mistake. When retrieving the genomic DNA sequences some proteins failed to find a match and were also dropped from the analysis. Finally, removal of duplicate sequences at the final step before tree calculation may have led to a reduction in diversity of organisms but not proteins. The pfam family PF00909 is perhaps a better resource for acquiring sequences but the number of proteins is prohibitively large (76,000 proteins on 29/07/25). Therefore, clustering these protein sequences and taking the representative sequence from each cluster may provide the greatest diversity of

protein groups. This may come with the caveat of reducing organism diversity as closely related sequences will not be included. It also brings the risk of a representative sequence failing the TM region or genomic DNA step and thus a whole group not being included in the final analysis.

7.3.2 From Tree to Transport: How Phylogeny Informs Ammonium Transporter Mechanism

Although the physiological functions of ammonium transporters have been studied extensively, their underlying transport mechanisms are broadly categorised as electrogenic or electroneutral. More recently, a third functional category has emerged, proteins that bind NH_4^+ but do not transport it (Pflüger *et al.*, 2018, 2024). These mechanistic distinctions have been investigated using different systems, most notably *X. laevis* oocyte expression assays (Brito *et al.*, 2020; Caner *et al.*, 2015b; Loqué *et al.*, 2009; Ludewig, Wiren, *et al.*, 2002; Nakhoul *et al.*, 2006) and solid-supported membrane electrophysiology (Mirandela *et al.*, 2019; Pflüger *et al.*, 2018, 2024; Williamson *et al.*, 2020, 2022). Interestingly, all proteins studied via SSME have so far been either electrogenic or non-transporting sensors, whereas oocyte-based assays have reported both electrogenic and electroneutral transporters, though the latter system has known methodological limitations (see Chapter 1, Section 1.4.5).

These mechanistic differences are intriguing, especially considering that all members of the ammonium transporter superfamily share highly conserved functionally important structural features, such as the Phe Gate, Twin-His motif, and a hydrophobic pore. This raises the possibility that mechanistic divergence may not be as widespread as assumed, or conversely, that subtle sequence variation leads to functional specialisation.

The phylogenetic tree developed in this work reveals distinct clades of ammonium transporters, which now provide an evolutionary framework for investigating mechanistic differences (Figure 1.9). This classification opens the door to systematic testing: Are all proteins in the “sensor” group truly non-transporting? Are all Rhesus proteins genuinely bidirectional? Do all transporters within a given clade share the same underlying mechanism?

Future functional studies can be improved by combining this phylogenetic insight with advances in experimental methodology. For example, generating right-side-out (RSO) vesicles directly from overexpressing cells ensures native membrane orientation and

consistent topology. Creating inside-out (ISO) vesicles via methods such as French press disruption allows for direct comparison of transport directionality. This controlled setup is a major improvement over reconstitution approaches where membrane insertion orientation is mixed and often ambiguous. Such methods will be especially valuable for systematically assessing bidirectionality, particularly in proteins like Rh50, where unpublished data suggest that mutations in the Twin-His motif impair bidirectional transport (Bizior A., unpublished data³).

By integrating phylogenetic structure with precise functional assays, we can now begin to map evolutionary divergence onto mechanistic function, transforming our understanding of ammonium transporter biology.

In Chapter 6 I found that in both Rh50 and AmtB mutations to the Phe Gate led to a lack of transport but not a change in selectivity against K^+ . This suggests that despite the differences in Phe Gate orientation between the two, their role appears to be similar, further adding evidence of a potential conserved mechanism.

7.4 Future work

7.4.1 Continuation of investigating the Phe gate variants

Owing to time constraints I was not able to complete the full investigations into the Phe Gate that I had planned. The next steps would be to create the single Phe-Ala mutants in AmtB which has already been completed by Peter Henderson a new PhD student in the Javelle lab. K^+ selectivity measurements should also be performed on all mutants.

7.4.2 Creation of vesicles from yeast and mammalian cells

One of the primary motivations for generating vesicles directly from the cells used to overexpress our target protein was to enable the functional characterisation of eukaryotic ammonium transporters. Achieving this would require adapting the bacterial vesicle preparation protocol for application in yeast systems. *S. cerevisiae* has been widely used for heterologous expression of ammonium transporters from diverse

³ A. Bizior thesis "Mechanistic characterisation of the Rh50 ammonium transporter protein from *Nitrosomonas europaea*. (2022).

organisms, including plants, insects, bacteria, and archaea, for functional studies (for review, see Williamson *et al.*, 2023, and references therein). Furthermore, established protocols exist for generating spheroplasts from yeast using zymolyase, after which cell disruption can be performed via osmotic lysis or mechanical methods such as French press (Kaback, 1970).

However, attempts to express mammalian Rh50, both in its native sequence and in codon-optimised forms, were unsuccessful in *E. coli* and *S. cerevisiae* systems, yielding insufficient protein levels for crystallisation or functional analysis. As an alternative, stable transfection of RhCG into HEK293 cells proved successful, facilitating both crystallisation of the protein (Gruswitz *et al.*, 2010) and its reconstitution into artificial liposomes for activity assays (Mouro-Chanteloup *et al.*, 2010). HEK293 cells have also been employed to generate vesicles for SSME using nitrogen decompression as a method of cell disruption (Bazzone *et al.*, 2022). In that study, the human glucose transporter SGLT1 was successfully characterised. Direct measurement of SGLT1 using SSME provided new mechanistic insights not previously accessible with traditional two-electrode voltage-clamp (TEVC) recordings in *X. laevis* oocytes. SSME offers particular advantages for transporters with low activity, as it enables high signal-to-noise measurements that are often difficult to achieve using TEVC.

Increasing the diversity of proteins we can directly measure will allow us to investigate human Rhesus variants associated with disease like RhAG F65S which is thought to cause OHS by losing selectivity to NH_4^+ and allowing leakage of monovalent cations (Bruce *et al.*, 2009; Stewart *et al.*, 2011). When measured on SSME, the equivalent F55S mutation in NeRh50 was found to be inactive (Bizior A., unpublished data⁴). This difference highlights the need to move away from using model proteins like AmtB and Rh50 and investigate the actual protein causing the pathologies. Taken together, the combination of heterologous expression in *S. cerevisiae* or HEK293 cells with SSME-based functional assays represents a powerful and scalable approach for the mechanistic characterisation of ammonium transporters. This strategy provides a clear path forward to systematically investigate the diverse members of the Amt/Mep/Rh

⁴ A. Bizior thesis "Mechanistic characterisation of the Rh50 ammonium transporter protein from *Nitrosomonas europaea*. (2022).

family, including those that have been historically difficult to express or analyse using traditional methods.

7.4.3 Structural analysis using CryoEM

To date, the structures of eight ammonium transporters have been resolved all using X-ray crystallography with the proteins stabilised in detergent micelles. These structures consistently reveal a highly conserved overall architecture, with no observable conformational differences between the ammonium-bound and unbound states. This lack of visible conformational change may reflect the tendency of crystallisation to capture a single, energetically favourable conformation, potentially obscuring dynamic states relevant to the transport cycle. To overcome this limitation and gain insight into possible conformational dynamics, cryogenic electron microscopy (CryoEM) was employed. Unlike X-ray crystallography, CryoEM allows the protein to be embedded within a lipid environment more closely resembling the native membrane, such as via peptidiscs, which stabilise the protein using native or synthetic lipids rather than detergent. This approach offers the potential to capture multiple conformational states and better reflect the physiological context of the transporter. (Carlson *et al.*, 2018). I therefore purified WT AmtB and Rh50 and in collaboration with Dr Georgia Isom and Dr Benjamin Cooper at the William Dunn School of Pathology in Oxford University, we have solved the structures of these proteins using CryoEM to a resolution of 3.1Å for AmtB (Figure 7.1) and 2.9Å for Rh50 (Figure 7.2A). This is the first time that structures have been solved using this method for any ammonium transporter.

For WT Rh50, water molecules can be seen in the structures. This is important as the mechanism of transport depends on the two water wires to carry the proton through the hydrophobic pore. It also means that the effect of mutations on the hydration of the pore can be seen. Also, the pore hydration of the WT is different to AmtB; due to the 90° turned first Phe gate water molecules are able to penetrate ~2Å deeper into the pore (Hoyhtya *et al.*, 2020; Lupo *et al.*, 2007).

A conformational dynamic has also been observed for the first time for any ammonium transporter structure (Figure 7.2B). NeRh50 appears to tense up, drawing the cytoplasmic C terminal helices toward the transmembrane region and twisting the TM helices closer together. It is not yet known which state is in response to ammonium.

Finally, lipids have been seen previously bound to AmtB (Laganowsky *et al.*, 2014; Mirandela *et al.*, 2019), in the cryoEM structure annular lipids have also been seen to be bound to the Rh50 structure. The lipids included an inverted lipid bound between each monomer (Figure 7.2C). These lipids are thought to be crucial for the structure and function of the proteins.

This research is being continued by Peter Henderson a PhD student in the Javelle group. His research will focus on the structure of AmtB and Rh50, the Phe gate variants and he is also performing yeast complementation assays. It is hoped that his work will elucidate a clear conformational change in the presence of ammonium and further our knowledge about these intriguing proteins

7.5 Conclusion

This research has, for the first time, provided a justifiable rooting of the phylogenetic tree for the Amt/Mep/Rh superfamily of ammonium transporters. This novel rooting enabled new evolutionary insights, including the hypothesis that multiple ammonium transporters were already present in LUCA, a finding not previously proposed. Notably, previous studies reconstructing LUCA's proteome did not include ammonium transporters (Sousa, Nelson-Sathi and Martin, 2016; Weiss *et al.*, 2016), likely due to the methodological constraints of focusing only on monophyletic protein families.

In addition to the phylogenetic analysis, I began to develop a technique to measure the activity of *E. coli* AmtB using SSME, employing a new method involving the direct generation of vesicles from the cells used for overexpression. This approach improves upon previous reconstitution-based techniques by preserving native membrane topology and enabling more consistent protein orientation.

I further demonstrated that the conserved Phe Gate plays a critical role in ammonium transport in both *E. coli* AmtB and *N. europaea* Rh50, despite their structural and evolutionary divergence. This finding suggests a conserved mechanism across distinct subfamilies, reinforcing the functional relevance of this motif throughout the superfamily.

Collectively, this work has addressed the core aims of the project: constructing a robust phylogenetic framework, uncovering evolutionary relationships and potential ancestral origins, developing methodological tools for functional analysis, and

exploring mechanistic features conserved across diverse ammonium transporters. Importantly, this is the first comprehensive phylogenetic study of the Amt/Mep/Rh superfamily, and it provides a valuable foundation for future research. In parallel, by adapting the SSME technique to work with vesicles derived from different expression systems, this research opens the door to functionally characterising a broader range of transporters, including those from eukaryotic sources, that have previously been difficult to study.

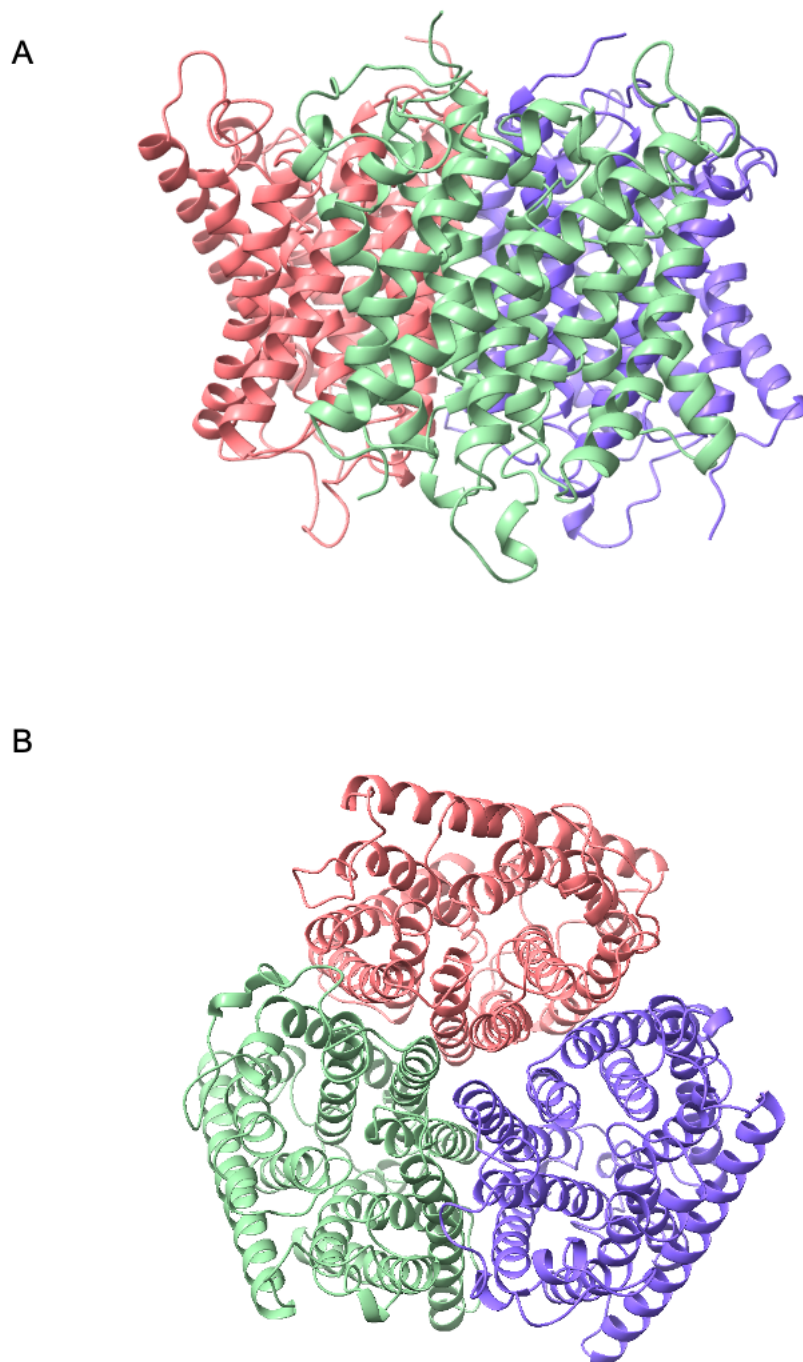


Figure 7.1 **Ribbon structure of AmtB solved using CryoEM at 3.1Å.** The side (A) and top (B) views of AmtB show the monomers and that this structure is the same as those solved using X-ray crystallography

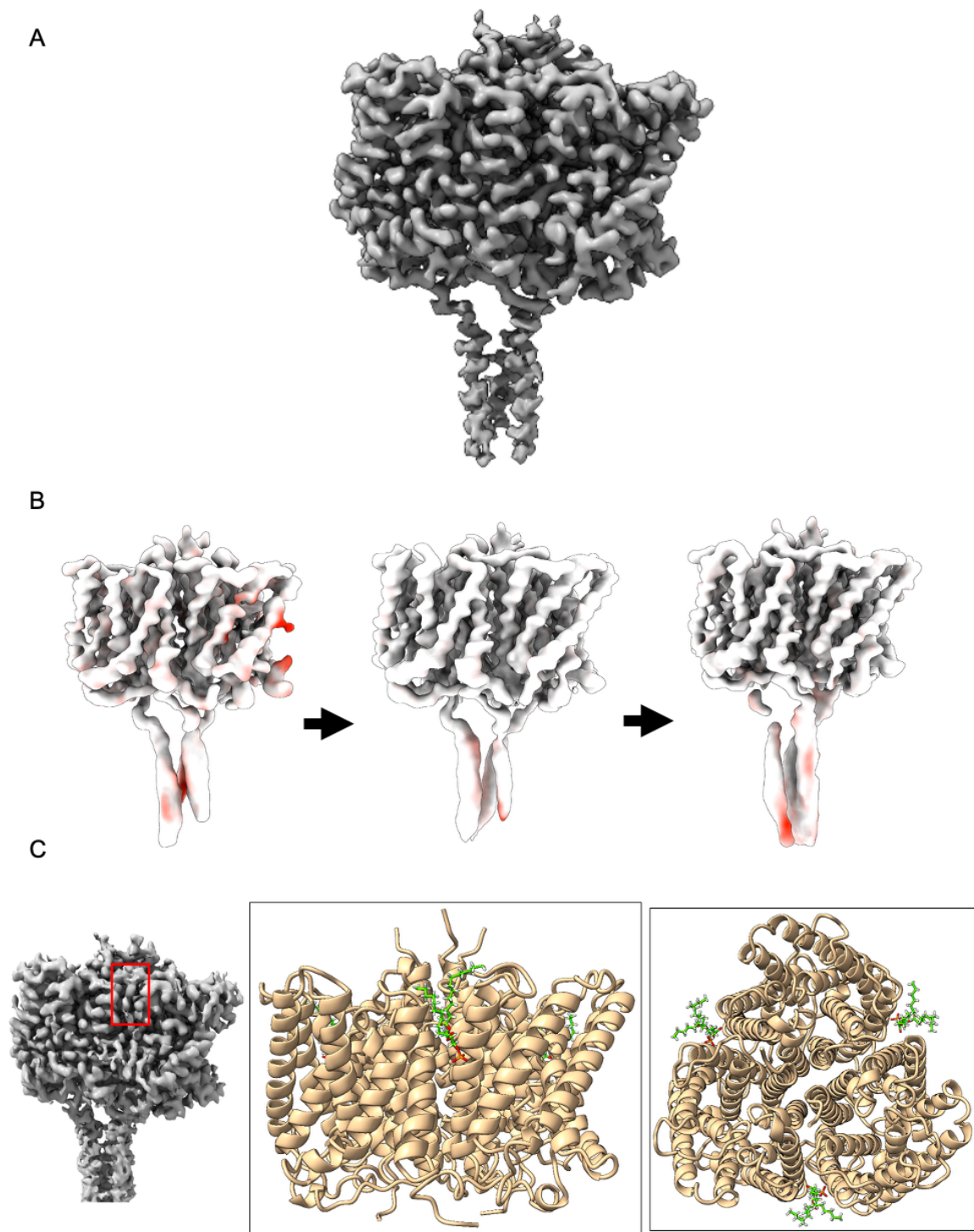


Figure 7.2 **CryoEM maps of Rh50.** A) Shows a map of the structure of Rh50 at 2.9Å. B) Shows the intermediate states in the conformational change in Rh50. C) Shows the location of the inverted lipid bound between each monomer in the trimer in Rh50.

Chapter 8: References

8 Chapter 8 References

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Appendix

9.1 Additional figures

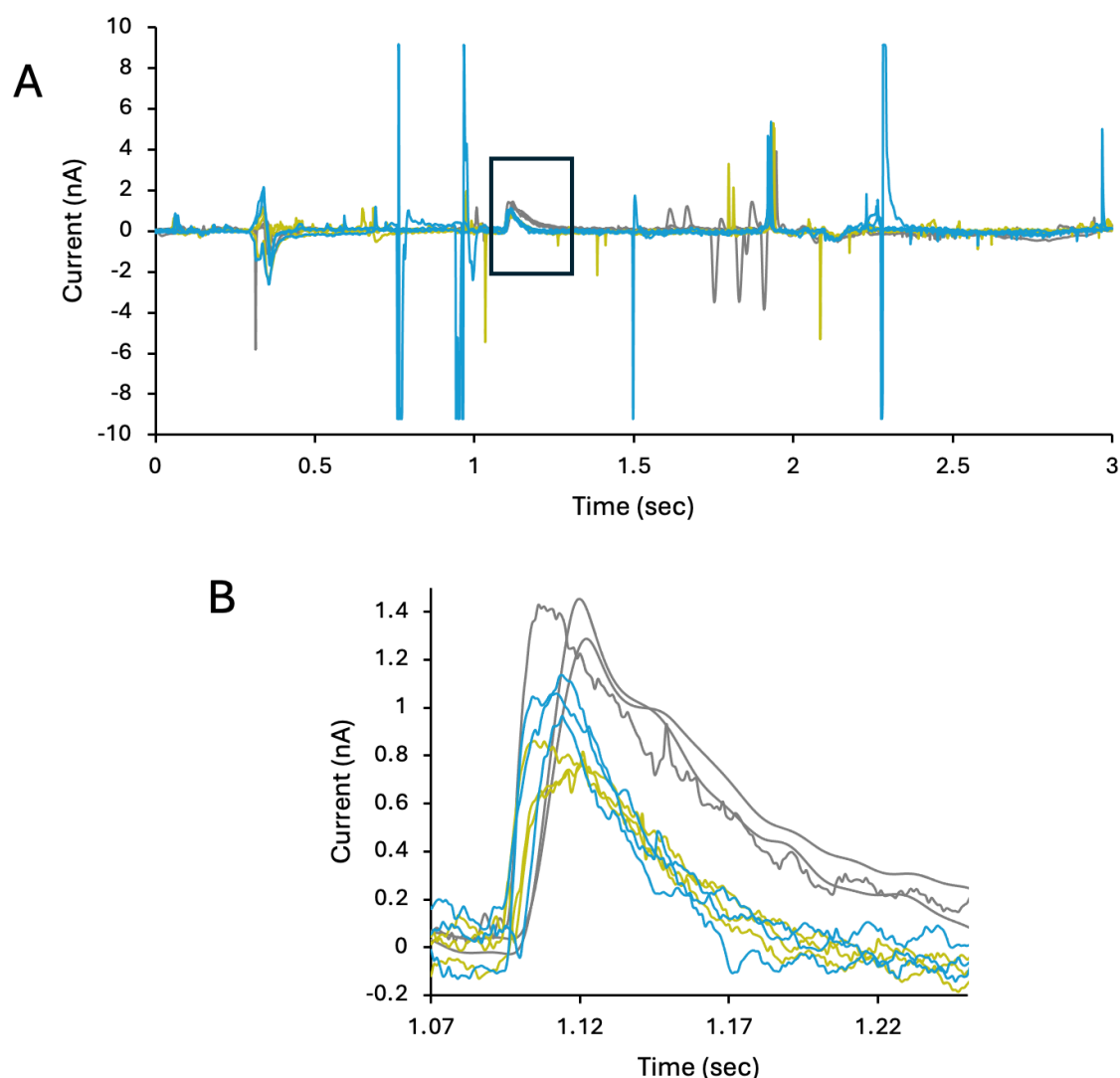


Figure 0.1 **SSME analysis of vesicle responses to a 200 mM NH_4^+ pulse.** Representative overlaid SSME traces are shown for undiluted vesicles containing AmtB prepared from C43 cells (yellow), empty vesicles (blue), and artificial liposomes reconstituted with purified AmtB (grey). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

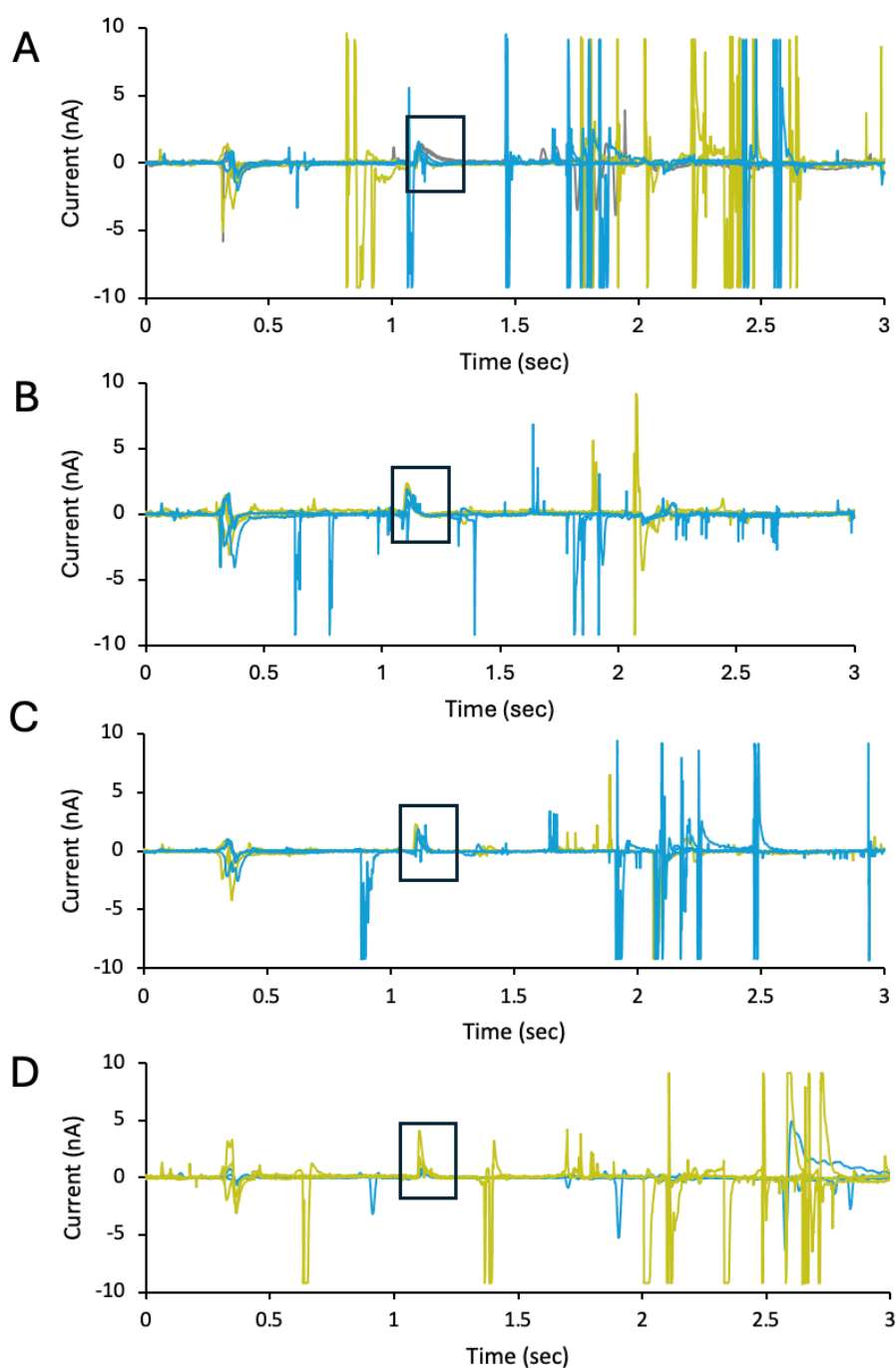


Figure 9.2 SSME analysis of diluted vesicle responses to a 200 mM NH_4^+ pulse. Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from C43 cells (yellow), empty vesicles (blue), and artificial liposomes reconstituted with purified AmtB (grey). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. Each panel shows the full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles. The boxed area indicates the area shown in Figure 9.3.

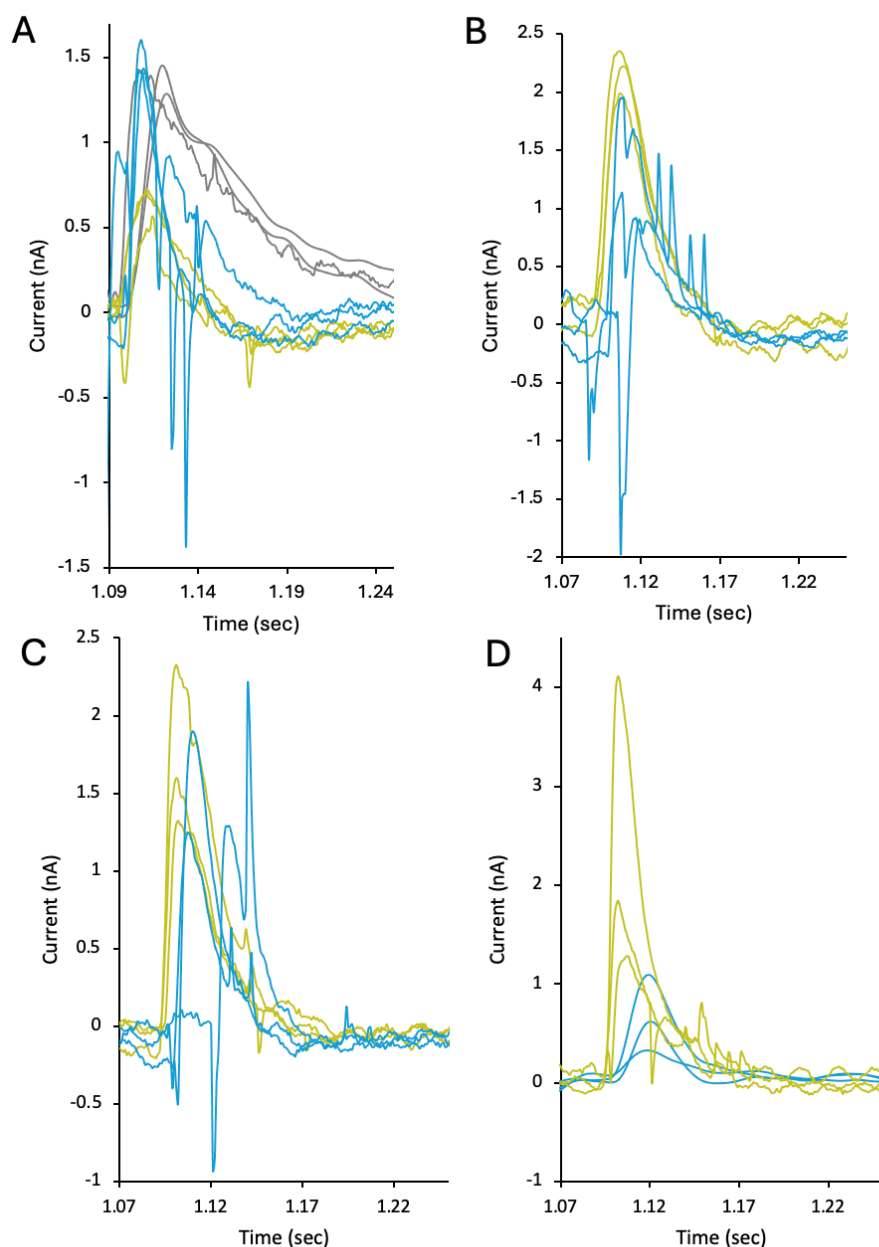


Figure 9.3 **Expanded view of SSME analysis after pulsing vesicles with 200 mM NH_4^+** . Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from C43 cells (yellow), empty vesicles (blue), and artificial liposomes reconstituted with purified AmtB (grey). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles.

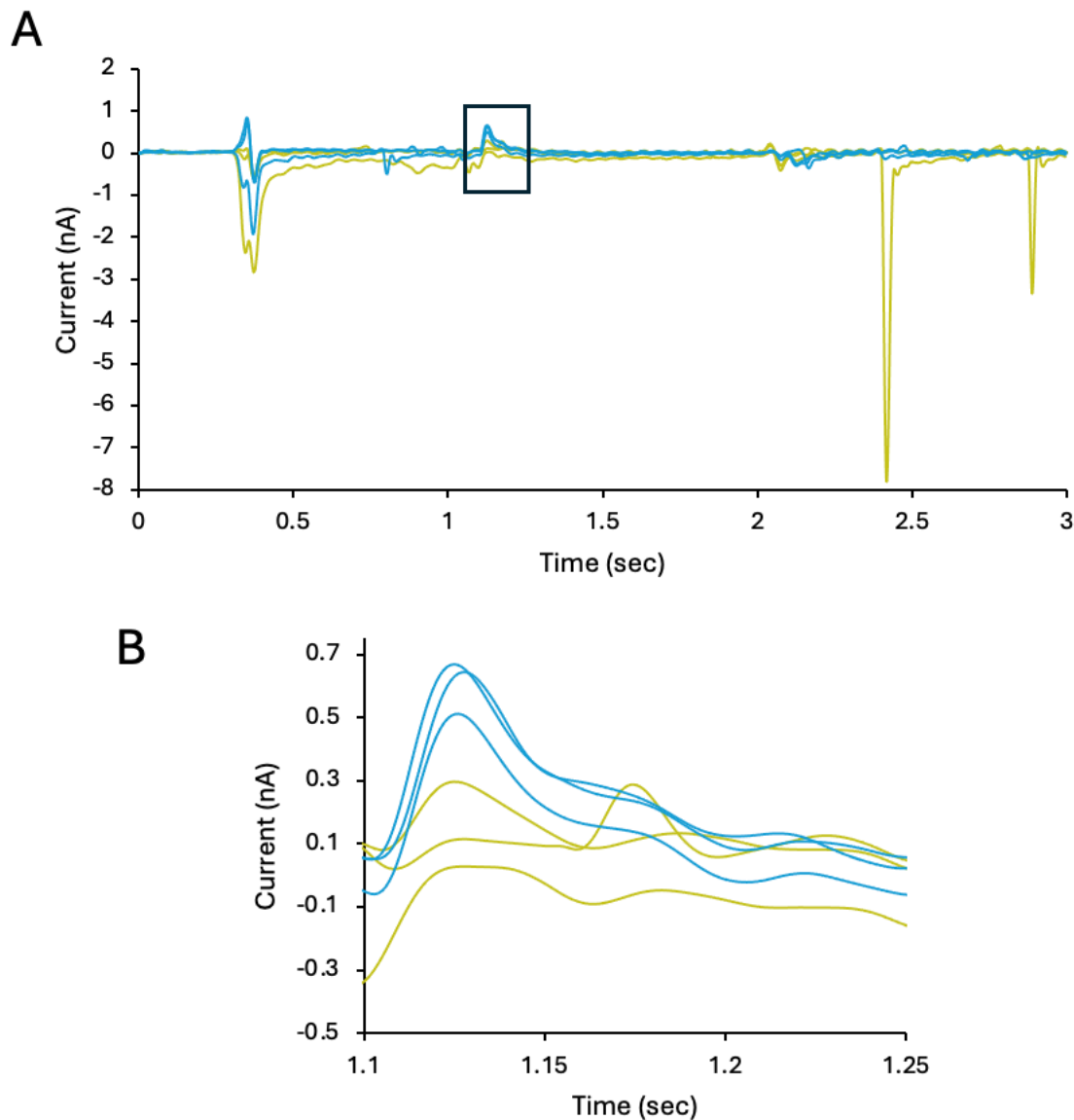


Figure 9.4 **SSME analysis of vesicles following pulse of 200 mM NH_4^+** . Representative overlaid SSME traces are shown for 2x diluted vesicles prepared from C43 cells grown with 20 mM NH_4^+ added to culture medium, AmtB containing (yellow) and empty vesicles (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

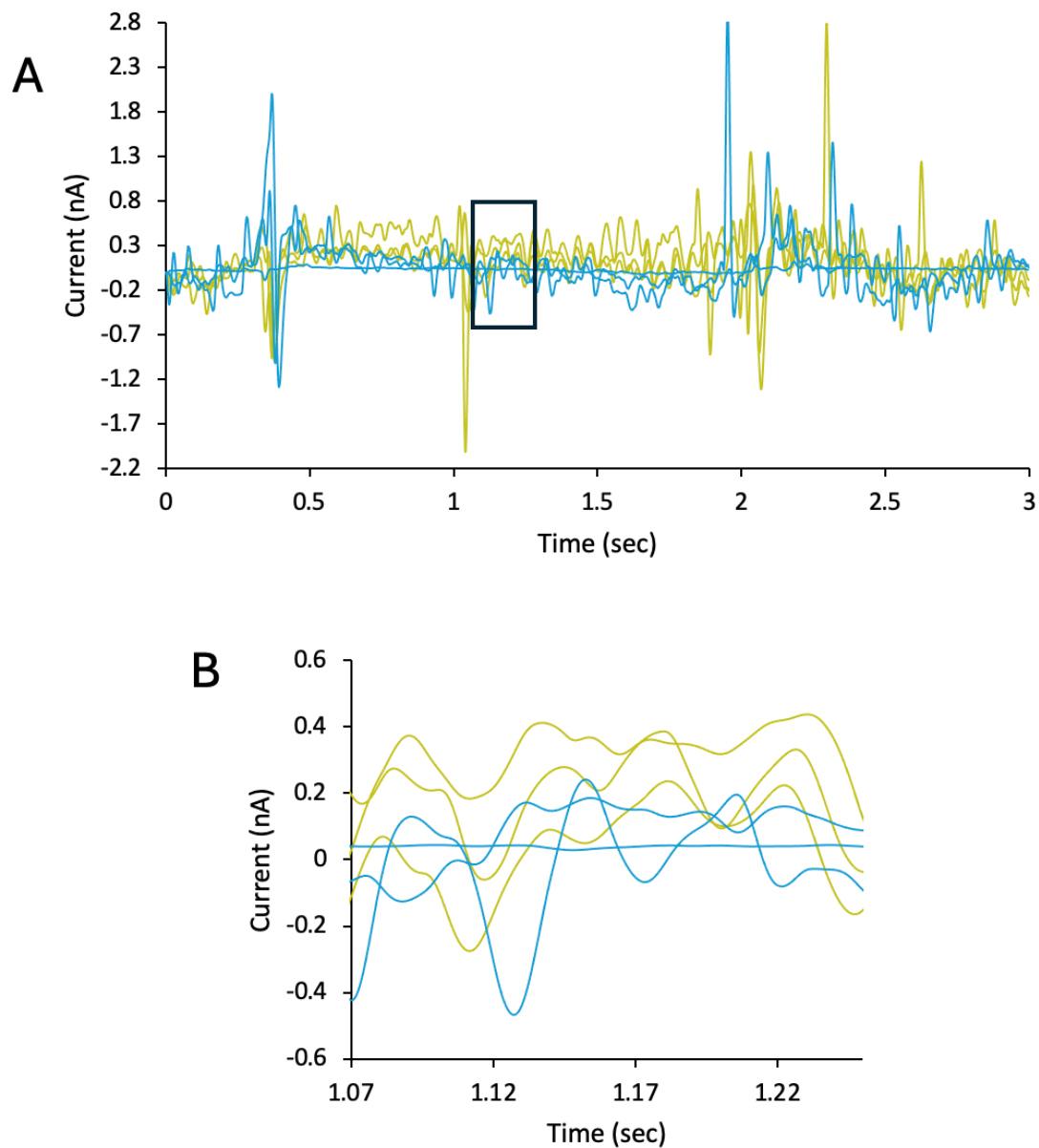


Figure 9.5 **SSME analysis of vesicles following a pulse of 200 mM K⁺**. Overlaid traces are shown for 2x diluted vesicles prepared from GT1000 cells containing AmtB (yellow) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

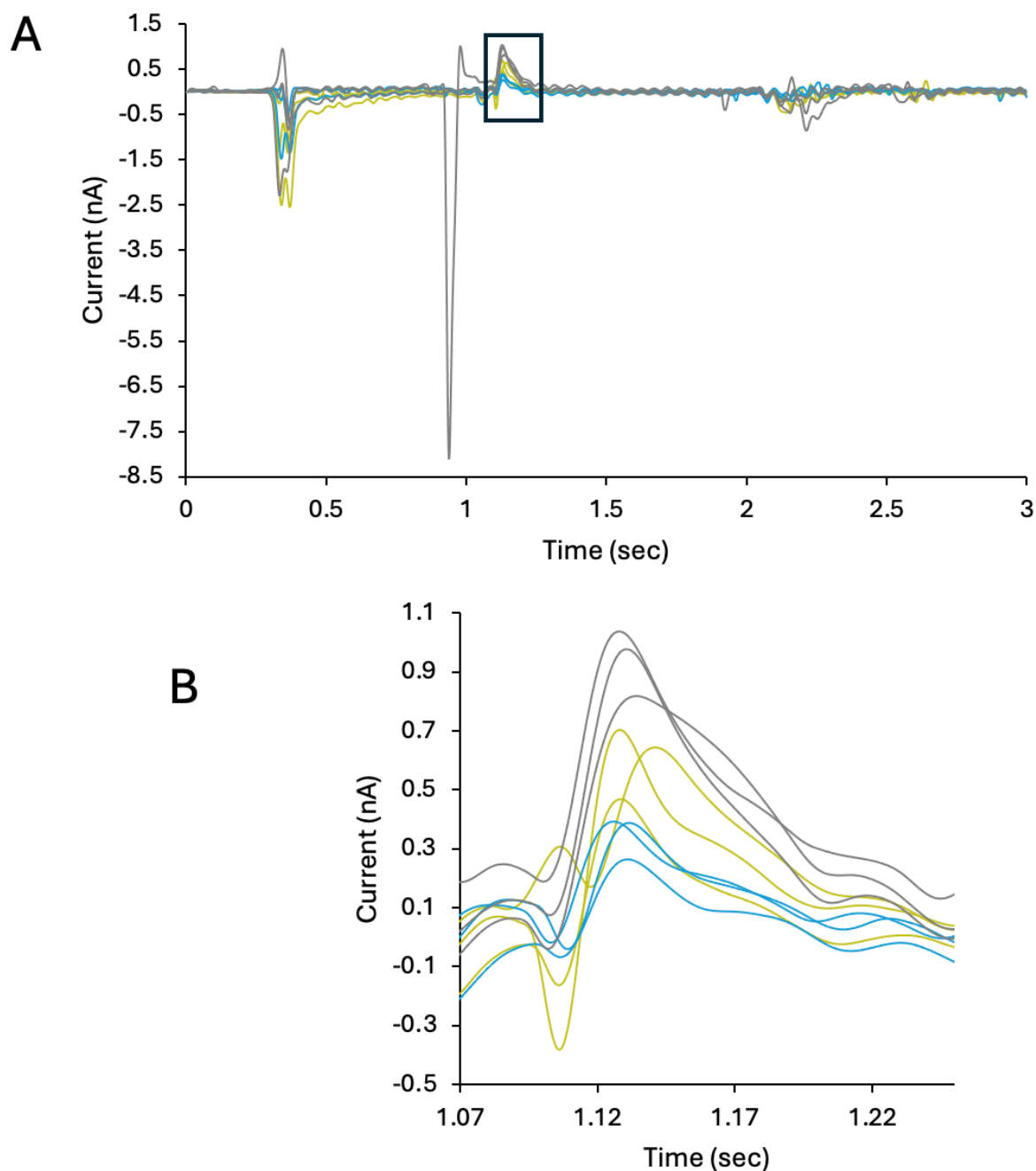


Figure 9.6 **SSME analysis of vesicle response to 200 mM NH_4^+ pulse.** Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow), from pJT6E plasmid (grey) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

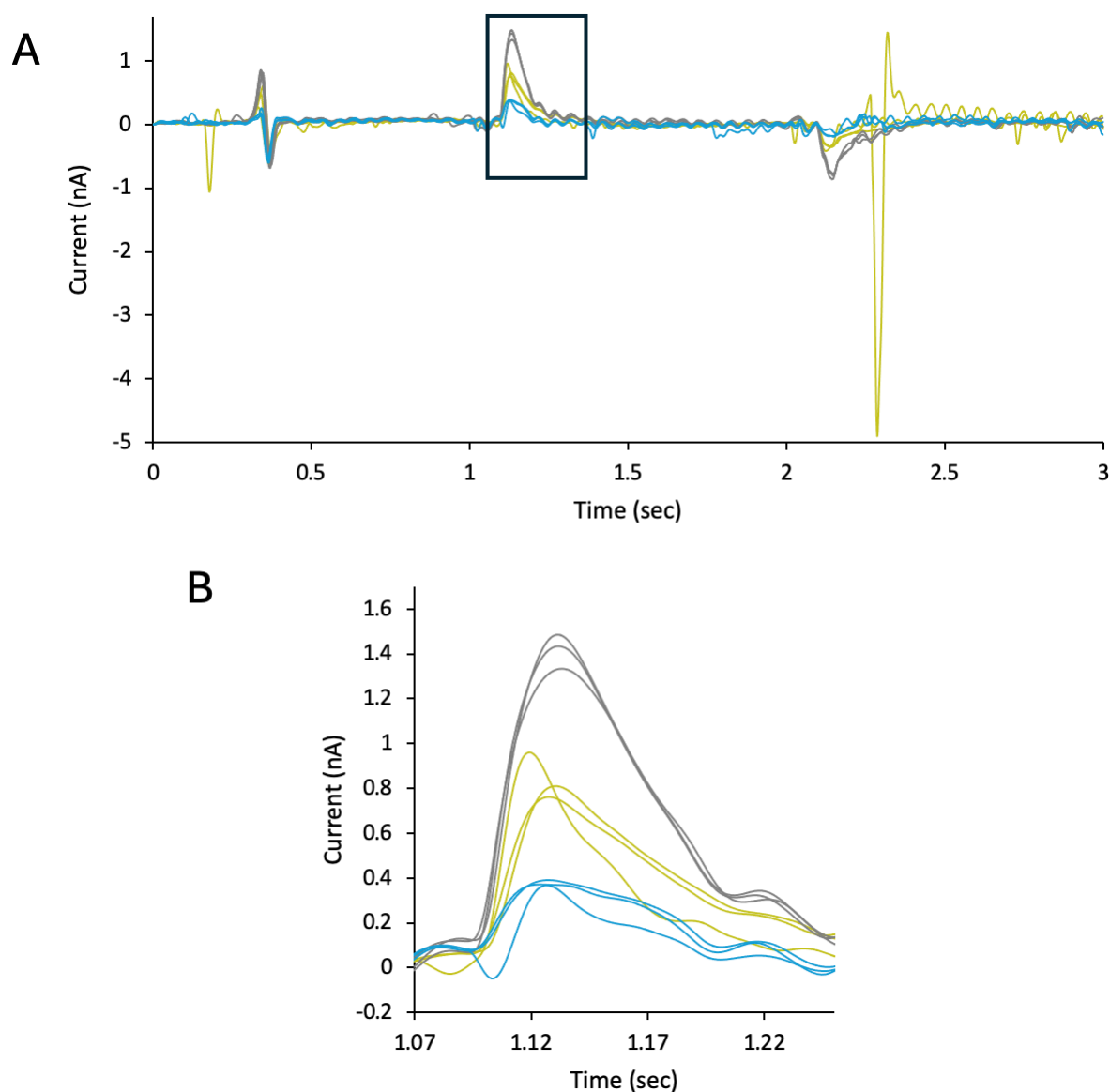


Figure 9.7 **SSME analysis of vesicle response to 200 mM MeA pulse.** Overlaid traces are shown from undiluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow), from pJT6E plasmid (grey) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

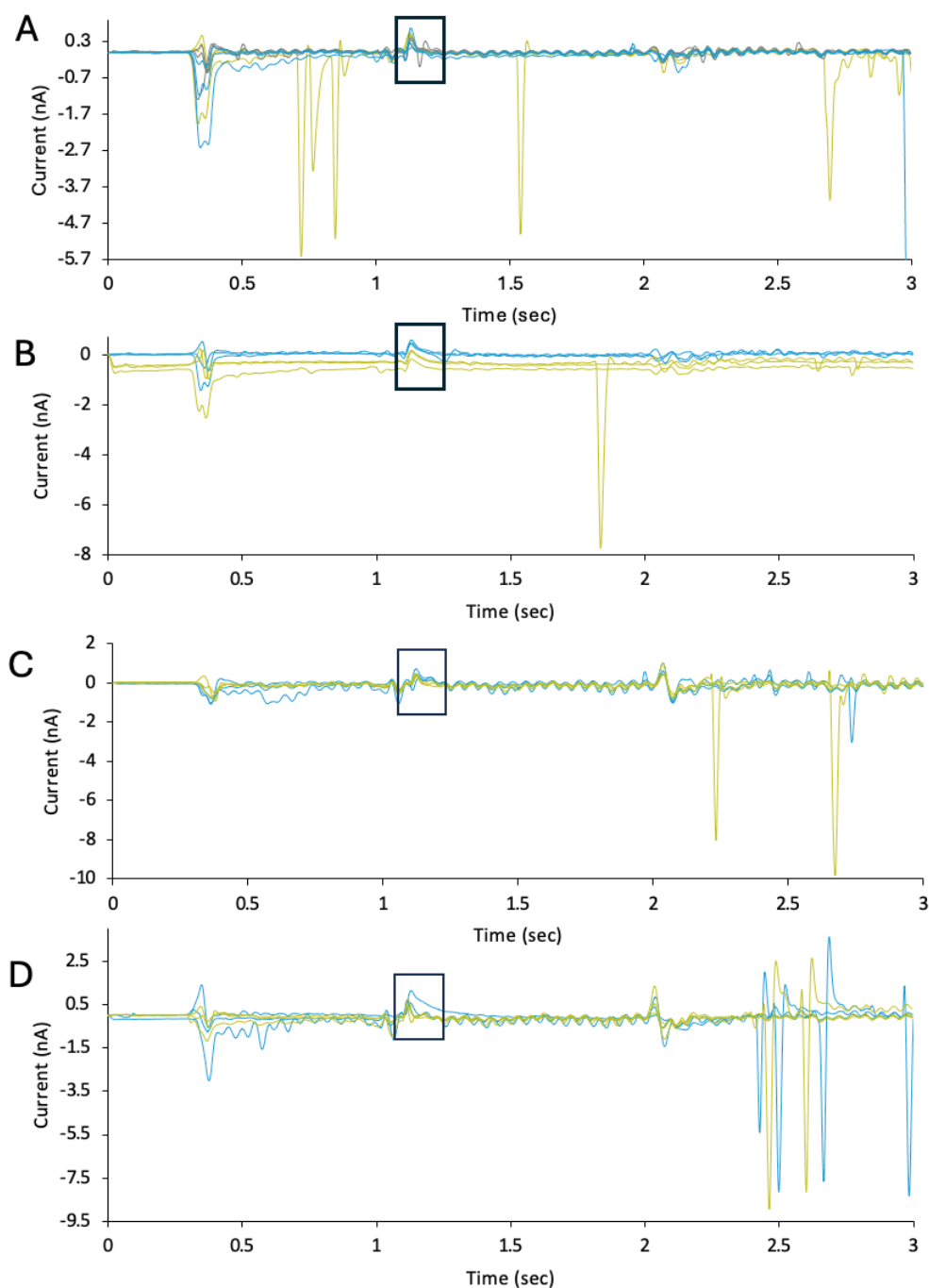


Figure 9.8 **SSME analysis of diluted vesicle responses to a 200 mM NH_4^+ pulse.** Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from GT1000 cells from pJT5 plasmid (yellow), pJT6E plasmid (grey). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. Each panel shows the full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles. The boxed area indicates the area shown in Figure 9.9.

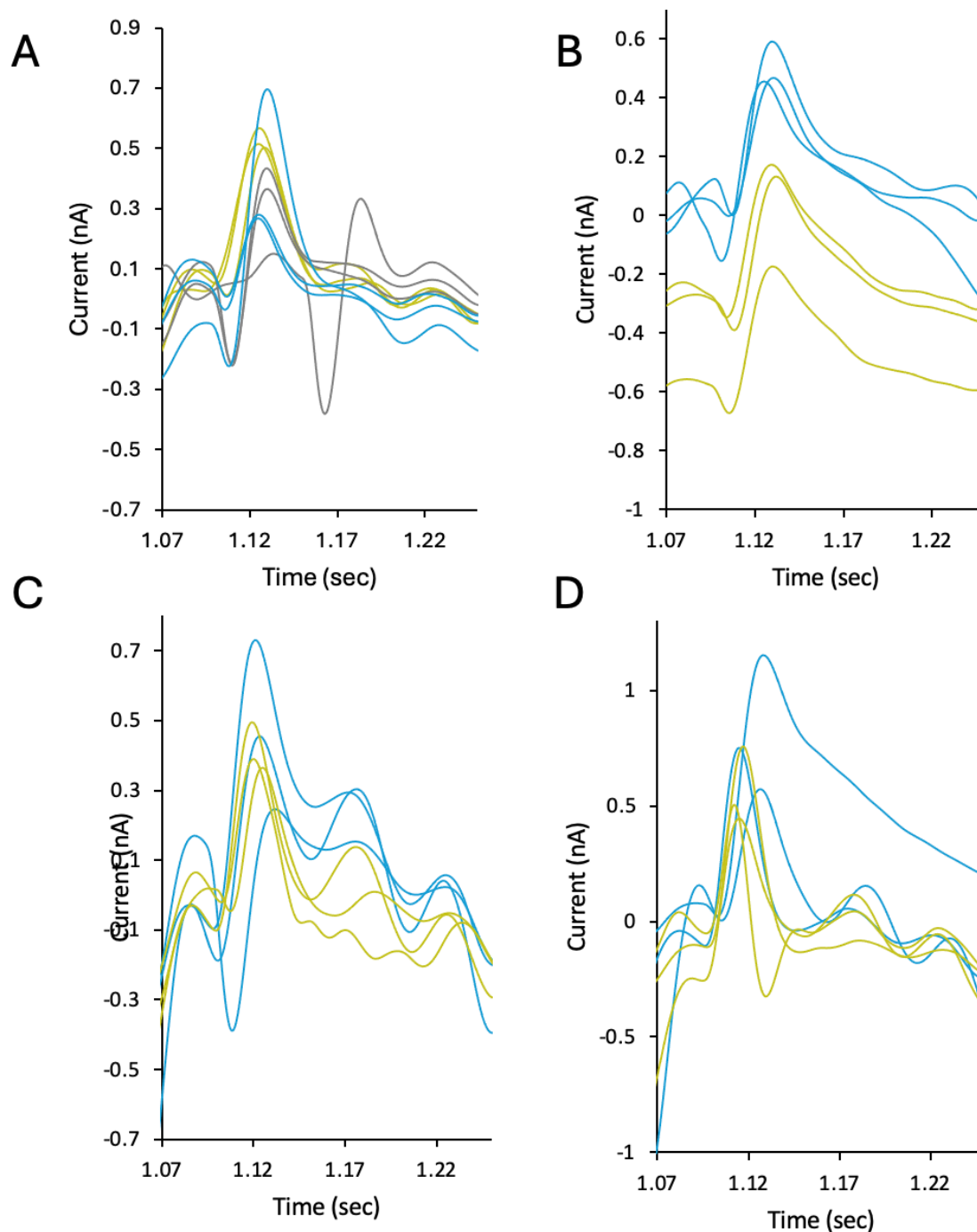


Figure 9.9 **Expanded view of SSME analysis after pulsing diluted vesicles with 200 mM NH_4^+ .** Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from GT1000 cells from pJT5 plasmid (yellow), pJT6E plasmid (grey). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles.

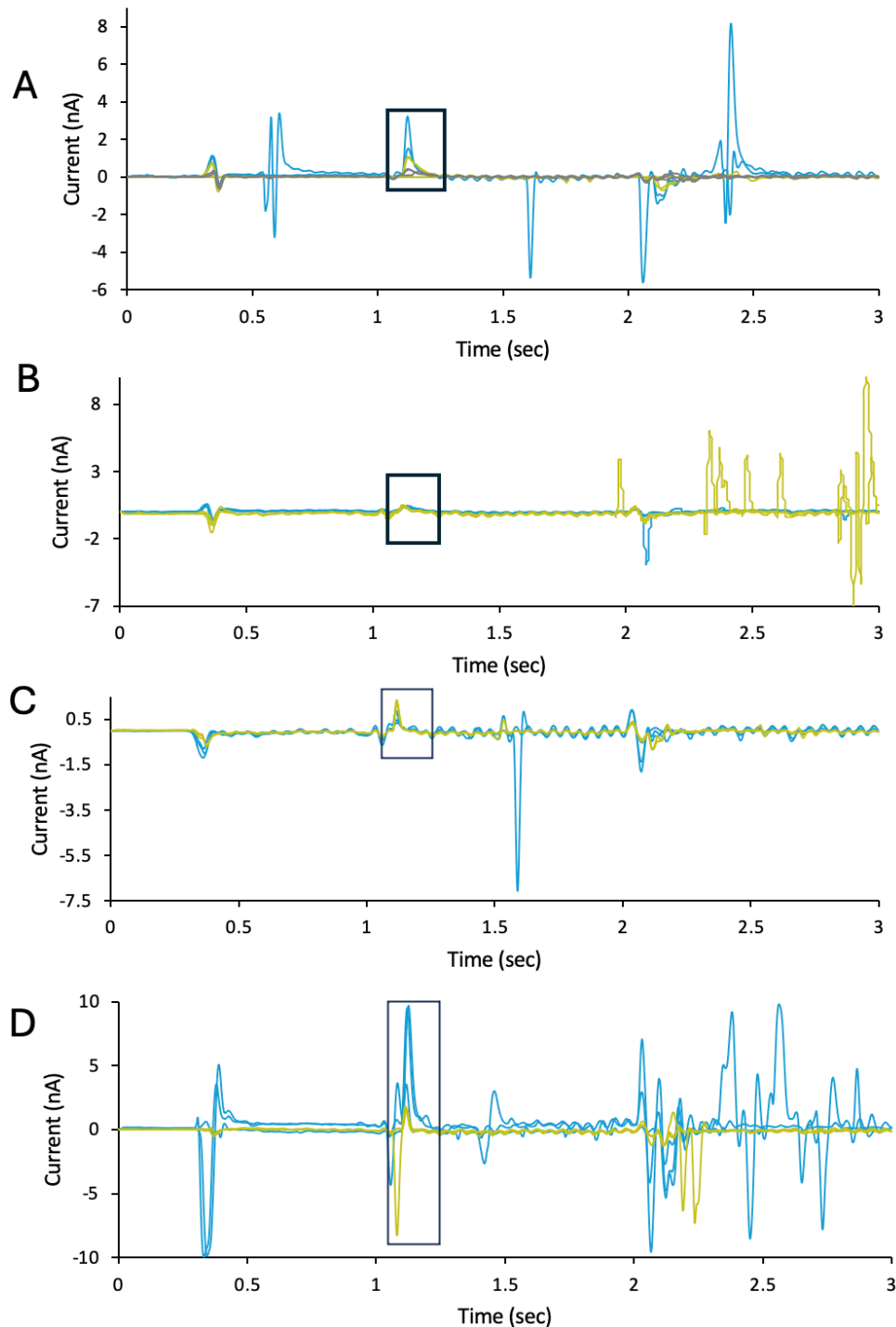


Figure 9.10 **SSME analysis of diluted vesicle responses to a 200 mM MeA pulse.** Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from GT1000 cells from pJT5 plasmid (yellow), pJT6E plasmid (grey) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. Each panel shows the full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles. The boxed area indicates the area shown in Figure 9.11.

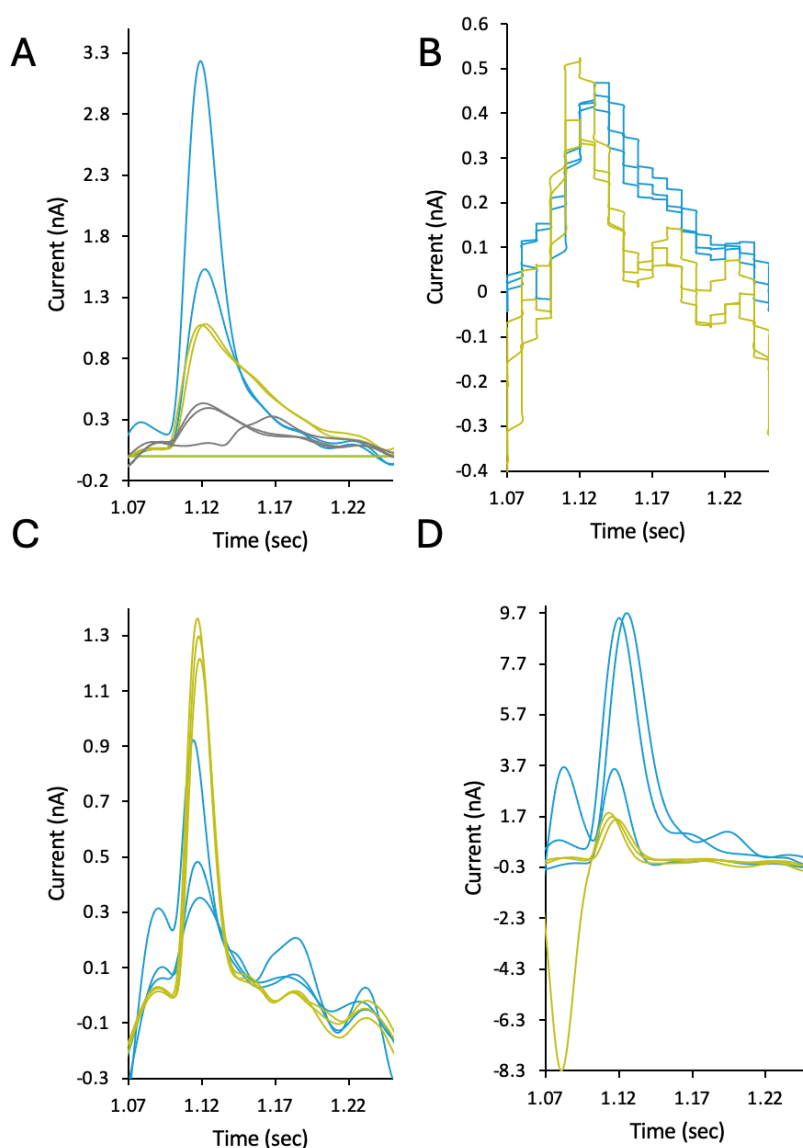


Figure 9.11 **Expanded view of SSME analysis after pulsing vesicles with 200 mM MeA.** Representative overlaid SSME traces are shown for vesicles containing AmtB prepared from GT1000 cells from pJT5 plasmid (yellow), pJT6E plasmid (grey) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. A) 2x diluted, B) 5x diluted, C) 10x diluted and D) 50x diluted vesicles.

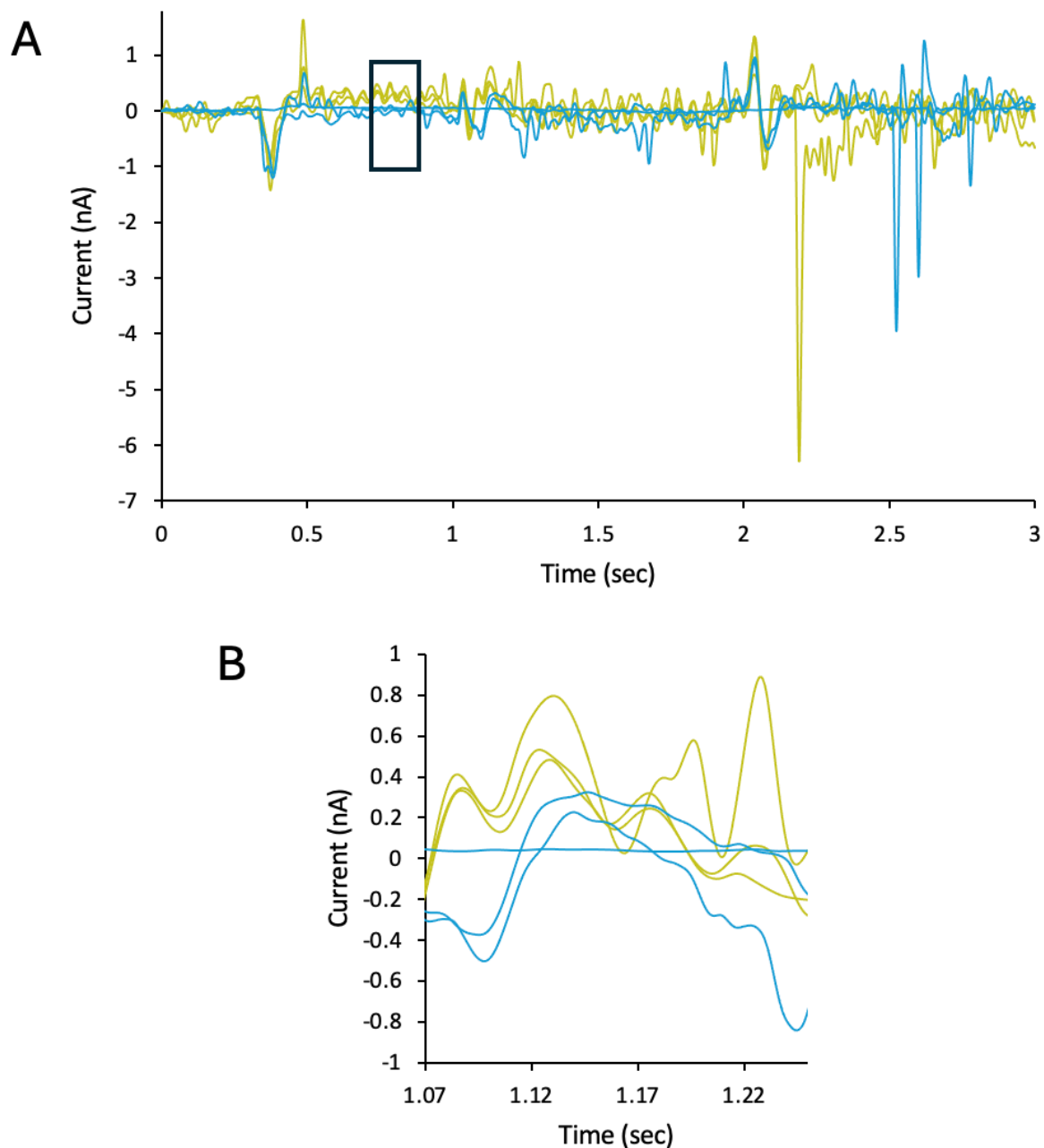


Figure 9.12 **SSME analysis of vesicle response to 200 mM NH_4^+ pulse in a sodium phosphate buffer background.** Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

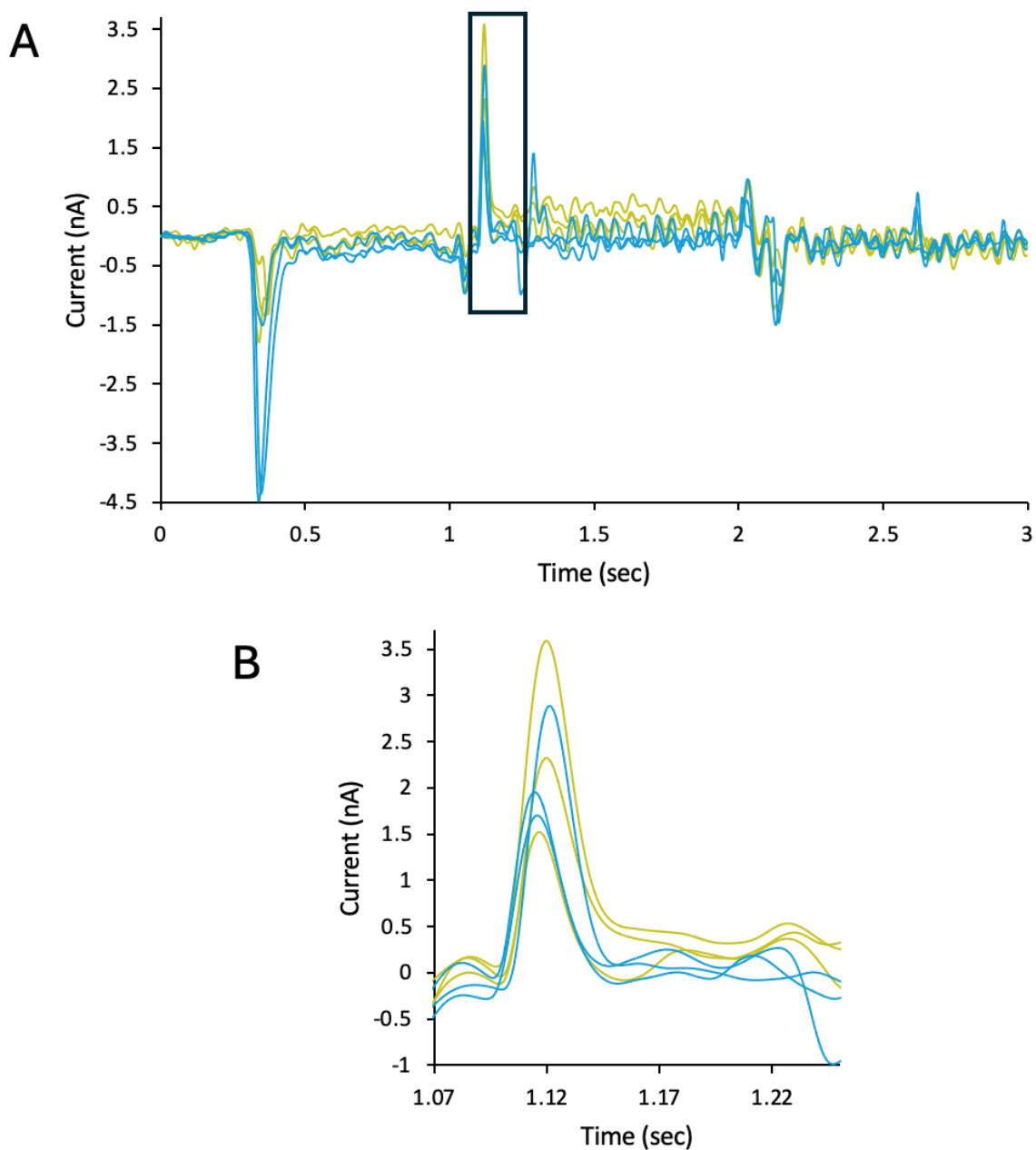


Figure 9.13 **SSME analysis of vesicle response to 200 mM NH_4^+ pulse using ionically imbalanced activating and non-activating buffers.** Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

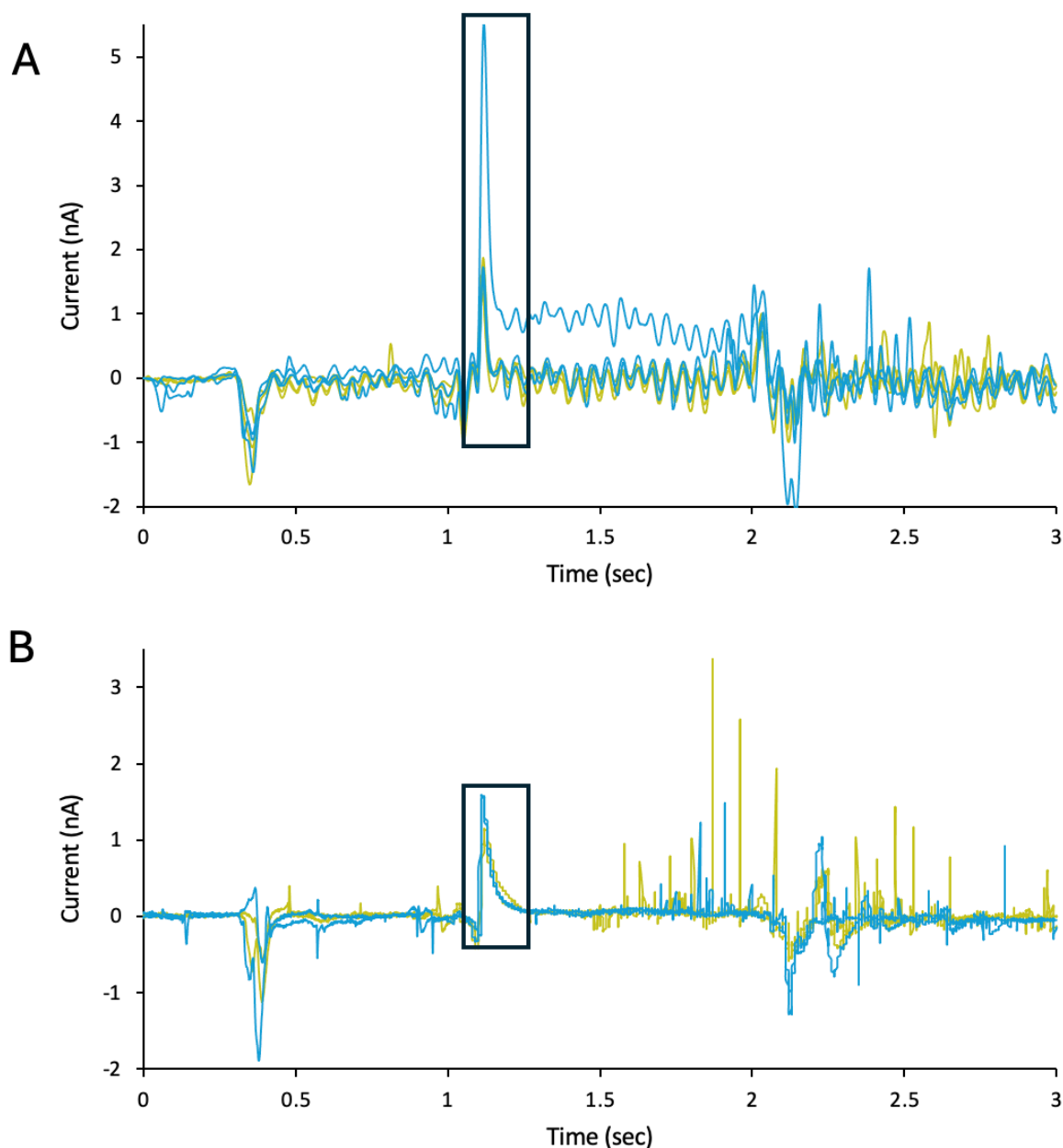


Figure 9.14 **SSME analysis of vesicles pulsed with either 200 or 100 mM NH_4^+ using a tris buffer background.** Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow) and empty (blue). The full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement is shown for A) 200 mM NH_4^+ and B) 100 mM NH_4^+ . The boxed area indicates the sections shown in Figure 9.15. The 200 mM pulse condition was measured using at least three independent sensors, and one representative trace from each sensor is shown, while the 100 mM pulse condition was measured with two sensors thus two traces are displayed.

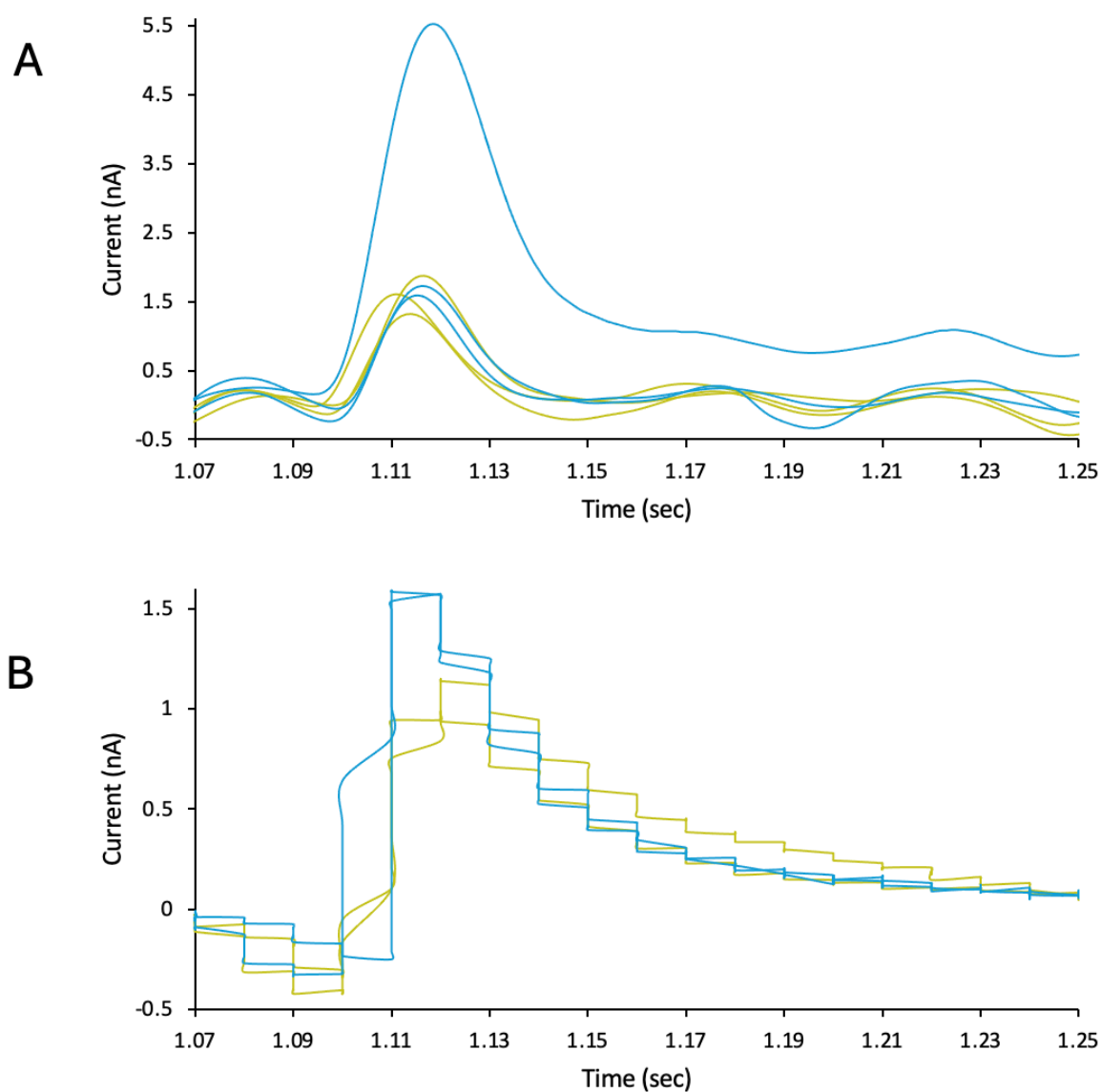


Figure 9.15 **Expanded view of SSME analysis of vesicles pulsed with either 200 or 100 mM NH_4^+ using a tris buffer background.** Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow) and empty (blue). A) 200 mM NH_4^+ and B) 100 mM NH_4^+ . The 200 mM pulse condition was measured using at least three independent sensors, and one representative trace from each sensor is shown, while the 100 mM pulse condition was measured with two sensors thus two traces are displayed.

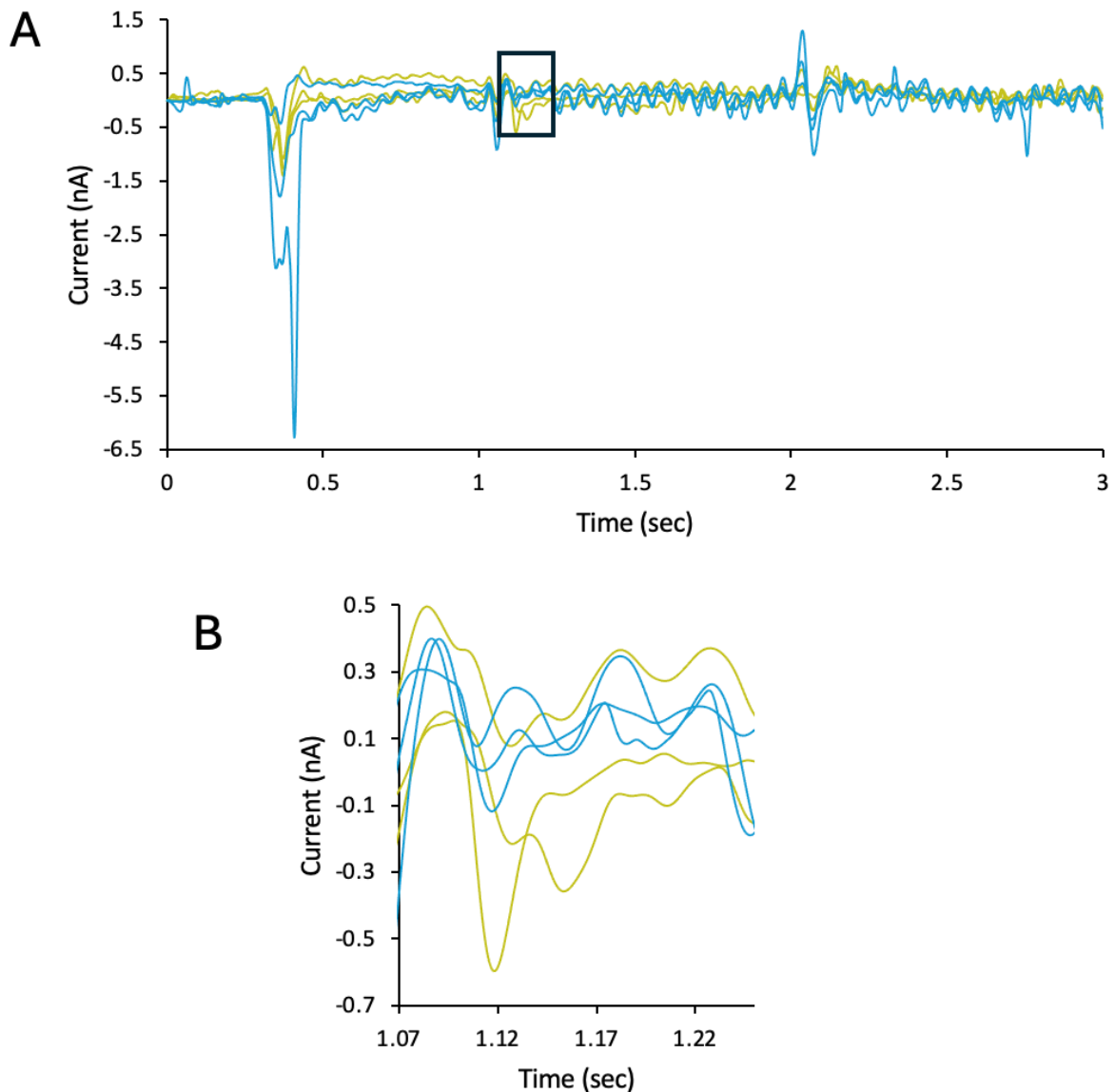


Figure 9.16 **SSME analysis of vesicle response to buffers used by Wacker *et al* (2014)**. Overlaid traces are shown from 2x diluted vesicles made from GT1000 cells containing AmtB from pJT5 plasmid (yellow) and empty (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

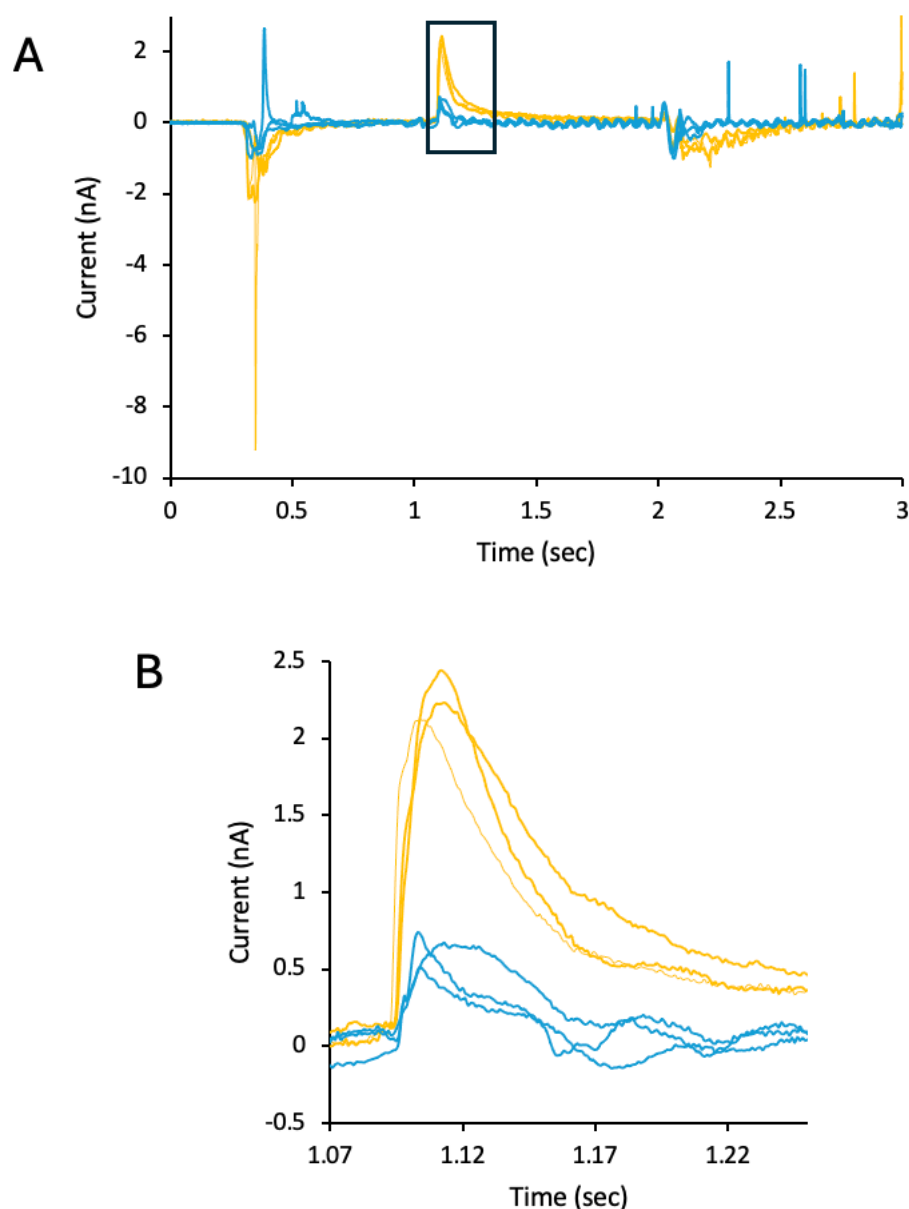


Figure 9.17 **SSME analysis of WT AmtB response to 200 mM NH_4^+ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT AmtB LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

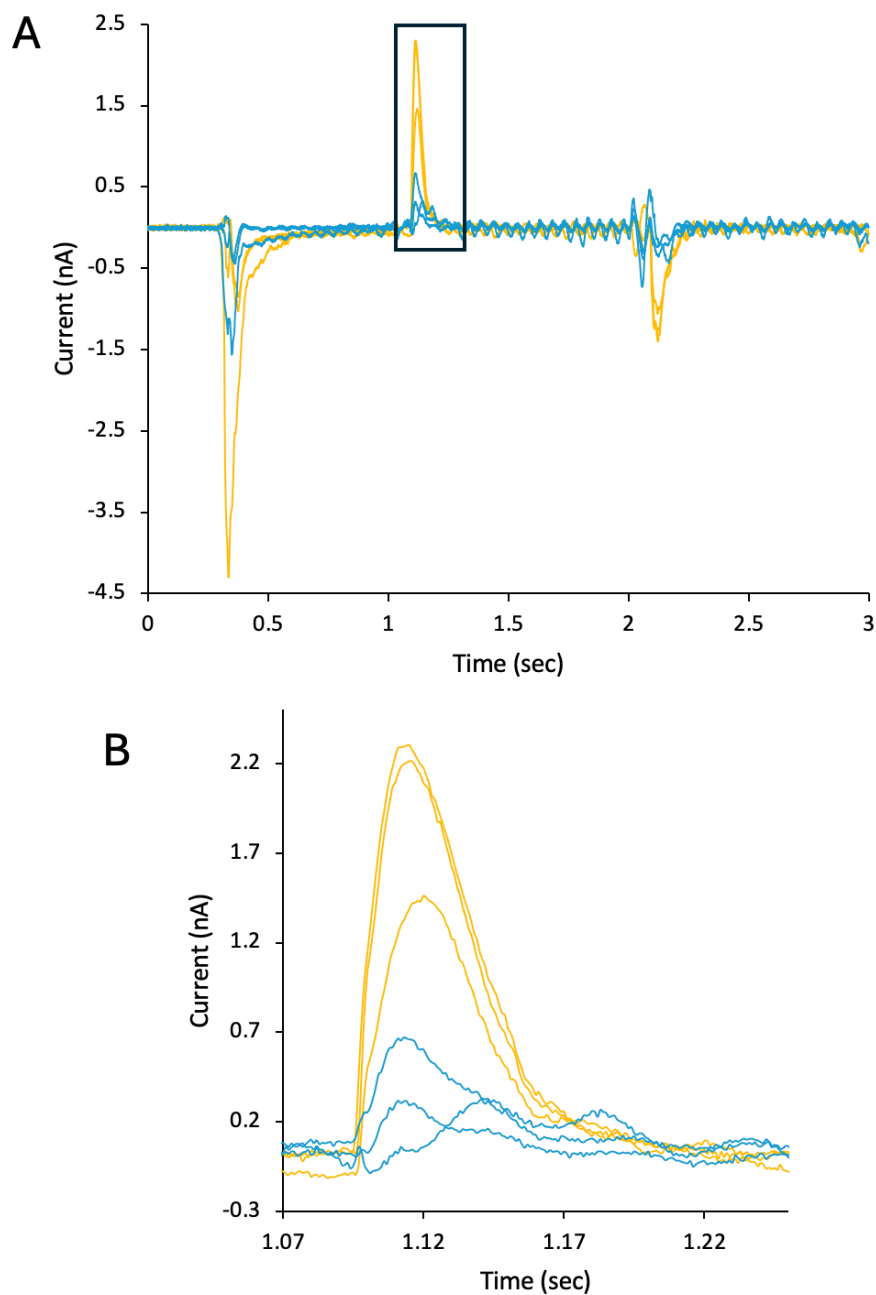


Figure 9.18 **SSME analysis of WT Rh50 response to 200 mM NH_4^+ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT Rh50 LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

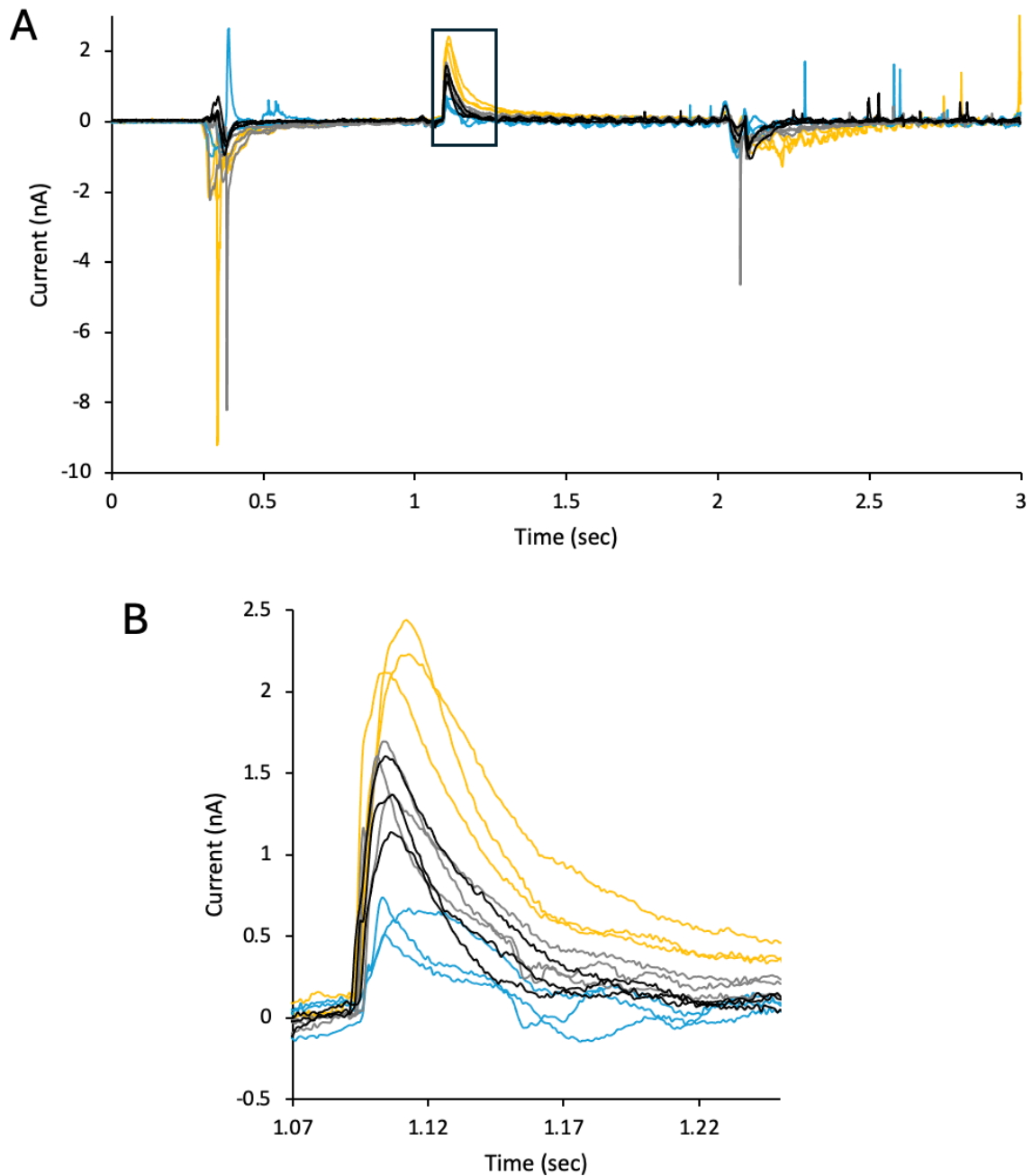


Figure 9.19 **SSME analysis of WT AmtB response to 200 mM NH_4^+ pulse in different LPRs.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT AmtB LPR5 (yellow), LPR10 (grey), LPR50 (black) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

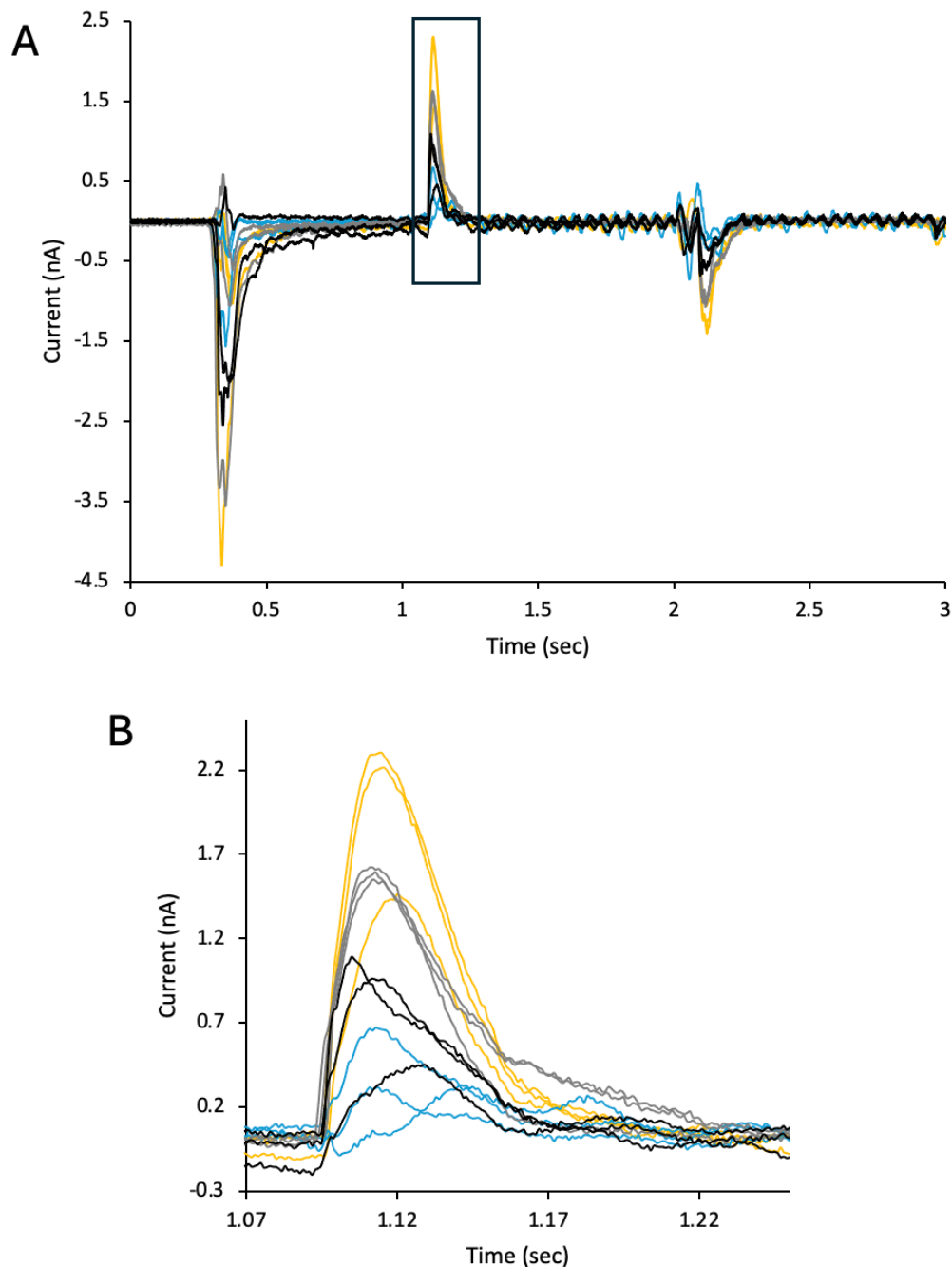


Figure 9.20 **SSME analysis of WT Rh50 response to 200 mM NH_4^+ pulse in different LPRs.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT Rh50 LPR5 (yellow), LPR10 (grey), LPR50 (black) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

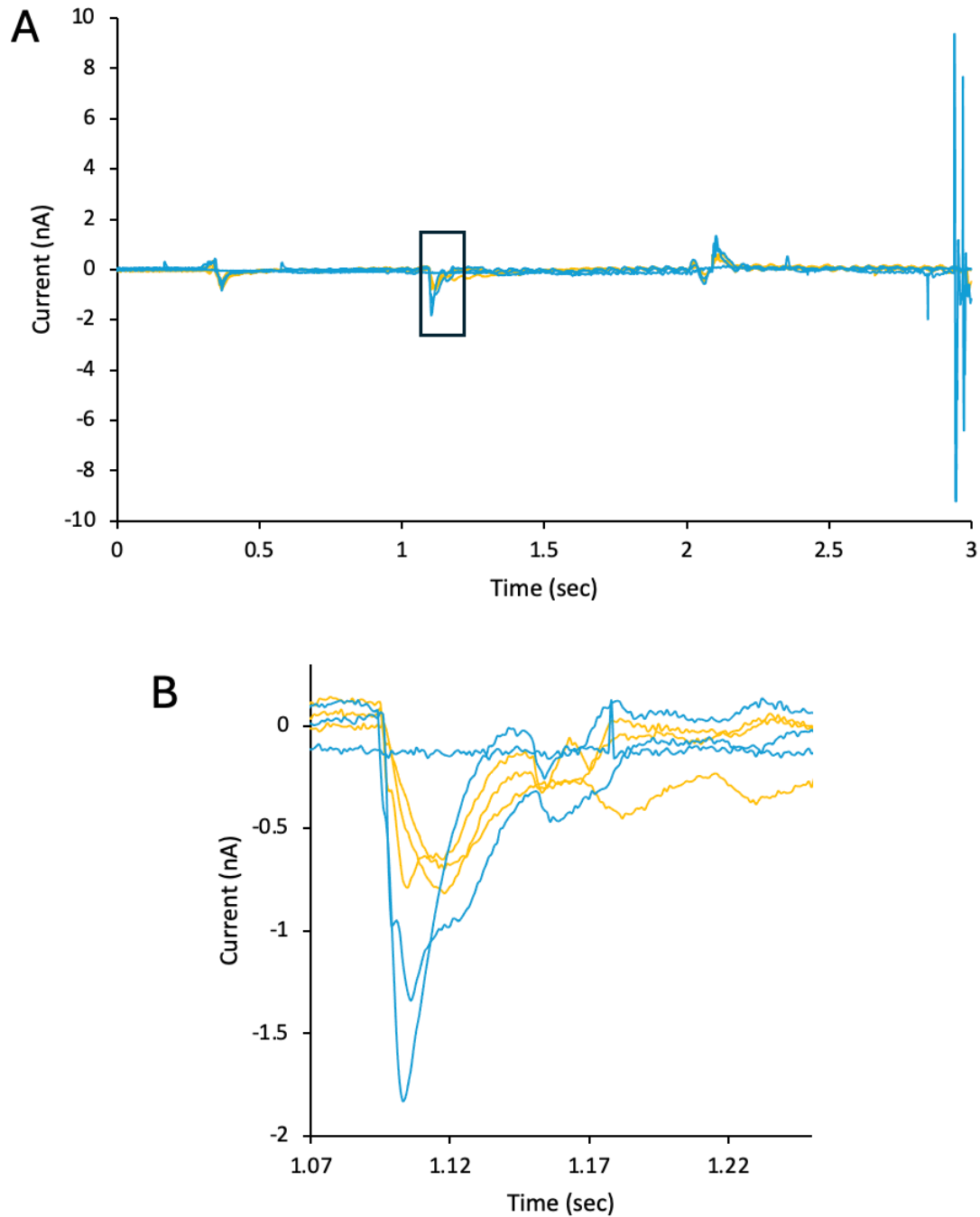


Figure 9.21 **SSME analysis of WT AmtB response to 200 mM K⁺ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT AmtB LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

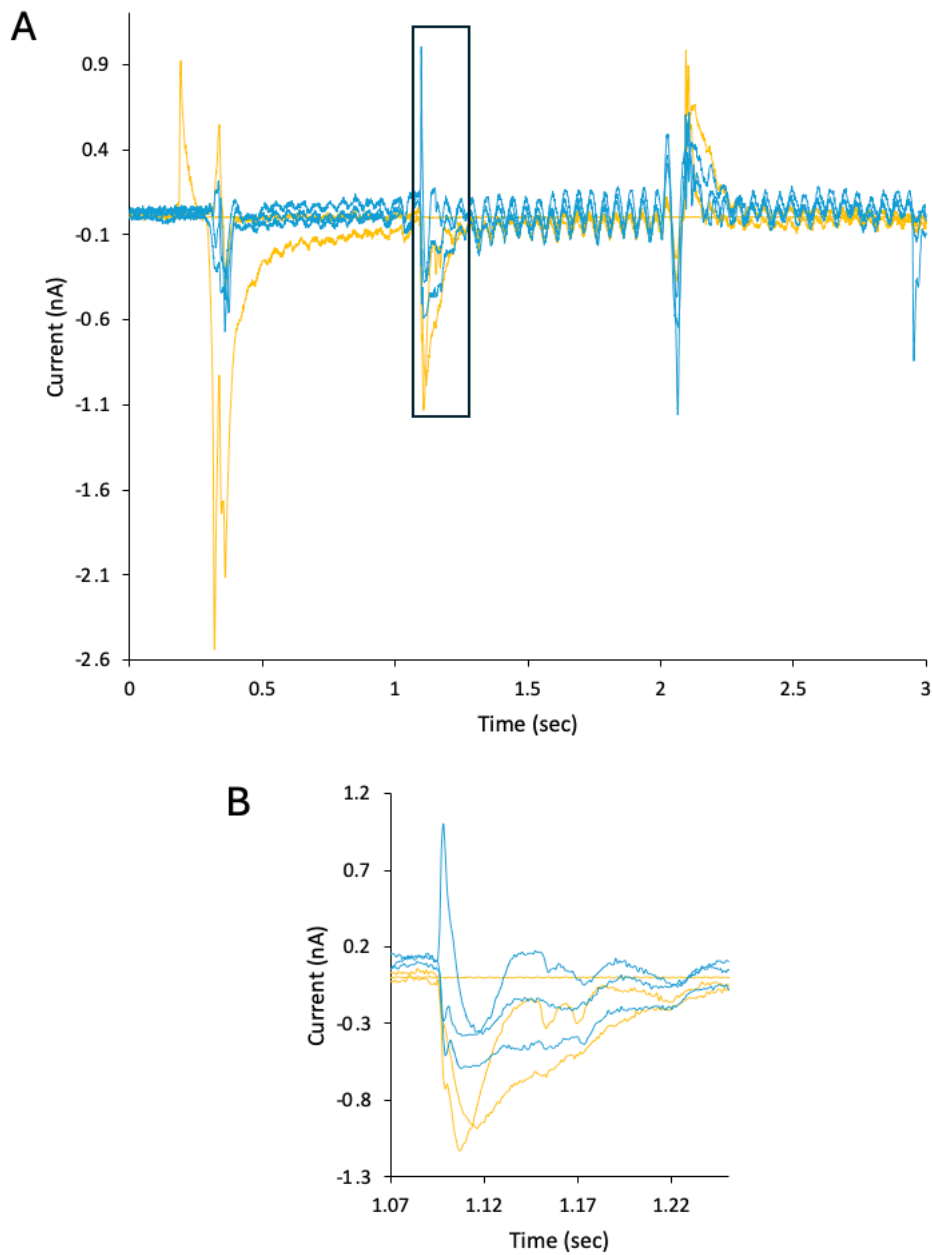


Figure 9.22 **SSME analysis of WT Rh50 response to 200 mM K⁺ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified WT Rh50 LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

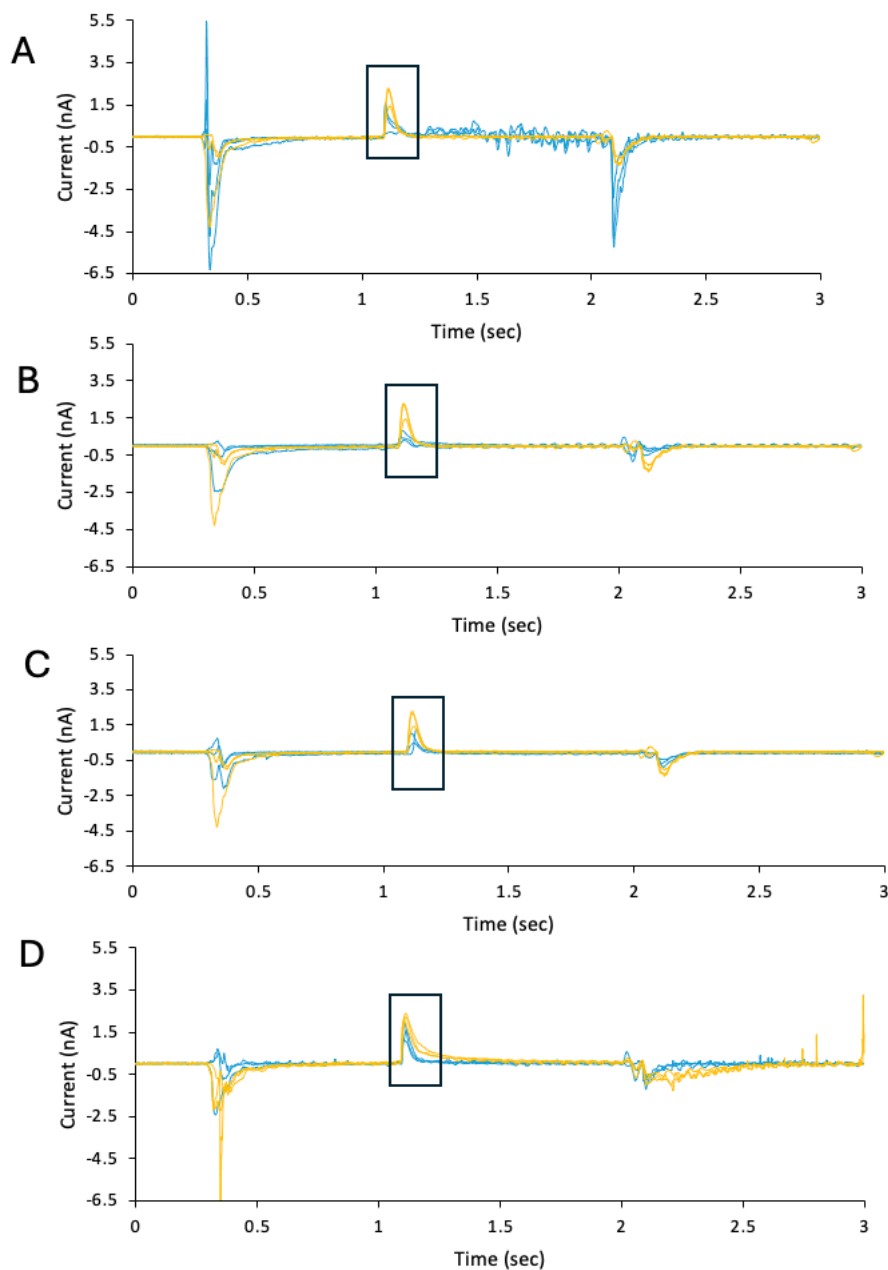


Figure 9.23 **SSME analysis of response of Phe Gate mutants to a 200 mM NH_4^+ pulse.** Overlaid traces showing the full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement for A) Rh50 F110A (blue) and WT Rh50 (yellow) B) Rh50 F218A (blue) and WT Rh50 (yellow), C) Rh50 FAFA (blue) and WT Rh50 (yellow) and D) AmtB FAFA (blue) and WT AmtB (yellow). The boxed area indicates the sections shown in Figure 9.24. Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown.

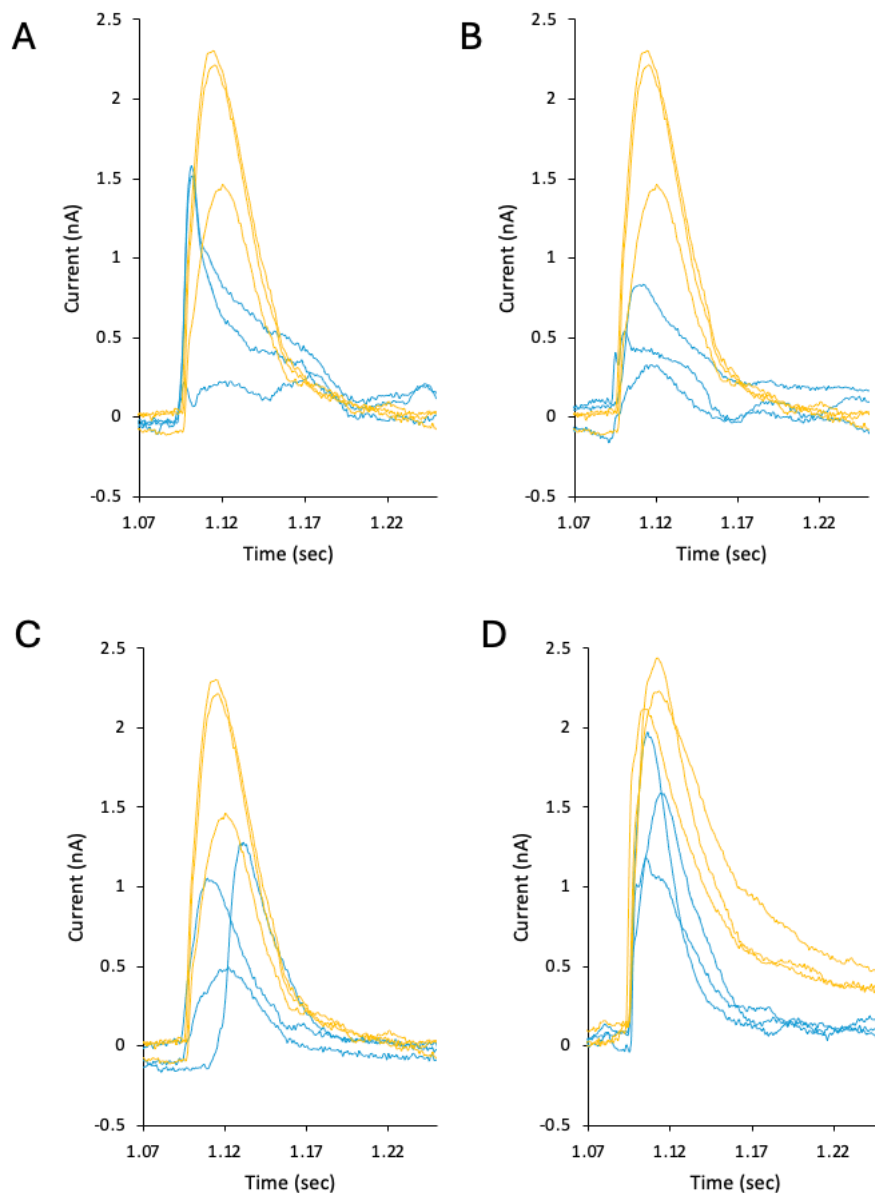


Figure 9.24 **Expanded view of SSME analysis of response of Phe Gate mutants to a 200 mM NH_4^+ pulse.** Overlaid traces showing the full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement for A) Rh50 F110A (blue) and WT Rh50 (yellow) B) Rh50 F218A (blue) and WT Rh50 (yellow), C) Rh50 FAFA (blue) and WT Rh50 (yellow) and D) AmtB FAFA (blue) and WT AmtB (yellow). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown.

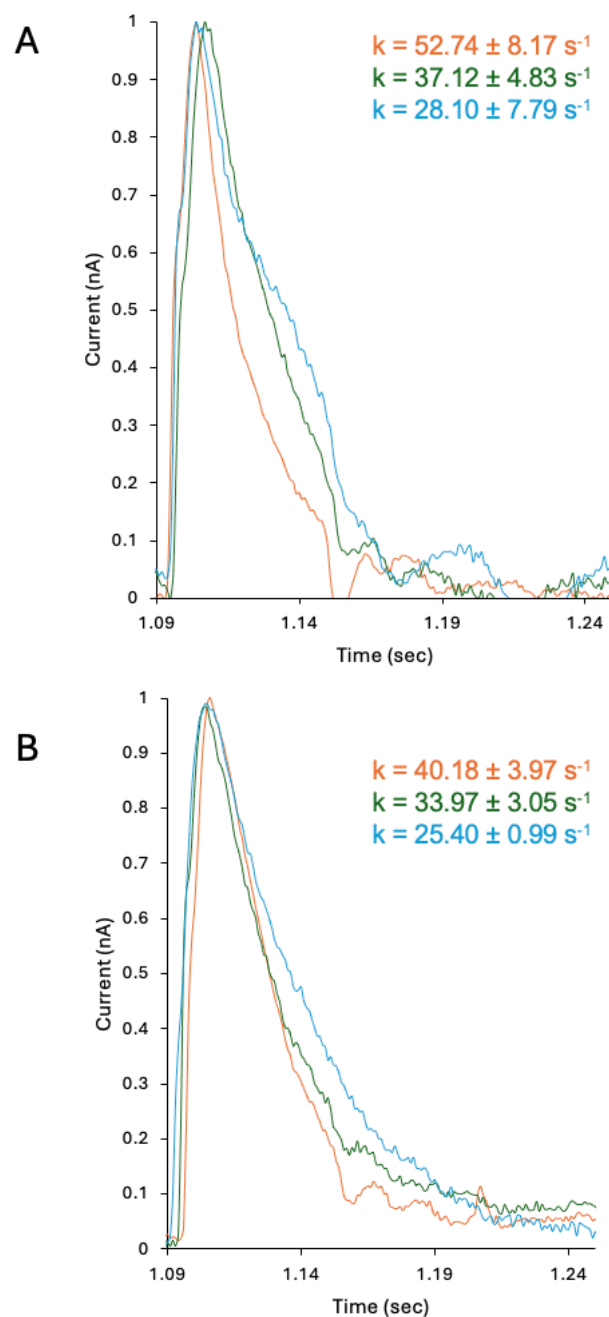


Figure 9.25 **Normalised currents following 200 mM NH_4^+ pulse for different LPRs.** The maximum currents measured for the three LPR conditions were normalised to 1 to facilitate comparison of the decay rate constants. Representative overlaid traces, showing the shape and decay values for A) WT AmtB LPR5 (orange), LPR10 (green) and LPR50 (blue) and B) WT Rh50 LPR5 (orange), LPR10 (green) and LPR50 (blue).

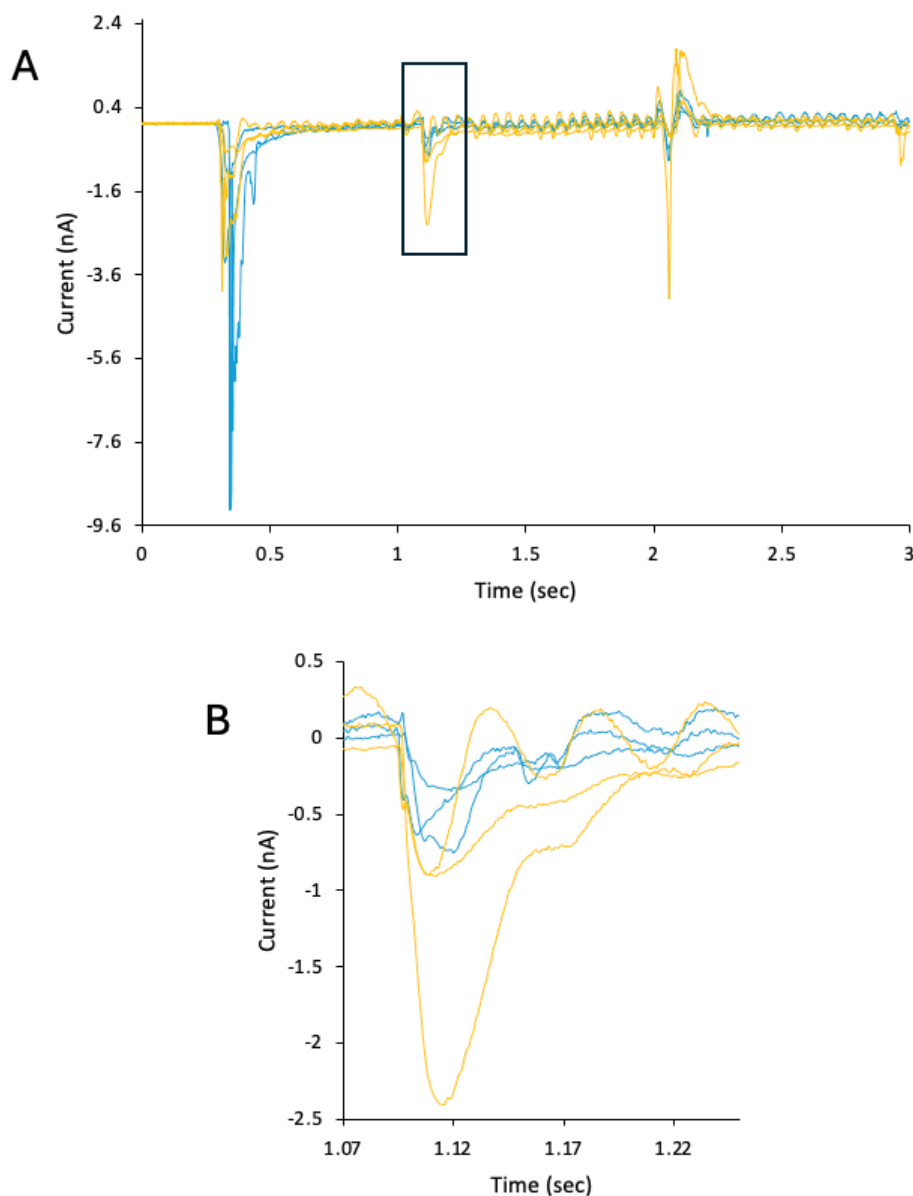


Figure 9.26 **SSME analysis of AmtB FAFA mutant response to 200 mM K⁺ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified AmtB FAFA LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

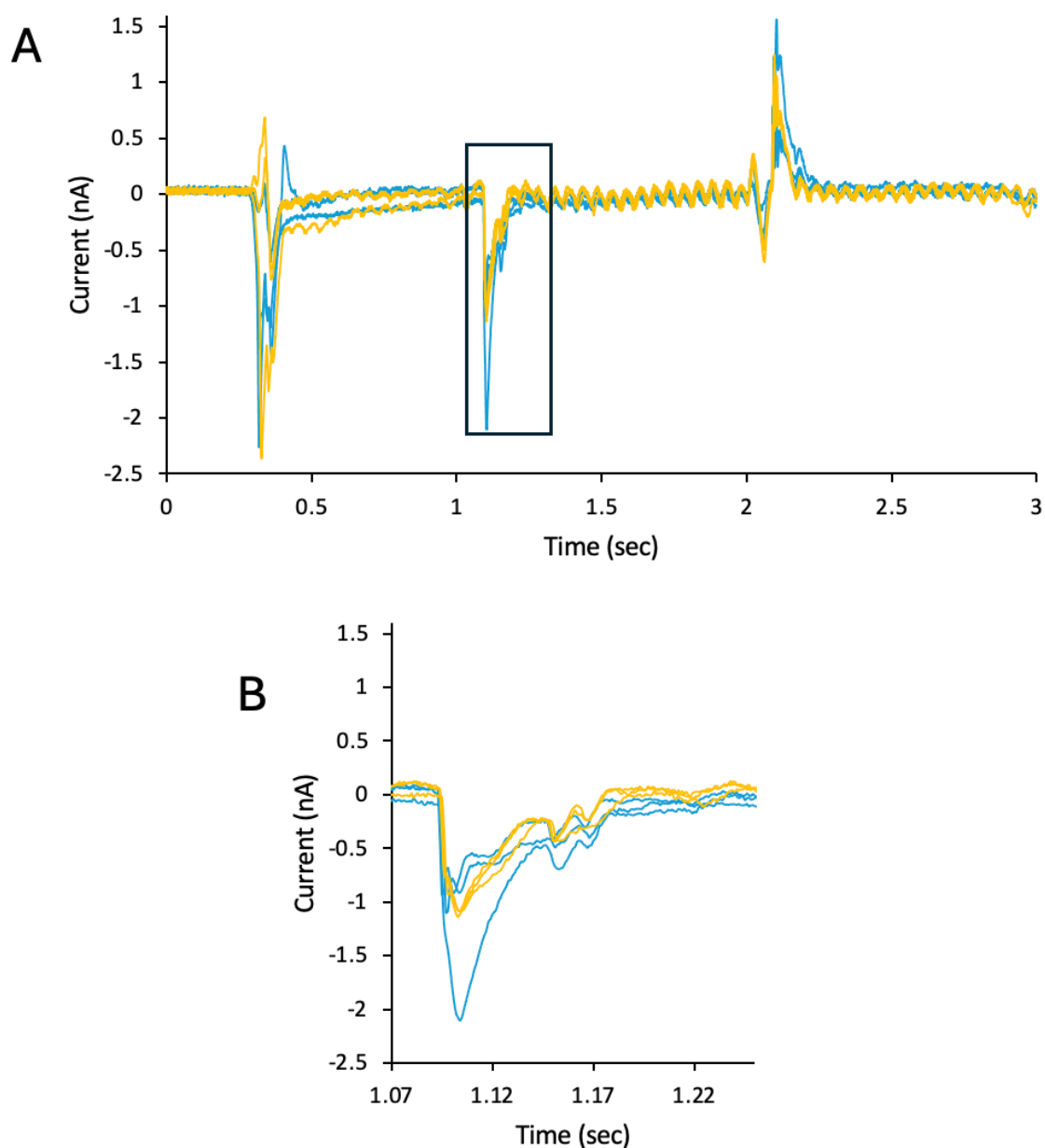


Figure 9.27 **SSME analysis of Rh50 F218A mutant response to 200 mM K⁺ pulse.** Overlaid traces are shown from artificial liposomes reconstituted with purified Rh50 F218A LPR5 (yellow) and empty liposomes (blue). Each condition was measured using at least three independent sensors, and one representative trace from each sensor is shown. (A) Full 3 s recording showing the pre-phase, activation phase, and post-phase of the measurement. The region shown in (B) is indicated by the boxed area. (B) Expanded view of the response immediately following application of the activating buffer pulse.

9.2 Publications

Publication 1:

“Coexistence of Ammonium Transporter and Channel Mechanisms in Amt-Mep-Rh Twin-His Variants Impairs the Filamentation Signalling Capacity of Fungal Mep2 Transceptors.”

Publication 2:

“Prokaryotic ammonium transporters: what has three decades of research revealed?”

Publication 3:

“Biological ammonium transporters from the Amt/Mep/Rh superfamily: mechanism, energetics, and technical limitations.”

Publication 4:

“Biological ammonium transporters: evolution and diversification.”



Coexistence of Ammonium Transporter and Channel Mechanisms in Amt-Mep-Rh Twin-His Variants Impairs the Filamentation Signaling Capacity of Fungal Mep2 Transceptors

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Gordon Williamson, Ana Sofia Brito, and Adriana Bizior contributed equally to this work. Author order was determined by the time they have joined the project: most ancient first. Anna Maria Marini, Mélanie Boeckstaens, and Arnaud Javelle contributed equally to this work. Author order was determined by the time they have joined the project: most recent first.

ABSTRACT Ammonium translocation through biological membranes, by the ubiquitous Amt-Mep-Rh family of transporters, plays a key role in all domains of life. Two highly conserved histidine residues protrude into the lumen of the pore of these transporters, forming the family's characteristic Twin-His motif. It has been hypothesized that the motif is essential to confer the selectivity of the transport mechanism. Here, using a combination of *in vitro* electrophysiology on *Escherichia coli* AmtB, *in silico* molecular dynamics simulations, and *in vivo* yeast functional complementation assays, we demonstrate that variations in the Twin-His motif trigger a mechanistic switch between a specific transporter, depending on ammonium deprotonation, to an unspecific ion channel activity. We therefore propose that there is no selective filter that governs specificity in Amt-Mep-Rh transporters, but the inherent mechanism of translocation, dependent on the fragmentation of the substrate, ensures the high specificity of the translocation. We show that coexistence of both mechanisms in single Twin-His variants of yeast Mep2 transceptors disrupts the signaling function and so impairs fungal filamentation. These data support a signaling process driven by the transport mechanism of the fungal Mep2 transceptors.

IMPORTANCE Fungal infections represent a significant threat to human health and cause huge damage to crop yields worldwide. The dimorphic switch between yeast and filamentous growth is associated with the virulence of pathogenic fungi. Of note, fungal Mep2 proteins of the conserved Amt-Mep-Rh family play a transceptor role in the induction of filamentation; however, the signaling mechanism remains largely unknown. Amt-Mep-Rh proteins ensure the specific scavenging of NH_4^+ through a mechanism relying on substrate deprotonation, thereby preventing competition and translocation of similar-sized K^+ . Our multidisciplinary approaches using *E. coli* AmtB, *Saccharomyces cerevisiae*, and *Candida albicans* Mep2 show that double variation of the family-defining Twin-His motif triggers a mechanistic switch from a specific transporter to an unspecific ion channel with both mechanisms still coexisting in single variants. Moreover, we show that this mechanistic alteration is associated with loss of signaling ability of Mep2, supporting a transport mechanism-driven process in filamentation induction.

KEYWORDS *Candida albicans*, *Escherichia coli*, *Saccharomyces cerevisiae*, ammonium assimilation, fungal filamentation, secondary transporter mechanism

Cellular ammonium transport is facilitated by the ubiquitous Amt-Mep-Rh superfamily, members of which have been identified in every branch of the tree of life (1). The physiological relevance of Amt-Mep-Rh proteins extends beyond their role in

Editor Michael Lorenz, University of Texas Health Science Center

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The authors declare no conflict of interest.

Received 4 October 2021

Accepted 26 January 2022

Published 1 March 2022

ammonium acquisition as a nitrogen source. In fungi, for instance, in the presence of very low ammonium concentrations, specific Amt-Mep-Rh transporters, the Mep2-like proteins, have been proposed to act as transceptors required for the development of filamentous growth, a dimorphic switch associated with the virulence of pathogenic fungi (2). However, it is currently unclear how the signal that leads to the pseudohyphal growth is initiated by Mep2-like transporters.

Despite the relevance of this protein family, the transport mechanism of Amt-Mep-Rh has remained largely elusive for decades. Crystal structures of various family members revealed a trimeric organization with a narrow conducting transport pore through each monomer lined with hydrophobic residues (3–8). While it was next shown that Amt-Mep-Rh proteins mediate NH_3 transport after NH_4^+ deprotonation (9), other functional studies demonstrated that *Archaeoglobus fulgidus* Amt1 and 3, *Escherichia coli* AmtB, and several plant Amts can mediate electrogenic ammonium transport (10–13), questioning the fate of the proton released by NH_4^+ fragmentation. We recently showed that two water wires connect the periplasmic side with the cytoplasmic vestibule of EcAmtB (12). These two water wires are interconnected via the H168 residue of the Twin-His, a highly conserved motif constituted of two histidine residues protruding into the lumen of the pore (12, 14). We showed that, after deprotonation of NH_4^+ at the periplasmic side, the two interconnected water wires and the Twin-His motif enable H^+ transfer into the cytoplasm. A parallel pathway, lined by hydrophobic groups within the protein core, facilitates the simultaneous transfer of uncharged NH_3 .

Despite its high level of conservation, the Twin-His motif presents some variations in fungal Amt-Mep-Rh proteins (14–16). Specifically, the first histidine is present in Mep2 transceptors, whereas the Mep1- and Mep3-type proteins feature a natural occupation by a glutamic acid at the first histidine position, defining two functional Amt-Mep-Rh subfamilies in fungi. More recently, using functional characterization in *Xenopus* oocytes, we revealed that *Saccharomyces cerevisiae* Mep2 mediates electroneutral substrate translocation, while Mep1 performs electrogenic transport, and we proposed that the specific transport mechanism of Mep2 could be responsible for the signal leading to filamentation induction (17). We therefore sought to analyze in more detail the impact of Twin-His variation on the transport mechanism of Amt-Mep-Rh proteins.

Here, we report that altering the Twin-His motif within the pore of *E. coli* AmtB, *S. cerevisiae* and *Candida albicans* Mep2 does not impair ammonium transport function but abolishes the pore selectivity against potassium. We further demonstrate that this loss of selectivity is the result of a mechanistic switch from a transporter-like activity to a channel-like activity governed by a change in the hydrophobicity of the pore. Our findings show that the mechanism of substrate transport ensures the high specificity of the transport. Finally, we show that the mechanistic alteration impacts on the ability of fungal Mep2 proteins to act as transceptors in the development of pseudohyphal growth, supporting the hypothesis that the transport mechanism of Mep2-like proteins is connected to the signal leading to yeast filamentation.

RESULTS

Altering pore hydrophobicity does not disrupt transport activity of AmtB. To determine the effect of altering the hydrophobicity of the pore on transport activity, we first looked at the effect of single acidic substitutions H168E and H168D within the Twin-His motif of AmtB, mimicking the glutamate substitution present in the fungal Mep1/3-type transporters. We purified and reconstituted both AmtB variants into liposomes and measured their activities *in vitro* using solid supported membrane electrophysiology (SSME) and *in vivo* by yeast complementation assays. In proteoliposomes containing AmtB^{H168D} or AmtB^{H168E}, an ammonium pulse of 200 mM elicited very high-current amplitudes of 14.23 nA and 22.28 nA, respectively, compared to 3.38 nA observed for the wild type (WT) (Fig. 1A). To confirm that the transient currents correspond to the translocation of ammonium into the proteoliposomes and not to a simple interaction between ammonium and the protein, we investigated the effect of varying

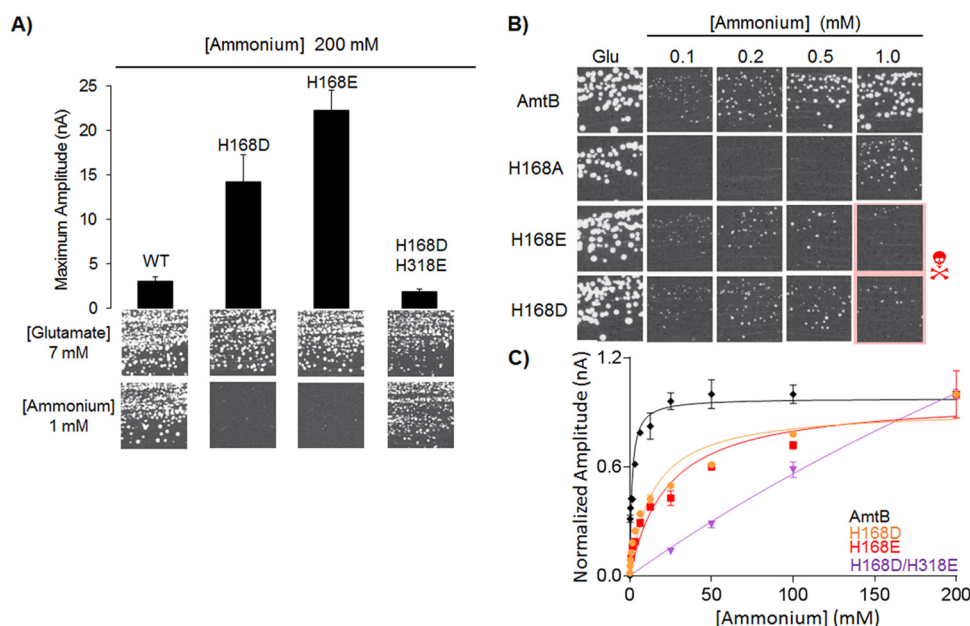


FIG 1 Effect of Twin-His substitution on AmtB ammonium transport activity. (A) (Upper panel) Maximum amplitude of the transient currents measured using SSME following a 200 mM ammonium pulse. (Lower panel) Yeast complementation test after 3 days at 29°C, on potassium glutamate (Glu, positive growth control) or ammonium as the sole nitrogen source. The strain 31019b (*mep1Δ mep2Δ mep3Δ ura3*) was transformed with the pDR195 plasmid, allowing expression of the various AmtB variants. (B) Yeast complementation test, after 5 days at 29°C, in the presence of a range of ammonium concentrations (0.1 mM to 1.0 mM) or glutamate. The red skull shows inhomogeneous growth (C) Kinetics analysis for the transport of ammonium using SSME. The maximum amplitudes recorded after a 200 mM ammonium pulse have been normalized to 1.0 for comparison.

the protein density in the liposomes on the transient current. It is expected that increasing the protein density in the liposomes will prolong the decay time of the current if the current represents a complete transport cycle (18). The lifetime of the currents measured for both variants was dependent on the liposomal lipid:protein ratio (LPR), indicating that the currents indeed account for a full translocation cycle (Table 1). Additionally, we measured an increase of the catalytic constants (K_m) for both variants compared to the WT (Fig. 1C, Table 2).

Expressed in a *S. cerevisiae* triple-*mepΔ* strain deprived of its three endogenous Mep ammonium transporters, both variants were unable to restore growth on 1 mM ammonium after 3 days, suggesting a loss of function (Fig. 1A). However, after 5 days, an inhomogeneous weak growth was observed (Fig. 1B). Previous work has demonstrated that noncontrolled ammonium influx can be toxic to *S. cerevisiae* (19, 20). To test if this inhomogeneous growth could be linked to toxic ammonium influx, the yeast complementation assay was repeated with a lower range of ammonium concentrations (0.1 to 1.0 mM) (Fig. 1B). The complementation ensured by both AmtB^{H168D} and AmtB^{H168E} variants was improved by decreasing the ammonium concentrations. These

TABLE 1 Decay time constants (s^{-1}) of transient currents triggered after an ammonium, methylammonium, or potassium pulse of 200 mM in proteoliposomes containing AmtB variants at various LPR^a

Variant	Ammonium		MeA		Potassium	
	LPR 10	LPR 5	LPR 10	LPR 5	LPR 10	LPR 5
WT	13.4 ± 1.5	18.7 ± 1.0	3.6 ± 0.5	8.3 ± 1.2	NC	NC
H168D	31.4 ± 1.3	38.1 ± 4.1	17.7 ± 2.1	26.4 ± 4.2	NC	NC
H168E	45.5 ± 4.8	53.9 ± 1.6	27.0 ± 1.9	36.4 ± 3.1	NC	NC
H168D/H318E	15.8 ± 1.1	17.8 ± 0.7	10.5 ± 2.5	13.9 ± 1.8	2.3 ± 0.4	4.9 ± 0.2

^aNC, no transient current recorded.

TABLE 2 K_m (mM) of AmtB variants for ammonium, methylammonium, and potassium, using SSME^a

Variant	Ammonium	MeA	Potassium
WT	0.8 ± 0.1	49.86 ± 4.76	NC
H168D	14.02 ± 4.08	55.09 ± 12.67	NC
H168E	12.87 ± 7.79	86.00 ± 9.46	NC
H168D/H318E	NA	NA	NA

^aNC, no transient current recorded; NA, not applicable.

results suggest that the acidic substitution at position 168 drastically increases the ammonium flux through AmtB, rendering it toxic to yeast cells. We did not previously observe ammonium toxicity with the AmtB^{H168E} variant expressed in yeast (15). In those experiments, AmtB^{H168E} was expressed under the control of another promoter, *MET25*, repressible by methionine supplementation. The presence of ammonium under its sulfate salt may lead to partial repression of the *MET25* promoter. In the present experiment, the variants are expressed under the control of the *PMA1* promoter, a stronger promoter than *MET25*. Therefore, enhanced expression of the variants in the present experiment could explain the observed ammonium toxicity.

A double H168D/H318E mutation has previously been shown to impact AmtB specificity (21). To further probe the impact of pore hydrophobicity on the activity and specificity of AmtB, we substituted both residues of the Twin-His motif with acidic residues (AmtB^{H168D/H318E}). SSME measurements revealed that a 200 mM NH_4^+ pulse elicited an LPR-dependent transient current with a maximum amplitude of 1.84 nA in proteoliposomes containing AmtB^{H168D/H318E}, a 1.8-fold reduction compared to WT AmtB (Fig. 1A, Table 1). Additionally, a catalytic constant (K_m) for AmtB^{H168D/H318E} could not be determined, as saturation could not be achieved, even following an ammonium pulse of 200 mM (Fig. 1C, Table 2). When expressed in the *S. cerevisiae* triple-*mepΔ* strain, AmtB^{H168D/H318E} was able to restore cell growth on low ammonium concentrations (Fig. 1A). These data demonstrate that while AmtB^{H168D/H318E} is still functionally active, its activity seems to be reduced compared to WT AmtB. The absence of saturable kinetics further suggests that the variant behaves like a channel rather than having transporter-like activity. We previously observed this type of behavior when we replaced the Twin-His motif with alanine residues, although the mechanism of this switch remained elusive (12).

Previously, methylammonium (MeA) was used as a substrate analogue for ammonium. However, it triggers transient currents of only 15 to 20% of those elicited by ammonium, indicating strong substrate discrimination by AmtB (11, 16, 22). To determine if this level of discrimination is maintained in the Twin-His variants, the AmtB^{H168D} and AmtB^{H168E} variants were subjected to a pulse of 200 mM MeA during SSME. Both AmtB^{H168D} and AmtB^{H168E} exhibited increased activity compared to WT AmtB (9-fold and 12-fold increase in current amplitude, respectively). The double mutant AmtB^{H168D/H318E} showed a maximum amplitude comparable to WT AmtB (Fig. 2A), but it was not possible to determine a catalytic constant, again due to the lack of saturation (Fig. 2B, Table 2). These data indicate that the mutations do not alter the ability of AmtB to discriminate between MeA and ammonium *in vitro*. Yeast complementation was carried out in parallel. MeA cannot be metabolized by yeast cells and is toxic at high external concentrations (15, 23). The native AmtB and all the Twin-His variants allowed growth of triple-*mepΔ* *S. cerevisiae* cells on glutamate medium, but not when it was supplemented with MeA (Fig. 2A). This shows that all the variants are also active in transporting MeA in yeast.

Altering pore hydrophobicity abolishes transport selectivity. The findings reported above showed that altering the hydrophobicity of the pore does not inactivate AmtB but has a substantial impact on the transport mechanism. Thus, we next investigated whether the substitutions also affect the ammonium/MeA selectivity of AmtB against competing ions. We focused on K^+ , as it has an ionic radius similar to that of NH_4^+ . A 200 mM K^+ pulse failed to elicit a measurable current in AmtB^{H168D} or AmtB^{H168E} proteoliposomes but

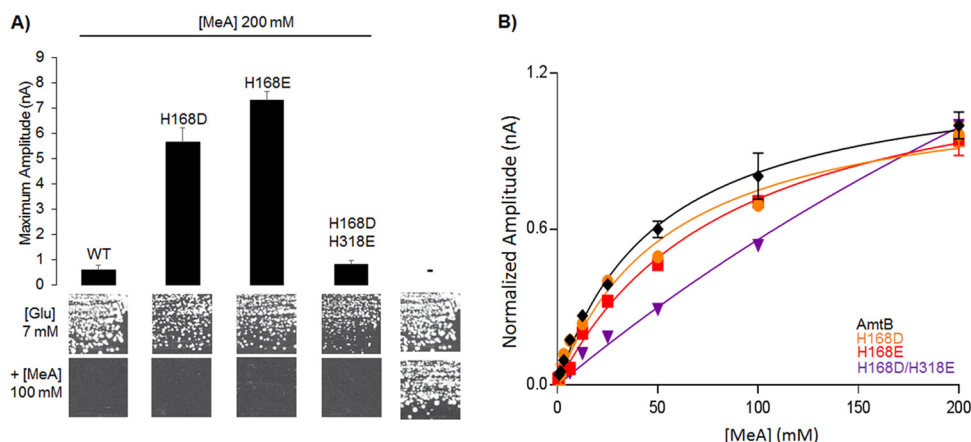


FIG 2 Effect of Twin-His substitution on AmtB methylammonium transport activity. (A) (Upper panel) maximum amplitude of the transient current measured using SSME following a 200 mM methylammonium (MeA) pulse. (Lower panel) Yeast complementation test, after 3 days at 29°C, on solid minimal medium containing, as the sole nitrogen source, potassium glutamate 7 mM (glu, positive growth control) supplemented or not with 100 mM methylammonium. The strain 31019b (*mep1Δ mep2Δ mep3Δ ura3*) was transformed with the empty pDR195 plasmid (–), or with pDR195 allowing expression of the various AmtB variants. (B) Kinetics analysis for the transport of methylammonium using SSME. The maximum amplitudes recorded after a 200 mM MeA pulse have been normalized to 1.0 for comparison.

was able to trigger a clear transient current in AmtB^{H168D/H318E} (Fig. 3A). The amplitude and decay time of the currents recorded for AmtB^{H168D/H318E} are LPR-dependent, confirming that they are caused by K⁺ translocation and are not only the result of protein-substrate interaction (Fig. 3B, Table 1). It was not possible to determine a catalytic constant for AmtB^{H168D/H318E}, again due to the lack of saturation, indicating a channel-like rather than a transporter-like activity (Fig. 3C, Table 2). Moreover, all the variants, but not the WT, were able to complement the growth defect of a triple-*mepΔ trkΔ S. cerevisiae* strain,

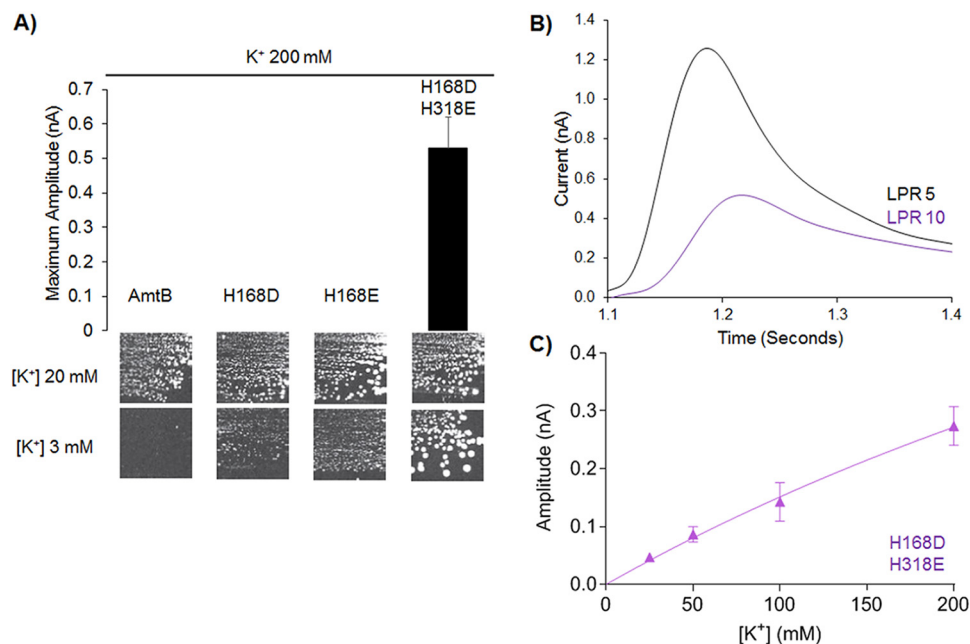


FIG 3 Potassium (K⁺) transport in AmtB Twin-His variants. (A) (Upper panel) Maximum amplitude of the transient current measured using SSME following a 200 mM potassium pulse. (Lower panel) Yeast complementation test, after 5 days at 29°C, in the presence of 20 mM or 3 mM potassium. The #228 strain (*mep1Δ mep2Δ mep3Δ trk1Δ trk2Δ leu2 ura3*) was transformed with the pDR195 plasmid allowing expression of the various AmtB variants. (B) Transient currents measured using SSME following a 200 mM potassium pulse with AmtB^{H168D/H318E} reconstituted into proteoliposomes at a LPR 5 (black) or 10 (purple). (C) Kinetics analysis for the transport of potassium using SSME.

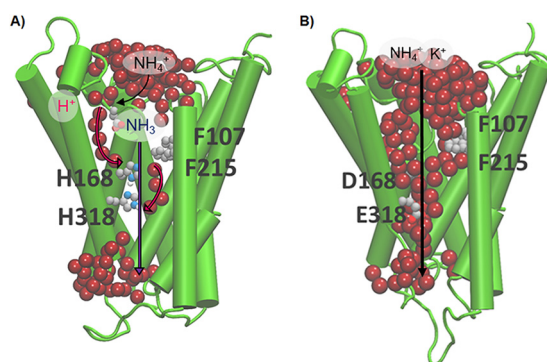


FIG 4 Schematic comparison of transport in WT AmtB and AmtB^{H168D/H318E}. (A) Molecular dynamic simulation of the AmtB monomer, showing the interconnected water wires (represented as red spheres). Following sequestration of NH₄⁺ at the periplasmic face, NH₄⁺ is deprotonated, before passing the “Phe-Gate,” representing the first hydrophobic barrier, and H⁺ and NH₃ follow two separate pathways to join the cytoplasm (magenta arrows depict the pathway for H⁺ transfer, dark blue arrows for NH₃) facilitated by the presence of two internal water wires. (B) Molecular dynamic simulation of the AmtB^{H168D/H318E} monomer, showing the pore filled with water molecules. Due to the increased hydration within the pore, periplasmic NH₄⁺ and K⁺ are translocated directly through the central pore to the cytoplasm.

which lacks the three endogenous ammonium (Mep) transporters and the 2 major potassium (Trk) transporters, in the presence of a limited concentration of K⁺ (Fig. 3A). The complementation is clearly improved when AmtB^{H168D/H318E} is expressed compared to AmtB^{H168D} or AmtB^{H168E}, showing that the single variants are also able to translocate potassium, albeit at a lower rate than AmtB^{H168D/H318E}. This could explain why we measured a current after a K⁺ pulse in the proteoliposomes containing AmtB^{H168D/H318E} but not with AmtB^{H168D} or AmtB^{H168E}. We reason that there could be an inverse relationship between the hydrophobicity of the central pore and the selectivity of AmtB.

Loss of AmtB selectivity is due to increased pore hydration accompanied by a mechanistic change. To understand the loss of substrate selectivity observed in the AmtB variants and the switch from transporter-like to channel-like activity observed in the SSME recordings with the AmtB^{H168D/H318E} variant, molecular dynamics simulations were conducted. Our previously proposed model for the transport mechanism of AmtB suggests that, after deprotonation of NH₄⁺ at the periplasmic side, two interconnected water wires enable H⁺ transfer into the cytoplasm. A parallel pathway, lined with hydrophobic groups within the protein core, facilitates the simultaneous transfer of NH₃ (Fig. 4A) (12).

Molecular dynamics simulations based on alterations of the Twin-His motif by the introduction of charged residues show that this destabilizes and widens the pore, causing it to fill with water and forming a continuous aqueous channel (Fig. 4B). This represents a significant change from the ordered single-file chains of water molecules, separated by the Twin-His motif, observed in the simulations with the WT. The formation of a wide aqueous pore at the expense of discrete water chains disrupts the mechanism of transport and compromises the ability of AmtB to act as a specific transporter, since the newly flooded pore can enable the direct passage of hydrated cations.

To test if the single-file water wires are indeed disrupted by the substitutions, we employed a D₂O-based SSME assay. Because the strength of a covalent bond involving deuterium increases compared to hydrogen, proton mobility is reduced by 30% for each D₂O molecule compared to H₂O (24). If the water wires are intact and are required for the transport mechanism, replacement of H₂O with D₂O is expected to result in the complete abolishment of current when measured by SSME. If, however, the mechanism does not require the water wires, the substitution of H₂O with D₂O should not affect the observed current following an ammonium pulse. As previously observed in WT AmtB, no current was measured under D₂O conditions (Fig. 5) (12). In AmtB^{H168D} and AmtB^{H168E}, the current observed following a 200 mM ammonium pulse was diminished by about 4-fold in the presence of D₂O compared to H₂O, but not completely

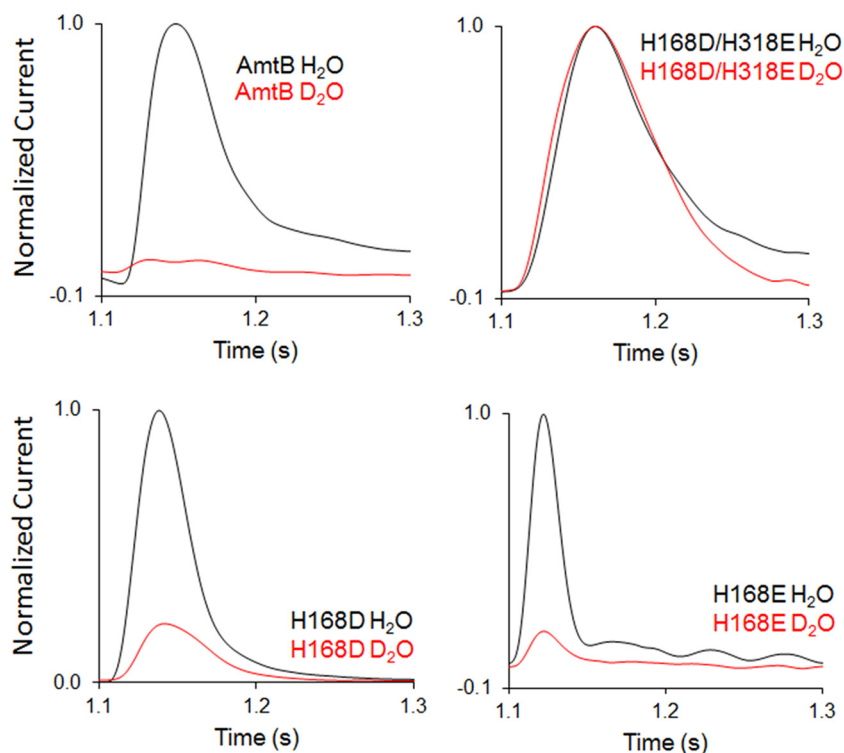


FIG 5 Hydrophobicity of the AmtB transport pore governs mechanistic switch. Transient currents measured using SSME following a 200 mM ammonium pulse on sensors prepared with solutions containing either H₂O (black) or D₂O (red) in WT AmtB, AmtB^{H168D/H318E}, AmtB^{H168D}, or AmtB^{H168E}. D₂O currents have been normalized to respective H₂O currents.

abolished (Fig. 5). The stark reduction of current in the presence of D₂O indicates that proton hopping remains a mechanistic feature within AmtB^{H168D} and AmtB^{H168E}. However, the lack of complete abolition implies that some charge translocation is occurring by another mechanism. These data suggest that two transport mechanisms are used by these variants, one depending on NH₄⁺ deprotonation, and the other allowing the occasional passage of hydrated NH₄⁺ or K⁺, as shown in Fig. 4. This result is in agreement with the capacity of these variants to transport K⁺ in yeast (Fig. 3A).

In contrast, in AmtB^{H168D/H318E}, a 200 mM ammonium pulse elicits transient currents of similar magnitude with either D₂O or H₂O (Fig. 5). This indicates that the central mechanism of proton hopping observed in WT AmtB is no longer a mechanistic feature of AmtB^{H168D/H318E} which gains the ability to directly transport NH₄⁺ without deprotonation. These findings demonstrate that hydrophilic substitutions within the Twin-His motif gradually lead to a switch in the transport mechanism of AmtB protein from transporter to channel-like, which is observed in AmtB^{H168D/H318E}. In the latter, NH₄⁺ is translocated as an intact cation in its hydrated form, abolishing transport specificity (Fig. 4). These data also explain why the ammonium/MeA/potassium transport activity cannot be saturated in proteoliposomes containing AmtB^{H168D/H318E} and why this variant is highly efficient in potassium transport (Fig. 1, 2, and 3, Table 2).

The Twin-His motif is involved in the transport specificity of ScMep2. Previously, we showed that alanine mutations in the Twin-His motif (AmtB^{H168A} and AmtB^{H318A}) also alter the selectivity of the AmtB pore, resulting in the ability of the variants to translocate K⁺ ions (12). To understand the general importance of the hydrophobicity of the central pore in substrate selectivity, we simultaneously compared Twin-His variants of *S. cerevisiae* Mep2 with single or double mutations in alanine or glutamate residues in terms of ammonium, methylammonium, and potassium transport functionality. We chose a double Twin-His mutation to double E because the paralogue ScMep1 has an E at the His₁ position and E at the His₂-1 position. We wished to remain as close as possible

to a yeast protein situation known to be functional (ScMep1). We first tested if the *S. cerevisiae* triple-*mep* Δ strain expressing ScMep2^{H194A}, ScMep2^{H194E}, ScMep2^{H348A}, ScMep2^{H348E}, ScMep2^{H194A/H348A}, or ScMep2^{H194E/H348E} was able to grow on low ammonium concentration, compared to cells expressing native ScMep2 as the positive control (Fig. 6A). We found that a single substitution of the first or second histidine of the Twin-His motif into alanine or glutamate does not affect in a major way the ammonium transport function of ScMep2 in a home-made buffered medium. However, the ScMep2 double Twin-His mutants (ScMep2^{H194A/H348A} and ScMep2^{H194E/H348E}) do not support the growth of the triple-*mep* Δ cells on low ammonium (Fig. 6A). Fluorescence microscopy using Mep2 variants fused to the pHluorin version of green fluorescent protein (GFP) reveals that all variants reach the cell surface, except the double Twin-His variants, which seem to be additionally stacked in the endoplasmic reticulum (Fig. S3A). This could indicate that the failure to complement results from a trafficking problem of the double Twin-His variants.

We also tested the capacity of ScMep2 variants to intoxicate cells in the presence of high MeA concentrations. Contrary to ScMep1, native ScMep2 is unable to intoxicate cells in the presence of MeA, proposed to be due to a lower maximal transport rate (25). Expression of ScMep2 variants with single His substitutions reduced growth of the triple-*mep* Δ cells in the presence of MeA, suggesting a higher transport flux through the variants or an altered transport mechanism increasing the sensitivity of the cells to the toxic compound (Fig. 6A) (15). The substitution of both histidines in the Twin-His motif was not accompanied by an increased sensitivity of the cells to MeA, which is in agreement with the absence of ammonium transport by the ScMep2^{H194A/H348A} and ScMep2^{H194E/H348E} variants (Fig. 6A). As for AmtB, the Twin-His substitutions of ScMep2 do not affect ammonium/MeA discrimination.

The capacity of the ScMep2 variants to transport K⁺ was next assessed in the triple-*mep* Δ strain further lacking its endogenous high-affinity potassium transporters Trk1/2 in the presence of limiting concentrations of K⁺ (Fig. 6B). In contrast to native ScMep2, expression of any of the ScMep2 Twin-His variants rescues cell growth in the presence of low K⁺ concentrations, with growth efficiency depending on the variant. Of note, the double Twin-His variants enable K⁺ transport function, even if the pHluorin-fused versions are at least partially blocked in the endoplasmic reticulum (Fig. S3B), thereby suggesting that sufficient proteins reach the plasma membrane to produce this phenotype. These data show that ScMep2 Twin-His variants can translocate K⁺, with variants possessing the H348A or H348E mutation being the most efficient in K⁺ transfer (Fig. 6B). These results indicate that, as for AmtB, the mutations in the twin-His motif of the central pore of ScMep2 alters the transport selectivity.

Coexistence of different transport mechanisms in ScMep2 variants. It is of note that the medium used to test the ammonium transport capacity of the AmtB and ScMep2 variants in yeast contains about 180 mM K⁺. Thus, it appears that single ScMep2 His-variants are able to ensure growth in the presence of 1 mM ammonium despite a very high K⁺ concentration, indicating that potassium does not efficiently compete with ammonium recognition and transport in these variants. This indicates that the single His substitutions allow the coexistence of two transport mechanisms, as proposed for AmtB^{H168D} and AmtB^{H168E}. This finding suggests that the first mechanism allows the selective transport of NH₃ after NH₄⁺ recognition and deprotonation (as in native Mep2) and is not sensitive to high K⁺. The second mechanism, created by the substitution, could act as an ion channel pathway allowing the passage of K⁺, and likely NH₄⁺, and would thus be sensitive to competition between both ions. This prompted us to test if the absence of growth under low-ammonium conditions of cells expressing ScMep2^{H194A/H348A} and ScMep2^{H194E/H348E} could be linked to high concentrations of potassium in the medium used. We performed growth tests with the triple-*mep* Δ *trk* Δ strain in a medium allowing us to simultaneously address the capacity of the variants to transport K⁺ and ammonium. As shown in Fig. 7, ScMep2^{H194E/H348E} allows cell growth in the presence of 3 mM ammonium and equal or lower K⁺ concentrations (1 mM and 3 mM), indicating that the ammonium transport mechanism of this variant is highly sensitive to the potassium concentration. Of note, cells

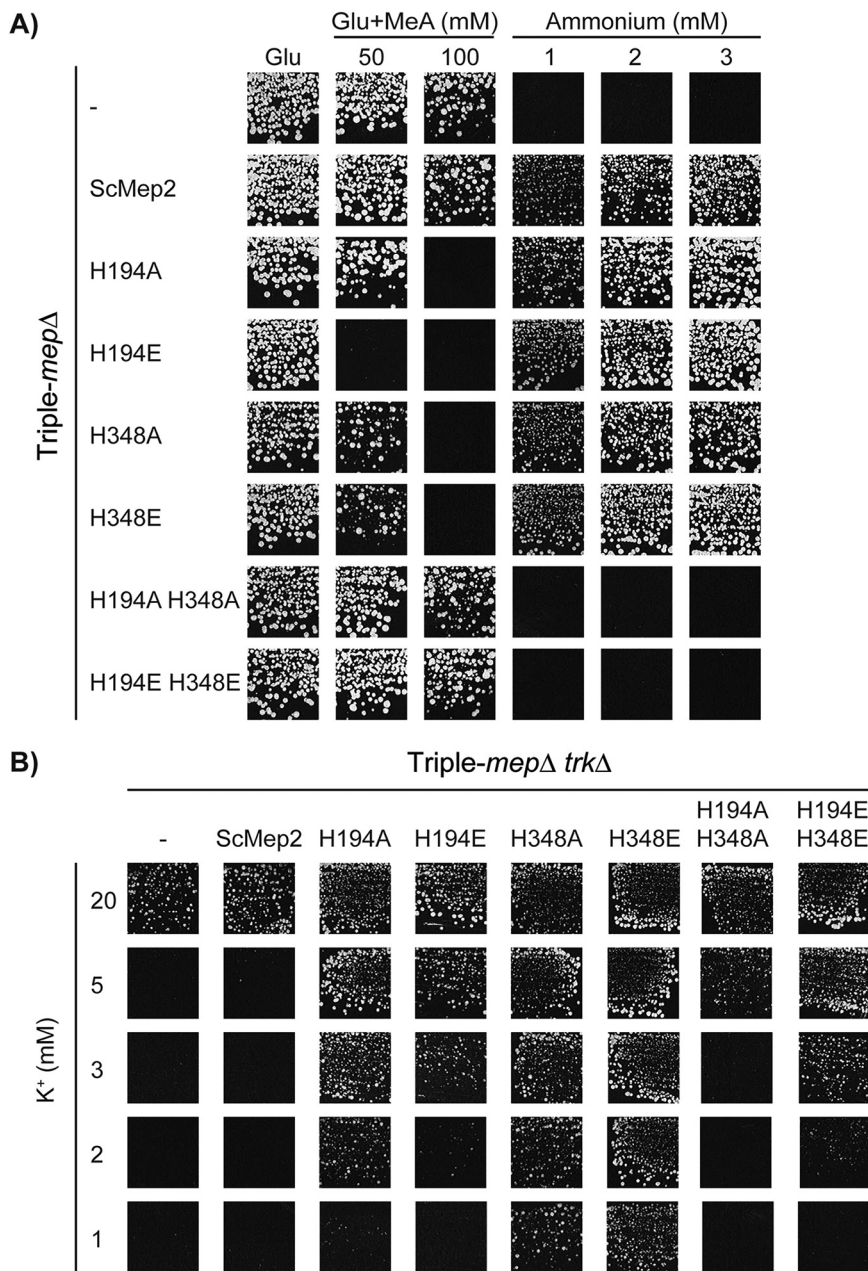


FIG 6 Effect of Twin-His substitutions on ScMep2 transport activity and specificity. (A) Growth tests, after 4 days at 29°C, on solid minimal medium containing, as the sole nitrogen source, ammonium 1, 2, and 3 mM or potassium glutamate (Glu, positive growth control) supplemented or not with 50 or 100 mM methylammonium (MeA). The *Saccharomyces cerevisiae* strain 31019b (*mep1Δ mep2Δ mep3Δ ura3*) was transformed with the pFL38 empty plasmid (–) or with YCpMep2, YCpMep2^{H194A}, YCpMep2^{H194E}, YCpMep2^{H348A}, YCpMep2^{H348E}, YCpMep2^{H194A/H348A}, or YCpMep2^{H194E/H348E}. (B) Growth tests, after 4 days at 29°C, on solid minimal medium containing different potassium concentrations (from 1 mM to 20 mM) and in the presence of sodium glutamate as the nitrogen source. The *Saccharomyces cerevisiae* strain #228 (*mep1Δ mep2Δ mep3Δ trk1Δ trk2Δ leu2 ura3*) was transformed with the pFL38 empty plasmid (–) or with YCpMep2, YCpMep2^{H194A}, YCpMep2^{H194E}, YCpMep2^{H348A}, YCpMep2^{H348E}, YCpMep2^{H194A/H348A}, or YCpMep2^{H194E/H348E}.

expressing the variant ScMep2^{H348E} appear to grow better when the ammonium concentration decreases specifically in the presence of 3 mM K⁺ and not at higher K⁺ concentrations. One possible explanation could be that upon lowering the concentration of the competitor K⁺ ion, ammonium uptake increases up to toxic levels, as previously observed with AmtB^{H168E} and AmtB^{H168D} variants (Fig. 1B).

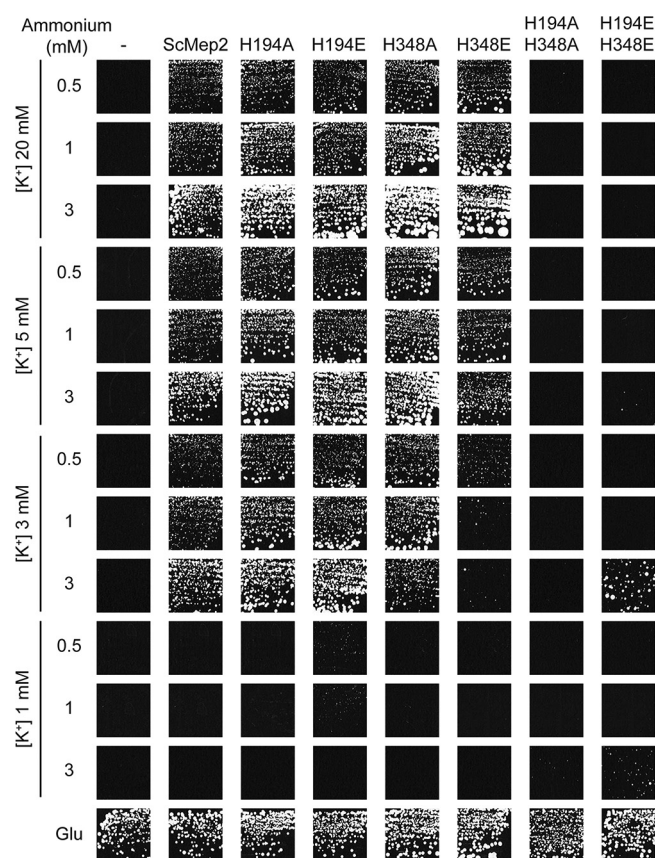


FIG 7 Different transport mechanisms in the ScMep2 Twin-His variants. Growth tests, after 12 days at 29°C, on solid minimal medium containing different potassium concentrations (from 1 mM to 20 mM) and in the presence of different ammonium concentrations (0.5, 1, or 3 mM) as the nitrogen source. The *Saccharomyces cerevisiae* strain #228 (*mep1Δ mep2Δ mep3Δ trk1Δ trk2Δ leu2 ura3*) was double transformed with the pFL46 and pFL38 empty plasmids (–) or with pFL46 and YCpMep2, YCpMep2^{H194A}, YCpMep2^{H194E}, YCpMep2^{H348A}, YCpMep2^{H348E}, YCpMep2^{H194A/H348A}, or YCpMep2^{H194E/H348E}. The same solid medium containing 20 mM potassium and potassium glutamate, as the sole nitrogen source, was used as the positive growth control (lower panel; the growth corresponds to day 5 at 29°C).

Consistent with the conclusions made for AmtB, these data support that simultaneous substitution of the two conserved histidines in ScMep2 results in the formation of a K^+/NH_4^+ channel pathway, while the transport mechanism dependent on NH_4^+ deprotonation is abolished. The observation that single histidine variants are able to ensure growth on low ammonium concentrations even in the presence of high potassium concentrations supports the hypothesis that both pathways, one based on ammonium deprotonation and the other linked to direct NH_4^+/K^+ transport, coexist in these variants.

Influence of the transport mechanism on the capacity of ScMep2 to induce filamentation. ScMep2, but not ScMep1 or ScMep3, is required for the dimorphic switch leading to yeast filamentation in the presence of very low ammonium concentrations (2). H194E and H348A mutations of ScMep2 were proposed to affect the filamentation signaling process under very low ammonium conditions (15, 17, 26). Here, we simultaneously compared the whole set of single and double mutants in the Twin-His motif of ScMep2 to assess the effect of the mechanistic switch from a transporter-like to a channel-like activity on the capacity of ScMep2 to induce filamentation on the appropriate synthetic low ammonium dextrose (SLAD) medium (100 μ M ammonium) (Fig. 8A). Synthetic high ammonium dextrose (SHAD) medium (1 mM ammonium) was also used to check for potential constitutive induction of filamentation. In parallel, the ammonium transport functionality of the variants was also evaluated by growth tests

on these media (Fig. 8B). We show that, contrary to native ScMep2, filamentation is not observed in the presence of any of the His variants (Fig. 8A), as previously observed with ScMep2^{H194E} and ScMep2^{H348A} (15, 17, 26). As the single His substitutions into alanine slightly affect the functionality of ScMep2 on SLAD (Fig. 8B), the conclusion concerning its signaling capacity appears tricky. ScMep2^{H194A/H348A} and ScMep2^{H194E/H348E} are not able to ensure growth on SLAD (Fig. 8B), probably due to the high potassium concentration (30 mM) compared to the reduced ammonium concentration (100 μ M) in this medium; therefore, we cannot draw conclusions on their capacity to induce filamentation. ScMep2^{H194E} and ScMep2^{H348E} have lost their signaling capacity, although they seem functional in ammonium transport function, revealing the importance of the pore hydrophobicity and/or the transport mechanism in the signaling process.

As potassium could compete with ammonium transport and a high potassium concentration is present in SLAD, we tested the capacity of the Twin-His variants to induce filamentation in a medium containing low potassium concentrations. Filamentation and growth tests were performed in a home-made medium, equivalent to yeast nitrogen base (YNB) medium and allowing control of potassium and ammonium concentrations (0.1 or 1 mM ammonium and a potassium concentration ranging from 0.1 to 20 mM) (Fig. 9A and B). Again, no filamentation is observed with the ScMep2 His variants (Fig. 9B). However, as shown in Fig. 9A, ScMep2^{H194A/H348A} and ScMep2^{H194E/H348E} are not able to sustain growth at 0.1 or 1 mM ammonium, even if the potassium concentration is reduced, suggesting that ammonium transport is strongly inhibited by potassium and is not sufficient to ensure growth. Of note, filamentous growth is not observed with cells expressing ScMep2 at very low potassium concentrations (0.1 mM, Fig. 9B). However, growth is also strongly impaired under this condition, indicating that a minimal potassium concentration is required to ensure optimal growth (Fig. 9A). ScMep2-dependent filamentation is also inhibited by increasing the potassium concentration, which could be due to the inhibition of ammonium translocation by potassium (Fig. 9B). This confirms that the simple presence of ScMep2 at the cell surface is not sufficient for its signaling property; translocation of the substrate is mandatory for signaling (15, 17, 27–29). Taken together, these data suggest that altering the transport mechanism of ScMep2 through Twin-His mutations is correlated with an impaired capacity of the protein to induce filamentation. This result supports the hypothesis that the capacity of ScMep2 to induce filamentation is associated with its substrate translocation mechanism (15, 17).

Coexistence of different transport mechanisms in CaMep2 variants and influence on filamentation. CaMep2 from *Candida albicans*, the orthologue of *S. cerevisiae* Mep2, is also required to induce filamentation of this human-pathogenic fungus (30). Expression of CaMep2 in *S. cerevisiae* cells deprived of endogenous ScMep2 restores filamentation, suggesting that a similar mechanism leads to filamentation in both species (17, 30). Here, we compared the impact of a set of Twin-His substitutions in CaMep2 by expressing the variants in *S. cerevisiae*. As observed with ScMep2, CaMep2 variants mutated in the first and/or second histidine CaMep2^{H188E}, CaMep2^{H342E}, and CaMep2^{H188E/H342E} are able to translocate K⁺ (Fig. 8C). Growth tests on SLAD and SHAD show that native CaMep2 and CaMep2^{H188E} are less functional in ammonium transport than ScMep2, while CaMep2^{H342E} and CaMep2^{H188E/H342E} are completely nonfunctional, at least in the presence of high potassium concentrations (Fig. 8B). None of the CaMep2 variants are able to allow filamentation (Fig. 8A). Hence, as for ScMep2, the capacity of CaMep2 to induce filamentation also appears to be associated with its transport mechanism.

DISCUSSION

The data presented show that substitutions within the Twin-His motif of Amt-Mep-Rh decrease the specificity of transport. While this was previously proposed in *E. coli*, *S. cerevisiae*, and *Arabidopsis thaliana* (12, 15, 31), it was assumed that the Twin-His motif was central for the selectivity of the transporters. In this context, the natural occurrence of a glutamic acid in the place of the first histidine in fungal Mep1 and Mep3, but not Mep2 protein, was unclear. Here, our findings lead us to propose a new

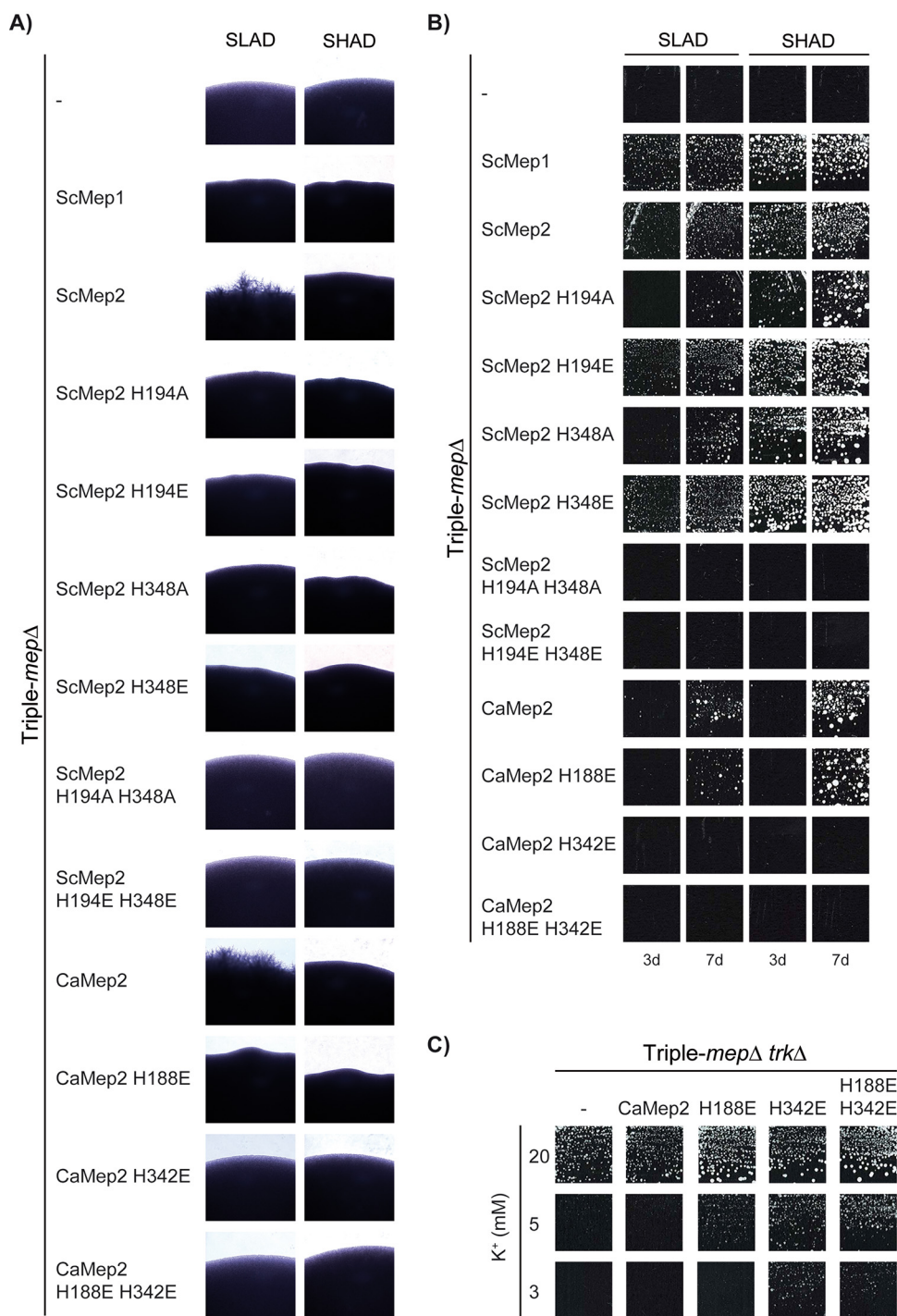


FIG 8 Effect of Twin-His substitutions on the capacity of ScMep2 and CaMep2 to induce fungal filamentation. Homozygous diploid triple-mepΔ *ura3* cells (strain ZAM38) were transformed with the pFL38 empty plasmid (–) or with YCpMep2, YCpMep2^{H194A}, YCpMep2^{H194E}, YCpMep2^{H348A}, YCpMep2^{H348E}, YCpMep2^{H194A/H348A}, YCpMep2^{H194E/H348E}, YCpCaMep2, YCpCaMep2^{H188E}, YCpCaMep2^{H342E}, or YCpCaMep2^{H188E/H342E}. (A) Pseudohyphal growth tests of high-density cell suspensions dropped on SLAD and SHAD media at day 7. (B) Growth tests of low-density cell suspensions on SLAD and SHAD media at days 3 (3d) and 7 (7d), at 29°C. (C) Growth tests, after 4 days at 29°C, on solid minimal medium containing different potassium concentrations (from 2 mM to 20 mM) and sodium glutamate as the nitrogen source. The *Saccharomyces cerevisiae* strain #228 (*mep1Δ mep2Δ mep3Δ trk1Δ trk2Δ leu2 ura3*) was transformed with the pFL38 empty plasmid (–) or with YCpCaMep2, YCpCaMep2^{H188E}, YCpCaMep2^{H342E}, or YCpCaMep2^{H188E/H342E}.

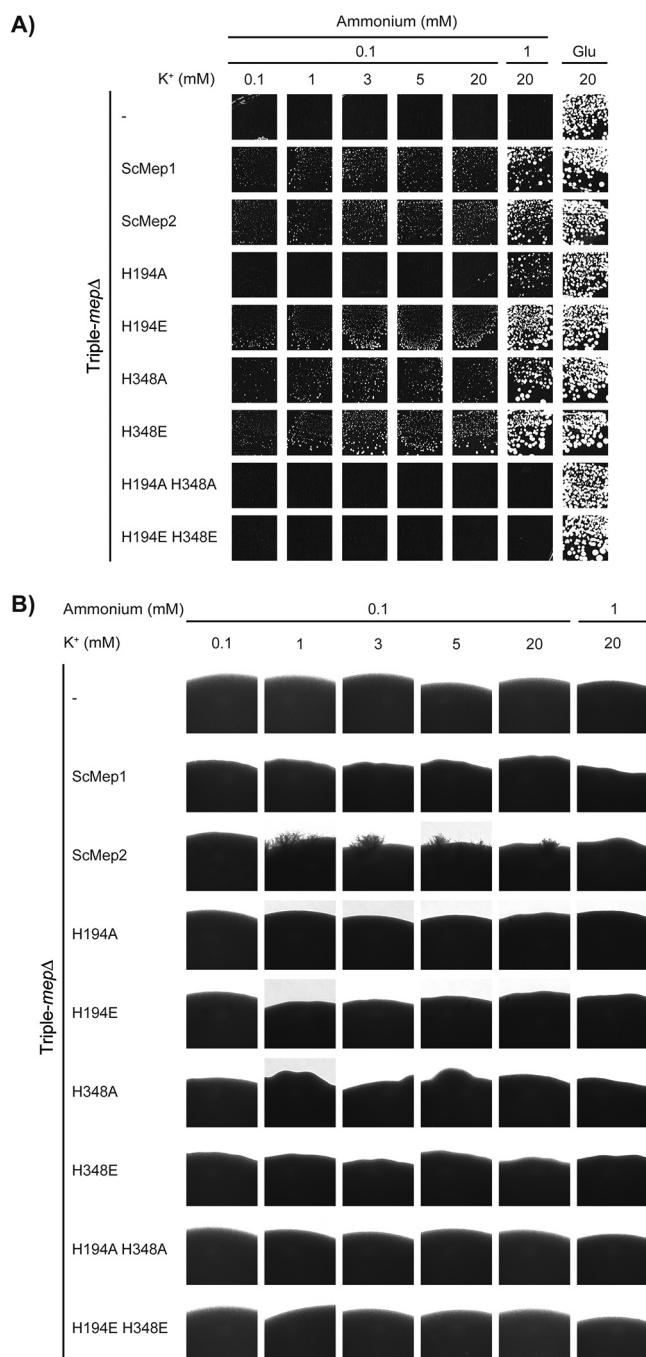


FIG 9 Effect of Twin-His substitutions on the capacity of ScMep2 to induce fungal filamentation at different K⁺ concentrations. Homozygous diploid triple-*mepΔ* *ura3* cells (strain ZAM38) were transformed with the pFL38 empty plasmid (–) or with YCpMep1, YCpMep2, YCpMep2^{H194A}, YCpMep2^{H194E}, YCpMep2^{H348A}, YCpMep2^{H348E}, YCpMep2^{H194A/H348A}, or YCpMep2^{H194E/H348E}. (A) Growth tests, after 7 days at 29°C, on 183 medium containing different potassium concentrations (0.1 mM to 20 mM) and 0.1 mM or 1 mM ammonium, as the nitrogen source. The same medium containing 20 mM potassium and sodium glutamate (Glu), as the sole nitrogen source, was used as the positive growth control. (B) Pseudohyphal growth tests of high-density cell suspensions dropped on 183 medium containing different potassium concentrations (from 0.1 mM to 20 mM) and 0.1 mM or 1 mM ammonium, as the nitrogen source. Cells were incubated 7 days at 29°C.

model that explains the loss of specificity associated with variations in the Twin-His motif. We show that the modification of the Twin-His motif is associated with a change in the pore hydrophobicity increasing its hydration pattern. These findings are supported by our previous X-ray structural analysis, where we showed that the introduction of a charged

residue at the Twin-His position drastically enhanced the pore hydration level (14). This increase in hydration governs a switch of the translocation mechanism from a specific transporter, based on substrate fragmentation, to an unspecific channel-like activity, where NH_4^+ is transported in its hydrated form and K^+ , an ion of similar size, is able to compete. The mechanism of substrate transport alone ensures the high specificity of the transporter. This is reminiscent of the formate/nitrite transporters that ensure transport selectivity by neutralizing the formate anion by protonation (deprotonation in Amt-Mep-Rh), followed by the passage of the neutral substrate through a lipophilic constriction zone (24). We anticipate that the selective mechanism here evidenced could also apply to Rh members of the Amt-Mep-Rh family, as human RhAG mutations are associated with overhydrated stomatocytosis (OhSt), a hemolytic anemia characterized by the loss of specificity and leakage of important monovalent cations (K^+ , Na^+) inside red blood cells (32).

In this context, it is important to note that the residues that delineate and stabilize the water wires in AmtB are highly conserved across the whole Amt-Mep-Rh family, which implies that the model proposed for ammonium conduction in AmtB could be conserved among members of the family that exhibit electrogenic activity (12). In line with this hypothesis, our previous results for NeRh50, a phylogenetically distant homolog of AmtB, demonstrate that its activity is electrogenic (12). In addition, other groups have reported electrogenic activity in other Amt-Mep-Rh members, including Amt1 and Amt3 from *Archaeoglobus fulgidus*, Amt1;1 from *Lycopersicon esculentum*, and some human Rh proteins (10, 33, 34). However, electroneutral activity has also been reported for a number of Amt-Mep-Rh proteins (13, 17, 33, 35). Notably, protein isoforms within a single species have been characterized as having different activity. For example, in *Arabidopsis thaliana*, whose genome encodes 6 *AMT* genes, divided in 2 subfamilies, activity of Amt1 members is electrogenic, while activity of Amt2 is electroneutral (for review see reference 36 and references therein). A similar split is observed in *S. cerevisiae* and *C. albicans* Mep2 and Mep1/3 proteins (15, 17). These results suggest that the family possesses more than one unique transport mechanism and, if so, it may be advantageous in order to maintain multiple physiological functions (17). At the moment, the molecular basis underpinning the diversity in transport mechanism in the Amt-Mep-Rh protein family is not fully solved. Our study, however, offers a hint to better apprehend this complex question. We have shown that a single mutation in ScMep2 and AmtB changes a specific transporter to an unspecific ion channel. This is reminiscent of the ubiquitous and very large CLC family of chloride translocators in which apparently similar architecture supports various mechanisms of transport—strict Cl^- channel, weak coupled Cl^-/H^+ exchanger, strict Cl^-/H^+ , or NO_3^-/H^+ antiporter (for review see reference 37). Hence, it is now necessary to further investigate the mechanism throughout the Amt-Mep-Rh family, alongside the energetics of the transport and the dynamics of the protein during the transport cycle.

Our results extend beyond the characterization of a new mechanism for selectivity and offer a gateway to better understand signaling mechanisms responsible for fungal filamentation. The mechanism of Mep2-mediated signaling in yeast filamentation remains largely unsolved. Two hypotheses are raised concerning the molecular mechanism of Mep2-mediated signaling. The first one is that Mep2 is a sensor, potentially interacting with signaling partners leading to the induction of filamentation (2). The other one proposes that the specific transport mechanism of Mep2 could underlie the signal leading to filamentation (15, 17). We showed that, in the Mep2 single Twin-His variants, an NH_4^+ deprotonation-dependent transport mechanism and a channel-like mechanism (direct NH_4^+ transport) coexist. We further showed that this mechanistic alteration impairs the capacity of the *S. cerevisiae* and *C. albicans* Mep2 protein to induce filamentation. Altogether, these results support the hypothesis of mechanism-dependent signaling in yeast filamentation. As previously suggested, the difference in the transport mechanism between fungal Mep2 and Mep2^{H/E} could influence in an opposite way the intracellular pH (15, 17). This pH modification could be the signal leading to the dimorphic change. While Mep2 would transport NH_3 after NH_4^+ deprotonation,

the current data indicate that the Mep2^{H194E} variant transports NH₄⁺, thereby leading to acidification. However, we cannot exclude that the mechanism alteration leading to the creation of an ionic channel in single Twin-His Mep2 variants precludes a conformational switch required to transmit the signal to partners. Filamentation is often related to the virulence of pathogenic fungi, such as the human pathogens *Candida albicans* (38), *Histoplasma capsulatum* (39), or *Cryptococcus neoformans* (40). In this context, our results are of particular importance, as the characterization of the conditions regulating the yeast dimorphism may be crucial to better understanding fungal virulence.

MATERIALS AND METHODS

Plasmids and mutagenesis. The plasmids used are listed in Table S1. AmtB mutants were generated using the Quikchange XL site-directed mutagenesis kit (Agilent Technologies), following the manufacturer's instructions. The *amtB* gene cloned into pET22b(+) was used as the template, as previously described (4).

Site-directed mutagenesis of *ScMEP2* was performed by GeneCust, using YCpMep2 as the template. The *CaMEP2* (orf19.5672) gene and the mutated *CaMEP2*^{H188E} and *CaMEP2*^{H342E} genes, with the *ScMEP2* promoter (−661 to −1) and terminator (1 to 262), were synthesized and cloned in pFL38 by GeneCust. Of note, compared to the equivalent plasmids used in reference 17, the *ScMEP2* promoter used in these new plasmids is longer (660 bp compared to 400 bp) and allows a better complementation in growth tests. Plasmid extraction from bacterial cells was performed using the GeneJET plasmid miniprep kit (Thermo Fisher). All constructs were verified by sequencing.

AmtB purification and solid supported membrane electrophysiology. AmtB(His)₆ cloned into the pET22b(+) vector was overexpressed in C43 cells (4, 41), purified (42), and inserted into liposomes (11) as previously described. The size distribution of proteoliposomes had an average diameter of 110 nm (Fig. S1). The elution profile of all variants and the wild type were identical, showing a single monodisperse peak eluting between 10.4 and 10.6 mL (Fig. S2). Then, 3-mm gold-plated sensors (Nanion Technologies) were prepared (18) and SSME measurements done (12) as previously described. See Text S1 for details.

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study are the 31019b strain (*mep1Δ mep2Δ mep3Δ ura3*) (43), the #228 strain (*mep1Δ mep2Δ mep3Δ trk1Δ trk2Δ leu2 ura3*) (44), and the ZAM38 strain (*mep1Δ/mep1Δ mep2Δ/mep2Δ mep3Δ/mep3Δ ura3/ura3*) (29). Cells were grown at 29°C. Cell transformation was performed as described previously (45).

For growth tests on limiting ammonium concentrations, yeast cells were grown on minimal buffered (pH 6.1) medium (167) containing potassium salts (46). For growth tests on limiting potassium concentrations, a similar minimal buffered (pH 6.1) medium (173) where potassium salts were replaced by sodium salts was used, and KCl was added at the specified concentration; 3% glucose was used as the carbon source. Nitrogen sources were added as required by the experiment and as specified in the text. The nitrogen sources used were 0.1% potassium glutamate, 0.1% sodium glutamate, or (NH₄)₂SO₄ at the specified concentrations referring to the ammonium moiety.

Pseudohyphal growth tests were performed as previously described (47). A suspension of diploid cells was patched onto synthetic low ammonium dextrose (SLAD) and synthetic high ammonium dextrose (SHAD) [0.68% yeast nitrogen base without amino acids and without (NH₄)₂SO₄, containing 3% glucose and 1% bacteriological agar (Oxoid)], respectively, supplemented with 50 μM or 0.5 mM (NH₄)₂SO₄. Pseudohyphal and growth tests on limiting potassium concentrations were performed on a home-made medium (183) equivalent to yeast nitrogen base medium without amino acids, (NH₄)₂SO₄ and potassium salts, and containing low NaH₂PO₄ concentrations. (NH₄)₂SO₄ and KCl were added as required by the experiment and as specified in the text. For growth tests, diploid cells were streaked on SLAD, SHAD, and 183 medium to follow the formation of colonies.

All growth experiments were repeated at least twice.

Molecular dynamics simulations. The AmtB trimer (PDB code 1U7G) (3) was processed using the CHARMM-GUI web server (48). Any mutations inserted during the crystallization process were reverted to the wild-type form. The studied mutations were introduced into the protein using PyMOL. The N termini and C termini of the subunits were capped with acetyl and N-methyl amide moieties, respectively. The protein was then inserted into a membrane patch of xy-dimensions 13 by 13 nm. Unless otherwise specified, a membrane composition of palmitoyl oleoyl phosphatidyl ethanolamine and palmitoyl oleoyl phosphatidyl glycine (POPE/POPG) at a 3:1 ratio was used in order to approximate the composition of a bacterial cytoplasmic membrane. We employed the CHARMM36 forcefield for the protein and counter ions (49). The water molecules were modeled with the TIP3P model (50). Water bonds and distances were constrained by the Settle method (51), and bonds involving hydrogen by the LINCS method (52). In simulations without ammonium, K⁺ and Cl[−] ions were added to neutralize the system and obtain a bulk ionic concentration of 250 mM. In simulations with ammonium, K⁺ was replaced by NH₄⁺.

After a steepest-descent energy minimization, the system was equilibrated by six consecutive equilibration steps using position restraints on heavy atoms of 1,000 kJ/mol.nm². The first three equilibration steps were conducted in a constant number, volume, and temperature (NVT) ensemble, applying a Berendsen thermostat (53) to keep the temperature at 310 K. The subsequent steps were conducted under a constant number, pressure, and temperature (NPPT) ensemble, using a Berendsen barostat (53) to keep the pressure at 1 bar. Production molecular dynamics simulations were carried out using a v-

rescale thermostat (54) with a time constant of 0.2 ps and a Berendsen barostat with semi-isotropic coupling. A timestep of 2 fs was used throughout the simulations.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.03 MB.

FIG S1, DOCX file, 2.1 MB.

FIG S2, DOCX file, 1.1 MB.

FIG S3, DOCX file, 1 MB.

TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Pascale Van Vooren for support and discussion. Special thanks go to Iain Hunter (Strathclyde Institute of Pharmacy and Biomedical Sciences) for invaluable discussions and help during this project.

A.B., G.W., and G.D.M. are supported by Ph.D. studentships from the University of Strathclyde. A.J. is supported by a Chancellor's Fellowship from the University of Strathclyde and Tenovus Scotland (S17-07). G.T. and U.Z. are supported by the Scottish Universities' Physics Alliance (SUPA). P.A.H. is supported by the Natural Environment Research Council (NE/M001415/1).

A.S.B. is a research fellow of the Fund for Scientific Research (F.R.S.-FNRS), A.M.M. is a senior research associate of the F.R.S.-FNRS and a WELBIO investigator, M.B. is a scientific research worker supported by WELBIO. A.M.M. received support for this work from F.R.S.-FNRS (CDR J017617F, PDR T011515F, PDR 33658167), the Fédération Wallonie-Bruxelles (Action de Recherche Concertée), WELBIO, and the Brachet Funds.

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Review Article

Biological ammonium transporters from the Amt/Mep/Rh superfamily: mechanism, energetics, and technical limitations

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The exchange of ammonium across cellular membranes is a fundamental process in all domains of life and is facilitated by the ubiquitous Amt/Mep/Rh transporter superfamily. Remarkably, despite a high structural conservation in all domains of life, these proteins have gained various biological functions during evolution. It is tempting to hypothesise that the physiological functions gained by these proteins may be explained at least in part by differences in the energetics of their translocation mechanisms. Therefore, in this review, we will explore our current knowledge of energetics of the Amt/Mep/Rh family, discuss variations in observations between different organisms, and highlight some technical drawbacks which have hampered effects at mechanistic characterisation. Through the review, we aim to provide a comprehensive overview of current understanding of the mechanism of transport of this unique and extraordinary Amt/Mep/Rh superfamily of ammonium transporters.

Introduction

Ammonium is a vital source of nitrogen for bacteria, fungi and plants, and a toxic metabolic waste for animals. Hence, ammonium transport across biological membranes is a process of fundamental importance in all living organisms. In 1994, the first genes encoding ammonium transporters were identified in *Saccharomyces cerevisiae* (mep1 for methylammonium permease) [1]. Two other Mep proteins (Mep 2 and 3) were later identified [2]. A parallel study identified and cloned Amt1 from the small flowering plant *Arabidopsis thaliana* by the expression of cDNA library in *S. cerevisiae* triple Δmep mutants that could not grow on medium containing ammonium as sole nitrogen source [3]. Later, it was shown that the rhesus protein (Rh) is an Amt ortholog in vertebrates and that yeast *mep* mutants could be complemented functionally with the human Rh glycoprotein which can therefore act as an ammonium transporter [4,5]. Since then, members of the Amt/Mep/Rh protein family have been identified in almost all sequenced organisms; they constitute a unique and highly specific family of ammonium transporters [6,7].

EcAmtB structure

To date, the *Escherichia coli* AmtB is the paradigmatic, most intensely studied member of the Amt/Mep/Rh superfamily of transporters with more than 20 high resolution structures reported in the Protein Data Bank (PDB) [8]. Structurally, AmtB forms homotrimers. At the centre of each monomer, is a potential periplasmic NH_4^+ binding site, delineated by the residues S219, W148, F107 and D160, followed by a hydrophobic pore (Figure 1). The binding site is separated from the pore by the partly stacked phenyl rings of residues F107 and F215, termed the 'Phe-gate'. The high conservation of this 'dynamic gate' throughout the Amt/Mep/Rh family hints at an important mechanistic role, which is presently not

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Received: 13 October 2023
Revised: 12 December 2023
Accepted: 21 December 2023

Accepted Manuscript online:
22 December 2023
Version of Record published:
12 January 2023

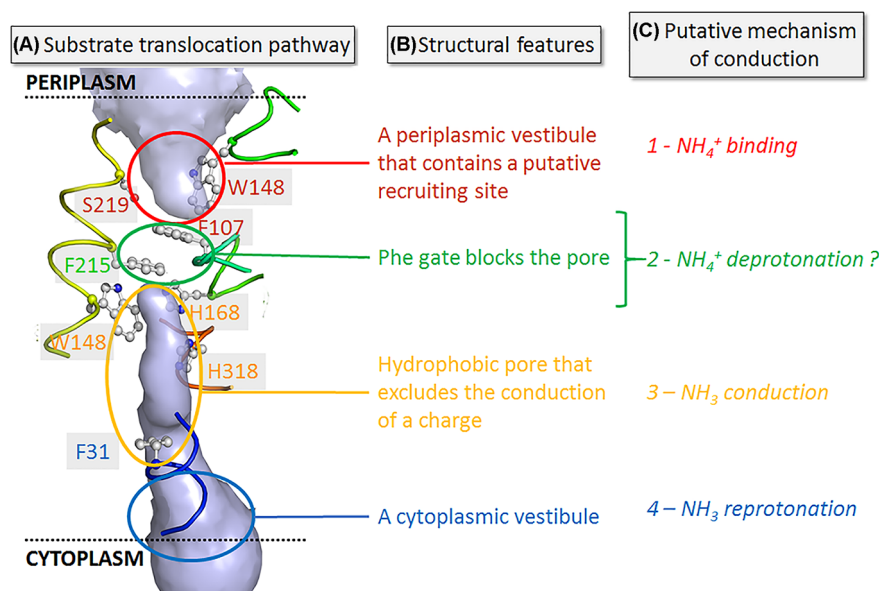


Figure 1. Substrate translocation path of AmtB

(A) The pore region of one monomer is shown with the water-accessible volume represented in space filling representation (light-blue) (adapted from [16]). (B) Four mechanistically distinct segments: (i) periplasmic ammonium binding site, (ii) phenylalanine gate, (iii) central pore with twin-His arrangement and (iv) cytoplasmic vestibule can be discriminated. Selected, highly conserved residues are shown in ball-and-stick representation for the ammonium binding site (red), phenylalanine gate (green), central pore (yellow) and cytoplasmic vestibule (blue) where parts of the two transmembrane helices TM5 (His168) and TM10 (His318) are also shown. (C) Location and mechanism of the four steps in the putative translocation mechanism deduce from the structure.

understood. The side chains of two highly conserved histidine residues (H168 and H318 in AmtB), protrude into the lumen forming the so-called 'twin-His' motif (Figure 1). The strong hydrophobic nature of the pore suggests a high energy barrier for the conduction of NH_4^+ . Therefore, the tentative consensus on the overall transport mechanism was that NH_4^+ binds to the transporter and gets deprotonated to NH_3 before being translocated into the hydrophobic lumen of the channel [9–12].

High resolution structures have been solved during the last two decade for various Amt/Rh proteins. In a recent review, we compared all the known structures of prokaryotic Amts [8]; hence, in the context of the present review, we will compare the structure of AmtB with the structure of eukaryotic Mep and Rh protein.

Comparison of ScMep2 and CaMep2 structure

In 2016, two separate crystal structures of fungal Mep proteins, Mep 2 from *S. cerevisiae* and Mep2 from *Candida albicans*, were reported simultaneously [13]. Compared with each other, these proteins were highly similar (root mean square deviation of 0.7 Å) and thus will be discussed together (Figure 2). The crystal structures revealed the general architecture of the fungal Mep proteins to be highly similar to their prokaryotic counterparts: conserving archetypal trimeric organisation and pseudo-two-fold similarity across the 11TM helices in the monomers. In addition, the key structural features of the translocation pathway (S1 binding site, Phe-gate, twin-His motif) are all conserved (Figure 2).

Comparison of CaMep2 and EcAmtB structure

Significant deviation between Mep2 and previous bacterial structures was noted at 3 regions and could signify potential functional divergence. The first of these divergent regions is the N-terminus, which is extended by 20–25 residues compared with bacterial Amt. This causes the N-terminus to contact the ECL5 (Extracellular Loop 5) of the neighbouring monomer, substantially widening the extracellular domain and creating a more distinct binding site (Figure 3). The authors proposed that these changes would increase the stability of the Mep2 trimer however, as mutants lacking the N-terminus tail grew as well as WT on minimal media, they could not verify a functional role for the N-terminus. The Mep2 structures also differ from other ammonium transporters at the cytoplasmic exit of the translocation channel. In Mep2, the cytoplasmic end of TM2 is unwound, creating an extended intracellular loop 1

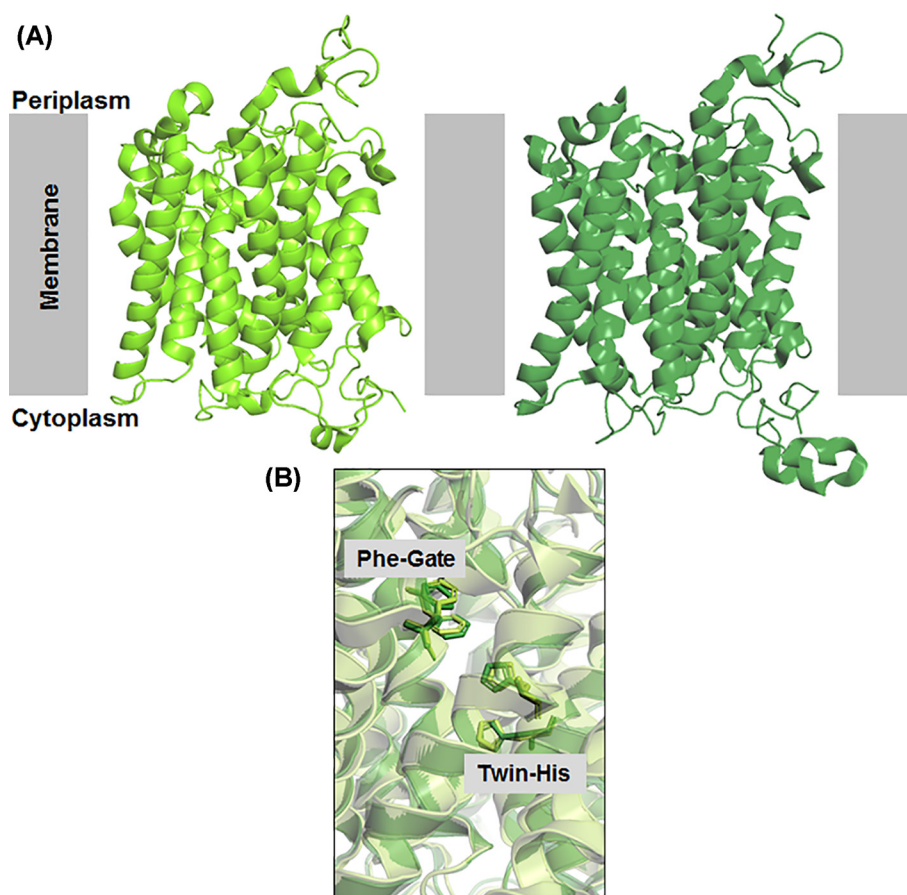


Figure 2. Comparison of fungal Mep2

(A) Cartoon models of X-ray crystal structures of Mep2 transceptors viewed from the side for *S. cerevisiae* Mep2 (light green) and *C. albicans* Mep2 (dark green). (B) Overlay of translocation pathway for ScMep2 (light green) and CaMep2 (dark green), showing the conservation of the Phe-gate and twin-His motif.

(ICL) that is shifted inwards compared to its bacterial counterparts (Figure 3). This alters the positioning of several residues, and ultimately results in a hydrogen bond interaction between Y49 (located at the C-terminus of TM1) and H342 of the twin-His motif which is not present in prokaryotic Amt. In addition, the ICL which links TM1-5 and TM6-10 is shifted by ~ 10 Å, blocking the channel on the cytoplasmic side.

Crystal structure of human RhCG

In 2010, the crystal structure of RhCG – found in epithelial cells of the renal collecting ducts – was resolved at 2.1 Å [14]. This demonstrated that human Rh also adhered to a trimeric organisation and confirmed conservation of this conformation across the Amt/Mep/Rh superfamily (Figure 4). RhCG retains the pseudo-two-fold symmetry between TMH1-5 and TMH6-10 seen in bacterial Amt and Rh proteins, but also features a 12th N-terminal TMH. This helix, termed TM0, lies at the subunit interface of the trimer and varies in length across the different Rh isoforms, suggesting functional differentiation. As with the bacterial NeRh50, no S1 binding site was observed in the structure of RhCG. However, the authors suggested that acidic residues within the extracellular vestibule (E166, D218, D278, and E329) could serve to recruit NH_4^+ . The phenylalanines of the Phe-gate are conserved, but in RhCG the outer phenylalanine (F130) does not obstruct the pore, resulting in an ‘open’ conformation with no barrier between the vestibule and the hydrophobic central pore (Figure 4). As with previously discussed members of the family, the twin-His motif is conserved and protrudes into the centre of this pore. Interestingly, Rh proteins share a common feature not seen in Amt or Mep proteins: a ‘shunt’ on the cytoplasmic face of the proteins (Figure 4). Whilst the function, if any, of the ‘shunt’ is unclear, the authors hypothesised that it may represent an alternative path for NH_4^+ entry, and NH_3 delivery into the hydrophobic portion of the pore.

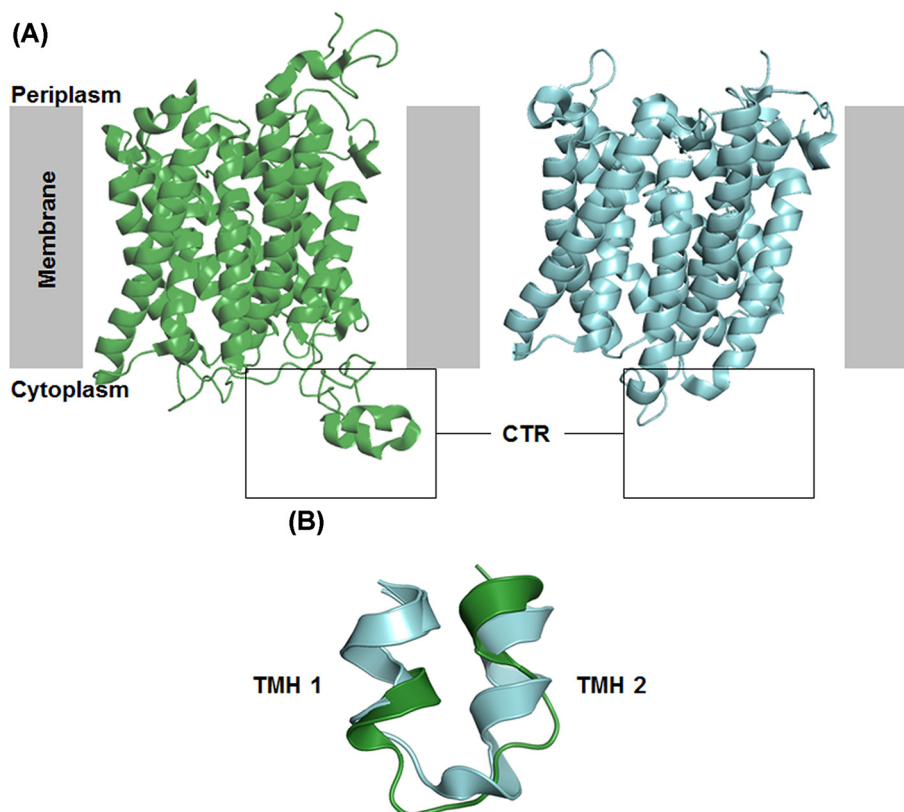


Figure 3. Comparison of *C. albicans* Mep2 and *E. coli* AmtB

(A) Cartoon models of X-ray crystal structures of Mep2 transceptors viewed from the side for *C. albicans* Mep2 (dark green) and *E. coli* AmtB (cyan). The extended CTR has been boxed for comparison. (B) ICL1 in EcAmtB (cyan) and CaMep2 (dark green), showing unwinding and inward position of the fungal protein.

Remarkably, the ubiquitous Amt/Mep/Rh family have strictly maintained their overall structure (Figure 5), despite individual members sharing low amino acid sequence identity.

Highly conserved structure but functional diversification

Despite sharing a common structure, the Amt/Mep/Rh proteins have become involved in a diverse range of physiological processes spanning all domains of life, with new reports describing their involvement in biological processes published regularly, including selective ammonium transport across biological membranes, the mitigation of ammonia toxicity (due to nitrogen metabolic waste or environmental NH_3), osmoregulation, maintenance of the physiological acid–base balance, ammonia detection in sensory structures (for organism development, finding a mate, a host, or food), and enhancing sperm survival and overall male fertility [8,15]. It is tempting to hypothesise that the different physiological functions gained by these proteins may be explained, at least in part, by differences in the energetics of their translocation mechanisms. Indeed, the energetics of ammonium transport through biological membrane can be very different. In solution, ammonium is in equilibrium between NH_3 and NH_4^+ with an acid dissociation constant of 9.28, which means that at a physiological pH, more than 99% of ammonium is present in its charged form. Because NH_3 is uncharged, it can diffuse freely through the membrane bilayer down its chemical gradient, independently of the membrane potential. Following diffusion and because it is a strong base, NH_3 triggers alkalinisation of the compartment it enters (Figure 6). In contrast, NH_4^+ is a weak acid, so the compartment is acidified as it accumulates (Figure 6). Additionally, NH_4^+ cannot easily diffuse through the membrane, due to its charge. As a result, the translocation of NH_4^+ through biological membranes is highly dependent on the membrane potential.

While structural and mechanistic understanding of the superfamily has expanded greatly over the last few decades [8], the energetic properties of Amt/Mep/Rh-mediated translocation remains poorly understood. Therefore, in this

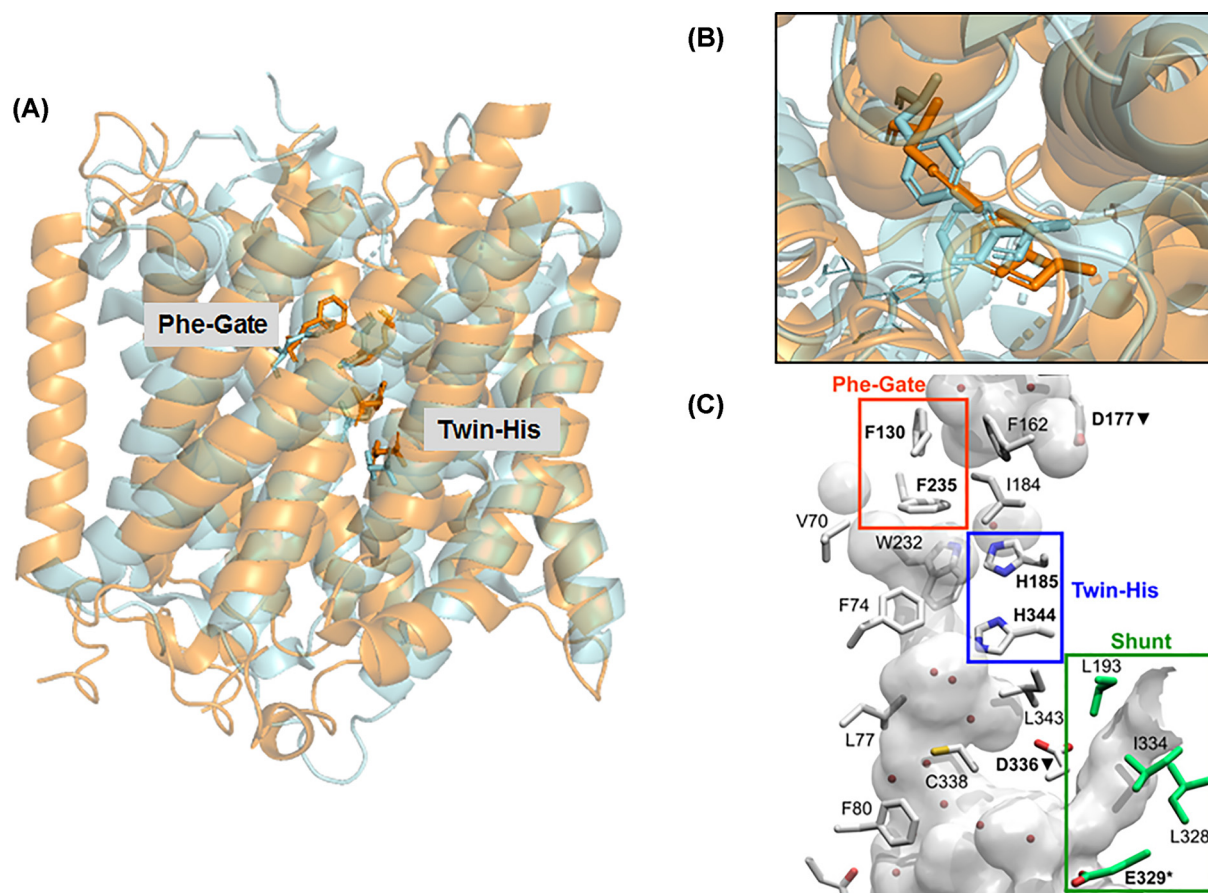


Figure 4. Comparison of AmtB and RhCG

Overlay of a single monomer of AmtB (cyan) or RhCG (orange) as inserted into the membrane (A) Residues of the Phe-gate, and twin-His motif are displayed in the same colour as their respective monomer. (B) Comparison of the Phe-gate as viewed from the periplasm. (C) Side-view of the RhCG channel and shunt, as presented in [14].

review, we will explore our current knowledge of energetics of the Amt/Mep/Rh family, discuss variations in observations between different organisms, and highlight some technical drawbacks which have hampered effects at mechanistic characterisation.

Is transport active or passive?

Before the identification of genes encoding bona fide ammonium transporters

The classical textbook definition of passive versus active transport is that passive transport down an electrochemical gradient occurs spontaneously, either by simple diffusion through the lipid bilayer or by facilitated diffusion through channels and passive carriers. In contrast, active transport occurs against the electrochemical gradient, and thus energy is required to empower transporter proteins to pump the solute against its electrochemical gradient. The first step to understand the energetics of Amt, Mep, and Rh proteins is to ascertain whether they act as passive or active translocator. In other words, do the transporters passively equilibrate the substrate alongside the existing chemical gradient, or do they have to actively overcome the electrochemical gradient? Classical approaches to discriminate between passive or energy-dependent transport mechanism focus on studying the kinetics of substrate accumulation in cells, cell-derived vesicles, patch-clamped oocytes, or proteoliposomes [16,17].

Early studies using these approaches, developed assays to measure ammonium accumulation in whole-cell samples of Gram-negative, Gram-positive bacteria, cyanobacteria and ectomycorrhizal fungi [17–20]. This is a complicated process for ammonium due to its high flux requirements for biosynthesis. However, the consensus view was that in these organisms they can accumulate ammonium inside the cell at concentrations 60–3000 times greater than

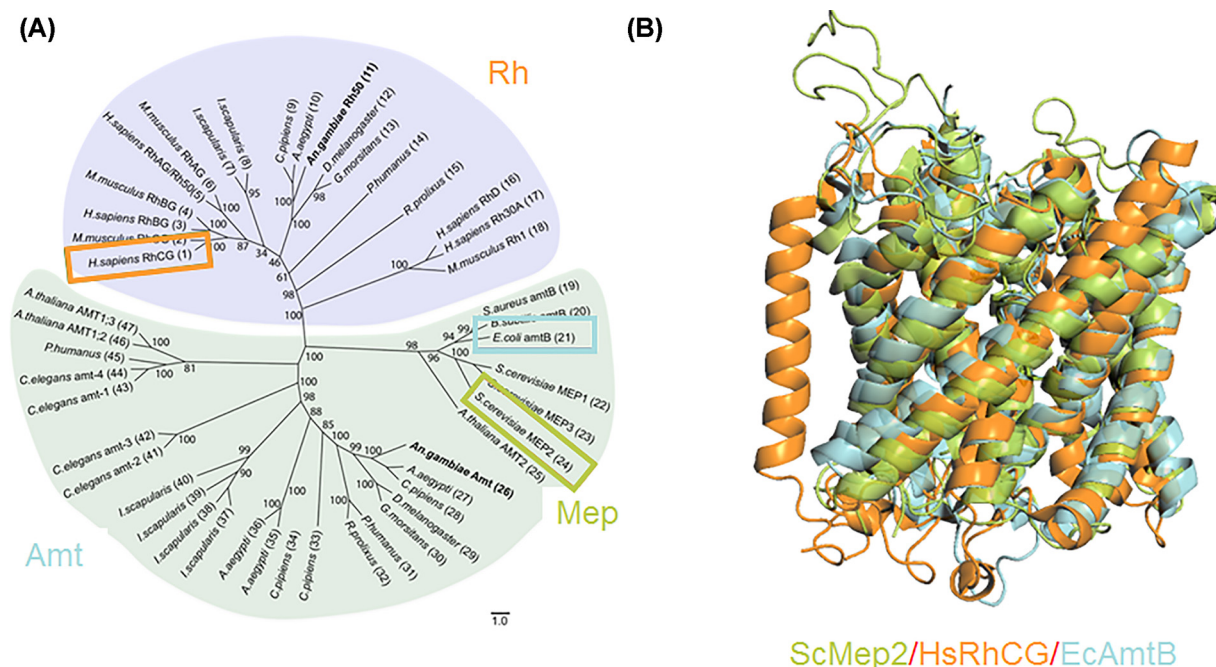


Figure 5. The Amt/Mep/Rh family is ubiquitous and highly structurally conserved

(A) Neighbour-joining tree comparing ammonium transporters. Rhesus and Amt separate into distinct clades, whilst Mep appears within the Amt grouping. One example each of *E. coli* Amt (blue), *S. cerevisiae* Mep2 (orange), and *H. sapiens* RhCG (green) have been highlighted. (adapted from [54]) Scale bars are 0.5% and 1% corrected distance. (B) Structural Conservation of Amt/Mep/Rh protein. A monomer from *E. coli* Amt (blue), *S. cerevisiae* Mep2 (orange), and *H. sapiens* RhCG (green) aligned as inserted in the membrane. To highlight the conservation of the translocation pathway, the proteins have been oriented with the N-terminus at the top and C-terminus at the bottom.

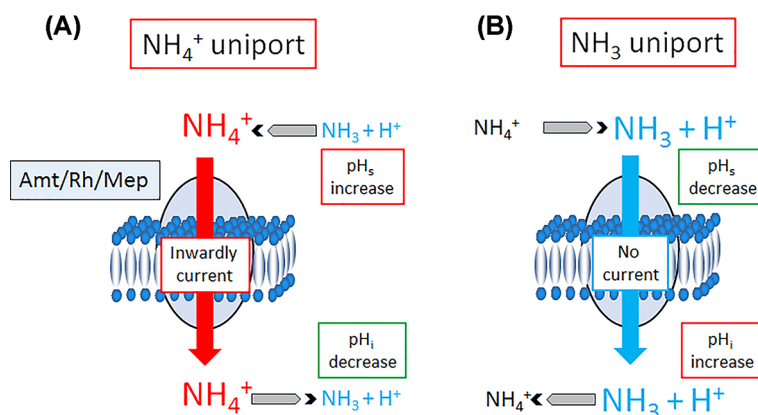


Figure 6. Influence on the current, surface pH (pH_s) and internal pH (pH_i) in oocyte as a function of the type of transport mechanism

(A) If an Amt/Rh/Mep protein act as an NH_4^+ uniporter, it is expected to be associated with an inwardly orientated current and increase of the pH at the external surface (pH_s) of the membrane and a decrease of the pH inside the compartment (pH_i). (B) If an Amt/Rh/Mep protein act as an NH_3 uniporter, it is expected that the translocation is electroneutral, and associated with a decrease of the pH at the external surface (pH_s) of the membrane and an increase of the pH inside the compartment (pH_i).

the extracellular concentration [17]. This clearly indicated an active transport process as such ratios could not be achieved by passive diffusion (see [17] for review). At the time of these studies, the genes encoding these ammonium transporters had not been identified, which limited more detailed characterisation. These first genes encoding *bona*

Table 1 Transport Mechanism and energetics in plant AMTs

Organism/Protein	Mechanism*	Possible energetic	Reference
Tomato/LeAMT1;1	1	pH gradient independent $\Delta\psi$ dependent	[55]
Tomato/LeAMT1;2	1	pH gradient independent $\Delta\psi$ dependent	[56]
Tomato/LeAMT1;1	1 or 2	$\Delta\psi$ dependent	[57]
Arabidopsis/AtAMT1;1	1	pH gradient independent $\Delta\psi$ dependent	[58]
Tomato/LeAMT1;2	1 or 2	pH gradient independent $\Delta\psi$ dependent	[59]
Arabidopsis/AtAMT1;2	1 or 2	$\Delta\psi$ dependent	[60]
Arabidopsis/AtAMT1;1	1	pH gradient independent $\Delta\psi$ dependent	[61]
Wheat/TaAMT1;1	2	Proton gradients stimulate. $\Delta\psi$ independent	[62]
Lotus/LjAMT2;2	4	Proton gradients stimulate. $\Delta\psi$ independent	[63]
Arabidopsis/AMT2	4	pH gradient independent $\Delta\psi$ independent	[64]
Bean PvAMT1;1	3	Proton gradients stimulate. $\Delta\psi$ dependent	[65]
Arabidopsis/AtAMT1;2	2	Not determined	[66]
Rice/OsAMT1;1	1	pH gradient independent $\Delta\psi$ dependent	[67]
Liverwort/MpAMT1;2	2	pH gradient dependent $\Delta\psi$ dependent	[68]
Arabidopsis/AtAMT1;1, 1;2, 1;3 and 2;1	2 or 4	Not determined	[69]

*Mechanism: 1- NH_4^+ uniport, 2- NH_4^+ deprotonation followed by NH_3/H^+ conduction, 3- NH_4^+/H^+ symport, 4- NH_4^+ deprotonation followed by the conduction of NH_3 only.

Table 2 Transport mechanism and energetics in NeRh50 proteins*

Mechanism*	Supporting evidence	Possible energetics	References
5&	- Electroneutral transport pH dependent - Resistance to MeA toxicity	pH gradient dependent $\Delta\psi$ independent	[72,75,76]
6	- Presence of CO_2 binding pocket in crystal structure	CO_2 concentration gradient	[77]
5	- MD simulation computing potentials of mean force and permeabilities for NH_3	NH_3 chemical gradient	[78]
2	- Electroneutral transport in liposomes - Independent of pH gradient	pH gradient independent $\Delta\psi$ independent	[12]

*Mechanism: 2 - NH_4^+ deprotonation following by NH_3/H^+ conduction, 5 - NH_3 uniport, 6 - CO_2 transport, &- the activity may be bidirectional activity.

fide ammonium transporters would be identified 10 years later in 1994 [1,3]. In the years following this discovery, the superfamily of Amt/Mep/Rh transporters have been identified in all domains of life [6,8].

After the identification of genes encoding bona fide ammonium transporters

This marked a paradigm shift in our understanding of the mechanism of ammonium transport. Instead of complying with the previous view that biological ammonium transporters actively translocate their substrate, a series of studies by Sydney Kustu speculated that instead Amt and Mep increased the rate of equilibration of uncharged ammonia across the cytoplasmic membrane using metabolic coupling with the glutamine synthetase in bacteria or acid trapping in the vacuole in yeast [21,22]. The authors also proposed that Amt and Mep may translocate substrate bidirectionally [23].

A passive transport mechanism was further substantiated when the first structure of the *Escherichia coli* ammonium transporter AmtB was published, revealing (i) a highly hydrophobic pore that would not accommodate NH_4^+

and (ii) the transporter structure was essentially unchanged when crystallised with or without the substrate, supporting the hypothesis of a channel with passive-like activity as opposed to active transport which is often associated with large conformational change during the transport cycle [9–11,16,24]. In addition, Khademi et al. [9], measured alkalisation inside AmtB-containing artificial liposomes after an ammonium pulse, suggesting passive diffusion of NH_3 (Figure 4) [9]. This was further supported in 2009 when Musa-Aziz et al. [25], expressed the *E. coli* ammonium transporter AmtB in oocytes and monitored the intracellular pH (pH_i) and extracellular surface pH (pH_s) following an ammonium pulse. They observed that a 0.5- or 5-mM ammonium pulse was associated with a sharp fall of pH_s . They concluded that this was due to the protons released near the membrane after NH_4^+ deprotonation followed by NH_3 diffusion through AmtB (Figure 6) [25]. This should be associated with a drastic alkalisation inside the oocyte due to the reprotonation of NH_3 releasing a hydronium (OH^-) ion (Figure 6), but paradoxically they measured a decrease of pH_i . However, numerous experimental observations contradict this model of AmtB acting as an NH_3 uniporter. First, in a systematic effort we failed to reproduce the experiments published by Khademi et al. [16]. Secondly, Westerhoff and coworkers, using a very sophisticated systems biology approach, calculated that passive transport of NH_3 would not be sufficient to sustain *E. coli* growth [26,27]. Thermodynamically, intracellular $[\text{NH}_4^+]$ would not be able to exceed extracellular $[\text{NH}_4^+]$ whilst maintaining an inward nitrogen flux. Therefore, if transport were passive, it would be expected that in an ammonium limited environment with low pH, *E. coli* would not be able to grow [26,27]. However, *E. coli* does grow and continues to use ammonium as a nitrogen source in these conditions. In fact, AmtB is exclusively expressed under nitrogen limited conditions, when passive conductance would be insufficient. So, their conclusion was that AmtB should actively transport NH_4^+ (either net NH_4^+ transport or NH_3/H^+ co-transport) [26,27]. This hypothesis was experimentally proven using Solid-Supported Membrane Electrophysiology (SSME) in an *in vitro* assay with the purified protein reconstituted into artificial liposomes with two AmtB from *Archaeoglobus fulgidus* and *E. coli* AmtB [12,28,29]. Using this SSME assay, it was clearly shown that the transport was electrogenic, equivalent to a net transport of a NH_4^+ molecule. The flux of NH_4^+ was estimated to be between 30 and 300 NH_4^+ ions per second per trimer [28], placing it in the flux range of secondary active transporters and far below a flux of 10^6 – 10^8 molecules of substrate transported per second expected for a passive channel. By combining this assay with a series of AmtB variants, the mechanism was further dissected, revealing a unique two-lane pathway for electrogenic NH_4^+ transport in the archetypal member of the family, the transporters AmtB from *E. coli* [12,30]. The pathway underpins a mechanism by which charged H^+ and neutral NH_3 are carried separately across the membrane after NH_4^+ deprotonation. Hence there is now a consensus that microbial AmtB are active transporter [8,12,31]. This raised the question of the energetics of the translocation mechanism.

What is the energy that drives the transport?

Although there is a growing consensus that most AMT/Mep/Rh proteins are active transporters, it is unclear how transport is powered. In this section, we will discuss the current hypotheses and associated evidence for the energy underpinning transport in different systems.

Ammonium transport energetics in bacteria and cyanobacteria

Three main hypotheses have been put forward: ATP as a primary metabolic source of energy, metabolic trapping, or the proton motive force (PMF).

The hypothesis that the energy is provided by ATP is the easiest to formally exclude. Firstly, ATP-driven carrier proteins importing substrate into bacterial cells depend on the activity of an associated periplasmic substrate binding protein. However, spheroplasts of *Clostridium pasteurianum*, *Anacystis nidulans* and *E. coli* [32,33] are still active in transporting the radioactive ammonium substrate analogue [^{14}C]-methylamine (MeA). Secondly, the structure of bacterial AmtB proteins show that they lack the nucleotide binding domain typical of ATP-driven transporters [9–11,24,34].

Metabolic trapping is an attractive hypothesis, but evidence in support of this mechanism is scarce. A metabolic coupling between the Glutamine synthetase (GS) and AmtB in *E. coli*, *S. typhimurium*, and the free-living cyanobacterium *Anabaena variabilis* have been proposed [21,35–37]. This system would allow cells to concentrate glutamine instead of ammonium, and thus mitigate the toxicity of the latter. However, the initial MeA uptake activities in *E. coli* wild-type cells and cells that do not express the GS is similar. Also, the initial uptake activity of MeA is not affected when the wild-type cells of *E. coli*, *A. variabilis* and the filamentous cyanobacteria *anabaena flos-aquae* are treated with the GS inhibitors L-methionine sulphone (MSF) or L-methionine-DL-sulphoximine (MSX) [35,36,38]. Hence, AmtB transport activity does not only depend on metabolic trapping in bacteria and cyanobacteria.

Table 3 Transport mechanism and energetics in RhAG proteins*

Mechanism*	Supporting evidence	Possible energetics	References
7&	Human RhAG - Yeast resistance to MeA toxicity, optimum an acidic pH - Ammonium excretion essay	pH gradient dependent	[5,82]
7	Human RhAG - Electroneutral transport in oocyte - Stimulated by outwardly pH gradient.	pH gradient dependent $\Delta\psi$ independent	[83]
5	Human RhAG - Rapid alkalinisation of RBC ghost cell after NH_4^+ pulse - low Arrhenius activation energy	NH_3 chemical gradient	[84]
1	Mouse RhAG - Rapid acidification of oocyte pH - Depolarization of the cell	$\Delta\psi$ dependent	[85]
8	Human RhAG - Acidification in HeLa cell after ammonium pulse - Alkalinisation after an MeA pulse	Not none	[74]
8	Mouse RhAG - Acidification in oocyte after ammonium pulse - Alkalinisation after an MeA pulse	$\Delta\psi$ dependent pH sensitive	[85]
5	Human RhAG - Oocyte pHs fall after ammonium pulse on oocyte	Potentially $\Delta\psi$ independent or NH_4^+ electrochemical gradient	[25,86]
8	Mouse RhAG - Transport both $\text{NH}_4^+/\text{NH}_3$ and MeA^+/MeA but; - Electrogenic transport is favour for ammonium - Electroneutral transport is favour for MeA	$\Delta\psi$ dependent NH_3 chemical gradient pH dependent	[87]
6	- Intracellular pH changes - Surface pH changes - Ammonia- CO_2 competition experiments - Translational knockdowns of Rh proteins	CO_2 concentration gradient	[86,88,89]

*Mechanism: 1 - NH_4^+ uniport, 3 - NH_4^+/H^+ symport, 5 - NH_3 uniport, 6 - CO_2 transport, 7 - NH_4^+/H^+ antiport, 8 - Transport of both NH_3 and NH_4^+ , &- the activity may be bidirectional activity.

Table 4 Influence on the current, surface pH (pH_s) and internal pH (pH_i) in cells as a function of the type of transport mechanism*

Mechanism	Current	pH_s	pH_i
NH_4^+ uniport	yes	Increase	Decrease
NH_3 uniport	No current	Decrease	Increase
Transport both	yes	Increase first then recovers if NH_4^+ flux is higher than NH_3 Decrease first then recovers if NH_3 flux in higher than NH_4^+	Decrease first then recovers if NH_4^+ flux is higher than NH_3 Increase first then recovers if NH_3 flux in higher than NH_4^+

* pH_s : pH at the external surface of the membrane. pH_i : pH inside the compartment

An ion electrochemical gradient combines the membrane potential ($\Delta\psi$) and the ion concentration gradient. These components can either work additively, to increase the driving force on the ion across the membrane or can conflict and lower the driving force. In the case of the proton motive force (PMF), the two components: the inwardly orientated proton gradient, and $\Delta\psi$ with the inside negative with respect to the outside may favour an ammonium/proton symport mechanism. An early series of experiments point toward the PMF driving transport. Kleiner and co-workers showed that in *C. pasteurianum* and *Klebsiella pneumoniae*, inhibition of the ATPase, which maintains the PMF, also inhibits the MeA transport [32,39]. The use of protonophore Carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone (FCCP) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) on intact cells of *E. coli* or *Corynebacterium glutamicum* also collapses the PMF as well as MeA uptake [40].

The PMF has two components: the proton gradient, and the membrane potential ($\Delta\psi$). $\Delta\psi$ is the dominant PMF component [41] and numerous lines of evidence point toward $\Delta\psi$ being the specific driving force for ammonium translocation. Using our SSME *in vitro* assay [12], we were able to demonstrate that in the absence of ammonium, a proton pulse in liposomes containing purified AmtB did not trigger a discernible current. Additionally, in the presence of ammonium, an inward-orientated pH gradient did not increase AmtB activity. This indicated that the proton gradient is not required for the activity of *E. coli* AmtB [12]. These data further show that AmtB cannot act as an uncoupler, which raises the question of proton selectivity and the coupling between NH_3 and H^+ transfer. However, the driving force created by the 200 mM ammonium concentration gradient used in these experiments is much bigger than the imposed ΔpH (out pH = 5.0, inside pH = 8.0), therefore interpretation of these data should be cautious [12].

The generation of an artificially high membrane potential by addition of valinomycin to K^+ -loaded cells of *C. pasteurianum* increase the MeA uptake [32]. Addition of the protonophores CCCP or Methyltriphenylphosphonium (TPMP) selectively decreases both $\Delta\psi$ and MeA transport in the cyanobacteria *Anabaena cylindrica* [42]. Collapsing specifically $\Delta\psi$ using valinomycin and a K^+ gradient inhibit MeA transport in *Azotobacter vinelandii* [43]. MeA uptake studies in *C. glutamicum* cells demonstrated a high steady-state accumulation in the cytoplasm in the absence of any significant pH gradient [40,44]. Based on these data, the authors deduced that the membrane potential represents the driving force for ammonium transport in bacteria [40,44]. Similar conclusions were reached for AmtB from *E. coli*, when Fong *et al.* [45], observed accumulation of MeA ion (MeA^+) in response to the electrical potential across the membrane [45]. In *A. fulgidus*, the thermodynamics of the different possible transport mechanisms of Amt-1 using free energy calculations were studied [46]. They concluded that the import of ammonium is likely predominantly driven by the $\Delta\psi$ since the transfer free energy of ammonium is much more sensitive to this component of the PMF than to the pH gradient. Maeda and co-workers [27] conducted a tour de force of engineering and modelling of a complex network for the ammonium transport and assimilation network in *E. coli*, involving AmtB, the regulatory proteins GlnK and GlnB, and the central N-assimilating enzymes and incorporated the highly complex interactions in to the model. Their simulations clearly shows that the $\Delta\psi$ in whole cells is required for ammonium uptake [27].

In the filamentous cyanobacterium *Anabaena flos-aquae*, MeA transport is completely abolished in the presence of 2 μM of the protonophore FCCP [38]. This suggested that ammonium transport depends mainly on the membrane proton gradient. However, FCCP is known to have some pleiotropic effects on cellular metabolism, including complete dissipation of the $\Delta\psi$ or depletion of intracellular ATP pool, therefore any interpretation of these data should be cautious [47].

Taken together, all these studies seem to indicate that at least for prokaryotic Amts, the membrane potential $\Delta\psi$ is essential for the transport activity.

Energetics in Eukaryotes

Ammonium transport energetics in yeast

The Baker's Yeast, *S. cerevisiae*, possesses acidic vacuoles, where the pH is maintained between 5.5 and 6.2, (approximately 0.8 to 1.5 pH units lower than the cytosol) by two key players: the V-ATPase and V-type H^+ -ATPase [48]. The MeA transport activity of Δvph1 cells, which cannot assemble the V-ATPase, or Δvma1 cells which do not encode for a catalytic subunit of the ATPase is completely inhibited [22]. This originally led to the conclusion that energetic ammonium transport by the Mep proteins depends on trapping in the acidic vacuole. However, later studies seem to indicate that this process is more complicated and have provided a body of evidence indicating that the Mep2-type ammonium transporter had a different mechanism of transport compared to Mep1 and Mep3-type. This first hint of this was the discovery that the optimum pH activity for Mep1 is pH 6 whereas it is pH 4 for Mep2 [49]. More importantly, functional characterisation in oocytes of the frog *Xenopus* revealed that Mep2 mediates electroneutral substrate translocation while Mep1 conducts electrogenic transport [49]. A pHluorin reporter assay showed that the *in vivo* transport activity of Mep2 and Mep1 affected yeast cytosolic pH differently. After an ammonium pulse, the cells expressing only Mep1 showed a transient drop of the cytosolic pH but this was less marked in cells expressing only Mep2 [49]. It seems therefore clear that the mechanism of Mep2-type transporter differs from the Mep1/3-type, suggesting that the energetics of the transport may also be different. This would also explain why Mep2 acts as a signal transducer in the pseudohyphal growth signalling cascade, but Mep1/3 do not [31,50]. Later studies have reinforced this view. The MeA transport activity of Mep2 and Mep1 have been measured in the presence or absence of 50 μM of the protonophore CCCP. In cells expressing Mep2 only, MeA uptake was reduced to near background levels while transport was only marginally impacted in the cells expressing only Mep1 [49]. This suggests that the energetics of Mep1 and Mep2 are indeed different. However, it has been shown that protonophores (including FCCP and CCCP)

have pleiotropic effects in yeast. Indeed, these protonophores do not only collapse the proton electrochemical gradient in mitochondria and across the plasma membrane, but also trigger a cytosolic acidification [51]. Hence, the popular use of these ‘protonophores’ to equilibrate H^+ concentration across membranes appears to be an unreliable procedure that may lead to false and confusing interpretation. This makes it difficult to conclude with confidence that Mep2 depends on the proton gradient and Mep1 does not.

Ammonium transport energetics in ectomycorrhizal fungi

The ectomycorrhizal fungi *Paxillus involutus* and *Hebeloma cylindrosporum* encode for three AmtS transporters [18,20,52]. In *P. involutus*, the ammonium and MeA transport activity have an optimum pH between 4 and 5.5 [18]. The uptake systems of ectomycorrhizal fungi therefore seem to be adapted to the acidic pH of forest soil. To determine the energetic nature of ammonium translocation in these species, MeA transport activity has been measured in the presence of various metabolic inhibitors [18]. The protonophores CCCP and 2,4-dinitrophenol (DNP) strongly inhibit MeA uptake and NaN_3 , which reduces the internal pH by 1 or 2 units, results in moderate inhibition. These results indicate that MeA transport in *P. involutus* depends on the electrochemical proton gradient. The GS inhibitor MSX had no effect on the uptake of MeA, which suggests that metabolic trapping by the GS is not necessary for the transport activity. Addition of diethyl pyrocarbonate (DEPC), a histidine-specific alkylating reagent that modifies the histidine residues within proteins strongly inhibited MeA uptake, suggesting that a histidine residue would be instrumental in the translocation cycle. This was validated by later work which showed that the highly conserved ‘twin-His motif’, present at the centre Amt/Mep/Rh50 pore, is crucial for uptake activity [12,24,31].

Rotenone, SHAM and KCN, respectively, inhibitors of Complex I, alternative pathway and Complex IV of the mitochondrial respiratory chain, did not affect MeA uptake, nor did the mitochondrial ATPase inhibitor, arsenate, and glycolysis inhibitor, NaF, which indicates that the transport system was not directly under metabolic control. Gramicidin, a Na^+/K^+ ionophore, did not affect the transport of MeA, indicating that these cations did not take part in the uptake activity [18].

Take together these results indicate the PMF plays a role in ammonium transport in fungi, like in bacteria, but it is unclear if the $\Delta\psi$ or proton gradient is the dominant factor.

Ammonium transport energetics in insects

Insects detect their hosts and/or food by using ammonium olfactory detection to sense ammonium present in animal/human breath and sweat or decaying organic matter [53]. As part of this sensory apparatus, numerous insects express both Amt and Rh proteins in the same cell [Follstaedt, 2003 #199; Durant, 2019 #6257; Durant, 2020 #6258; Pitts, 2014 #6229]. The malaria vector mosquito, *Anopheles gambiae* encode for two ammonium transporters belonging to the Amt and Rh subfamily, AgAmt and AgRh50 (AgRh50 encodes two spliced transcripts, AgRh50a and AgRh50b) [54]. The three proteins were expressed in oocytes and current were monitored in response to ammonium or MeA pulse ranging from 0.5 to 0.2 mM. Although all three proteins were able to restore growth to a transport deficient *S. cerevisiae* strain, only AgAmt produced a membrane potential dependant current in response to an ammonium pulse [54]. These results indicates that AgAmt acts as an electrogenic NH_4^+ transporter, while the two AgRh are electroneutral symporter or antiporter.

Ammonium transport energetics in plants

In plants, four separate mechanisms, each with distinct energetic characteristics, have been proposed for ammonium transport (Table 1).

1. NH_4^+ uniport,
2. NH_4^+ deprotonation followed by NH_3/H^+ conduction,
3. NH_4^+/H^+ symport,
4. NH_4^+ deprotonation followed by the conduction of NH_3 only.

The first characterisation of the transport cycle for a plant AMT was carried out on the tomato transporters LeAMT1;1 and LeAMT1;2 [56,70]. These tomato transporters were expressed in *Xenopus* oocytes and characterised using electrophysiological assays. For both proteins, ammonium pulses elicited currents that depended on the membrane potential but not the proton gradient. The K_m of both transporters varied with the applied voltage: as the voltage become more negative, the K_m measured for NH_4^+ decreased. From this, the authors concluded that LeAMT1;1 and LeAMT1;2 specifically bind NH_4^+ and mediate its potential-driven translocation (Table 1). This view was supported

by later work which used a 3D model based on the structure of the *E. coli* AmtB combined with mutagenesis studies and revealed a functional ammonium binding site in the external vestibule of LeAMT1;1 [57].

In 2006, characterisation of another plant Amt, AtAMT1;1 from *Arabidopsis thaliana*, was reported [58]. The authors demonstrated that AtAMT1;1 can restore growth in Δmep *S. cerevisiae* (which are incapable of sustaining growth with ammonium as a sole nitrogen source) and that AtAMT1;1 activity in yeast is not pH-dependant. Additionally, they recorded an ammonium dependent current in oocytes expressing AtAMT1;1, indicating that the transport is electrogenic. By combining these data, the authors concluded that AtAMT1;1, like LeAMT1;1, acted as a membrane-potential-dependent NH_4^+ uniporter [58].

However, it is important to point out that for both AtAMT1;1 and LeAMT1;1, the data presented does not differentiate between mechanism 1 (NH_4^+ uniport) and mechanism 2 (NH_3/H^+ translocation following NH_4^+ deprotonation). In fact, in a later study based on electrophysiological analysis of tomato LeAMT1;1 and LeAMT1;2 expressed in oocyte, the authors recognized that they can't differentiate between the mechanism 1 and 2 (Table 1) [57,71]. In 2018, Ariz *et al.* [69], developed a detailed approach to explore this question. By using the natural chemical-physical properties of the N-isotopic signature linked to $\text{NH}_4^+/\text{NH}_3$ interconversion, Ariz *et al.* [69] showed that *S. cerevisiae* cells expressing AtAMT1;2, 1:2, 1:3 and AtAmt2:1 were depleted in ^{15}N relative to ^{14}N when compared with the external ammonium source [69]. They show that this isotope fractionation can only be explained by the deprotonation of NH_4^+ before the translocation of NH_3 . However, the authors were unable to assess whether after deprotonation, the proton was translocated to the cytoplasm (mechanism 2) or recycled back to the periplasm (mechanism 4). By combining the deprotonation [69] with the electrogenicity measured in oocytes expressing plants Amt (Table 1 and references herein) it seems likely that AtAMT1;1, 1:2, 1:3, and LeAMT1;1, utilise mechanism 2. In contrast, the electroneutrality associated with the deprotonation favours the mechanism 4 for Lotus and Arabidopsis AMT2 activities (Table 1 and references herein).

Remarkably, the puzzle of plant AMTs mechanism of transport was not over. In 2014, a studies on Bean PvAMT1;1 propose that the transporter act as a NH_4^+/H^+ symporter (Mechanism 3, Table 1 [65]). The authors heterologously expressed the transporter in oocytes and three main lines of evidence indicates the symporter activity in oocyte expressing PvAMT1;1, firstly, submillimolar ammonium and pH pulse triggered an inward currents, secondly, the amplitude of the current was proportional to the membrane voltage apply, finally, the ammonium pulse was associated the acidification of the oocyte cytoplasm.

Taken together, these data suggest that, despite the variety of mechanisms reported, the importance of the proton gradient and/or membrane potential is a common feature of plant AMTs.

Energetics of ammonium transport in Rh proteins

As the most diverged member of the family, Rh have been proposed to have distinct functions compare to Amt/Mep proteins. However, yeast complementation assays [5,72] and functional studies [73,74] proved that Rh proteins facilitate ammonium transport. The hydrophobic nature of the bacterial NeRh50 and human RhCG pore revealed by X-ray crystallographic, suggested transport of ammonia (NH_3) [14,75] but electrogenic transport of NH_4^+ has been repeatedly noted (Tables 2, 3, 5 and 6).

In keeping with the classically enigmatic nature of Rh proteins no less than eight different mechanisms, each with different energetic characteristics have been hypothesised. In this section, each of the proposed mechanisms of ammonium transport via bacterial and animal Rh50 proteins will be reviewed.

1. NH_4^+ uniport
2. NH_4^+ deprotonation following by NH_3/H^+ conduction
3. NH_4^+/H^+ symport
4. NH_4^+ deprotonation following by NH_3 transport only
5. NH_3 uniport
6. CO_2 transport
7. NH_4^+/H^+ antiport
8. Transport of both NH_3 and NH_4^+

Table 5 Transport mechanism and energetics in RhBG proteins*

Mechanism*	Supporting evidence	Possible energetics	References
7	Human RhBG - Ammonium pulse is associated with current in oocyte, not MeA pulse - ^{14}C -MeA uptake increases by outwardly pH gradient. - NH_4^+ transport induce pH_i alkalisation.	$\Delta\psi$ dependent	[94]
1	Mouse RhBG - ammonium pulse associated with current and induce a pH_i acidification	$\Delta\psi$ dependent	[95]
1 or 8	Mouse RhBG - ammonium pulse associated with current and decrease pH_i - MeA pulse associated with current and increase pH_i	$\Delta\psi$ dependent NH_3 chemical gradient	[96]
8	Mouse RhBG - electrogenic transport is pH dependent - electroneutral transport is pH-independent	$\Delta\psi$ dependent NH_3 chemical gradient pH dependent	[97]
5	Human RhBG - Oocyte pH_s fall after ammonium pulse on oocyte	Potentially $\Delta\psi$ independent or NH_4^+ electrochemical gradient	[86]
8	Mouse RhBG - Transport both $\text{NH}_4^+/\text{NH}_3$ and MeA^+/MeA but; - Electrogenic transport is favour for ammonium - Electroneutral transport is favour for MeA	$\Delta\psi$ dependent NH_3 chemical gradient pH dependent	[87]
5&	Human RhBG - ammonium pulse associated with alkalisation of cell expressing RhBG - pH_i acidification when cell loaded with ammonium were pulse with ammonium-free buffer	NH_3 chemical gradient	[73]
7	Mouse RhBG - Electroneutral transport of MeA in oocyte - Stimulated by outwardly pH gradient.	pH gradient dependent $\Delta\psi$ independent	[98]

*Mechanism: 1 - NH_4^+ uniport, 5 - NH_3 uniport, 7 - NH_4^+/H^+ antiport, 8 - Transport of both NH_3 and NH_4^+ , &- the activity may be bidirectional activity.

Ammonium transport in *Nitrosomonas europaea* Rh50

The chemolithoautotroph *Nitrosomonas europaea* is an Ammonia Oxidizing Bacteria (AOB) and it gains all its energy from the oxidation of ammonia to nitrate. A general assumption was that ammonia (NH_3) rather than ammonium (NH_4^+) is the substrate for ammonia oxidation in *Nitrosomonas* [79]. The *N. europaea* genome encodes a single gene (*rh50*) belonging to the Amt/Mep/Rh family of ammonium transporters [6]. When expressed in *S. cerevisiae*, it mediates pH-dependent and bidirectional MeA transport, indicating that either neutral NH_3 is transported alone, or NH_4^+/H^+ are exchanged [72,76]. Weidinger et al. [72] also demonstrated that the MeA transport activity of *N. europaea* positively correlated with transcriptional level of *rh50* [72]. Another study compared MeA influx in *N. europaea* wild-type and *rh50* KO strains and observed decreased uptake rates in mutant cells [76]. Interestingly, it appears that AOB have developed a survival strategy at the molecular level by transcriptional regulation: under starvation conditions (deprivation of NH_3 and CO_2), nearly 70% of *N. europaea* genes are down-regulated. The expression level of ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are maintained, while genes related to oxidative stress and transcription of Rh50 are up-regulated [80]. This suggests that Rh50 might be involved in the survival strategy of *N. europaea*, where it can sustain prolonged starvation periods, while preparing for the ammonia uptake and oxidation when it becomes available [81]. The presence of a highly hydrophobic pore identify in the crystal structure of NeRh50, prevents charge translocation, suggesting further that NeRh50 might translocate NH_3 and/or CO_2 [75,77]. However, a subsequent molecular dynamics study, simulating spontaneous full permeation events of ammonia and carbon dioxide across Rh50 revealed that the protein increases NH_3 , but not CO_2 permeability in its crystallographic conformation, without the requirement for a major conformational change, indicating it functions as a channel rather than a transporter [78]. It was concluded that NeRh50 was highly representative of

Table 6 Transport mechanism and energetics in RhCG proteins*

Mechanism*	Supporting evidence	Possible energetics	References
7&	Human RhCG (formerly RhGK) - Yeast resistance to MeA toxicity, - Ammonium excretion assay	pH gradient dependent	[100]
8	Human RhCG Electrogenic transport Current depend on [NH ₃]	$\Delta\psi$ dependent	[101]
5&	Human RhCG - ammonium pulse associated with alkalinisation of cell/liposome/erythrocyte vesicles expressing RhBC - pH _i acidification when cell loaded with ammonium were pulse with ammonium-free buffer	NH ₃ chemical gradient	[73,102]
7	Mouse RhCG - Electroneutral transport of MeA in oocyte - Stimulated by outwardly pH gradient.	pH gradient dependent $\Delta\psi$ independent	[98]
5	Human RhCG - Electroneutral transport of ammonium and MeA in oocyte - Increase flux when increase external pH - MeA transport assay in yeast	NH ₃ chemical gradient	[71]
5	Human RhCG - Alkalinisation inside liposomes containing purified RhCG after an ammonium pulse	NH ₃ chemical gradient	[14]
5	Human RhCG - Oocyte pH _s fall after ammonium pulse on oocyte	Potentially $\Delta\psi$ NH ₄ ⁺ electrochemical gradient	[86]
5	Mouse RhCG - pHs decrease upon ammonium pulse, but not with MeA	NH ₃ chemical gradient	[87]
4	Human RhCG - Molecular and quantum mechanical calculations	Potentially $\Delta\psi$ independent or NH ₄ ⁺ electrochemical gradient	[103]

*Mechanism: 3 - NH₄⁺/H⁺ symport, 4 - NH₄⁺ deprotonation following by NH₃ transport only, 5 - NH₃ uniport, 7 - NH₄⁺/H⁺ antiport, 8 - Transport of both NH₃ and NH₄⁺, &- the activity may be bidirectional activity.

all Rh proteins; therefore, it was widely assumed that human Rh proteins would also act as NH₃ channels. In 2020 however, it was shown by *in vitro* SSME assay that NeRh50 supports electrogenic activity [12].

Ammonium transport in RhAG proteins

In 2000, the Rhesus-associated glycoprotein (RhAG) found in erythrocytes and often used for blood typing, was identified as a distant relative of existing Amt/Mep ammonium transporters through sequence homology [4]. Soon after this RhBG and RhCG glycoproteins found in almost all other tissues were shown to be involved in ammonia transport and metabolism [90,91], but the understanding of their true substrate or mechanism was lacking.

In 2000, Marini et al. [5], observed that expression of human RhAG in *Δmep* yeast conferred resistance against MeA toxicity, which provided the first evidence that RhAG mediates bidirectional transport of MeA [5]. This observation was consistent with a study showing that RhAG might be involved in ammonium efflux [92]. Two years later, Westhoff and colleagues expressed RhAG in *Xenopus* oocytes and MeA uptake was found to be independent of the membrane potential but dependent on an outwardly orientated gradient of protons, suggesting electroneutral NH₄⁺/H⁺ exchange [83]. Two years later, Westhoff et al. [82], extended and confirmed their conclusion by showing that the excretion of MeA through RhAG expressed in yeast was pH-dependent, being optimal at acidic pHs, which according to the NH₄⁺/NH₃ pK_a of 9.25, suggest that RhAG functions is a bidirectional NH₄⁺/H⁺ antiporter [82]. Ever since, many studies have tried to elucidate the mechanism of RhAG-mediated substrate translocation. Pierre Ripoche tackled the question with a different approach and measured the pH inside human red blood cell ghost from genetic variants with defects of RhAG expression using stopped flow spectrofluorometry [84]. They measured alkalinisation inside the ghost cells in response to ammonium pulses, indicating the fast entry of NH₃ (Figure 6).

The Arrhenius activation energy for the translocation process is low, therefore they concluded that RhAG act as a NH_3 channel [84]. Therefore, the work of Westhoff [93] and Ripoche et al [84] offers two opposing lines of evidence relating to the substrates for RhAG and no clear way to determine which was correct. However, a year later it was proposed that RhAG was able to transport both NH_3 and NH_4^+ [74]. In this study, the authors pulsed HeLa cell with ammonium and monitor the intracellular pH (pH_i). They observe the classical biphasic response for a cell membrane permeable to both NH_3 and NH_4^+ . In phase one, there is an initial pH_i alkalization due to rapid passive entry of NH_3 , which steals a proton from an intracellular water molecule, forming NH_4^+ and releasing a hydroxide (OH^-) ion, increasing pH_i . This is followed by phase two - wherein NH_4^+ slowly enters the cells and dissociates to form NH_3 and a proton H^+ , which slowly acidifies the cell and mediates recovery of pH_i (Figure 6 and Table 4). They repeated the same experiment in HeLa cells expressing RhAG and observed that the amplitude of the pH_i variation during the two phases were greater when compared the HeLa cell not expressing RhAG. Using a mathematical model to adjust the permeabilities to fit the pH_i profiles, they found that RhAG expression resulted in a three-fold and a two-fold increase of the NH_4^+ and NH_3 permeability respectively and concluded that RhAG was able to transport both NH_4^+ and NH_3 [74].

In 2009 and 2013, Musa-Aziz et al. [25,86] expressed human RhAG in *Xenopus* oocytes and measured that an ammonium pulse was associated with an acidification of the surface pH (pH_s). They interpret this as due to the protons released outside near the membrane after NH_4^+ deprotonation following NH_3 diffusion through RhAG (Figure 6 and Table 4) [25,86]. Later, Caner et al. [87] completed this work and measured pH_i , pH_s , and whole cell currents following an ammonium pulse using an ion-selective microelectrodes and two-electrode voltage clamp in oocyte expressing RhAG [87]. The objective was to elucidate if RhAG acts as a NH_4^+ (or MeA^+) electrogenic uniport, NH_3 (or MeA) electroneutral uniport, or translocate both NH_4^+ and NH_3 (and MeA^+/MeA). When they pulsed the oocyte expressing RhAG with ammonium, they observed a current, an increase in pH_s , (contrary to the observations of Musa-Aziz and colleagues who observed a pH_s decrease [25,86]) and an increase in pH_i , indicating that RhAG acts as an NH_4^+ uniporter. However, both pH_s and pH_i slowly recover, which shows that RhAG transports both NH_4^+ and NH_3 but the flux is higher for NH_4^+ than NH_3 (Figure 6 and Table 4). Interestingly, when they used MeA, a current was measured but the pH responses were inverted: first the pH_s decreases and then the pH_i increases before both begin to recover. Hence it seems that Rh protein expressed in *Xenopus* oocytes handle ammonium and MeA differently; the electrogenic flux of the charged species dominate the electroneutral diffusion of the uncharged species for ammonium and inversely for MeA [87].

Ammonium transport in RhBG protein

In a first study aimed at elucidating the mechanism of human RhBG translocation, Uwe Ludewig [94] recorded the current induced by ammonium and MeA pulse in *Xenopus* oocytes expressing RhBG using a two-electrode voltage-clamp. In addition, MeA uptake and intracellular pH_i measurements were performed. The results showed that MeA translocation in *Xenopus* oocytes expressing RhBG is electroneutral and enhanced by increasing an outwardly orientated H^+ gradient. Moreover, an ammonium pulse did not elicit a current but did result in internal alkalisation. These results lead to the conclusion that human RhBG acts as an NH_4^+/H^+ antiporter [94].

Studies on *Rhbg* knockout (KO) mice demonstrated lowered urinary ammonium excretion, while HCl-induced acidosis increased RhBG protein expression in healthy mice [99]. The transport mechanism of mouse RhBG and RhCG have been studied by electrophysiology in *Xenopus* oocytes. It was shown that oocytes expressing RhBG can transport MeA and that this activity is independent of the membrane potential. Moreover, the transport was dependent on an outward-directed pH gradient, suggesting an NH_4^+/H^+ antiporter activity, in agreement with Uwe Ludewig's data on human RhBG [94,98]. Interestingly, while adapting Uwe Ludewig's technique on mouse RhBG, Nakhoul et al. [95] reported that an ammonium pulse on oocytes expressing RhBG triggers a $\Delta\psi$ dependent current and a significant pH_i decrease. These data suggest, contrary to the conclusion drawn before by Uwe Ludewig, that RhBG acts as a strict NH_4^+ transporter [95].

Nakhoul and colleagues clarified this apparent discrepancy in a series of three papers published between 2010 and 2015 [87,96,97]. Firstly, in 2010 Nakhoul et al. used ion-selective microelectrodes and voltage-clamp experiments to measure currents and intracellular pH changes in whole cells after an ammonium or MeA pulse [96]. After an ammonium pulse, they measured a current and acidification inside the oocytes, consistent with their 2005 paper, which seemed to confirm that RhBG acts as an NH_4^+ uniporter (Figure 6) [95,96]. However, while a MeA pulse induced a current as ammonium did, it also induces alkalisation of inside the oocyte instead of acidification. Therefore, they concluded that as for RhAG (see above), RhBG handles ammonium and MeA differently: NH_4^+ is transported

electrogenically but MeA can either be transported electrogenically (as charged MeA^+) or in an electroneutral manner (via diffusion of neutral MeA) [96]. The authors then expanded their experimental design to include surface pH measurements in addition to current and pH_i and probed the effect of pH on the activity of RhBG-expressing oocytes. The pH_i of the oocytes was acidified by 0.2–0.3 units using butyrate, while the extracellular pH (pH_o) was adjusted to either 6.5, 7.5, or 8.2 prior to pulsing with substrate. Their data show that the electrogenic transport (of either NH_4^+ or MeA^+) is pH sensitive: stimulated by alkaline pH_o but inhibited by acidic pH_i or pH_o , however the electroneutral translocation of MeA is not affected by pH [97]. In a third paper Nakhoul *et al.*, expanded their experimental design further: this time including an electrode to measure pH at the surface of RhBG-expressing oocytes (in addition to the previous measurements) [87]. These data demonstrate that RhBG can facilitate electroneutral transport of NH_3 and thus the demarcation between electrogenic and electroneutral transport of MeA^+/MeA in RhBG is also extended to ammonium. As with RhAG, RhBG handled the two substrates differently: the electrogenic flux of charged NH_4^+ dominated over the electroneutral diffusion of neutral NH_3 while the inverse was true for MeA (Figure 6 and Table 4) [87]. From this, they deduced that the electroneutrality previously observed by Ludewig *et al.* [94], in RhBG-expressing *Xenopus* oocytes was due to the fact that they used MeA and not ammonium, hence the electrogenic transport of MeA^+ was masked by the diffusion of MeA [94]. Surprisingly in 2013, Geyer and colleagues [86] observed an acidification of oocyte pH_s expressing RhBG, concluding to an NH_3 uniport mechanism (Figure 6) [86].

A completely different approach was developed by Pierre Ripoché in Paris [73]. In this study, they measured the pH_i of HEK-293 (human embryonic kidney) and MDCK (Madin–Darby canine kidney) cells expressing RhBG or RhCG following an ammonium or MeA pulse. For both Rh proteins, an ammonium or MeA pulse triggers a pH_i alkalisation; moreover, when the cells were loaded with ammonium and pulse with an ammonium-free buffer, they measured a pH_i acidification. This suggests clearly that RhBG and RhCG act as a bidirectional NH_3 channel [73].

3-5-4-RhCG Protein

In a triple *mep*-deficient yeast strain, human RhCG was shown to function as a bidirectional NH_4^+ transporter [5]. Bakouh *et al.* [97] subsequently expressed RhCG in *Xenopus* oocytes and used voltage-clamp experiment at various external pHs to control both NH_4^+ and NH_3 concentrations independently of each other. They measured an inwardly orientated current following an ammonium pulse, revealing electrogenic transport. This current was drastically increased when the external NH_4^+ concentration was kept constant while the external concentration of NH_3 was increased 3-fold by increasing external pH by 0.5 unit. They concluded that RhCG induced NH_4^+ transport depending on $[\text{NH}_3]$ but could not identify the underlying mechanism [101]. In a later study, it was shown that RhCG expressed in *Xenopus* oocytes transport MeA independently of the membrane potential. Moreover, the transport was also dependent on an outward-directed pH gradient, suggesting an NH_4^+/H^+ antiporter activity [98].

In 2006, Mayer and colleagues [71] expressed human RhCG in *Xenopus* oocytes and in yeast and combined electrophysiology, with MeA uptake assays, and pH_i measurements to characterise the protein activity. Their results show that RhCG transports both ammonium and MeA and that uptake was strongly favoured as extracellular pH increased. This clearly indicated that RhCG mediates electroneutral NH_3 transport [71] (Table 6). This conclusion was further supported in 2013 when Geyer and colleagues [86] observed an acidification of *Xenopus* oocyte pH_s expressing RhCG following an ammonium pulse, also indicating an NH_3 uniport-type mechanism (Figure 6) [86]. This was confirmed two years after, in *Xenopus* oocytes expressing RhCG, where Caner *et al.* [87] could not measure an inward current following a pulse of either ammonium or MeA, but they were able to measure a significant acidification of pH_s with ammonium but not with MeA [87] (Table 6). These data seem to indicate that RhCG transports NH_3 , but not MeA. Surprisingly however, NH_3 transport by RhCG is not accompanied by alkalisation in *Xenopus* oocytes (Figure 6). This is likely due to heterologous expression in oocytes results in ammonium being handled differently compared to other cells (see paragraph below ‘Technical limitations studying ammonium transport’). Nevertheless, the authors concluded that RhCG acts as an NH_3 uniporter [87].

Work using stopped-flow spectrophotometry to measure pH_i changes in HEK-293 cells expressing RhCG, erythrocyte vesicles and liposomes containing purified RhCG was found to show that ammonium and MeA pulses are associated with a pH_i decrease, indicating that RhCG translocates NH_3 [73,102]. Measurements conducted at different temperatures revealed that the translocation mechanism exhibits a low Arrhenius activation energy, suggesting a channel-like rather than transporter-like mechanism for RhCG (Table 6) [73,102].

In 2010, the high-resolution structure of human RhCG was published alongside characterisation of the protein reconstituted into functional liposomes [14]. The authors measured an alkalisation in the lumen following an ammonium pulse, and thus concluded that RhCG is an NH_3 uniporter (Table 6) [14]. Notably, they used the same

experimental design used six years prior to conclude that EcAmtB was also an NH_3 uniporter [9]. However, a later systematic effort failed to reproduce these data [16]. Following the publication of the high-resolution structure of RhCG [14], a team lead by Simon Bernèche used molecular and quantum mechanical calculations to characterise the mechanism of RhCG translocation [103]. They showed that RhCG recruits NH_4^+ which is subsequently deprotonated via the 'twin-His' motif in the pore and the proton is transferred back to the extracellular vestibule through a hydrogen bond network, resulting in the net transport of NH_3 [103].

Rh proteins and CO_2 transport

Analysis of evolutionary conservation and diversification suggested that Rh may have adapted to transport neutral CO_2 , in place of or in parallel to NH_3 , as a means of maintaining an appropriate pH homeostasis [6]. The first experimental report claiming Rh involvement in CO_2 transport came from studying expression of the RH1 gene in the green alga *Chlamydomonas reinhardtii* [104]. The authors showed that expression of RH1 increased in cells grown in air supplemented with 3% CO_2 and concluded that Rh1 and, by extension, Rh proteins are the long-sought gas channels for CO_2 [104]. A subsequent study provided further evidence for this claim by showing that inhibiting expression of Rh reduces the response to extracellular CO_2 in *C. reinhardtii* [105]. In another study, which used direct ammonia- CO_2 competition experiments affected both ammonia and CO_2 excretion in the Zebrafish, *Danio rerio*, suggesting that Rh proteins may serve as channels for both CO_2 and NH_3 [89]. Identification of a potential CO_2 binding pocket in the NeRh50 crystal structure, further suggested that Rhesus proteins might act as gas channels for NH_3 and/or CO_2 [77]. These findings prompted the investigation of human Rhesus proteins and their involvement in CO_2 transport [88,106]. Analysis of red blood cell ghost membrane vesicles deficient in RhAG expression and knockout mice displaying well-characterized protein defects, showed that even a small drop in the production of this protein significantly reduces CO_2 exchange [88,106]. It was found that, RhAG accounts for ~50% of CO_2 transport [88]. This was also supported in another study showing that RhAG represents a channel for extra-renal transport of CO_2 molecules based on changes in surface pH in *Xenopus* oocytes expressing RhAG and aquaporin AQP1 [107]. However, RhAG demonstrated four-fold greater preference for passage of NH_3 versus CO_2 leaving the results rather open ended.

At the same time, experimental characterisation NeRh50 *in vivo* provided no evidence of a CO_2 -dependent growth effect in a KO mutant [76]. Another argument against Rh involvement in CO_2 transport was presented in a later report monitoring transmembrane CO_2 flux by imposing a CO_2 concentration gradient across planar lipid bilayers and detecting the resulting small pH shift in the immediate membrane vicinity. The study concluded that Rh protein facilitated transport of CO_2 through biological membranes is highly improbable [108]. In addition, no putative CO_2 binding pockets were detected in the crystal structure of human RhCG, which was proposed to be representative for all Rh homologues [14]. Finally, a recent study showed that during long equilibration molecular dynamics simulations the CO_2 molecules do not show any tendency to diffuse across the periplasmic vestibule of either bacterial or human Rh50 protein [109].

Technical limitations studying ammonium transport

A recurring theme through this review is that despite using the same, or similar techniques, various authors (ourselves included) have produced conflicting evidence regarding the mechanism of Amt/Mep/Rh transport. As such, the various methodologies used over the past four decades to explore the questions of specificity and mechanism of conduction of Amt/Mep/Rh protein require a reasoned critical appraisal. In this section, we want to look beyond the heated debates concerning the Amt/Mep/Rh transport mechanism and instead highlight some potential technical drawbacks. We believe that understanding the limitations of methodology, combined with the application of current and emerging approaches will fuel an expansion in our mechanistic understanding of this extraordinary superfamily of transporters.

Choice of experimental system

Xenopus laevis oocytes

Amt/Mep/Rh proteins are present in a diverse range of organisms, and thus are placed in a variety of biological and physiological contexts. As a result, it is likely that Amt/Mep/Rh from different organisms fulfil different functions and thus differ mechanistically. Despite this, the majority of the studies are carried out *in vivo* using intact cells (typically *E. coli*, *S. cerevisiae*, *X. laevis* oocytes, or human cells) or cell-derived vesicles (red blood cell ghost, *E. coli* spheroplasts or vesicles). The parameters used as proxies for the detection and measurement of ammonium conduction (pH change, electric current, uptake of labelled analogue) are likely to be affected by other physiological

phenomena and are thus difficult to control and interpret with confidence. The best example is the use of *X. laevis* oocytes to study biological ammonium transporters. As mentioned in the introduction, oocytes possess endogenous ammonium-activated channels and thus handle ammonium in an unusual manner compared with the native hosts of the tested transporters [110]. Detailed study of ammonium handling in *Xenopus* oocytes has revealed three key ways in which they differ from other cells [25]. With the acid/basic equilibrium $\text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+$ with a pKa of 9.25 in mind (see also Figure 6), first, after an ammonium pulse, the pH inside the oocyte falls, while in other cells passive diffusion of NH_3 through the membrane is associated with intracellular alkalinisation (Figure 6). Second, abrupt removal of external ammonium immediately after an ammonium pulse leads to alkalinisation inside *Xenopus* oocytes. Normally, however, this should lead to acidification, as NH_3 would be expected to diffuse out, displacing the $\text{NH}_4^+/\text{NH}_3$ equilibrium towards the right, and yielding a proton [25]. Third, removal of external ammonium following a pulse would be expected to trigger a sharp increase of the pH at the surface of the oocyte due to the acid/basic $\text{NH}_4^+/\text{NH}_3$ equilibrium being completely displaced to the left. However, the authors did not measure any significant pH_s variation [25]. In an attempt to explain this erratic behaviour of oocytes towards ammonium, the authors developed between five and seven different models which are too extensive to reproduce here. *Xenopus* oocytes seems to be particularly adept at avoiding ammonium-induced increases in pH_i, most likely as an adaptation to protect the egg from toxic levels of ammonium arising from the decay of organic matter in pond water [25]. The poorly elucidated behaviour of ammonium handling in oocytes, combined with variations in the use of ammonium and/or methylammonium, specific parameter measured, and detection method may explain how the same basic approach has given rise to such various conclusions on the translocation mechanism of Rh proteins.

Heterologous expression in prokaryotes

Heterologous expression of Amt/Mep/Rh proteins in bacterial systems comes with its own limitations. The ¹³N radionuclide has a short half-life (~10 min), so radiolabelled NH_4^+ is not viable for biological studies. As a result, [¹⁴C]-MeA uptake assays have long been used to assess Amt activity *in vivo* [111]. Bacterial glutamine synthetase (GS) is able to convert MeA into methylglutamine and thus introduce it into the larger cellular metabolism [112]. Hence in [¹⁴C]-MeA uptake assays, free intracellular MeA is removed upon washing the cell and consequently the radioactivity measured is only from metabolized products of MeA. As a result, kinetic parameters determined in this way do not relate to MeA translocation but rather are representative of its assimilation [113]. Hence, any kinetic data obtained from MeA uptake assays *in vivo* must be analysed and interpreted very carefully before drawing conclusions on the translocation mechanism [114]. Jayakumar and Barnes [113], followed by Javelle et al. [36], developed an assay that does not require a washing step. The benefit of this assay is that it allows determination of both free and metabolized intracellular MeA and (using a GS mutant) allows uncoupling of MeA uptake from subsequent assimilation [113,114]. However, the absence of a washing step also makes the assay extremely sensitive to pipetting error, hence can vary from experimenter to experimenter.

Artificial proteoliposomes

An *in vitro* assay set up with purified protein would not suffer from such problems and would also make it easier to vary experimental parameters like substrate concentration, pH, and presence of potential inhibitors. In particular, it allows assessment of the activity of variant proteins designed to test mechanistic hypotheses under controlled conditions. Such an assay has been developed by purifying and reconstituting Amt protein from *A. fulgidus* and *E. coli*, along with Rh50 from *N. europaea* and human RhCG into artificial liposomes [12,28,29,102]. The problem here is that it is impossible to control the exact quantity and the orientation of the protein inside the liposomes. In these studies it was demonstrated that AfAmt was inserted 100% inside out (IO), while for EcAmtB and NeRh50 it was a mixture of IO and right side out (RSO). This complicates interpretation as measurements include activity resulting from both influx and efflux at the same time. Also, these assays use enormous ammonium concentration (5–200 mM), which creates a significant driving force that might force mechanistic steps that would not occur at physiologically concentrations or mask steps that do occur physiologically. Another problem with these system is the lipid composition of the proteoliposomes. Electrophysiology measurements coupled with MD simulations revealed that phosphatidylglycerol is an essential cofactor for AmtB activity and, in its absence, AmtB cannot complete the full translocation cycle [115]. Another study further investigated the lipid selectivity of AmtB from *E. coli* in heterogeneous nanodiscs. Gas-phase ejection and solution-phase detergent ‘flash’ extraction analysis of AmtB revealed that the protein has a few tight binding sites for phosphatidylcholine, is selective for binding phosphatidylglycerol overall, and is nonselective for phosphatidylethanolamine [116]. Thus far, lipid-dependency has only been demonstrated for AmtB, but given the conserved structure of the family it is likely to be a universal feature for the family. Hence it is important to identify the optimum lipid composition for the specific ammonium transporter under investigation.

X-ray crystallography and MD simulation

They were big expectations that high resolution structures would help to understand the mechanistic of these transporters. High resolution structures have been solved during the last decade for various Amt/Mep/Rh proteins (for review see [8] and reference herein). Unfortunately, the anticipated elucidation of the transport mechanisms from these 3D structures has not been met, as all the structures are very similar when generated in the presence or absence of ammonium and show the same inward-facing state of the protein. There are no significant differences in the crystal structures of Amt/Mep and Rh proteins that can clearly account for different mechanism of translocation. Consequently, all structural model based on authentic 3D structures will also all look similar and are largely uninformative. The incredible 'structural rigidity' has also complicated the application of MD simulations aimed at interrogating hypotheses on the mechanism of transport. Resulting in an aspect that has largely ignored – the dynamics associated with the translocation mechanism in these proteins. It is, therefore, essential to develop an alternative approach to protein crystallography to observe different conformations and obtain more structural information. In this context, developing a combined approach using the stabilisation of the protein in a native lipid environment combined with Cryo-electron microscopy and neutron/X-ray scattering may offer a way forward [115].

Methylammonium is an imperfect substrate analogue

The use of ^{14}C -MeA transport essay, as discussed above, has become an almost universal tool for assessing Amt activity *in vivo* in bacteria, yeast, *Xenopus* oocytes and fungi [1,8,18,94]. However, numerous observations raise clear concerns over the validity of MeA as a substrate analogue in any system (either *in vivo* or *in vitro*) to solve mechanistic questions in ammonium transporters. In some variant proteins, the activity measured with MeA as a substrate does not mirror the activity measured using ammonium [12,31,114,117,118]. This is particularly apparent in *Xenopus* oocytes, where it has become clear that Rh proteins handle ammonium and MeA differently [87,94–96]. It is possible that the degree of discrimination between ammonium and MeA differs between individual proteins: limiting potential comparisons between different transporters. Indeed, Amt/Mep/Rh proteins selectively transport a very small molecule (NH_4^+), adding a methyl group to that is a significant increase in size. This will affect how the speed and efficiency of pore entry and translocation. Additionally, the pK_a of methylammonium is ~ 10.6 , such that the equilibrium of the substrate and the analogue when interacting with the protein are not the same, which can lead to problems during the translocation cycle.

Activity measurements are incomplete

Different studies have measured different phenomena as a proxy for ammonium transport activity. The two most common approaches are to monitor for a change in pH (either inside the vesicle/oocyte or its surface) or measuring an electric current, but both introduce complications. Measuring only current can hide the diffusion of the neutral species as it has been the case for RhBG expressed in *Xenopus* oocytes [87,94] and is also problematic with electrophysiology on liposomes [28,29]. Conversely, measuring only pH_i variation may favour the detection of electroneutral over electrogenic components of the translocation mechanism (Table 4 and Figure 6). Another problem with pH_i measurements lies in the detection method: often a fluorescent dye or pH-sensitive electrodes. A disadvantage of conventional electrodes is that the impalement depth is hard to quantify and pH-sensitive electrodes may measure bulk cytosolic pH rather than the submembraneous pH [94]. This may, in part, explain why a study monitoring intracellular pH of *Xenopus* oocytes expressing RhBG via fluorescent dye reported that an ammonium pulse induced an alkalization of intracellular pH [94], while all studies using microelectrodes to monitor pH_i changes Rh-expressing *Xenopus* oocytes detected acidification following an ammonium pulse [87,95–97,101].

Concluding remarks

Through the development and integration of a wide range of *in vivo* and *in vitro* techniques, our understanding of the extraordinary superfamily of Amt/Mep/Rh has been transformed over the past 30 years. Despite this, the energetics of transport and structural dynamics of the protein during the translocation cycle, remain largely elusive and controversial. This is due, in part, to historical underappreciation of the nuanced physiological differences between individual members of the family. In the pursuit of achieving a 'complete picture' understanding of the mechanism of Amt/Mep/Rh, we failed to appreciate that our current understanding is a collage of evidence. In addition, limitations with existing methods may allow subtle features of the proteins to go unnoticed or actively mask them from detection. Thus, it is time to explore new and rapidly developing biophysical and biochemical techniques to solve the fundamental mechanistic questions about Amt/Mep/Rh family. In this context, a standardised approach based on the capture of pure protein from the native lipid environment (e.g., using SMALP) coupled with a finely controlled and adaptable

in vitro measurement system (such as SSME) could pave the way to the next breakthrough in our understanding of this remarkable superfamily of transporters.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

A.B., G.W. and T.H.: PhD studentships from the University of Strathclyde. A.J.: Chancellor's Fellowship from the University of Strathclyde and Tenovus Scotland (S17-07). P.A.H. would like to acknowledge funding from BBSRC [grant numbers BB/T001038/1 and BB/T004126/1] and the Royal Academy of Engineering Research Chair Scheme for long-term personal research support [grant number RCSR2021 \ 11 \ 15].

Open Access

Open access for this article was enabled by the participation of University of Strathclyde in an all-inclusive *Read & Publish* agreement with Portland Press and the Biochemical Society under a transformative agreement with JISC.

CRediT Author Contribution

Gordon Williamson: Writing—original draft, Writing—review & editing. **Adriana Bizior:** Writing—original draft, Writing—review & editing. **Thomas Harris:** Writing—review & editing. **Leighton Pritchard:** Writing—review & editing. **Paul A. Hoskisson:** Funding acquisition, Writing—review & editing. **Arnaud Javelle:** Conceptualization, Supervision, Funding acquisition, Writing—original draft, Writing—review & editing.

Acknowledgements

Special thanks to Prof. Iain Hunter (Strathclyde Institute of Pharmacy and Biomedical Sciences), Dr Mélanie Boeckstaens (Free University of Brussels, Gosselies, Belgium) and Prof Anna-Maria Marini (Free University of Brussels, Gosselies, Belgium) for invaluable discussions and help during this project.

Abbreviations

AMO, ammonia monooxygenase; AOB, Ammonia Oxidizing Bacteria; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DEPC, diethyl pyrocarbonate; DNP, 2,4-dinitrophenol; FCCP, p-(tri-fluoromethoxy)phenyl-hydrazone; HAO, hydroxylamine oxidoreductase; ICL, intracellular loop; MSF, L-methionine sulphone; MSX, L-methionine-DL-sulphoximine; PMF, proton motive force.

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Prokaryotic ammonium transporters: what has three decades of research revealed?

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Abstract

The exchange of ammonium across cellular membranes is a fundamental process in all domains of life. In plants, bacteria and fungi, ammonium represents a vital source of nitrogen, which is scavenged from the external environment. In contrast, in animal cells ammonium is a cytotoxic metabolic waste product and must be excreted to prevent cell death. Transport of ammonium is facilitated by the ubiquitous Amt/Mep/Rh transporter superfamily. In addition to their function as transporters, Amt/Mep/Rh proteins play roles in a diverse array of biological processes and human physiopathology. Despite this clear physiological importance and medical relevance, the molecular mechanism of Amt/Mep/Rh proteins has remained elusive. Crystal structures of bacterial Amt/Rh proteins suggest electroneutral transport, whilst functional evidence supports an electrogenic mechanism. Here, focusing on bacterial members of the family, we summarize the structure of Amt/Rh proteins and what three decades of research tells us concerning the general mechanisms of ammonium translocation, in particular the possibility that the transport mechanism might differ in various members of the Amt/Mep/Rh superfamily.

INTRODUCTION

The transport of ammonium (NH_4^+) across cell membranes is a fundamental process in all domains of life. After N_2 , ammonium is the most prevalent nitrogenous compound on earth, and for most prokaryotes, fungi and plants, it is a preferred nitrogen source [1–3]. For ammonia-oxidizing bacteria (AOB), ammonium provides energy and reducing power [4]. In contrast, for mammals, ammonium is the end product of nitrogen metabolism [2], and a key metabolite in the control of systemic acid–base balance [5, 6]. In humans, elevated concentrations of ammonium can lead to health conditions, including neurological disorders, and growth retardation in neonates and children [7]. Thus, efficient ammonium excretion is essential in detoxification of erythrocytes, kidney and liver cells [8].

For years, the prevailing view was that neutral NH_3 species passively diffused across the membrane down its concentration gradient [9]. However, for many plants and bacteria, passive diffusion rates would be insufficient to meet cellular nitrogen requirements [10]. The pK_a of the $\text{NH}_4^+/\text{NH}_3$ equilibrium is 9.25, thus at physiological pH, 99% of ammonium is protonated. In addition, lipid membranes were found to be impermeable to ions [11, 12], suggesting the presence of specific ammonia/ammonium ($\text{NH}_3/\text{NH}_4^+$) transport systems.

The first evidence of the existence of ammonium translocation systems was reported while investigating amino acid transport during nitrogen starvation response in the filamentous fungi *Penicillium chrysogenum* [13]. The authors found that the activity of a non-specific amino acid permease was inhibited by ammonium, suggesting the presence of a dedicated ammonium uptake system. This was further confirmed by measuring the uptake of the ammonium analogue: ^{14}C -labelled methylammonium (MeA). It was shown that MeA uptake occurred against the concentration gradient and was inhibited by ammonium, confirming the presence of a specific membrane transport system for ammonium [14].

Received 17 November 2021; Accepted 24 June 2023; Published 14 July 2023

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Keywords: bacterial ammonium transport; transport mechanism; transporter proteins.

Abbreviations: Amt, ammonium transporter; AUC, analytical ultracentrifugation; CF, 5-carboxy fluorescein; DDM, dodecyl-beta-maltopyranoside; dRTA, distal renal tubular acidosis; EP, electrophysiology; GE, growth experiment; HK, histidine kinase; kDa, kilo Dalton; LacZ, beta-galactosidase; MD, molecular dynamic; MeA, methylammonium; Mep, methylammonium permease; OHSt, overhydrated hereditary stomatocytosis; PhoA, alkaline phosphatase; RBC, red blood cell; Rh, rhesus protein; SANS, small angle neutron scattering; SAXS, small angle X-ray scattering; SEC, size exclusion chromatography; SSME, solid supported membrane electrophysiology; TA, ^{14}C -methylammonium transport assay; TMH, transmembrane helices; WT, wild-type; WW, water wire.

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A major advance in the field came with the molecular identification of the first bona fide genes encoding ammonium transporters, named *Mep1* in the yeast *Saccharomyces cerevisiae* [15] and *Amt1* in the plant *Arabidopsis thaliana* [16]. Two additional Mep transporters, Mep2 and Mep3, were identified and subsequently characterized in *S. cerevisiae* [17]. The first prokaryotic Amt gene was sequenced and characterized in *Corynebacterium glutamicum* [18]. Soon after, numerous Amt/Mep family members were identified and characterized in a wide range of micro-organisms, including the bacterium *Escherichia coli*, the Archaeon *Archaeoglobus fulgidus*, the yeast *Candida albicans* and the ectomycorrhizal fungi *Paxillus involutus* and *Hebeloma cylindrosporum* [19–24].

In 1997, based on a sequence similarity of 20–25% between the human red blood cell rhesus protein (RhAG) and existing Amt/Mep family members, the Rh proteins were identified as potential mammalian ammonium transporters [25, 26]. Soon afterwards, RhAG and RhGK (now called RhCG), a homologue expressed in human kidney cells, were shown to facilitate ammonium uptake in a *S. cerevisiae* triple-*mepΔ* strain, deprived of its three endogenous Mep ammonium transporters. This provided the first functional evidence that Rh proteins are able to translocate ammonium [27]. In parallel, the ammonium transport activity of RhBG, a third member of the Rh50 glycoprotein group, was characterized in human and mouse nonerythroid tissues [28, 29]. Rh homologues were later identified in many lower organisms [30, 31], including the green alga *Chlamydomonas reinhardtii*, the worm *Caenorhabditis elegans* and the nitrifying bacterium *Nitrosomonas europaea* [32–34].

The physiological relevance of Amt/Mep/Rh proteins extends beyond their role in ammonium acquisition as a nitrogen source. In the baker yeast *S. cerevisiae* and the human pathogen *C. albicans*, for instance, the Mep2 protein was found to act as a sensor required for the initiation of filamentous growth [35]. This dimorphic change is an essential process in the virulence of *C. albicans* [36, 37]. The function of Rh proteins was first revealed using a mouse knockout model; in renal excretion of ammonium, Rhcg absence causes a deregulation of blood pH, leading to lethality according to the intensity of the applied acid load [38]. This phenotype in mouse knockouts of Rhcg is reminiscent of a human syndrome called distal renal tubular acidosis (dRTA) [39]; however, so far, no relation between RhCG malfunction and dRTA has been shown.

Correlations between Rh proteins mutation/dysfunction and human pathologies have also been documented. In red blood cells (RBCs), RhAG mutations are associated with Rh deficiency syndrome (Rh_{null} or Rh_{nod}), characterized by the lack of Rh antigens on RBCs [40, 41]. Overhydrated hereditary stomatocytosis (OHSt) is a rare dominantly inherited haemolytic anaemia, characterized by leakage of important monovalent cations (K^+ , Na^+) through malfunctioning RhAG [42, 43]. In addition, the *rhbg* and *rhcg* genes have been proposed to act as potential tumour suppressors in human oesophageal squamous epithelial cancers [44] and mouse brain tumours [45]. Finally, *rhcg* has been identified as a candidate gene for early-onset major depressive disorder [46].

Many functional studies aimed at elucidating the mechanism of transport by the bacterial Amt/Rh proteins lead to a considerable controversy regarding the substrate and mechanism of transport [47]. Hence in this review, concentrating on bacterial transporters, we will present the structure of Amt/Rh proteins and discuss in detail our current knowledge of the mechanisms of ammonium translocation in prokaryotes.

MECHANISM OF AMMONIUM TRANSPORT

Various mechanisms were proposed before the structure of ammonium transporters was determined in 2004. The transport mechanisms proposed before 2004 are summarized in Table 1, followed by a detailed discussion of the structure of bacterial Amt/Rh and, finally, an exploration of whether our expectations of solving the mechanism of transport based on these structures has been met.

In 1985, the prevailing view was that these systems function in active transport of NH_4^+ to concentrate it within the cell [48]. A decade later, Kleiner's ruminations could be tested following the initial identification of genes encoding *bona fide* ammonium transporters [15, 16]. Rather than providing elucidation of the mechanism, however, the lines of investigation quickly gave rise to a variety of differing perspectives and conclusions.

Some of the earliest characterization of Amt/Mep/Rh-mediated transport and accumulation of [^{14}C]methylammonium (MeA), an ammonium analogue, supported proposals that the family were capable of energy-dependent concentration of the ammonium ion, NH_4^+ [16, 17].

This view would be challenged by work from Sydney Kustu's laboratory who generated an *E. coli* strain with a disrupted *amtB*. The resulting mutant matched wild-type (WT) growth at pH 7 across a range of ammonium concentrations, but was slower at pH 5 under ammonium limitation (<1 mM NH_4^+). The pKa of ammonium is 9.25, so the relative concentration of the uncharged species decreases at acidic pH, and this was taken as evidence that AmtB recognizes NH_3 [49]. In addition, they reported that *Salmonella typhimurium* 'metabolically trapped' NH_3 : metabolizing it immediately rather than accumulating it in the cytoplasm. Follow-up work revealed that, when grown on alternative N-sources, *S. typhimurium* can use AmtB to excrete intracellular ammonia [49, 50]. Taken together, the authors argued that AmtB was a passive channel that merely

Table 1. Reported mechanisms in Amt/Mep/Rh before 2004

Organisms	Proteins	Systems	Methods	Mechanisms	References
<i>L. esculentum</i>	Amt1;1	Oocyte	EP	$\Delta\psi$ -driven NH_4^+ uniporter	[53, 54]
	Amt1;2	Oocyte	EP	$\Delta\psi$ -driven NH_4^+ uniporter	[54]
<i>C. glutamicum</i>	AmtB	<i>C. glutamicum</i> cells	TA	$\Delta\psi$ -driven NH_4^+ uniporter	[52]
<i>H. sapiens</i>	RhAG	Oocyte	TA	Electroneutral NH_4^+/H^+ antiporter	[58]
		Red blood cells	TA	NH_4^+ exporter	[57]
	RhBG	Oocyte	EP/TA/pH	Electroneutral NH_4^+/H^+ antiporter	[55]
	RhCG	Oocyte	EP/pH	Transport of NH_4^+ and NH_3	[56]
<i>S. cerevisiae</i>	Mep1-3	Yeast	TA	NH_3 channel	[51]
<i>S. typhimurium</i>	AmtB	<i>S. typhimurium</i> cells	GE	NH_3 channel	[89]
<i>E. coli</i>	AmtB	<i>E. coli</i> cells	TA/GE	NH_3 channel	[49]

EP, electrophysiology; TA, ^{14}C -methylammonium transport assay; pH, pH measurement; GE, growth experiment.

enhanced the pre-existing rate of NH_3 diffusion. The channel hypothesis was extended to eucarya, with reports that NH_3 was the natural substrate of the MEP proteins of *S. cerevisiae*. Here, Soupene *et al.* explained previous observations of MeA accumulation as the result of energy-dependent sequestration of MeA into acidic vacuoles. This was evidenced by a significant decrease in MeA accumulation in yeast strains deficient in vacuolar H^+ -ATPase, which is responsible for the acidification of said compartments [51]. Within the discussion, the authors speculate that Rh proteins might also be channels, but for CO_2 rather than NH_3 .

Taken in isolation, these studies suggest a growing case for Amt/Mep/Rh proteins as simple channels. However, contemporaneous studies were also providing support for a more involved transporter-like role for the Amt/Mep/Rh family.

In 2001, Meier-Wagner *et al.* characterized AmtB from *C. glutamicum* using ^{14}C MeA uptake assays [52]. They showed that AmtB-mediated methylammonium uptake was dependent on membrane potential and reported that the K_m for methylammonium was unaffected by a pH shift of 2.5. This supported the conclusion that methylammonium (and by extension ammonium) was the substrate, not methylamine/ammonia [52]. Ludewig *et al.* provided more direct evidence for active transport in plant Amt proteins. Specifically, they expressed AMT1;1 and AMT1;2 from *Lycopersicon esculentum* in *Xenopus laevis* oocytes and measured charge translocation in the presence of ammonium via a voltage clamp [53, 54]. The currents were dependent on both voltage and NH_4^+ concentration, and unaffected by changes in pH. From this, the authors concluded that these proteins were NH_4^+ uniporters.

Xenopus oocytes were subsequently used to probe the mechanism of human RhBG and RhCG. RhBG was reported to be electroneutral, with the authors concluding that it was likely an NH_4^+/H^+ antiporter [55]. In contrast, an NH_4^+ -dependent current was observed for human RhCG, supporting a similar NH_4^+ uniport mechanism to *L. esculentum* AMTs. The authors observed that NH_3 concentration altered the affinity of RhCG, hinting at a more complex mechanism involving transport of both species [56]. Finally, mixed results were obtained for RhAG. Transport assays carried out on RhAG-expressing oocytes supported NH_4^+/H^+ antiporter activity, while a comparable assay in RBCs suggested that RhAG had the capacity to export NH_4^+ [57, 58].

As portrayed by these studies, the early 2000s featured a confluence of disparate hypotheses pulling the field in different directions (Table 1). The controversy was the result of the lack of quantitative kinetic data characterizing the activity of Amt/Mep/Rh proteins at the single channel level. Essentially all functional studies were carried out using intact cells, cell-derived vesicles or substrate analogues. In all of these systems, the parameters measured to analyse substrate conduction (pH change, electric current, uptake of labelled analogue) are likely to be affected by other physiological phenomenon, requiring careful controls [47].

Hence from the contemporary literature, it was difficult to confidently proclaim that Amt/Mep/Rh were channels or transporters, or even that a single mechanism was conserved across the family. Thus, it was hoped that the eventual purification of active protein and solving of crystal structures would provide clarity and elucidate the truth of the mechanism.

STRUCTURAL CHARACTERIZATION

Membrane topology and secondary structure

The topological organization of Amt proteins was first determined *in vivo* for the ammonium transporter AmtB from *E. coli* using alkaline phosphatase (PhoA) and β -galactosidase (LacZ) reporter fusion proteins [59]. The results supported an 11 TM helices (TMH) model with the N-terminus facing the periplasm and the C-terminus located in the cytoplasm [59]. Prior sequence analysis had predicted a 12th helix [24], but these results suggested that the first theoretical helix was a signal peptide that would be cleaved following correct folding or insertion of the protein into the membrane [59]. The high-resolution structure (see paragraph below) demonstrated that the 11 TMH model and general topology observed in AmtB is conserved across Amt/Mep proteins; human rhesus proteins, however, have been shown to possess 12 TM helices, with both the N-terminus and C-terminus located in the cytoplasm [60].

Tertiary and quaternary structure

Investigation into the oligomeric state of Amt/Mep/Rh was prompted by the observation of crosstalk between the different Mep isoforms in *S. cerevisiae*, where expression of an inactive Mep1 inhibited the activity of Mep2 and Mep3 [61]. Subsequently the *E. coli* AmtB, the first Amt/Mep protein to be purified, was subjected to a combination of analytical ultracentrifugation (AUC) and size-exclusion chromatography (SEC). Through these techniques it was determined that, when solubilized in dodecyl- β -maltopyranoside (DDM), AmtB purifies as a homotrimer with a molecular mass of ~140 kDa [62]. This does not correspond to the expected mass of the trimer (~130 kDa), likely due to partial unfolding of hydrophobic domains following SDS binding [63]. In 2004, the first ordered 2D crystals of AmtB were obtained, with cryo-electron microscopy, generating a low resolution (12 Å) projection map, and atomic force microscopy was used to obtain higher resolution topographs [64]. Both methods supported the trimeric structure of AmtB and suggested that each monomer exhibited pseudo-twofold symmetry

These results validated that the first high-resolution crystal structures of AmtB were solved [65, 66]. The general structure of AmtB was also later characterized using a combination of small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) to obtain detailed structural information on AmtB stabilized in the detergent DDM [67]. These structures confirmed the trimeric organization of AmtB and provided an unprecedented insight into the structure of the translocation pathway, enabling investigation into the mechanism of ammonium transport. Over the last two decades, high-resolution crystal structures have been solved across the Amt/Mep/Rh family, including rhesus proteins from *N. europaea* and *Homo sapiens*, fungal Mep2 from *S. cerevisiae* and *C. albicans*; and Amt from *Archaeoglobus fulgatus* and *Candidatus Kuenenia stuttgartiensis* [60, 68–71].

As AmtB is the archetypal member of the family, with over 20 high-resolution structures in the Protein Databank (PDB), its structure will be discussed in detail, followed by a series of comparisons to other solved structures of bacterial Amt/Rh proteins.

Structure of *Ec*AmtB

Consistent with predictions [62, 64], AmtB crystalized as a homotrimer, with each monomer conforming to the 11 TMH model (Fig. 1) [59, 65, 66]. Crucially, these high-resolution structures led to the identification and characterization of the potential substrate translocation pathway located in the centre of each monomer and the identification of four key features of mechanistic interest within the translocation pathway (Fig. 1):

- (1) An NH_4^+ -binding site within a vestibule in the periplasmic face of the protein named S1 site.
- (2) A constriction imposed by two stacked phenylalanine residues that separates the S1 site from the hydrophobic pore, named the 'Phe-gate'.
- (3) A second potential binding site named S2 followed by a narrow pore lined with hydrophobic residues and containing the characteristic 'twin-His' motif of the family.
- (4) A cytoplasmic vestibule.

S1-binding site

The high-resolution structure revealed a putative NH_4^+ -binding site nestled at the bottom of a periplasmic vestibule in each monomer [65, 66]. As AmtB is only expressed under ammonium-limited conditions, the S1-binding site would be necessary to ensure a high affinity for the substrate and confer efficient scavenging for ammonium at low concentration in the surrounding environment [72]. The binding site (S1) is delineated by the residues S219, W148 and F107 (Fig. 1). At this site, NH_4^+ could be stabilized via π -cation interactions with the phenyl rings of W148 and F107 alongside hydrogen bonding with S219 [65, 66]. Javelle *et al.* demonstrated that thallium (which has a similar size and coordination to ammonium) can bind at this position, and that thallium competitively inhibits MeA uptake, strongly supporting that the S1 is capable of binding NH_4^+ [73].

D160 lies in the proximity of the S1 site (Fig. 2) and is highly conserved, implying a functional or structural role [59, 74]. However, the crystal structures rule out a role in binding, as the carboxy function of D160 is buried too deeply in the pore to directly create an electrostatic interaction with NH_4^+ [65].

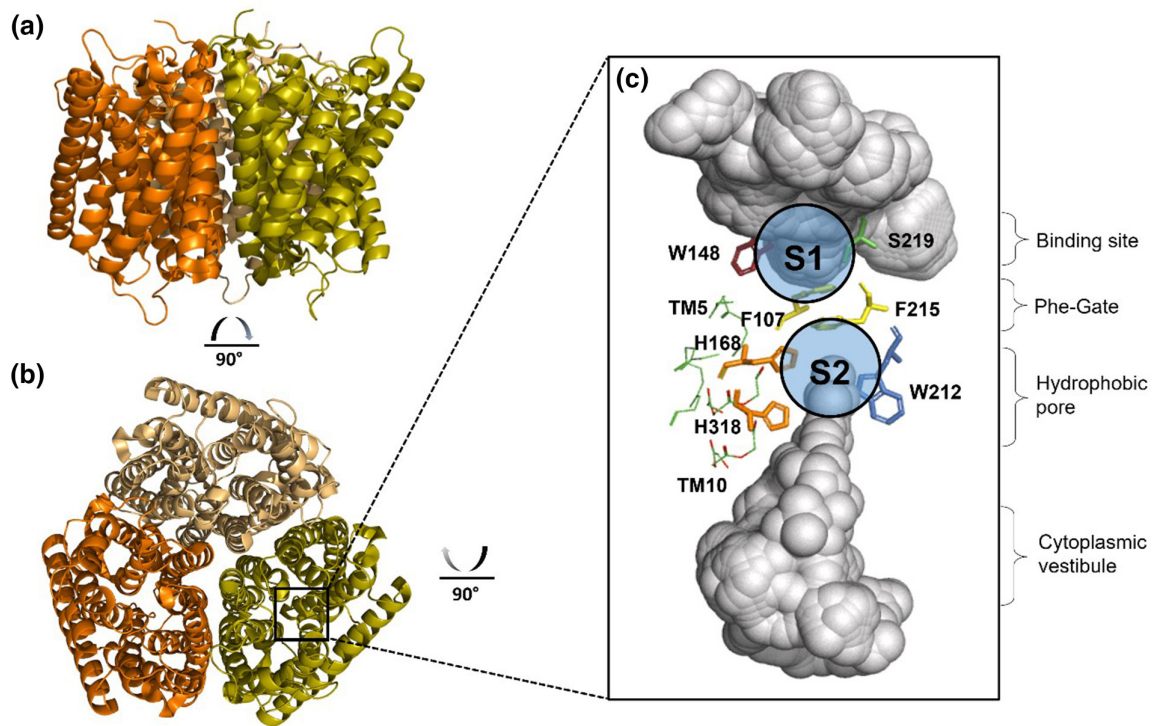


Fig. 1. Structure of AmtB from *E. coli*. (a) View of the AmtB trimer from the side, and (b) from the top, with each monomer represented in olive, orange, or yellow. (c) Expanded view of the pore region of a single monomer with the water-accessible volume represented in grey. Highly conserved residues are shown in ball and stick representation for the ammonium-binding sites S1 and S2 (blue circle), phenylalanine gate (yellow) and central pore (grey), with displayed parts of the two transmembrane helices TM5 (His168) and TM10 (His318).

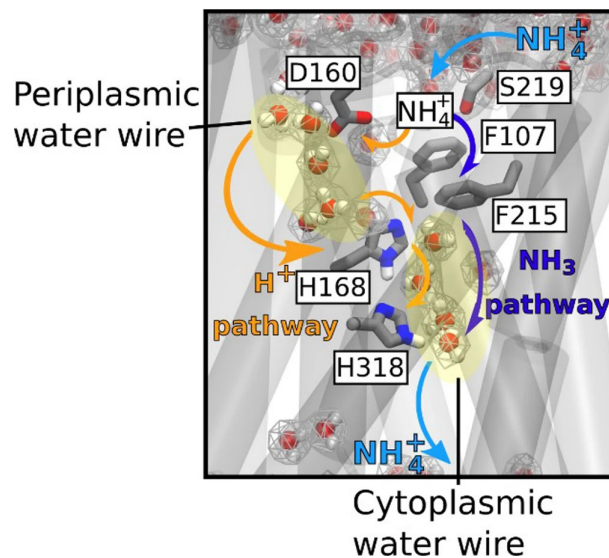


Fig. 2. Mechanism of electrogenic NH_4^+ translocation in AmtB. NH_4^+ is deprotonated at the periplasmic face of the protein, allowing passage of uncharged NH_3 molecules through the central part of the hydrophobic channel (depicted by dark blue arrow). A parallel passage of H^+ into the cytoplasm is enabled by the polar conduction route formed by two internal water wires (WWs) that span across the membrane H^+ (depicted by orange arrows). NH_3 re-protonation likely occurs near the cytoplasmic exit. Reproduced from [76].

Phe-gate

The second key structural feature of AmtB is the Phe-gate. Positioned immediately after the S1-binding site, the stacked phenyl rings of F107 and F215 form a constriction that completely blocks access to the pore from the S1 site (Fig. 1) [65]. The arrangement of F107 and F215 was identical in all the AmtB crystal structures (including AmtB co-crystallized in the presence of ammonium or different variants), but the authors suggested that the rings would have to be dynamic, as they completely block the translocation pathway [73, 75].

Hydrophobic pore and twin-His motif

Immediately after the Phe-gate, a potential second binding site, named S2, mirrors the S1 site present in the periplasmic vestibule (Fig. 1) [65, 66, 73]. This potential binding site is delineated by the residues F215, W212 and H168. At this position, NH_4^+ may be stabilized via π -cation interactions with the phenyl rings of W212 and F215 alongside hydrogen bonding with H168 and a water molecule in the pore. The S2 site is followed by a narrow and strongly hydrophobic pore that leads to the cytoplasm. The hydrophobic nature of the pore represents a highly energetic barrier against the movement of ions [66]. Two highly conserved histidine residues, H168 and H318, protrude into the lumen of this pore, forming the family's characteristic twin-His motif [75]. Whilst the hydrophobic residues in the pore can be variable, the twin-His motif is highly conserved, indicating a functionally important role [66, 75].

Remarkably, a series of four residual electron densities within the hydrophobic pore were recorded only after soaking the crystal in ammonium sulfate; indicating that they were ammonium molecules [65]. In contrast, another study observed these densities regardless of the presence or absence of ammonium [66]. Since at 1.35 Å molecules of H_2O and NH_3 are not readily distinguished, it was hypothesized that these densities are equally likely to be water molecules [66]. These densities would later form the foundation of different hypotheses in the mechanism (see below [76, 77]).

Cytoplasmic vestibule

The translocation pathway terminates at a vestibule in the cytoplasmic face of the protein. Unlike the periplasmic vestibule, no discernible binding site was observed in the structure. In addition, there was no barrier between the vestibule and the pore, resulting in a clear asymmetry between the periplasmic and cytoplasmic ends of the channel. Interestingly, AmtB was crystallized in two different space groups, with the cytoplasmic vestibule in different conformational states in each crystal. In one conformational state, the residues around the N-terminus of TM10 are positioned differently, such that they obstruct the cytoplasmic exit and create a purely hydrophobic environment [66]. In the other conformational state, the residues do not obstruct the pore exit and result in a more polar 'open' configuration [66]. This evidence of conformational change within the vestibule suggested a functionally relevant role – which is still unknown. A later extensive site-directed mutagenesis study of the *E. coli* AmtB transporter revealed that the C-terminal region might mediate co-operativity between the three subunits of the protein, indicating conformational changes in the C-terminal tail and potentially the cytoplasmic vestibule [78].

Additional Amt/Rh structures

AmtB in complex with GlnK

In *E. coli*, the activity of AmtB is regulated via a physical interaction with the PII protein GlnK [74, 79, 80]. The PII protein family, the most important family of signal transduction proteins in prokaryotes, forms homotrimers with a compact barrel-like shape. A flexible loop, named the 'T-loop', protrudes from the upper face of the barrel. The X-ray structure of the GlnK–AmtB complex has been simultaneously resolved by two different groups [60, 81]. GlnK and AmtB form a GlnK3 : AmtB3 complex at a 1:1 ratio. Interestingly, the C-terminal tail of AmtB and the T-loop of GlnK are structurally defined in the complex due to the stabilizing interaction between the two proteins [60, 81], whereas they are not in the individual crystal structures [65, 66]. The T-loop of GlnK in the complex is composed of two anti-parallel β -strands and this conformation allows a perfect fitting of the loop into the cytoplasmic vestibule of AmtB, blocking its activity.

A. fulgidus Amt1

The crystal structure of *Af*Amt1, an ammonium transporter from the Archaeon *A. fulgidus*, was first solved in 2005 [68]. The general topology of *Af*Amt1 was highly similar to that of AmtB, following the same 11 TM model – with only the signal sequence missing. In addition, *Af*Amt1 retains the pseudo-twofold symmetry seen in AmtB, loosely mirroring TM1-5 and TM6-10 [65, 68].

Similarly to the S1 binding site identified in AmtB, *Af*Amt1 also possessed a serine (S208) and tryptophan (W137), which could stabilize a molecule of NH_4^+ in the vestibule (Fig. 3). Whilst Andrade *et al.* [68] observed a strong electron density in this region, they were unable to correlate this density with the presence or absence of ammonium. The paired phenylalanine residues beneath the periplasmic vestibule were conserved in *Af*Amt1 and, as with AmtB, were positioned such that their rings occluded access to the channel. Interestingly, Andrade *et al.* observed higher flexibility in the sidechain of F204, leading them to conclude that the residues move during the translocation event. While Khademi *et al.* [65] observed several density peaks within the hydrophobic

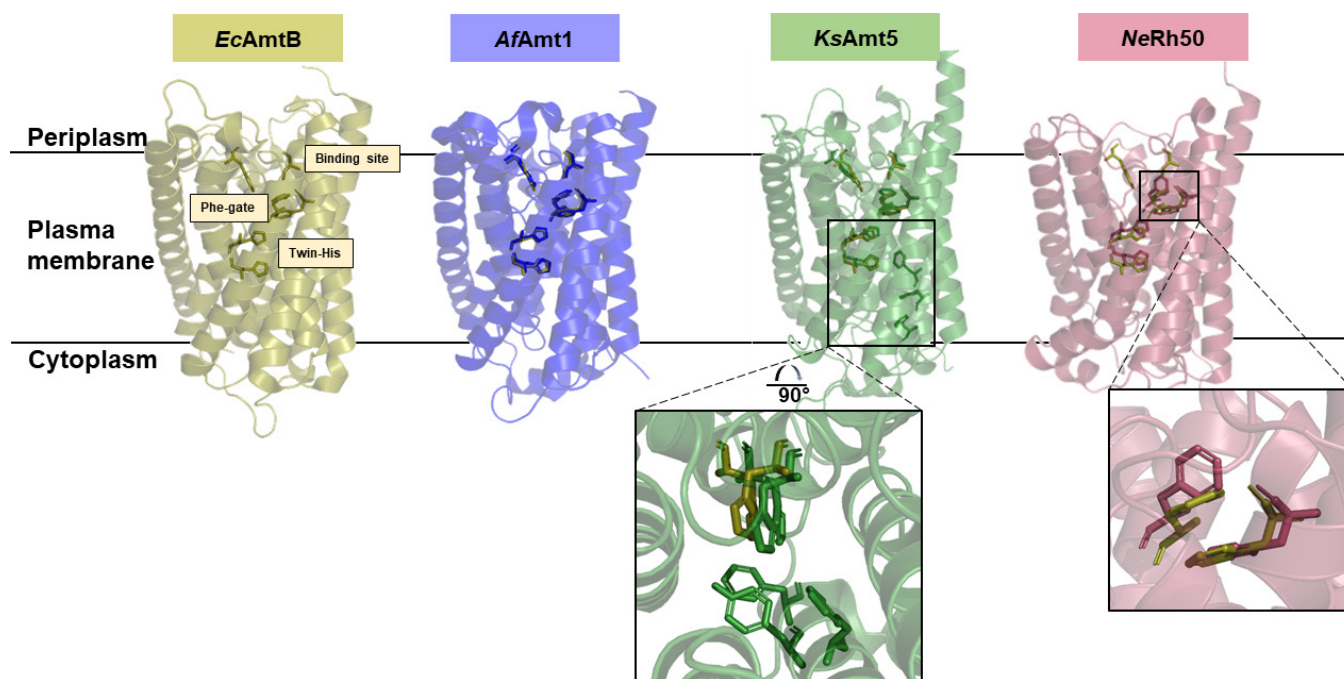


Fig. 3. Comparison of the translocation pathway of ammonium transporters present in bacterial representatives. Single monomer of AmtB from *E. coli* (yellow), Amt1 from *A. fulgatus* (blue), KsAmt5 from *K. stuttgartiensis* (green) and Rh50 from *N. europaea* (red). Crucial residues forming the binding site, phenylalanine gate and twin histidine motif from AmtB are overlaid with corresponding residues in the rest of bacterial representatives for comparison. Most critical differences are highlighted by boxes.

pore after soaking the crystal in ammonium sulfate and Zheng *et al.* [66] observed the densities in both the absence and presence of ammonium, Andrade *et al.* [68] did not observe densities in either condition.

Ca. Kuenenia stuttgartiensis Amt5

The solving of the crystal structure of KsAmt5 from the anaerobic ammonium-oxidizing bacterium *Ca. Kuenenia stuttgartiensis* revealed that the general topology of KsAmt5 is similar to that previously described for AmtB and AfAmt1, although the structure differs in key ways [70].

Firstly, while the binding site (W144/S227), the Phe-gate (F103/F223), and the twin-His motif (H171/H326) are all conserved, the crystal structure revealed a slight shift ($\sim 0.8 \text{ \AA}$) of the twin-His motif into the channel. Moreover, three additional bulkier side chains (F27, Y30, F34), non-conserved in other Amt proteins, were identified in the translocation pore (Fig. 3) [70]. This arrangement seems to occlude the translocation pore (Fig. 3, box), potentially preventing NH_4^+ translocation. Indeed, using solid supported membrane electrophysiology (SSME) [82], Pflüger *et al.* demonstrated that KsAmt5 binds ammonium but does not translocate it across the membrane [70]. In the structure, the second histidine (H326) residue is linked via H-bonding across two putative water molecules to an unprecedented NH_4^+ -binding site. At this position two tightly coordinated ammonium cations have been identified. Unlike in other proteins, it is positioned within the membrane and could represent a selective high-affinity NH_4^+ -binding site. The authors suggested that in KsAmt5 the substrate binds onto the high-affinity binding sites, leading to structural rearrangements that trigger signalling [70]. These marked structural and functional differences imply a distinct role for this Amt.

Compared to AmtB and AfAmt1, KsAmt5 features an extended C-terminal tail with high similarity to the histidine kinase (HK) domain observed in bacterial two-component systems [70]. Although the overall crystal structure obtained was high resolution, the HK domain was highly disordered due to its relative flexibility and thus remained undefined. Despite this, Pflüger *et al.* demonstrated HK-mediated phosphorylation to be ammonium concentration-dependent, with a significant increase under nitrogen limitation conditions (5–10 mM) [70].

Thus, KsAmt5 represents a system wherein an Amt protein has been repurposed as a specific ammonium receptor modulating signal transduction to its HK in response to NH_4^+ concentration. This unusual arrangement may have evolved to complement the unique physiology of anammox bacteria, where ammonium catabolism is highly dependent on the ammonium concentration within its unique organelle, the anammoxosome. Whilst KsAmt5 is the first to be characterized, other naturally occurring

sequences combining Amt with other protein domains have been observed [83]. It is possible that similar examples will be observed in other bacteria.

***N. europaea* Rh50**

In 2007 the first high-resolution (1.3 Å) structures of a rhesus protein from the chemolithotrophic ammonium-oxidizing obligate bacterium *N. europaea* were published [69, 84]. This was a milestone moment, as it not only allowed for structural insight into Rh50 itself, but also enabled comparison to the structures of AmtB [65, 66], AfAmt1 [68] and KsAmt5 [70].

Previous *in silico* work predicted that Amt and Rh had 11 and 12 TM helices, respectively [24]. However, the crystal structure of NeRh50 revealed only 11 TM helices, which Lupo *et al.* hypothesized was due to TM helix 12 being cleaved off when expressed in *E. coli* [69].

Of the key residues seen in the AmtB S1-binding site (F107, W148, S219), only the phenylalanine is conserved in NeRh50 (Fig. 3). However, the vestibule structure is not conserved, and no ammonium-binding site is seen in NeRh50. The Phe-gate is conserved but altered in comparison to that seen in AmtB (Fig. 3). F110 is tilted in NeRh50, creating an opening for a water molecule 2 Å deeper in the pore. A similar pocket is present directly above the first histidine of the twin-His motif and contains two water molecules [69, 84]. Beyond this change, the twin-His motif is conserved and similar in both Rh and Amt.

Interestingly, while residual electron densities have been seen in the hydrophobic pore of AmtB, they were not observed here [66, 69]. The cytoplasmic vestibules of AmtB and Rh50 are very similar, but the asymmetry between the periplasmic and cytoplasmic vestibules in Rh50 is less pronounced due to the lack of the S1-binding site (Fig. 3).

In contrast to prior speculation, based on immunoprecipitation analysis of the erythrocyte Rh complex [85], that the human rhesus proteins would form heterotetramers [85], Lupo *et al.* concluded that they are certainly trimeric [69]. This was based on the trimeric structures of EcAmtB and NeRh50, and comparative analysis of their respective monomer interface regions. Soon afterwards, the crystal structure of mammalian RhCG was resolved by Gruswitz *et al.* [60]. The protein was proved to retain the trimeric organization and the pseudo-twofold symmetry between TMH1-5 and TMH6-10 observed in AmtB [60], confirming previous assumptions [69].

DID THE PROTEIN STRUCTURES SOLVE THE TRANSPORT MECHANISM MYSTERY?

Despite the wealth of structural information, the mechanism of ammonium transport has remained elusive for 10 years following the publication of the structure of AmtB. All the structures show a very similar conformation, reflecting the inward-facing state of the protein, irrespective of the presence or absence of ammonium. Moreover, the hydrophobicity of the pore acts as an energetic barrier for ion translocation and this structure is highly conserved across the family [60, 65, 68, 69, 71]. This apparent structural inflexibility, combined with the hydrophobicity of the pore, lead to the initial conclusion that the Amt/Mep/Rh family were electroneutral NH₃ channels, rather than active transporters, which generally involve large conformational changes during the translocation cycle [86]. This assumption was supported by *in vivo* and *in vitro* experiments. Khademi *et al.* adapted a fluorescence-based assay to measure the influx of ammonia into proteoliposomes containing AmtB by monitoring the pH-sensitive fluorescence of 5-carboxy fluorescein (CF). Following mixing of CF-loaded proteoliposomes with NH₄Cl, the internal pH rose, reflecting influx of NH₃ (Table 1) [65]. However, these results have never been reproduced despite extensive effort, questioning the validity of the initial conclusion [47].

Javelle *et al.* developed an 'unwashed' MeA transport assay (for technical details of the unwashed assay, see [80]). These data report that strains expressing AmtB show a complete linear dependence over a range of external MeA concentrations, indicating a passive diffusion of the substrate down an electrochemical gradient, in agreement with a channel-like activity rather than transporter-like activity (Table 2 [80]). The same technique was used to assess the mechanism of Rh50 transport, with a similar result to the that obtained for AmtB, leading the authors to conclude that Rh50 from *N. europaea* also acted as a channel rather than a transporter [87]. In addition, the restoration of ammonium-dependent growth to a yeast *Δmep* mutant by Rh50 was more effective at higher pH values, providing further evidence in support of Rh50 translocating NH₃ rather than NH₄⁺ (Table 2 [87, 88]).

In parallel to NH₃ transport, Rh proteins were proposed to facilitate CO₂ across the plasma membrane. The Rh1 protein of the green alga *C. reinhardtii* was proposed to function as a CO₂ channel [34, 89], and human Rh30/RhAG proteins to contribute to the CO₂ permeability in RBCs [90–92]. While the evidence was merely transcriptional, hence indirect, for Rh1 from *C. reinhardtii* and conflicting data have been obtained for human Rh proteins, the identification of a potential CO₂-binding pocket in NeRh50 crystal structure supported the idea that rhesus proteins might act as gas channels for CO₂ in addition to NH₃ [84]. At the same time, *in vivo* characterization of NeRh50 showed no evidence of a CO₂-dependent growth effect in a knockout mutant [88]. Another argument against Rh involvement in CO₂ was presented in a later report monitoring transmembrane CO₂ flux, which concluded that protein-facilitated transport of CO₂ is highly improbable [93]. In addition, a CO₂-binding pocket was not detected in the crystal structure of human RhCG, which was proposed to be very representative of all Rh homologues [60]. Finally, a

Table 2. Summary of transport mechanism in bacterial ammonium transporters after 2004

Organism	Protein	System	Method	Mechanism	References
<i>A. fulgidus</i>	Amt-1	Proteoliposomes	EP	Electrogenic transport	[98]
	Amt-3	Proteoliposomes	EP	Electrogenic transport	[98]
<i>K. stuttgartiensis</i>	Amt-5	Proteoliposomes	EP	NH ₄ ⁺ binding only	[70]
<i>E. coli</i>	AmtB	Proteoliposomes	pH	NH ₃ transport	[65]
		<i>E. coli</i> cells	TA	Channel-like transport	[80]
		Proteoliposomes	EP	Electrogenic transport	[67, 76]
<i>N. europaea</i>	Rh50	Washed assay	TA	NH ₃ transport	[87]
		Proteoliposomes	EP	Electrogenic transport	[76]

EP, electrophysiological measurements; TA, ¹⁴C]-methylammonium transport assay; pH, pH measurement.

study showed that during long-equilibration molecular dynamics (MD) simulations CO₂ molecules do not show any tendency to diffuse across the periplasmic vestibule of either bacterial or human Rh50 proteins [94].

FINALLY; THE ACTIVITY OF BACTERIAL AMT AND RH PROTEINS IS ELECTROGENIC!

The view that Amt/Mep/Rh proteins conducted a neutral species was first experimentally challenged for some plant Amt and Rh proteins [95, 96], with charge translocation observed for both AMT1;1 from *Lycopersicon esculentum* and human RhAG expressed in *Xenopus laevis* oocytes [53, 95]. Concerning bacteria, in 2011, using a systems biological approach, Boogerd *et al.* provided evidence that AmtB-mediated import must be active for intracellular NH₄⁺ concentrations to sustain bacterial growth [97]. The first direct evidence for electrogenic transport of ammonium in bacteria was proposed after *in vitro* analysis of Amt1 and Amt3 from *A. fulgidus* reconstituted in artificial liposomes using SSME (Table 1) [98]. Afterward, the activity of AmtB and NeRh50 was also found to be electrogenic using the same electrophysiological approach (Table 1 [76, 99].

The apparent conflict between the crystal structures, in which certain structural features suggest that an electrogenic transport would be disfavoured, and functional observations of electrogenic transport reignited the controversies regarding the transport mechanism of Amt/Rh, centring on a new paradox: how can a charge travel through a hydrophobic pore?

Before answering this question, it is important to explore the different mechanisms that may explain the electrogenicity of transport. Three potential mechanisms are possible: NH₄⁺ uniport, NH₄⁺/H⁺ symport, NH₄⁺ deprotonation and subsequent symport of NH₃ and H⁺. Of these, the translocation of NH₄⁺ is the least likely, due to the energetic barriers preventing ion conduction through a hydrophobic pore. Additionally, it has been shown that ‘open-pore’ mutants of AmtB (constructed with the Phe-gate removed) become inactive in transporting MeA and do not gain permeability to either H⁺ or K⁺ [73]. This demonstrates that widening access to the pore does not allow for ion conduction through AmtB and suggests that another mechanism and/or residues must be implicated in transport.

Mechanisms of deprotonation

A breakthrough came from a study that experimentally demonstrated the importance of NH₄⁺ deprotonation in ammonium transport by some Mep and Amt proteins [100]. Ariz *et al.* used the natural chemical/physical properties of the N-isotopic signature linked to NH₄⁺/NH₃ interconversion, and showed that only *S. cerevisiae* cells expressing some Amt/Mep proteins were depleted in ¹⁵N relative to ¹⁴N when compared to the external ammonium source [100]. They showed that this isotope fractionation can only be explained by the deprotonation of NH₄⁺ before the translocation of NH₃ by Amt/Mep proteins. However, the mechanism of deprotonation is still a matter of debate.

Deprotonation at the S1 site

Three mechanisms have been proposed for NH₄⁺ deprotonation at the S1-binding site. The first hypothesis was proposed by Khademi *et al.* [65]. They found that when bound to the S1 site, the pKa of NH₄⁺ decreased below 6, which favours the deprotonation of a NH₄⁺ by a water molecule. They also measured a NH₃ flux through the pore of AmtB using a fluorescence-based assay to measure the pH variation in proteoliposomes containing AmtB after an ammonium pulse. Initially in agreement with this hypothesis, Fritz Winkler argued that the deprotonation happens before reaching the hydrophobic pore, based on a Michaelis–Menten kinetics model, and deduced that the most likely deprotonation occurs at the S1-binding site via water molecules, thus the proton should be released in the periplasm [10]. Further computational simulations substantiate this hypothesis: combining

the potential of mean force with data from thermodynamic integration to calculate the apparent pKa of $\text{NH}_4^+/\text{NH}_3$ along the translocation pathway, it was deduced that the most likely proton acceptor was a water molecule around the S1 site [101, 102], releasing the hydronium ions back in the bulk solution. However, it has now clearly been demonstrated that Amt/Rh-mediated transport in bacteria is electrogenic, which contradicts this potential mechanism of deprotonation [67, 76, 98].

Secondly, computational modelling suggested that at the S1 site, S219 fulfils a dual role in ammonium transport: firstly coordinating NH_4^+ via H-bond formation between the hydroxyl oxygen of S219 before acting as a proton acceptor to facilitate deprotonation [103]. This model has remained relatively untested – but Javelle *et al.* showed that AmtB^{S219A} had enhanced, rather than reduced, MeA and ammonium uptake compared to the WT when tested using MeA transport assays or SSME, respectively [73]. These results suggest that under the *in vivo* and *in vitro* conditions used in these studies, recruitment at S1 was not rate limiting and that the hydroxyl moiety of the S219 residue is unlikely to be involved in the deprotonation mechanism.

The third possibility for deprotonation at the S1 site is that the carboxyl moiety of the D160 residue acts as the proton acceptor. Multiple sequence alignments of Amt/Mep/Rh proteins show that D160 is highly conserved, potentially indicating an important functional role [59, 74, 104]. It was demonstrated by *in vivo* MeA uptake that the AmtB^{D160A} variant was unable to transport ammonium, whereas AmtB^{D160E} was capable of maintaining 70% of the WT activity, suggesting that the D160 residue is essential for AmtB activity [74]. However, it is important to note that this study was conducted *in vivo* using MeA as a substrate, and there are numerous examples where the activity of AmtB variants measured *in vivo* using MeA as a substrate does not mirror the activity measured in *in vitro* systems using ammonium [76, 77, 105], for example AmtB^{D160E} (see below). This shows that MeA is a poor substrate analogue. In contrast, the AmtB X-ray structure revealed later that the distance between the D160 residue and the binding site is not favourable for a direct interaction with the substrate [65, 66]. Further computational studies, using umbrella sampling simulations, indicate that the residue D160 is important for the structural stability of the periplasmic vestibule but not involved in the mechanism of ammonium transduction [106]. However, this contradicted MD simulation studies previously performed by Luzhkov and co-workers showing that the D160 residue, separated by 8 Å from the binding site, was able to interact with ammonium [107]. A potential indirect role of D160 on NH_4^+ deprotonation has also been proposed: a hydrogen bond wire between NH_4^+ at the S1 site and the carboxylate group of D160 via two water molecules was observed using MD simulation. Thus, the authors concluded that D160 is most likely the proton acceptor from NH_4^+ [108, 109]. More recently, combining SSME analysis, yeast complementation assay and advanced MD simulation revealed that AmtB^{D160A} and AmtB^{D160E} variants are transport-deficient, suggesting that residue D160 plays a central role in the transport mechanism [76]. Moreover, the fact that the conservative D to E substitution at position 160 impairs ammonium transport via AmtB indicates that D160 not only participates in electrostatic interactions with NH_4^+ at the S1 site, as previously hypothesized [102, 107], but is also involved in the translocation mechanism. Furthermore, residue D160 was proven to contribute to stabilizing the water wire that bridges the S1 site to the twin-His motif in the hydrophobic pore (see below and Fig. 2). Thus, while D160 is clearly important, the extent of its role in the transport mechanism and, more specifically, in NH_4^+ deprotonation is still a matter of debate.

Deprotonation at the Phe-gate

In 2008, it was noted that the F107A mutation has no effect on AmtB activity [73]. However, an AmtB^{F215A} variant could not transport ammonium or MeA. Similarly, replacing the F215 with H, E, S or W residues also abolished transport activity. This result demonstrated the importance of the F215 residue and it was speculated that the deprotonation of ammonium may occur during the passage through the Phe-gate [73]. This hypothesis was further substantiated by computational calculation; the apparent pKa for NH_4^+ changes from 4 to –16 pKa units (ΔpKa compared to normal $\text{NH}_4^+/\text{NH}_3$ pKa at the S1 and S2 sites) when moving from the S1 to the S2 site, which indicates that ammonium may be deprotonated only after entering the constricted part of the channel pore, near the Phe-gate position [107]. However, no evidence of the direct role of the residue F215 in the deprotonation has been obtained so far.

Deprotonation at the S2 position

A 2006 MD study indicated that the carbonyl group of the A162 residue can be orientated toward either the S1 or the S2 site (Fig. 1), forming an H-bond with NH_4^+ [110]. This result suggested that the A162 residue acted as a 'substrate guide' from the binding site across the Phe-gate to the S2 site. A 180 degree flip of the Phe-gate was frequently observed during these MD simulations; hence it was assumed that the deprotonation occurred after the S1 site and near to the S2 site. The deprotonation mechanism deduced by Nygaard and co-workers proposed that NH_4^+ is translocated between the S1 and S2 site by the movement of A162, which became perfectly aligned with the G163 : NH bond by interacting with the O₈ of the D160 residue. This new amino acid configuration allowed the deprotonation of NH_4^+ by A162 and its transfer to D160. Once a proton has been transferred from NH_4^+ to the O₈ of the D160 residue, all amino acids involved become neutral and the C-O-H group of A162 is reorientated to the S1-binding site and the proton released in the periplasmic vestibule via a water molecule [110]. However, as already pointed out above, this would imply an electroneutral activity for ammonium translocation, which is not the case [76, 98].

Lamoureux and co-workers, using MD simulations of AmtB, have shown that a wire composed of three water molecules was present along the hydrophobic pore, corresponding to the position (S2–S4) of the residual electron densities observed in the original structure published previously [65, 111]. By using protonation states and potential mean force calculations, the authors suggested that the deprotonation occurs at the S2 site. Two mechanisms have been proposed: firstly, a deprotonation via the H168 residue of the twin-His

motif, and transfer of the proton in the lumen via the twin-His motif, or secondly, a deprotonation by the water molecule present in the vicinity of the S2 site and transfer of the proton in the lumen through the water wire via a Grotthuss mechanism (Decoursey, 2003) [112, 113]. However, a study combining MD with functional assays (using a growth experiment or MeA uptake assay) was carried out by Mike Merrick's group, which challenged the deprotonation hypothesis via the H168 residue [105]. Numerous variants of the twin-His motif of AmtB and Mep2 from *S. cerevisiae* were analysed and it was shown that single His mutants (AmtB^{H168A} or H318A and Mep2^{H194A} or H348A) were still capable of growth on minimal medium containing 1 mM of ammonium as the sole nitrogen source, but were unable to translocate MeA inside the cell [105]. The apparent discrepancy between both *in vivo* assays (growth analysis and MeA uptake) was explained by MD simulations, which revealed that the single histidine H168A variant allowed ammonia to pass across the pore but not MeA [105]. Recently, the analysis of a series of variants in which alanine residues were introduced alone or in combination in the twin-His motif showed that AmtB switches from transporter to channel-like activity in the absence of the twin-His motif, directly translocating hydrated NH_4^+ through the pore [76]. Taken together, these results suggest that the H168 residue is not essential for NH_4^+ deprotonation, but further analysis is still needed to understand the exact role of the twin-His motif in Amt/Mep/Rh activities.

How does a charge travel through a hydrophobic pore?

As we have seen, although the mechanism of deprotonation remains elusive, there is increasing evidence that suggests deprotonation and co-transport of NH_3/H^+ as a likely mechanism for ammonium translocation, although the route the proton takes to get into the cytoplasm remains to be determined. Based on MD simulations, it was proposed that the pathway for protons, following NH_4^+ deprotonation, involves translocation via a Grotthuss mechanism through water chains formed in the pore or passage through the 'twin-His' motif [111]. A recent study combining *in silico*, *in vitro* and *in vivo* approaches, offers an attractive model and experimental evidence explaining the fate of the proton following NH_4^+ deprotonation [76]. Through extended MD simulation, two interconnected water wires (WWs), bridged by the twin-His motif, connecting the periplasmic and cytoplasmic regions of the protein, were identified in AmtB. These results led to the hypothesis that the WWs allow for a continuous pathway for proton transfer from the S1 NH_4^+ sequestration region to the cytoplasm. Using SSME assays and the reduced deuteron mobility of heavy water (D_2O), it has been experimentally proven that WWs are indeed essential for proton conduction during the AmtB transport cycle [76]. Collectively, these results indicate that in AmtB NH_4^+ undergoes deprotonation, allowing the passage of uncharged NH_3 molecules through the central part of the channel lined by the hydrophobic groups into the cytoplasm, while H^+ ions are translocated via a parallel pathway outlined by the WWs. The passage of NH_3 and H^+ is followed by subsequent reprotonation in the cytoplasm driven by intracellular pH (Fig. 2) [76]. This mechanism defines a new principle of achieving transport selectivity against competing ions in a biological transport process. In the same study, Williamson *et al.* provided evidence that the activity of the *N. europaea* Rh50 ammonium transporter is in fact electrogenic (Table 1), as opposed to the previously proposed NH_3 channel [76, 87, 88]. While the sequence similarity within the Amt/Rh family is low, the segments involved in the proposed transport mechanism are highly conserved (Fig. 4); hence, the authors proposed that this mechanism could be shared between various members of the Amt/Mep/Rh family [76].

CONCLUSION AND PERSPECTIVES: IS THERE A COMMON MECHANISM IN THE AMT/MEP/RH PROTEIN FAMILY?

It is now clear that the activity of bacterial ammonium transporters studied so far is electrogenic. An original model explaining how a charge can be translocated via the hydrophobic pore has been proposed [76]. However, whether this is a conserved mechanism of transport amongst the Amt/Mep/Rh family remains an open question. Reviewing the mechanistic information to date for non-bacterial Amt/Mep/Rh and its implications is a huge undertaking and is beyond the scope of this review, although some insight can be drawn from the work to date.

Firstly, the residues that delineate and stabilize the WWs (Fig. 2) in AmtB are highly conserved across the whole Amt/Mep/Rh family (Fig. 4). This indicates that the model proposed by Williamson *et al.* may be conserved amongst all members of the family that exhibit electrogenic activity [76]; however, it is clear that some Amt, Mep and Rh proteins may exhibit an electroneutral activity.

The transceptor function of Mep2 in the filamentation signalling in *S. cerevisiae* may hint at a different mechanism of transport [77, 114]. Currently, it is unknown how the signal that leads to the pseudohyphal growth is initiated, but two possibilities are currently envisaged: Mep2 binds to a partner protein to illicit the signal, or Mep2 conducts ammonium via a separate mechanism to Mep1/3 to transduce the signal. It has been shown recently using electrophysiology in *Xenopus* oocytes expressing either *S. cerevisiae* Mep1 or Mep2 transporter that their activity is electrogenic and electroneutral, respectively [114]. In addition, the first experiments with purified Mep2 reconstituted into artificial liposomes produced no observable current using SSME measurement, also suggesting an electroneutral transport mechanism (Boeckstaens *et al.*, unpublished results). The molecular basis of the difference between electrogenic Mep1 and electroneutral Mep2 remains, however, completely unknown.

Plant AMTs are also the subject of much research. In plants, the mechanism of ammonium transport is best understood in *A. thaliana*, where the genome encodes six AMT genes, divided into two sub-families, AMT1 and AMT2. The AMT1 sub-family have been

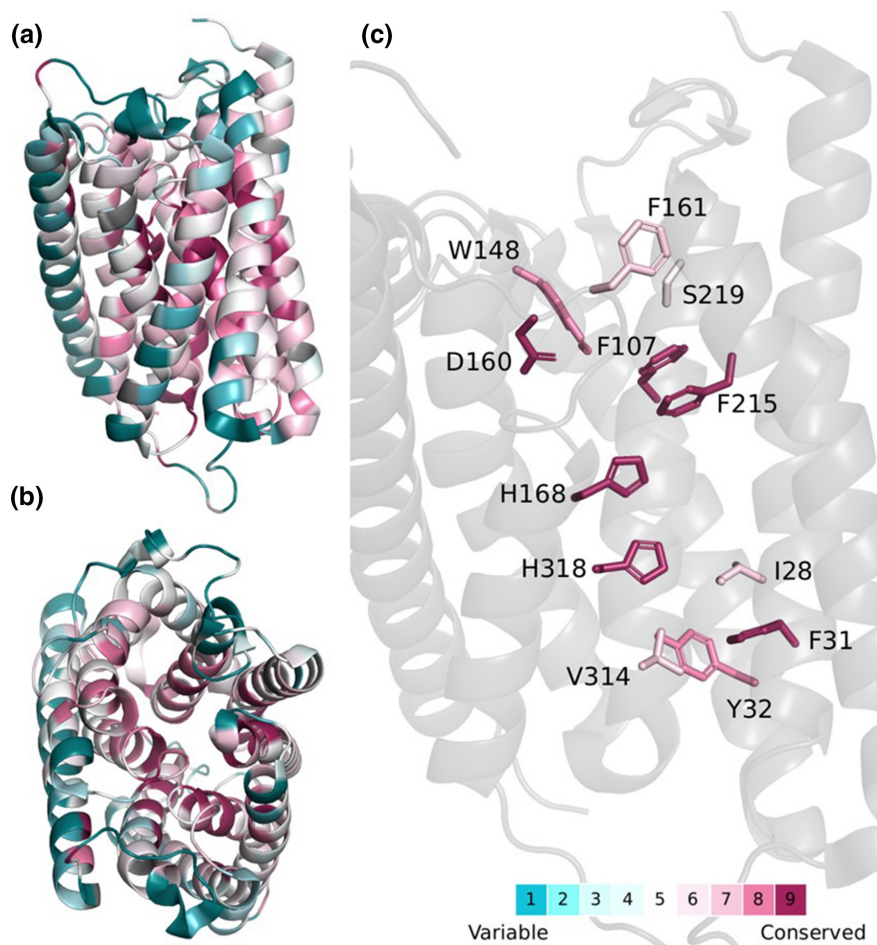


Fig. 4. Evolutionary conservation of the proton and hydrophobic pathways for H^+ and NH_3 translocation in AmtB. Evolutionary conservation scores indicate that the residues that line both the water wires and the internal hydrophobic pathway show a large degree of conservation among homologous proteins. Evolutionary conservation scores were estimated using the ConSurf server [119]. In brief, a multiple sequence alignment (MSA) was built of homologues collected from the SWISS-PROT database. Homologues were identified by five iterations of HMMER, producing an MSA of 64 sequences. The evolutionary conservation scores are projected onto an AmtB monomer (PDB code: 1U7G), with some helices removed for clarity. (a) Side view. (b) Top view from periplasmic side. (c) Close-up view of key residues involved in the two-lane mechanism. Reproduced from [76].

demonstrated to facilitate electrogenic transport in *A. thaliana* and in other plants, although it has been shown that the AMT2 family activity is electroneutral. For a review, see Ludewig *et al.* and Hao *et al.* and references therein [115, 116].

As the most distant member of the Amt/Mep/Rh family, distinct functions have repeatedly been hypothesized for the Rh proteins. A subset of the family (Rh30) has evolved to lose transport activity and instead act as antigens on the surface of erythrocytes. Rh50 proteins are known to be transporters, but their substrate and mechanism remain debated. The Rh50 protein from *N. europaea* and the human RhAG and RhCG complement $\Delta mep1-3$ *S. cerevisiae* [27, 88], suggesting that they also transport ammonium. Attempts to elucidate the mechanism, however, have been conflicting. Initial patch-clamp physiology experiments wherein RhAG expressed in *Xenopus* oocytes led the authors to propose that RhAG is an NH_4^+/H^+ antiporter [58]. Contemporaneous studies reported that RhAG and RhBG were electroneutral, and RhCG was an electrogenic NH_4^+ uniporter [53]. However, later work using stopped-flow spectrophotometry to measure changes in pH following an ammonium pulse in erythrocyte vesicles from human and mouse genetic variants and liposomes containing RhCG concluded that RhAG and RhCG were both NH_3 channels [117, 118]. This is supported by more recent oocyte experiments, wherein no significant difference in current was observed in Rhcg-expressing oocytes compared to control [95]. Despite this lack of current, Rhcg resulted in increased surface acidification, leading the authors to conclude that Rhcg transported NH_3 but not NH_4^+ [95].

Hence, whether there is a conserved, or partially conserved, mechanism of transport amongst the Amt/Mep/Rh family remains open. Further, important questions remain open concerning the site of deprotonation, which seems to be a common mechanism of all Amt/Mep/Rh studied so far, and residues involved in the process. The energetics of the transport and the dynamics of the protein during

the transport cycle also remain open questions. Hopefully, the next three decades of research will bring answers to these questions, improving our understanding of the family of these proteins.

Funding information

A.J. would like to acknowledge funding from Tenovus Scotland (S17-07). P.A.H. would like to acknowledge funding from BBSRC (BB/T001038/1 and BB/T004126/1) and the Royal Academy of Engineering Research Chair Scheme for long-term personal research support (RCSRF2021\11\15).

Acknowledgements

The authors thank Professor Ulrich Zachariae (University of Dundee, Dundee, UK) for help in generating Figs 2 and 4; Dr Mélanie Boeckstaens (Free University of Brussels, Gosselies, Belgium) for help with the work done on Mep2 (purification and SSME measurement); and Professor Iain Hunter (University of Strathclyde, Glasgow, UK) for helpful comments and discussions. Special thanks go to Professor Mike Merrick (Emeritus Professor, John Innes Centre, Norwich, UK), Professor Fritz Winkler (Emeritus Professor, ETH Zurich, Switzerland) and Professor Anna-Maria Marini (Free University of Brussels, Gosselies, Belgium) for their invaluable supervision, guidance and helpful discussion over the last 24 years.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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STATE-OF-THE-ART REVIEW

Biological ammonium transporters: evolution and diversification

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Keywords

ammonium transporter; Amt/Mep/Rh; evolution; functional diversification; methylammonium permease; rhesus proteins

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(Received 26 September 2023, revised 14 December 2023, accepted 9 January 2024)

doi:10.1111/febs.17059

Although ammonium is the preferred nitrogen source for microbes and plants, in animal cells it is a toxic product of nitrogen metabolism that needs to be excreted. Thus, ammonium movement across biological membranes, whether for uptake or excretion, is a fundamental and ubiquitous biological process catalysed by the superfamily of the Amt/Mep/Rh transporters. A remarkable feature of the Amt/Mep/Rh family is that they are ubiquitous and, despite sharing low amino acid sequence identity, are highly structurally conserved. Despite sharing a common structure, these proteins have become involved in a diverse range of physiological processes spanning all domains of life, with reports describing their involvement in diverse biological processes being published regularly. In this context, we exhaustively present their range of biological roles across the domains of life and after explore current hypotheses concerning their evolution to help to understand how and why the conserved structure fulfils diverse physiological functions.

Introduction

The earth's atmosphere is composed of 70% nitrogen in the form of dinitrogen (N_2). Nitrogen is also the fourth most common element found in living organisms, as it is an integral part of DNA, RNA and proteins. Despite its abundance in the atmosphere and biological significance, few microorganisms can reduce N_2 to ammonia/ammonium (NH_3/NH_4^+) and in doing so make it biologically available [1]. Ammonium

(NH_4^+) is the second most common nitrogen species on the planet and is the preferred source of nitrogen for many bacteria, fungi and plants and is frequently a limiting nutrient for growth. Prior to the early 20th century, production of NH_4^+ for fertiliser relied on biological N_2 fixation from atmospheric nitrogen and thus was difficult to scale. The invention of the Haber-Bosch process made synthetic NH_3 production

Abbreviations

Amt, ammonium transporter; ATP-ase, adenosine 5'-TriPhosphatase, adenylypyrophosphatase; CV, contractile vacuole; HGT, horizontal gene transfer; KO, knockout; LUCA, last universal common ancestor; MeA, methylammonium; Mep, methyl ammonium permease; MRC, mitochondrion-rich cells; OHSt, overhydrated stomatocytosis; POPC, phosphatidylcholine; POPE, phosphatidylethanolamine; POPG, phosphatidylglycerol; Rh, Rhesus protein; Smalp, styrene maleic acid co-polymer lipid particles.

economically viable. Since then, Haber-Bosch reactions have become essential to feeding the planet and currently consume approximately 1% of the world's electricity. The NH_3 produced is used in industrial fertilisers, which have dramatically increased crop yield, supporting rapid growth of human population over the past century. However, most of the NH_3 never reaches the target crops, instead it is assimilated by soil microbes or redirected into the nitrogen cycle by ammonia-oxidising microorganisms [2].

Although ammonium is the preferred nitrogen source for microbes and plants, in mammalian cells it is a toxic product of nitrogen metabolism that needs to be excreted [3]. Thus, ammonium movement across biological membranes, whether for uptake or excretion, is a fundamental process in all living organisms. For years, it was generally accepted that ammonium acquisition by cells occurred by passive diffusion of NH_3 across the lipid bilayer which has a NH_3 permeability of $\sim 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ [4]. This was confirmed later when a low thermodynamic barrier of $14 \text{ kJ} \cdot \text{mol}^{-1}$ was measured for NH_3 passage through an artificial lipid bilayer made of a mixture of phosphatidylethanolamine (POPE) and phosphatidylcholine (POPC) [5]. However, biological membranes are not simple lipid mixtures but are instead complex assemblies made up of several types of lipid and sterols (especially in animals). NH_3 being a polar molecule (with a dipole moment of 1.47 D) permeates poorly through lipid bilayers and thus requires specific membrane transporters to facilitate its permeation through biological lipid bilayer [6]. In addition, the pKa value for the ammonium/ammonia ($\text{NH}_4^+/\text{NH}_3$) equilibrium is 9.25, hence at physiological pH, 99% of the ammonium is protonated [7]. Ions such as NH_4^+ cannot diffuse through the hydrophobic layer because of the high energy required to strip away the hydration shell ($80 \text{ kcal} \cdot \text{mol}^{-1}$ for NH_4^+ ; [8]). Specific ammonium transport systems have evolved to overcome this thermodynamic barrier, efficiently translocating NH_4^+ across cellular membranes.

While the state of the art concerning the structure and mechanism of biological ammonium transporters from the Amt/Mep/Rh superfamily was recently reviewed [9], the distribution of ammonium transporters in a range of organisms and their physiological and functional importance in this context was not explored. This is an important aspect as the structure of these transporters is highly conserved across the two domains of life, with representatives being experimentally studied in the major lineages—archaea, bacteria, fungi, plants and animals (Fig. 1). In this context we will explore current hypotheses concerning the

evolution of ammonium transporters to help to understand how and why the conserved structure fulfils diverse physiological functions.

Identification of the Amt/Mep/Rh family of biological ammonium transporters

First evidence of the existence of a specific ammonium transport system

While characterising a nonspecific amino-acid permease expressed by *Penicillium chrysogenum* and *Aspergillus nidulans* under “nitrogen starvation conditions”, it was observed that amino-acid translocation was inhibited by the presence of ammonia [10,11]. This was the first indication of a specific ammonium transport system in *P. chrysogenum* and *A. nidulans*. To confirm this, Hackette *et al.* [11], further characterised the ability of *P. chrysogenum* to uptake the ammonium substrate analogue ^{14}C radio-labelled methylammonium (MeA). The authors demonstrated that ammonium was a potent competitive inhibitor of MeA uptake activity. Since the K_i value for ammonium inhibition of MeA transport activity (approximately $0.25 \mu\text{M}$) was orders of magnitude lower than the K_m for MeA (1 mM) and because MeA cannot serve as a carbon or nitrogen source in *P. chrysogenum*, this uptake system was identified as an ammonium rather than MeA transporter [11]. Following the identification of an ammonium transport system in *P. chrysogenum*, MeA accumulation assays were used to detect the presence of ammonium transporters in *Escherichia coli* [12], nitrogen fixing-bacterium *Clostridium pasteurianum* [13], *Klebsiella pneumoniae* [13], and in the eukaryotic organism *Saccharomyces cerevisiae* [14], indicating the ubiquitous nature of these transport systems. These studies relied on whole cell transport assays and genes encoding *bona fide* ammonium transport systems remained unidentified for a further 20 years.

Identification of the genes encoding ammonium transporters.

The first genetic evidence of ammonium transporter clusters started with identification of 5 different loci specific to the ammonium uptake mechanism system in *A. nidulans* [15]. Later, Dubois and Grenson demonstrated that *S. cerevisiae* possesses at least two different ammonium transporter systems, named Mep1 and Mep2 (for Methylammonium permease because they were characterised using ^{14}C MeA) [16]. Mep1 and Mep2 were respectively characterised as low capacity/

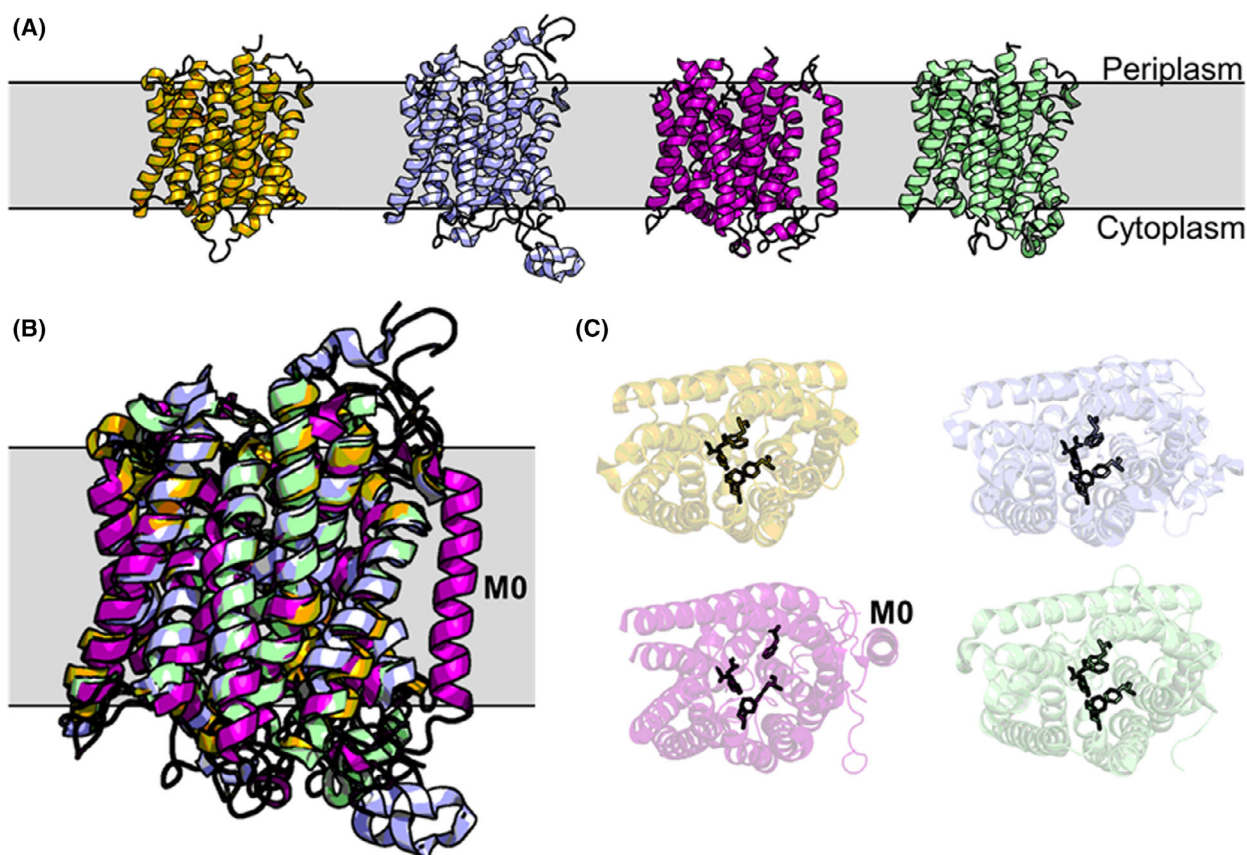


Fig. 1. Structural Conservation of Amt/Mep/Rh protein. A monomer from *E. coli* AmtB (olive), *C. albicans* Mep2 (light blue), *H. sapiens* RhCG (purple) and *A. fulgidus* Amt-1 (green) shown from the side view either alone (A) or aligned (B) as inserted in the membrane. To highlight the conservation of the translocation pathway, the proteins have been oriented with the N-terminus at the top and C-terminus at the bottom and select residues highlighted (C). The additional helix is present in RhCG is denoted as M0. Crystal structures for AmtB, Mep2, RhCG and Amt-1 were obtained from PDB (1u7g, 5af1, 3hd6 and 2b2f, respectively) and aligned in PYMOL 2.5.4. (Schrodinger, LLC, 2010, The PyMOL Molecular Graphics System, Version 2.5, New York, NY, USA).

high affinity and high capacity/low affinity transport systems. Two unlinked genetic mutations *MEP1* and *MEP2* were identified as separately abolishing the two functional activities, proving the existence of two distinct transporters [16]. The *S. cerevisiae* strain 26972c (containing both *MEP1* and *MEP2* mutations) was unable to grow in a medium containing 1 mM of ammonium as the sole nitrogen source. In 1994, Dr. Anne Marie Marini screened a low copy number plasmid library representing the total genome of the wild-type *S. cerevisiae* strain Σ 1278b for plasmids that complemented the growth defect of strain 26972c on media containing 1 mM ammonium as the sole nitrogen source [17]. From this, the authors identified and cloned the *MEP1* gene responsible for the Mep1 transport system [17] and two further *MEP* (*MEP2* and 3) were cloned 3 years later [18]. In parallel, using a cDNA library from *Arabidopsis thaliana* in the

S. cerevisiae strain 26972c, Dr. Olaf Ninnemann cloned the first plant ammonium transporter gene, *AMT1* [19]. A few years later, it was proposed that *GmSAT1* encoded for an ammonium transporter in soybean [20]. However, later work demonstrated that *GmSAT1* could not restore ammonium transport in yeast lacking all Mep proteins, but instead contributed to the regulation of Mep protein expression [21]. In a more recent work, Kaiser's research group acknowledged that *GmSAT1* encodes for a transcription factor [22]. The first bacterial ammonium transporter homologue to be identified via database screening was the *amtA* gene (at that time called *amt*) of *Corynebacterium glutamicum* [23]. Since then, apart from rare exceptions, all bacterial genomes sequenced have been annotated to contain at least one gene encoding an ammonium transporter protein, from either the Amt and/or Rh subfamily.

Identification of mammalian ammonium transporter genes

Using the Bakers Yeast *S. cerevisiae* Mep sequences in protein similarity searches, it was found that the human rhesus (Rh) proteins share 25% sequence identity with the Amt/Mep transporter family [24]. The Rh proteins in mammals form a multimeric protein complex comprising two types of protein: a polypeptide of 30 kDa (named Rh30) and a glycosylated protein of 50 kDa (named Rh50) [25,26]. The average amino-acid sequence identity between Rh30 and Rh50 is 36%. In humans, two Rh30 proteins (RhD and RhCE) and three Rh50 (RhAG, RhBG and RhCG) have been identified [27,28]. RhAG, RhD and RhCE are mainly localised at the membrane of the erythrocytes forming the group antigen [29] while RhBG and RhCG are mainly found in the kidney, liver, central nervous system, testes and intestine [30]. In 2000, human RhAG and RhCG (called RhGC at this time) were reported to restore growth in the *S. cerevisiae* mep knockout mutant, demonstrating that they are capable of ammonium transport activity [31]. From this, it was suggested that the rhesus proteins (Rh) represent a group of functional Amt/Mep orthologs in vertebrates. This was validated by subsequent functional expression analysis that showed that the glycosylated members of the Rh protein family (Rhag, Rhbg, Rhcg) from a range of mammals and fish are functional ammonia transporters [32–36].

Despite being prevalent in vertebrates, Rh homologues were later identified in many other organisms including the green alga *Chlamydomonas reinhardtii*, the worm *Caenorhabditis elegans*, and nitrifying bacterium *Nitrosomonas europaea* [37,38]. Both Rh and Amt genes have been found in the genomes of organisms as diverse as unicellular eukaryotic microbes (e.g. green alga, slime mould and water moulds) and invertebrate animals (e.g. nematodes, arthropods, echinoderms and ascidians) [39].

Evolution of Amt/Mep/Rh genes

Hypotheses for evolution of the Amt/Mep/Rh superfamily

Reconstruction of the phylogeny of the Amt/Mep/Rh proteins discriminates between alternative hypotheses for how they arose and helps us understand how their conserved structures support diverse physiological roles in various organisms. A central feature of Amt, Mep and Rh protein families is that they share a common structural core of transmembrane helices (Fig. 1)

[40–42]. Several evolutionary histories could plausibly have given rise to this outcome, bookended by two extreme hypotheses: a single ancestral family of transmembrane proteins may have given rise to Amt, Mep and Rh through duplication and followed by divergence into distinct protein families in response to diverse selective pressures, leading to the adaptive functions they now fulfil (Fig. 2), or the three Amt, Mep and Rh subfamilies arose independently, performing distinct physiological roles, with the maintenance of a functional transmembrane channel being the result of convergent evolution to a stable transmembrane structure (Fig. 2).

Amt/Mep/Rh subfamily assignment

The history of characterising these transporters has treated the superfamily as three subfamilies: Amt, Mep and Rh proteins. This classification is based on biochemistry, with the Amt and Mep proteins grouped as scavengers of ammonium from the environment, and the Rh proteins considered to translocate ammonium bidirectionally [43,44]. Amts are distributed across all domains of life but are absent from chordates [39], Mep proteins are mainly found in fungi [45], and Rh proteins are mainly found in animals but are also present in some prokaryotes and slime moulds [39,43,46]. Rh proteins are the only ammonium transporters present in chordates. This phylogenetic distribution does not follow the simple pattern of speciation and diversification from a monophyletic origin but is consistent with patterns of horizontal gene transfer, loss and duplication followed by functional diversification [45,46].

Some organisms encode for transporters from two different subfamilies, one scavenging and one regulatory, sometimes in the same cells (see § “Organisms expressing both Amt and Rh”). This raises the question of whether the last universal common ancestor (LUCA) already had both a scavenging and a regulatory protein. Alternatively, the LUCA may have had only a single transporter that later diverged multiple times into distinct functional lineages, to manifest as combinations of scavenger and regulatory proteins we see today.

Biological importance of the Amt/Mep/Rh proteins

The specific functional contexts of Amt/Mep and Rh transporters are as diverse as the organisms that possess them. A plethora of recent studies demonstrate that the structurally conserved transporters of this

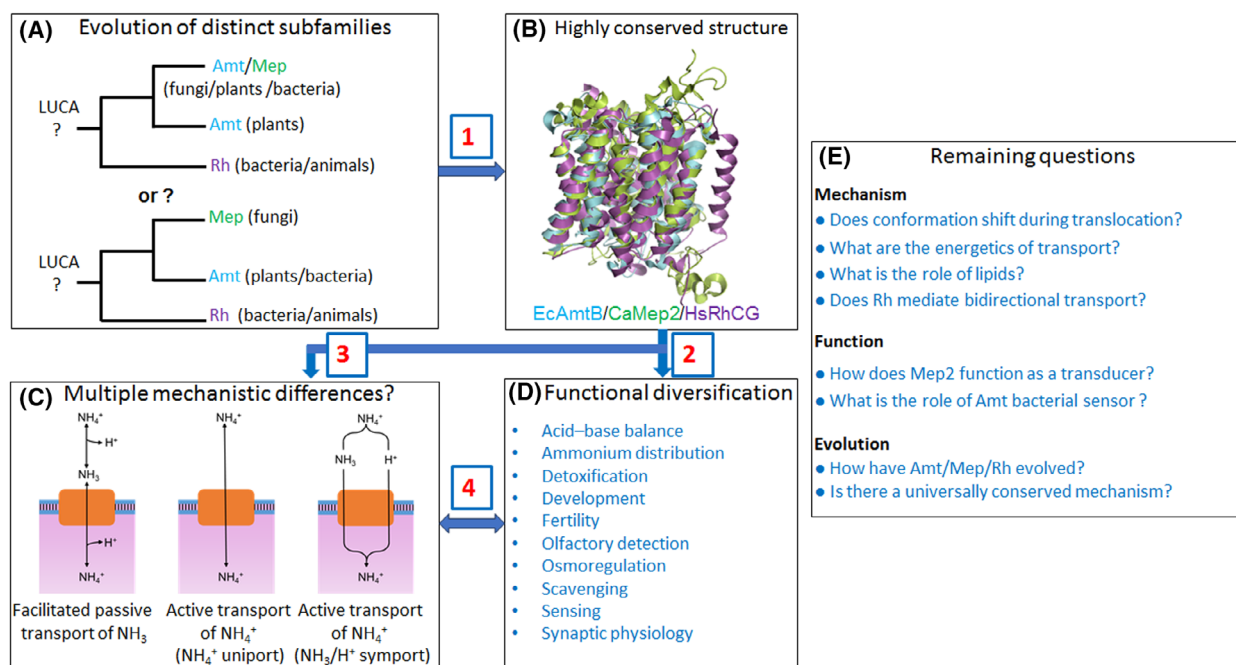


Fig. 2. Schematic analysis of the review which combine the inputs, some of the analysis conducted and the resultant outputs. (A) Reconstruction of the phylogeny of the Amt/Mep/Rh proteins discriminates between alternative hypotheses for how they arose but more comprehensive superfamily tree may support different interpretations of the subfamily. A single ancestral family of transmembrane proteins may have given rise to Amt, Mep and Rh through by divergence into distinct protein families in response to diverse selective pressures, or the three subfamilies arose independently with the maintenance of a functional transmembrane channel being the result of convergent evolution (1). (B) It is still unclear how the highly conserved structure can support (C) various mechanism of transport and (D) distinct functional role in different organisms (2–3). It is possible, as it has been demonstrated for the sensor activity of *Ca. K. stuttgartiensis* Amt5 [55] that a different mechanism, in which the transporter does not complete the full translocation cycle may underpin different physiological function (4). (E) the field continues to evolve and develop, and various pressing questions need to be explored to gain a glimpse into the internal diversity that has allowed ammonium transporters to become so essential to life.

superfamily are involved in various physiological processes. Currently, it is not clear what has driven the diverse functions of these transporters through evolution (Fig. 2).

Ammonium (Amt) transporters

Bacterial Amt

The ubiquity of Amt/Mep/Rh proteins means the biological context of their function is also extremely diverse. Heterotrophic bacteria that proliferate in nutrient-rich environments have access to many nitrogen sources and have evolved the means to utilise them efficiently. As a result, Amt is only required transiently, when ammonium or other nitrogen sources are scarce [47,48]. In many prokaryotes, nitrogen limitation triggers co-transcription of *amt* and *glnK*—a P_{II} signal protein that coordinates nitrogen regulation [49,50]. In this context Amt has two roles; (a) a high affinity scavenger of environmental ammonium; and

(b) part of the larger nitrogen regulation system within the cell [50,51].

This stands in contrast to more specialised organisms, which sacrifice metabolic flexibility in exchange for adapting to a more specific environmental niche. Ammonia oxidising microorganisms are one such group: having evolved to derive energy from oxidation of ammonia [52]. The three groups of aerobic ammonia oxidisers (ammonia-oxidising bacteria, ammonia-oxidising archaea and comammox bacteria) share a common metabolic problem: ammonia uptake is required for assimilation. As a result, ammonia oxidisers depend on constant maintenance of a delicate balance between ammonia uptake, assimilation and oxidation. Despite this common need, the distribution of ammonium transporters through ammonia oxidisers is varied, implying multiple solutions to the same problem. Amt predominates in ammonia-oxidising archaea, with all known species encoding at least two Amt proteins [53]. By contrast, only half of known ammonia-oxidising bacteria encode for an ammonium

transporter, and those that do exclusively express Rh proteins [53]. In comammox, the distribution is split, with members of clade A encoding Rh proteins and clade B encoding Amt proteins [46,54]. It is unclear if this split in distribution is the result of distinct evolutionary histories, or if Amt and Rh confer specific advantages depending on the environment. For example, it's been suggested that Amt contributes to the ability of *Candidatus Nitrosotalea devanattera*, an ammonia-oxidising archaeon, to grow in acidic environments [53].

Amt also appears to contribute to the adaptation of *Candidatus Kuenenia stuttgartiensis*, an anaerobic ammonia oxidiser. Of the seven putative Amt orthologues in this organism, one (Ks-Amt5) was demonstrated to have been repurposed as an ammonium sensor/transducer [55]. Compared to other Amt, Ks-Amt5 has an elongated cytoplasmic domain with sequence similarity to histidine kinases. The protein still selectively binds NH_4^+ , but it does not complete the translocation cycle, instead triggering an increase in histidine kinase activity and subsequent transduction of information to a currently unidentified response regulator [55].

Plant Amt

In plants, ammonium is a signal molecule involved in a variety of physiological functions, including pH homeostasis, regulation of gene expression, leaf chlorosis, oxidative stress and root development (for review, see [56] and references therein). Plant AMTs are split into two sub-families: AMT1 and AMT2. Members of the AMT1 group have been demonstrated to facilitate electrogenic transport in *A. thaliana* and in other plants [57]. In contrast, the AMT2 group have been reported to be electroneutral NH_3 transporters [58].

The Thale cress, *A. thaliana*, encodes six AMTs (AtAMT1;1–6), each of which is expressed differentially. AtAMT1;1 and AtAMT1;3 account for ~60% of the high affinity ammonium uptake into roots and are expressed in the rhizodermis (outermost layer of the root) and in root hairs [59]. AtAMT1;5 is also localised to the rhizodermis but is expressed only in response to nitrogen starvation [60]. Moving deeper into the root tissue, AtAMT1;2 is concentrated in endodermal and cortical cells [60]. AtAMT2;1 (the sole AMT2 representative in *A. thaliana*) is also expressed in the root, but its localisation depends on available nutrients. In nutrient solution lacking any nitrogen source, AtAMT2;1 is close to the root surface and is found within the rhizodermis and cortex, but when supplied with nitrate, its localisation shifts to the

endodermis. In the presence of ammonium, AtAMT2;1 is expressed in the pericycle, a deep region of the root that encircles the vascular tissue [61].

In contrast, AtAMT1;4 is a high affinity transporter restricted to pollen grains and the pollen tube [62,63] (Table 1). Similar differentiation of the AMT1 family has been observed in other plants, including *Lycopersicon esculentum*, *Oryza sativa*, *Pyrus betulaefolia* and *Populus trichocarpa* [64–67]. LeAMT1;1 and LeAMT1;2 are expressed in the roots of *L. esculentum*, while LeAMT1;3 is only expressed in the leaves [64]. In rice, northern blot analysis shows that OsAMT1;1 is constitutively expressed in the shoots, while OsAMT1;2 and OsAMT1;3 are root-specific and express in the presence or absence of ammonium [66]. In *P. betulaefolia*, PbAMT2 is found throughout the plant, while PbAMT3 is restricted to the leaves and is only expressed in response to phytohormones [67] (Table 1). Since then numerous genome wide and transcriptomic studies have confirmed that plant AMTs are differentially expressed in specific tissues (Table 2) [65,68–73].

Hence, in plants, AMTs are involved in the initial acquisition of ammonium from soil, transfer from root-to-shoot, transport in leaves, in the reproductive organs, and also in the root development [74]. However, the role of plant AMTs are not restricted to the acquisition and distribution of ammonium in various tissues.

It has been shown that plant AMTs (and mycorrhizal fungi) play a role in establishing the ecto- and endo-mycorrhizal symbiotic association. First, on the fungal side, it has been proposed that the high-affinity ammonium transporters from the ectomycorrhizal fungi *Hebeloma cylindrosporum*, *Paxillus involutus* and *Tuber borchii* may sense the environment and induce a switch in the mode of fungal growth observed during the formation of mycorrhiza [75–78]. Once the mycorrhizal association is established, the fungi will absorb and metabolise environmental nitrogen and transfer the products (including ammonium) to the plant [79,80]. In this context, it has been shown that specific ammonium transporters from *Sorghum bicolor* [81], *Lotus japonicus* [82], *Glycine max* [83] *Solanum lycopersicum* [84], *Populus tremulax tremuloides* [85] and in *Capsicum annuum* [86] are involved in the transfer of ammonium between the fungi and the plant in the mycorrhiza.

Finally, it has been demonstrated that specific Amt proteins play a role in plant-pathogen interactions. In *A. thaliana*, a T-DNA knockout mutant *amt1.1* displays enhanced resistance against the necrotrophic fungus *Plectosphaerella cucumerina* and reduced sensitivity to the hemibiotrophic bacterium *Pseudomonas syringae* [87]. In wheat, the infection by the fungal pathogen

Table 1. Examples of localisation and physiological relevance of Amt in plants. ND, not detected.

Species	Gene	Role	Localisation	References
<i>Arabidopsis thaliana</i>	AMT1;1	Uptake of ammonium from soil	Root (rhizodermis and root hairs)	[178]
	AMT1;2	Transport of ammonium to root core	Root (endodermis and cortex)	[60]
	AMT1;3	Uptake of ammonium from soil	Root (rhizodermis and root hairs)	[178]
	AMT1;4	Nitrogen nutrition of pollen	Pollen tube and pollen grains	[62,63]
	AMT1;5	Enhance transport under N limitation	Root (rhizodermis)	[60]
	AMT2;1	Shuttle ammonium from root to shoot	Low N: rhizodermis Nitrite: endodermis Ammonium: Pericycle	[61]
<i>Lycopersicon esculentum</i>	AMT1;1	Uptake of ammonium from soil	Root hairs	[64]
	AMT1;2	Uptake of ammonium from soil	Root hairs	
	AMT1;3	NH ₄ ⁺ retrieval from apoplasm	Leaves	
<i>Oryza sativa</i>	AMT1;1	Constitutive uptake of ammonium from soil and transport from root to shoot	Root and Shoot	[179]
	AMT1;2	Uptake of ammonium from soil into root	Root (exodermis, endodermis, and pericycle)	
<i>Pyrus betulaefolia</i>	AMT1;3	Uptake of ammonium from soil into root	Roots	[180]
	AMT1;1	Transport NH ₄ ⁺ to chloroplasts during photorespiration	Leaves	
	AMT2	Transport/assimilation of NH ₄ ⁺ throughout seedling	Throughout plant	
<i>Populus trichocarpa</i>	AMT3	Support N demand during leaf senescence	Leaves	[67]
	AMT1;1	Variable	Root, leaf, fruit, female flower, bud, petiole	[65]
	AMT1;2	Uptake from soil	Root	[65]
	AMT1;3	Uptake from soil	Root, leaf, stem	[181]
	AMT1;4	NH ₄ ⁺ capture during photorespiration	Roots, leaf, stem	[181]
		NH ₄ ⁺ capture during photorespiration		
	AMT1;5	Uptake from soil	Root, leaf, stamen	[65]
		NH ₄ ⁺ capture during photorespiration		
	AMT1;6	NH ₄ ⁺ capture during photorespiration	Leaf and female flower	[65]
	AMT2;1	Uptake of ammonium from soil	Shoot and leaf	[65]
	AMT2;2	Uptake of ammonium from soil	Root, petiole	[65]
	AMT3;1	NH ₄ ⁺ capture during photorespiration	Shoot and senescing leaf	[65]
	AMT4;1	Uptake from soil	Root, leaf, stem	[181]
	AMT4;2	-	ND	[65,181]
	AMT4;3	NH ₄ ⁺ capture during photorespiration	Leaf	[181]
	AMT4;4	Transport of ammonium throughout plant	Stem	[181]
	AMT4;5	Uptake of ammonium from soil	Fruit, root	[65]

Table 2. Genome-wide identification of plant AMT since 2004.

Organism	Number of Amts	Reference/year
Populus	6 AMT1 and 8 AMT2	[65]
Rapeseed	14 AMT1 and 6 AMT2	[71]
Cassava	6 AMT1 and 7 AMT2	[68,69]
Soybean	6 AMT1 and 10 AMT2	[72]
Tea	7 AMT1 and 9 AMT2	[72]
Sugarcane	6 AMT2 divided in 3 clusters	[73]
Apple	15 AMT divided into 4 clusters	[182]

Puccinia striiformis, specifically induces expression of the AMT2-type ammonium transporter gene *TaAMT2;3a* which facilitates the nitrogen uptake from

wheat leaves by *P. striiformis* thereby contribute to the infection of rust fungi [88]. Recently, it has been demonstrated that the ammonium transporter AMT1;1 promotes resistance to Sheath blight in rice via the regulation of diverse metabolic and signalling pathways [89].

Mep transporters

The three Mep isoforms encoded by *S. cerevisiae* allow for precise and adaptable control of ammonium uptake. Mep1-3 differ in terms of affinity Mep1 ($K_m = 5\text{--}10\ \mu\text{M}$) and Mep2 ($K_m = 1\text{--}2\ \mu\text{M}$) are high affinity transporters, while Mep3 is a lower affinity

transporter ($K_m = 1.4\text{--}2.1\text{ mM}$) [18]. As with other Amt/Mep/Rh members, Mep protein expression is regulated in response to nitrogen availability. Specifically, Meps are subject to differential regulation via Gln3p and Ntl1p, two general nitrogen regulatory factors [18]. In addition to their role as ammonium scavengers, Mep proteins trigger the formation of pseudohyphae in some species of filamentous fungi [90]. In *S. cerevisiae*, nitrogen limitation drives the yeast to transition from a unipolar budding phase and form pseudohyphae which explore the environment in search of nutrients [91]. *Mep2Δ S. cerevisiae* lose the ability to make this transition and do not form pseudohyphae, while deletion of *mep1* or *mep3* had no impact on filamentation [90]. In addition, ammonium transport in *mep2Δ S. cerevisiae* was unaffected—implying that Mep1 and Mep3 are sufficient for transport [90]. Thus, Mep2 has been nominated as a transceptor, sensing nitrogen limitation and initiating a signalling cascade that culminates in filamentation [42]. A series of recent studies support a signalling process driven directly by the transport mechanism of *S. cerevisiae* Mep2 transceptors [92–94]. As filamentation is associated with virulence in the human pathogens *C. albicans*, *Cryptococcus neoformans* and *Histoplasma capsulatum* [95–97], thus improved understanding of this process could aid disease treatment.

Remarkably, while both bacterial Ks-Amt5 (see above) and Mep2 have become involved in signalling, Mep2 has retained measurable transporter activity. Hence, Ks-Amt5 and Mep2 have leveraged the AMT protein for a similar purpose, but in different ways.

Rhesus proteins

Over the course of the last two decades, it has been shown that Rh protein are not restricted to humans, but are expressed in a wide variety of animals, slime moulds, and even bacteria where they fulfil different physiological function.

Rhesus (Rh) in bacteria

The chemolithoautotrophic bacterium *Nitrosomonas europaea* gains energy from the oxidation of ammonia to nitrite. Chain *et al.* [37] reported a putative ammonium transporter encoded by the *N. europaea* genome and surmised it mediated passive uptake at low pH. Later, Schmidt *et al.* [98] provided evidence that ammonium accumulation occurred alongside ammonia oxidation and suggested an active transport mechanism. Soon after, it was revealed that the ammonium transporter in *N. europaea* (rh1) belongs to the Rh

subfamily [39]. When expressed in *S. cerevisiae*, rh1 mediated pH-dependent, bidirectional MeA transport [43,44]. When purified and reconstituted in artificial liposome rh1, since renamed to NeRh50, transports ammonium electrogenically (Table 3) [92].

Rh in aquatic animals

Rh in crustaceans

Crabs are ammoniotelic which means they produce large quantities of ammonium through deamination of amino acids. Under these conditions, effective ammonia detoxification systems are essential to control cellular and fluid ammonia levels and maintain cellular functions. Ammonium excretion is a metabolically attractive detoxification strategy as it is less energetically expensive than converting ammonium to less toxic products, such as urea [99]. Hence, both terrestrial and aquatic crabs have evolved to actively excrete ammonium against the electrochemical gradient using their phyllobranchiate gills. The gills, comprised of a single-cell layer of epithelium covered by an ion-selective cuticle, are twice as permeable to ammonia than urea [99,100]. The biochemical basis of ammonium excretion through crab gills is a complex but was well-described phenomenon. Depending on the crab species, various transporters that normally translocate ions such as K^+ or Na^+ (Na^+/K^+ -ATPase, K^+ channels, $Na^+/K^+/2Cl^-$ -co-transporter, Na^+/H^+ exchanger), instead transport NH_4^+ through the basolateral membrane of gills epithelium cells. Here NH_3 diffuses into intracellular vesicles by acidic trapping due to the activity of the V-type H^+ -ATPase. The ammonia-loaded vesicles move through the gills epithelium cells via microtubules towards the apical membrane where vesicles fuse with the external membrane, releasing NH_3 into the subcuticular space. From here, gaseous NH_3 diffuses across the cuticle via amiloride-sensitive cation-permeable channel-like structures [101]. The discovery of Rh protein RhCM in the gills of the common littoral crab *Carcinus maenas* has somewhat completed the picture. The authors hypothesised that RhCM co-localised with the H^+ -ATPase within the intracellular vesicle membranes to support the proposed vesicular acid-trapping mechanisms of NH_3 (Table 3) [101]. In the Dungeness crabs *Metacarcinus magister*, RhMM is highly expressed in the gills and is upregulated in response to high environmental ammonia, indicating a role in ammonium excretion (Table 3) [102]. The Asian Blue Crab (*Portunus trituberculatus*) expresses the Rh protein PtRh in all tissues, but preferentially in the gills. RNAi silencing of PtRh significantly reduced ammonia excretion and altered the

Table 3. Examples of localisation and physiological relevance of Rh proteins^a.

	Species	Gene	Role	Localisation	References
Bacteria	<i>Nitrosomonas europaea</i>	NeRh50	Uptake from the environment	Cytoplasmic membrane	[39,92]
Mosquitos	<i>Aedes albopictus</i>	AaRh50	detoxification of excess ammonia of female adult	Midgut, fat body and Malpighian tubules	[127]
Crab	<i>Limulus polyphemus</i>	LpRh-1	maintenance of haemolymph ammonia	Gill epithelium	[104]
		LpRh-2			
	<i>Metacarcinus magister</i>	RhMM	Vesicular ammonium acid-trapping/excretion	Gills	[102]
	<i>Carcinus maenas</i>	RhCM	Vesicular ammonium acid-trapping/excretion	Gills	[101]
	<i>Portunus trituberculatus</i>	PtRh	ammonia excretion and detoxification	All tissue but enriched in gills	[103]
Fish	<i>Oryzias latipes</i>	Rhcg1	Ammonium excretion and osmoregulation	Gills and yolk sac skin	[111]
	<i>Danio rerio</i>	Rhbg	Ammonium excretion and osmoregulation	yolk sac of larvae/ adults	[34,112]
		Rhcg1		gill and kidney	
		Rhbg		Gills	
		Rhag		Gills/heart/kidney	
		Rhcg2		Gills	
	<i>Cyprinus carpio</i>	Rhcg3	Ammonium excretion	Gills	[109]
		Rhcg-a			
	<i>Oncorhynchus mykiss</i>	Rhcg-b	Ammonia excretion	All tissues	[108]
		Rh30-like 2			
		Rh30-like 3			
		Rhag			
		Rhbg-a			
	<i>Kryptolebias marmoratus</i>	Rhbg-b			[124]
		Rhcg1	Ammonia excretion in excretory tissues gills and skin	Brain, gills, liver and skin	
		Rhcg2		Gills and skin	
		RhBG		various tissues	
		RhCG1		Skin	
	<i>Eptatretus stoutii</i>	RhCG2		Gills	[107]
		Rhag	Potentially ammonium transport and excretion	Gills or liver	
		Rhbg		Gills	
		Rhcg1		Gills	
	<i>Takifugu rubripes</i>	Rhcg2	Ammonium excretion	Gill epithelium	[34]
		Rhag			
		Rhbg			
		Rhcg1			
	<i>Alcalicus grahami</i>	Rhcg2	Ammonium transport	Blood cell	[118]
		Rhbg	Ammonium excretion	Gills	
		Rhcg2			
	<i>Alcolapia alcalica</i>	Rhag	Ammonium transport	Blood cell	[120]
		Rhbg	Ammonium excretion	Gills	
		Rhcg2			
Fish/ amphibious traits	<i>Anablepsoides harti</i>	rhcg1	Excretion of ammonium onto skin when emersed to avoid intoxication	Skin	[125]
	<i>Aplocheilichthys lineatus</i>	rhcg2			
	<i>Cynodonichthys hildebrandi</i>				
	<i>Fundulopanchax gardneri</i>				
	<i>Kryptolebias marmoratus</i>				
	<i>Rivulus cylindraceus</i>				

Table 3. (Continued).

	Species	Gene	Role	Localisation	References
Human	<i>Homo sapiens</i>	RhAG	Sequestration of NH ₃ into erythrocytes for transport to detoxification organs	Erythrocytes	[183,184]
		RhBG	Renal transepithelial ammonia transport.	Liver, kidney, skin	[35,185,186]
		RhCG	Maintain acid–base homeostasis	Brain, kidney, testes	[187,188]
Mouse	<i>Mus musculus</i>	Rhag	Sequestration of NH ₃ into erythrocytes for transport to detoxification organs	Erythrocytes	[183,189]
		Rhbg	Renal transepithelial ammonia transport.	Liver, kidney, skin	[33,185]
		Rhcg	Maintain acid–base homeostasis	Brain, kidney, testes	

^aCell left blank when unknown.

expression of the Na⁺/K⁺-ATPase, K⁺ channels, Na⁺/K⁺/2Cl-co-transporter, Na⁺/H⁺ exchanger and V-type H⁺-ATPase genes which are involved in ammonia excretion. This indicates the PtRh protein is a primary contributor to ammonia excretion (Table 3) [103]. Further studies confirmed this role in the American horseshoe crab (*Limulus polyphemus*) which expresses two Rh protein isoforms (LpRh1 and LpRh2) in high abundance in gills tissues [104].

Rh in fish

Like crustaceans, most free-living fish are ammonotelic (ammonia secreting). After feeding, proteins are broken down in the gut, producing amino acids, mostly glutamate, which are further catabolised in the liver. Within the mitochondria of liver cells, amino acids are deaminated to produce ammonium, which must permeate the mitochondrial membranes prior to entering the bloodstream. This increases the ammonium concentration in blood to toxic levels, and thus, the ammonium needs to be excreted efficiently. As with crabs, fish use their gills as the primary site of NH₃ excretion down a favourable blood-to-water diffusion gradient and the Rh protein are critical to this process [105,106].

Rh in free-living fish—In saltwater fish, the role of Rh in NH₄⁺ transport was first hypothesised in 2007 by Nakada *et al.* [34]. The saltwater Japanese puffer fish *Takifugu rubripes* expresses four Rh proteins, fRhag, fRhbg, fRhcg1 and fRhcg2, all localised to specific regions in the gills epithelium, and all display ammonium transport when expressed in yeast. Based on the

Rh localisation Nakada *et al.* [34] proposed a preliminary model in which fRhag in the pillar cells functioned in cooperation with basolateral fRhbg and apical fRhcg2 in the gills pavement cells to facilitate ammonia efflux out of the gills, challenging the classic view of the passive ammonia diffusion through the gills into sea water (Table 3). Since then, the expression of Rh protein in the gills of saltwater fish have been reported in Hagfish. The Hagfish *Eptatretus stoutii* expresses three Rh orthologs, Rhag, Rhbg and Rhcg1 that may participate in excretion of ammonia across the gills in a similar fashion [107].

The Rh protein seems to play a similar role in ammonium translocation in freshwater fish. Soon after seven Rh proteins (Rh30-like 2, Rh30-like 3, Rhag, Rhbg-a, Rhbg-b, Rhcg1-a and Rhcg1-b) were identified in the rainbow trout *Oncorhynchus mykiss*. Rhbg and Rhcg1 and Rhcg2 were overexpressed in the gills in trout challenged with high external ammonia indicating that Rh glycoproteins may enhanced ammonia excretion [108]. The same results were observed in the common carp (*Cyprinus carpio*) which expresses two Rh proteins, (Rhcg-a and Rhcg-b) in the gills (Table 3) [109].

Freshwater fish need opposite ion transport mechanisms in their osmoregulatory organs compared to their saltwater counterparts, as the direction of the Na⁺ gradients and transepithelial potential in the latter are reversed. This means that fish are hyperosmotic to freshwater and hypoosmotic to saltwater. Hence to regulate their osmolarities, freshwater fish extract NaCl from the environment in their gills, while marine fish ingest seawater, absorb intestinal water by absorbing NaCl, and excrete the excess salt via gill transport mechanisms [110]. The mechanism of absorption of

Na in freshwater fish has been debated but it seems that Rh proteins may play a role in the process. The freshwater fish zebrafish *Danio rerio* expresses five Rh-related genes (*Rhag*, *Rhbg*, *Rhcg1*, *Rhcg2* and *Rhcg3*) (Table 3) [34]. The Japanese rice fish (*Oryzias latipes*) expresses two Rh, (*Rhbg* and *Rhcg1*). In both species, *Rhcg1* is expressed in gills and a new model for “NH₄⁺-dependent Na⁺ uptake” in mitochondrion-rich cells (MRC) in gills has been proposed. In this model, *Rhcg1* first binds and deprotonates NH₄⁺, the NH₃ is then excreted, leaving the proton in the MRC. This creates a proton gradient across the MRC membrane that an Na⁺/H⁺ exchanger use to import Na⁺ into the cells. Hence the Rh proteins are not only associated with ammonium homeostasis but also osmoregulation (Table 3) [111,112].

Rh in larval fish—While gills are the primary site of ammonia excretion in adult fish, they are generally not functional in larval fish immediately after hatching. In the larval life stage, many ammonotelic fish species rely on catabolism of yolk amino acid stores for energy [113]. This process also produces a large metabolic ammonia load, which the larvae must excrete to avoid overaccumulation. For a while, it was thought that fish embryos, larvae and juveniles were mainly ureotelic: using a functional ornithine urea cycle to produce urea from nitrogenous waste culminating in excretion of urea, not ammonium. However, it has since been found that Rh genes are expressed in the skin of larval freshwater zebrafish (Table 3) [114]. Numerous studies now show that, in larvae, ammonia excretion is facilitated by Rh protein expressed in the skin and that this role shifts to the gills as the fish matures [115].

The exception – *Alcolapia*—Fish of the genus *Alcolapia* represent an interesting case study with regards to ammonium transport. *Alcolapia* are found exclusively in the East African soda lakes Magadi and Natron. These lakes feature high salinity, temperatures between 30 and 42 °C, and pH between 9 and 12. In contrast to most physiological systems, this pH range favours NH₃ over NH₄⁺. As a result, sustaining NH₃ excretion from the gills is more challenging than in typical aquatic systems. Unsurprisingly, it was found that *Alcalicus grahami* (Magadi tilapia), the only fish living in Lake Magadi, was strictly ureotelic and excreted urea via the ornithine–urea cycle in the liver [116]. This allows *A. grahami* to circumvent the challenge of ammonia excretion at the gills and seemed to be a general adaptation of *Alcolapia* species to their

environment over the past 10 000 years, during which they diverged from their ancestors, to cope with the high external pH [117]. Therefore, it came as a surprise when in 2013 it was discovered that *A. grahami* expresses three Rh proteins: *Rhbg* and *Rhcg2* in gills and *Rhag* in gills and red blood cells. Exposure to high levels of ammonium upregulated both *Rhbg* and *Rhcg2* in gills, leading the author to hypothesis that the branchial Rh proteins facilitated ammonia efflux in response to ammonium stress [118]. *A. grahami* can encounter such stress as bacterial degradation of large amounts of flamingo guano in the lake that leads to very high local ammonia concentrations [119]. A recent study shows that after generations in the same aquarium under conditions designed to favour ammonotelism, *A. grahami* remains fully ureotelic while the closely related species *Alcolapia alcalica* started to excrete some nitrogenous waste as ammonia [120]. The authors explored Rh expression after this adaptation and measured that *Rhbg* and *Rhcg2* expression in the gills was much higher in *A. alcalica* than in *A. grahami*. Even after generations in non-extreme conditions, there is no recovery of *Rhbg/cg* expression in *A. grahami* which remains fully ureotelic. The authors speculate that a very strong transcriptional silencer is present in *A. grahami* and that it is an adaptation of the more hostile environment in the lake Magadi (where *A. grahami* live) compared to lake Natron (where *A. alcalica* live), with higher temperatures and salinity and lower flow rates [120].

Rh in fish that exhibit amphibious traits—The majority of free-living fish excrete ammonia, with some examples of urea excretion, from blood to water across the gills to evacuate nitrogenous metabolic waste products. Several genera of fish exhibit transitional lifestyles where they can spend significant periods of time in the terrestrial environment. While exposed to open air, gills cannot function normally as there is no water flow to excrete to. This is similar to problems faced during one of the greatest events in tetrapod evolution: the transition from an aquatic to a terrestrial habitat.

The mangrove killifish *Kryptolebias marmoratus* live along the coasts of Florida through to northern South America. *K. marmoratus* can tolerate prolonged periods of emersion (> 1 month), and spends this time living amongst moist leaf litter, detritus, or in hollowed logs [121]. It was known that many air-tolerant fish shift from ammonotelism to ureotelism during air-exposure [122]. Surprisingly, *K. marmoratus* does not undergo a shift towards ureotelism when emersed, instead continuing to release ammonia by NH₃

volatilisation [121]. In 2006, an 18-fold increase in ammonium concentration was measured on the cutaneous surface of *K. marmoratus* after 11 days of emergence [123]. The authors proposed that the site of ammonia excretion in emerged fish switched from primarily branchial to primarily renal and/or cutaneous by an unknown mechanism. In 2007, it has been shown that *K. marmoratus* expresses 3 Rh proteins: *RhBG*, *RhCG1* and *RhCG2*. Under normal conditions, all three were highly expressed in gills and only *RhBG* was also expressed in skin. After exposure to high environmental ammonia in water, the expression of *RhBG* was induced in many tissues (brain, eye, gonad, gut, kidney, liver and skeletal muscle), but surprisingly not in gills and skin. *RhCG1* expression extended to the skin. During high ammonia exposure, *RhCG1* and *RhCG2* appear to demonstrate tissue specificity with *RhCG1* and *RhCG2* mRNA expression levels dominating skin and gill *Rh* respectively. When exposed to air for 24 h *K. marmoratus* upregulate *RhCG1* and *RhCG2* by four- to six-fold in the skin. Taken together, these data indicate that the Rh proteins may be involved in the transport of ammonia across the epidermis for subsequent NH_3 volatilisation, as well as in aquatic ammonia excretion (Table 3) [124]. In order to determine if this strategy of NH_3 volatilisation via the skin when exposed to air is conserved, further studies extended to six phylogenetically diverse killifish; *Anablepsoides hartii*, *Cynodonichthys hildebrandi*, *Rivulus cylindraceus*, *Kryptolebias marmoratus*, *Fundulopanchax gardneri* and *Aplocheilichthys lineatus* [125]. All six species express *Rhcg1* and *Rhcg2* proteins in the skin and excrete ammonium by volatilisation when emerged (Table 3) [125]. The conservation of this mechanism could be the result of common ancestry, convergent evolution, or a combination of both. However, we have seen that even aquatic fish express Rh protein in the cuticle and already excrete ammonium via the skin while in the larval stage (see in “Rh in larval fish”). Almost all fish use Rh protein to excrete ammonia via the gills; hence, it would be surprising if cutaneous ammonia excretion initially evolve as an adaptation to terrestrial life. The more plausible hypothesis is that the first fish to explore land exploited an existing mechanism.

Rh in terrestrial animals

Rh in mosquitos—Female mosquitoes can deaminate more than 80% of the amino acids ingested from a blood meal, which produces toxic levels of systemic ammonia that needs to be detoxified [126]. In the tiger

mosquito (*Aedes albopictus*), the Rh gene *Aa/Rh50* is upregulated in midgut, fat body, and Malpighian tubules after a blood meal, indicating that *Aa/Rh50* plays an important role in detoxification of excess systemic ammonia during the gonotrophic cycle (Table 3) [127].

Rh in humans—In humans, interest in the Rh protein family was initially centred on their role as blood antigens and related involvement in haemolytic disease in the foetus and newborn [128]. Since then, it has become clear that Rh-mediated ammonium transport is essential in detoxification of erythrocytes, maintaining pH balance, and reabsorption of ammonium through the renal tubule epithelial cells [129,130]. Consequently, mutations in Rh are associated with numerous pathologies.

Genetic mutations of RhAG were found to cause Rh deficiency syndrome (Rh_{null} or Rh_{nod}) characterised by the lack of Rh antigens on red blood cells [25,131], or dominant Overhydrated Stomatocytosis (OHSt), a rare hereditary haemolytic anaemia, characterised by uncontrolled entry of monovalent cations (K^+ and Na^+) into erythrocytes [132,133]. In addition, a human RhAG was also linked to a subtype of migraine (Table 3) [134].

The physiological importance of non-erythroid RhBG and RhCG proteins, also became more evident in $\text{NH}_3/\text{NH}_4^+$ handling. Studies on *Rhbg* knockout (KO) mice demonstrated lowered urinary ammonium excretion, while HCl-induced acidosis increased RhBG protein expression in healthy mice [135]. Studies on *Rhcg* KO mice demonstrated impaired urinary NH_4^+ excretion when exposed to increased acid loads. It was noted that both, apical permeability to NH_3 and trans-epithelial $\text{NH}_3/\text{NH}_4^+$ transport, were reduced [136]. In mice, mutations in RhCG disrupt acid–base homeostasis, which has been linked to male infertility and distal Renal Tubular Acidosis [136], which can lead to development of kidney stones, and, in extreme cases, renal failure [137]. In humans, a genome-wide significant linkage analysis identifies the chromosome region 15q25.3–26.2 as the greatest linkage evidence to recurrent early-onset major depressive disorder. Further analysis of 176 cases and 176 control subjects identified RhCG as a candidate gene for early-onset major depressive disorder [138]. Finally, *rhbg* and *rhcg* genes were proposed to act as potential tumour suppressors, by inducing sharp down-regulation in human oesophageal squamous epithelial cancers [139] and mouse brain tumours [140].

Taken together it is clear that Rh proteins are employed for distinct strategies compared to

AMT/Mep in other organisms. However, it is unclear what permits this functional differentiation of Rh compared to other transporters. This is, in part, due to the lack of a nuanced understanding of the mechanism of Rh-mediated ammonium transport. To fully appreciate the extensive fundamental roles of Rh proteins in vertebrates, as well as remedy the significant biomedical consequences of their malfunction, it is essential this knowledge gap is filled.

Organisms possessing both Amt and Rh

Protist, *Dictyostelium discoideum*

The slime mould *Dictyostelium discoideum* colonise the leaf litter of temperate forests. In normal conditions, *D. discoideum* remains in a unicellular state and feeds on nearby bacteria and small microbial eukarya. When food becomes scarce, individual *D. discoideum* aggregate to form a multicellular “slug”, which follows environmental cues to migrate in search of food. Eventually, the slug will initiate terminal differentiation, culminating in the formation of a stalk supporting a sorus filled with spores, which are dispersed by wind [141,142]. Cells located at the anterior part of aggregates differentiate into pre-stalk cells, while the rest of the aggregates become pre-spore cells [142]. Environmental ammonium is one of the key signalling molecules in *D. discoideum* development: high environmental ammonium leads to slug migration whereas low ammonium promotes tip formation [143]. *D. discoideum* expresses three Amt (AmtA–C) and 2 Rh (RhA and B). AmtA is expressed in pre-stalk cells, AmtB in pre-spore cells, AmtC in pre-spore cells as well as at the tip of fingers and in the slug (Table 4) [144]. *amtA*[−] cells produced many small fruiting bodies even in the absence of exogenous ammonium and the germination of *amtA*[−] spores was compromised [145]. Cells *amtC*[−] are locked into their slug conformation, indicating that the cells perceived continual high local concentrations of ammonia [144]. Interestingly the *amtC*[−] phenotype can be rescued by deleting *amtA* suggesting that AmtC and AmtA play important but distinct roles in the developmental processes in *Dictyostelium* [146].

Amoebae and protozoa, including *Dictyostelium*, have developed a contractile vacuole (CV) to act as an osmoregulatory organelle, which enables them to adapt to osmotic shocks and survive their natural environment [147]. The RhA protein is amongst the few proteins strictly localised to the *D. discoideum* CV and it has been shown that C-terminal of RhA is extended to include a peptide signal that can localise chimeric protein in the CV; however, no osmotic related

phenotype is observed in *rhA*[−] cells and the role of Rh is still not understood [148,149].

Nematodes

Caenorhabditis elegans is a saprophytic soil ammoniotelic nematode that feeds continuously on bacteria [150,151]. *C. elegans* express at least 4 Amts and 2 Rh (CeRhr-1 and CeRhr-2) (Table 4). CeRhr-1 is predominantly expressed in the hypodermis [152]. Starvation is associated with a massive reduction of ammonium excretion and downregulation of CeRhr-2 whereas the expression levels of CeRhr-1 remained unchanged. Expression in a triple *mepΔ S. cerevisiae* strain confirmed that CeRhr-1 is capable to transport ammonium. Hence the authors suggest that CeRhr-1 is a housekeeping protein excreting ammonium during normal physiological fluctuations via the hypodermis. Future studies must confirm the cellular localisation of CeRhr-2 in the hypodermis and its ammonia transport capabilities [152]. The only information on *C. elegans* Amts come from a genetic screen that identify a group of genes, including Amt-2, that potentially function in a nutrient-sensing pathway to regulate the lifespan of *C. elegans* [153].

Aquatic animals

Ammonia can reach toxic levels in various aquatic habitats, including freshwater, seawater, and the water film surrounding soil particles. Invertebrates living in these environments use ammonia excretion as a strategy to facilitate their survival [154]. Hence, some crustaceans express both Amt and Rh proteins in the same cell as part of their survival strategies in hostile environments.

The razor clam *Sinonovacula constricta* expresses both ScAMT1 and ScRh protein. Silencing of ScAMT1 increases the ammonium concentration in the haemolymph [155] and it was previously shown that expression of ScRh in gills increases during ammonium stress, suggesting a role for both proteins in ammonium excretion (Table 4) [156]. In addition, silencing of ScAMT1 significantly increases the expression of ScRh, indicating a potential synergy between the activity of both proteins for optimum response to the stress [155].

Terrestrial animals

Insects, including vectors of disease, detect their hosts or food by sensing ammonium present in animal/human breath and sweat or decaying organic matter [157]. Hence numerous insects express both Amt and

Table 4. Examples of localisation and physiological relevance of Amt and Rh in the same organism.

	Species	Gene	Role	Localisation	References
Amoeba	<i>Dictyostelium discoideum</i>	AmtA	Developmental/ammonium transport	Pre-stalk cells	[144,145]
		AmtB		Pre-spore cells	[144]
		AmtC	Developmental/ammonium sensing	Pre-spore cell, stalk cells and slug	[144,146]
		RhA		Contractile vacuole	[148]
		RhB			
Insect	<i>Drosophila melanogaster</i>	<i>Amt</i>	Ammonium olfactory receptor	coeloconic sensilla	[158]
		<i>Rh50A</i>	Maintenance of muscle architecture/synaptic physiology	Larvae muscles	[160]
		<i>RhBC</i>			
	<i>Anopheles gambiae</i>	AgAmt	Ammonium chemosensing	Whole bodies/enriched in antennae	[190]
		AgRh50a	ammonia clearance in the head and body	Whole bodies	[161]
		AgRh50b	Ammonium chemosensing	Whole bodies/enriched in antennae	
	<i>Aedes aegypti</i>	<i>Rh50-1</i>	Ammonia trapping prior to excretion in urine	Malpighian tubules	[163,165]
		<i>Rh50-2</i>			
		<i>Amt1</i>	Secretion of NH_4^+ from anal sac Protect sperm	Anal papillae/sperm flagella	
		<i>Amt2</i>	Secretion of NH_4^+ from anal sac	Anal papillae	
Mollusc	<i>Sinonovacula constricta</i>	<i>ScAmt1</i>	Ammonium excretion	Gills	[155,156]
Nematode	<i>Caenorhabditis elegans</i>	<i>Rhr-1</i>	Ammonia excretion	Epidermis	[151]
		<i>Rhr-2</i>	Ammonia excretion	Hypodermis	[191]
		Multiple			
		<i>Amts</i>			

Rh proteins in the same cell as part of ammonium sensing and signalling to find a host, food, or a mate. *Drosophila melanogaster* is one such species, with both Amt and Rh documented [158–160]. An RNA-Seq screen of *Drosophila* antennae revealed the expression of an Amt in the neighbouring auxiliary cells of the olfactory receptor neurons contained within the coeloconic sensilla organ (Table 4) [158]. Electrophysiological analysis of ammonium neuronal response in coeloconic sensillum in wild-type versus mutant flies in which a transposon was inserted into the coding region of *Amt* reveal that Amt is essential for ammonia olfactory detection. This indicates that Amt in *D. melanogaster* gained a specific function acting as a non-canonical olfactory receptor (Table 4) [158,159]. The defective response observed in a *Drosophila Amt1* mutant is rescued by ectopic expression of the mosquito *Anopheles gambiae* AgAmt ortholog. Orthologs of AgAmt are found in virtually all insect species examined, suggesting that the role of insect Amt as olfactory receptors is conserved across insect species [158,159]. *D. melanogaster* also encodes two Rh50

isoforms, Rh50A and Rh50BC, expressed in larval muscles and enriched in the postsynaptic regions of the glutamatergic neuromuscular junctions (Table 4) [160]. The inactivation of Rh50A by RNAi led to muscular atrophy in larval stages and pupal lethality. Electrophysiological analysis shows that inactivation of both genes modifies the synaptic pH homeostasis and triggers an increase in the frequency of spontaneous excitatory postsynaptic potentials. Taken together these data show that both Rh50A and Rh50BC protein in *Drosophila* are important for muscle architecture maintenance and synaptic physiology [160].

The afrotropical malaria vector mosquito, *Anopheles gambiae* encodes for two ammonium transporters, AgAmt and AgRh50 (*AgRh50* encode two spliced transcripts, *AgRh50a* and *AgRh50b*; Table 4) [161]. Yeast complementation assays confirmed that all three proteins can transport ammonium. Interestingly, while the *AgRh50a* transcript was evenly distributed throughout the whole body, *AgAmt* and *AgRh50b* transcripts were highly enriched in antennae (Table 4). This suggests that a more critical role for *AgRh50a* in ammonia

clearance from the head and body, while *AgAmt* and the *AgRh50b* play a more specialised role in ammonia sensitivity of *A. gambiae* antennae, either by clearing ammonia from the sensillar lymph or by facilitating sensory neuron responses to environmental exposure [161]. Electrophysiological analysis of *AgAmt* and *AgRh50* expressed in *Xenopus* oocytes suggests that the activity of the former is electrogenic whereas it is electroneutral for the latter which also hints at distinct functionality for the two proteins. This also raises the possibility that the energetics of the translocation cycle differs between the proteins [161]. If so, it's possible that the different energetics of transport may underpin the specialised physiological functions of *AgAmt*, *AgRh50a* and *AgRh50b*.

The mosquito *Aedes aegypti*, the primary vector for human arboviral diseases including Zika, yellow fever, chikungunya and dengue virus expresses two Amt (*AeAmt1* and *AeAmt2*) and two Rh protein, *AeRh50-1*, and *AeRh50-2* [162]. In 2019 it was discovered that the larvae of *A. aegypti* upregulate expression of *Rh50-1*, and *Rh50-2* within a “physiological triad” of organs to efficiently excrete ammonium against a steep concentration gradient [163]. An attractive hypothesis would be that Amts and Rhs can functionally substitute for one another to ensure optimal ammonium excretion in its natural habitat. However, silencing of *AeRh50-1* alone was sufficient to decrease ammonium excretion whereas knockdown of *AeRh50-2* had no effect [164] and *AeAmt2* knockdown results in a significant decrease in ammonium efflux from the anal papillae [162]. Taken together these results indicate that both proteins cannot substitute for each other, and thus likely have separate functional roles.

More recently a completely new role for a mosquito Amt protein has been discovered. *AeAmt1* is highly expressed in spermatozoa flagella throughout development in males, insemination of females, and subsequent storage within spermathecae [165]. When *AeAmt1*-knockdown-males mate with WT females, the authors observed a significant reduction in the number of eggs laid and the percentage of those eggs that were viable. The authors concluded that *AeAmt1* is essential to protect the sperm from ammonia toxicity as they navigate the reproductive tract and speculate that this function could be conserved at least within the insect taxa [165].

Divergent functions, but highly conserved structure

Despite extensive sequence and functional diversity, the structure, and functional residues within the

superfamily are well conserved (Fig. 2). For example, a structural alignment across families between *N. europaea* Rh protein NeRh50 and *E. coli* Amt EcAmtB yields a root mean square deviation (rmsd) of 2.2 Å across 322 Cα (Fig. 1) [166]. Many organisms can code for both Rh and Amt, or Amt and Mep proteins [39,167]. Alignment of 17 pairs of Rh and Amt protein sequences from the same organism gave a mean pairwise amino acid identity score of 14% between the two [39]. This pattern of conservation could be consistent either with descent from a common ancestor of a highly adaptable single solution to ammonium transport, or with convergence onto a similar highly effective solution from more than one origin. However functional residues are found in the same sequential order and structural position in each transporter subfamily (Mep, Amt and Rh; Fig. 1; [40,41,55,166,168]). This contrasts with, for example, serine protease families that evolved to converge on a common catalytic triad (Ser-His-Asp). In these enzymes, the functional residues are in different sequence order and structural locations depending on the family [169]. This distinction implies that the transporter superfamily may be likely to share a single common origin.

Phylogenetic analysis

The focus of studies investigating the evolution of this family is usually on a single subfamily rather than the combined superfamily.

Amt phylogeny

Amts have been investigated with a focus on plants rather than bacteria [65,167]. Couturier *et al.* [65], focus on deducing the phylogenetics of the poplar tree's 14 Amts. This study aligned protein sequences and used neighbour joining to generate a tree for higher plant sequences. The ammonium transporters could be divided into two distinct groups, Amt1 and Amt2. These groups are postulated to have different mechanisms of ammonium transport and all plants studied so far have representatives from both groups [65].

McDonald and Ward [167] investigated the evolution of electrogenic transport, focusing on plant Amts. The maximum likelihood tree produced by manual alignment of nucleotide sequences was rooted with Rh proteins. The topology showed a basal Mep grade containing fungal, bacterial, archaeal and plant proteins. Eukaryotic Amts were divided into three clades. Amt clade 1 contained exclusively plants. Amt clade 2

contained mainly animal proteins from marine creatures with a handful of plants, the most basal being the algae *Asterochloris*. Amt clade 3 is broadly similar to Amt clade 2 in that there are animal sequences with some basal plants. Since, at the time, the only electrogenic transporters found had been from plants [57,59,170], the authors speculated that all sequences in the Amt clades were electrogenic [167].

Mep phylogeny

The Mep proteins have not been extensively studied. Only one paper fully investigates their origins [45]. Of the four ammonium transporting proteins found in the genome of the fungal partner in the lichen *Cladonia grayi*, two were more closely related to plant ammonium transporters, rather than those of the green algal symbiont of the lichen. The researchers attempted to deduce the origin of these plant-like proteins. Using a manual alignment of 513 nucleotide sequences containing a mix of Amt, Mep and Rh proteins, a maximum likelihood (ML) tree was generated. The tree was rooted with the Rh proteins and displayed a topology very similar to that constructed from electrogenic Amt_s. In this tree all proteins basal of the eukaryotic Amt_s were given the label Mep. These Meps were divided into four groups: the Mep grade (not monophyletic), Mep α , Mep β and Mep γ . The Mep grade contained bacterial sequences and was most basal in the tree. Mep α has putative archaeal origins and contained the plant-like fungal ammonium transporters. In this group were also the plant proteins along with bacterial and archaeal transporters. Consequently, the authors suggest separate HGT events for fungi and plants, dated at 800–700 mya and 900–450 mya respectively. Mep β is mostly bacterial with some eukaryotic organisms also present. Mep γ contains most of the fungal ammonium transporting proteins. The acquisition of Mep γ through HGT and subsequent functional displacement of the eukaryotic Amt_s found in animals and plants was suggested to be a defining trait of fungal evolution. The members of Mep γ also appear to have undergone multiple duplications suggesting an ancient HGT event. The more basal, non-monophyletic group of bacteria, called here the Mep grade by McDonald *et al.* [45] has also been referred to as part of a larger Amt family with the Mep α being a subfamily of it [161].

Current published literature clusters Mep proteins with Amt_s, suggesting a more recent common ancestor than to Rh. This has led to differences in the nomenclature with some researchers referring to bacterial Amt_s as Mep proteins [45,167] and others saying that

Mepα are a small subset of Amtα and may not even exist as a separate group [161]. Depending on the nomenclature used, some plants such as *A. thaliana* and *O. sativa*, and the fungal partner of *C. grayii*, may be said to code for both Amt and Mep proteins, only for Amtα (plants), or only for Mepα (fungi) [167].

Investigations into two Mep proteins isolated from *Aspergillus nidulans*, MeaA (likely homologue of Mep1) and MepA (likely homologue of Mep2) found that MepA may either not transport methylammonium or be capable of bidirectional transport as $\Delta meaA$ strains gained methylammonium resistance [171]. Phylogenetic analysis of these proteins found that they clustered with other Mep proteins and that the Mep proteins seemed to be themselves a sub-cluster branching off from the Amt group in the tree. A group of Rh proteins from *C. elegans* and the mammals *H. sapiens*, *Macaca mulatta* (the Rhesus macaque) and *Mus musculus* clustered together separately from Amt and Mep [171].

Rh phylogeny

Due to their clinical relevance, the evolution of the Rh group has had more focus [26,39,43,172,173]. Organisms ranging from prokaryotes to humans possess *rh* genes. While initially none were found in vascular plants and archaea, sequences have been found in Archaea that encode for only Rh proteins and sometimes both Rh and Amt, for example in *Methanomas-siliicoccus luminyensis* [46]. This, along with the finding that some other organisms including unicellular eukaryotic microorganisms and invertebrates express both Rh and Amt proteins (Table 4), suggests that Amt and Rh have co-existed over a long evolutionary period.

More detailed examination of Rh evolutionary relationships identified four distinct clusters: non-transporting Rh30 (RhD and RhCE), transporting Rh50 (RhAG, RhBG and RHCG) in vertebrates, and two ancestral clusters—Rhp2 in non-mammalian vertebrates and Rhp1 in microorganisms and invertebrates [39]. Rhp2 proteins are found more basally in the tree than Rhp1 suggesting invertebrates have lost the Rhp2 proteins. It was noted that all members of the Rh30 cluster have undergone greater change since the last common ancestor suggesting they may have diverged for a tissue-specific functional modification, at the same time losing their transporting abilities. This is postulated to have happened early in Teleost fish evolution owing to absence of Rh30 in all species ancestral to the zebra fish [39]. Using a number of evolutionary rate estimate methods, the date for

divergence was calculated at approximately 346–250 mya [26,174]. Rh50 genes accumulate nonsynonymous substitutions 2.6x more slowly than Rh30 genes [26,39] and therefore are likely to have arisen prior to Rh30. This split could have potentially been derived through duplication of an Rh50-like ancestor. Rhp1 is the most species-diverse cluster and its members are found in organisms expressing both the Rh and Amt proteins. Species of this group can have up to three Rh genes, thought to have arisen from gene duplication events [39].

Future investigations into the evolution of the Amt/Mep/Rh superfamily as a whole would benefit from the increase in the number of publicly available proteins belonging to this group. The increase in sequence numbers and diversity may lead to new insights into the history of this family.

Conclusion

The Amt/Mep/Rh protein family is remarkable in that they are conserved from bacteria to humans, and despite low sequence similarity they display high structural conservation. In this review, we described the diverse functional roles gained by ammonium transporters and discussed how the family evolved and spread to fulfil these roles. In doing so, we hope to have demonstrated that despite sharing a simple structure, ammonium transporters do much more than simple transport of ammonium across biological membranes. These proteins are essential for mitigating ammonia toxicity, supporting osmoregulation, maintaining acid–base balance, enabling sensory structures to “taste” ammonia, and even boost sperm survival and overall male fertility (Fig. 2).

Given the structural similarity of individual members of the Amt/Mep/Rh family, the mechanical basis of these different functions is unclear. It is tempting to hypothesise that variations in the energetics of translocation may, at least in part, explain the different physiological functions of these proteins. We have recently reviewed and discussed in depth this fascinating aspect of the proteins which is poorly understood and warrants its own discussion [175].

Future perspectives

To advance the field of Amt/Mep/Rh family biology, future work may benefit from considering a superfamily-wide perspective for studying the phylogeny of these transporters. Previously, Rhesus proteins have been used as outgroups phylogenetic trees made to investigate the whole superfamily [45,167]. This

approach implicitly assumes that there was early rapid divergence of Rh towards functions other than scavenging, and so they were the first subfamily to diverge. However Rh is well-represented in vertebrates, but rare in prokaryotes, and difference of function is not a definitive indicator of more ancient divergence as exemplified by the rapid evolution of Rh30 from Rh50 [26]. As discussed above this leaves inconsistencies in the distribution of organisms within the phylogeny, explained until now with repeated occurrences of HGT (Fig. 2). A more comprehensive superfamily tree may support different interpretations of the evolution of Amt, Mep and Rh.

The dynamics of these proteins during the translocation cycle has been largely ignored. To date, all the structures of Amt/Mep/Rh proteins are very similar when generated in the presence or absence of ammonium and show the same inward-facing state of the protein. There are no significant differences in the crystal structures of Amt, Mep and Rh proteins that can clearly account for functional differences. It is therefore essential to develop an alternative approach to protein crystallography to observe different conformations and obtain more structural dynamic information.

Using a newly developed mass spectrometry approach, it has been revealed that the ability of EcAmtB to resist unfolding is correlated with specific interaction with the lipid phosphatidylglycerol (POPG) [176]. It was further revealed, using electrophysiology measurements coupled with MD simulations, that POPG is an essential cofactor for AmtB activity and, in its absence, AmtB cannot complete the full translocation cycle [177]. These findings reinforce that a distinction must be made between lipids that merely bind from those that modulate membrane protein structure and/or function. In this context, developing a combined approach using the stabilisation of the protein in native lipidic environment using SMALP combining with Cryo-electron microscopy and neutron/X-ray scattering may be the way forward.

The Amt/Mep/Rh proteins are incredibly diverse in distribution and function. As the field continues to evolve and develop, we hope to gain a glimpse into the internal diversity that has allowed ammonium transporters to become so essential to life.

Acknowledgements

Special thanks to Prof Iain Hunter (Strathclyde Institute of Pharmacy and Biomedical Sciences), Dr Mélanie Boeckstaens (Free University of Brussels, Gosselies, Belgium) and Prof Anna-Maria Marini (Free University of Brussels, Gosselies, Belgium) for

invaluable discussions and help during this project. AB, GW and TH: PhD studentships from the University of Strathclyde. AJ: Chancellor's Fellowship from the University of Strathclyde and Tenovus Scotland (S17-07). PAH would like to acknowledge funding from BBSRC (BB/T001038/1 and BB/T004126/1) and the Royal Academy of Engineering Research Chair Scheme for long-term personal research support (RCSR2021\11\15).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualisation: AJ. Writing—original draft: AJ, LP, TH and GW. Writing—review and editing: All authors.

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