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Pre-Clinical Studies on Novel Lipid Therapeutics With Anti-Virulence and Antibiotic Synergising Potential Against *Pseudomonas aeruginosa* Isolates From Bronchiectatic Airways

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DECLARATION

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Abstract

Chronic infections of the airways remain the leading cause of mortality in cystic fibrosis. Antibiotic therapy has drastically improved survival in this patient cohort, however, this therapy seldom, if ever, results in bacteriologic eradication of the infecting organism and acts only to suppress infection. The efficacy of such therapy may also be undermimed by the emergence of drug resistant strains, some of which may also display enhanced virulence and this is further confounded by the fact that antibiotic development has virtually drawn to a halt in recent decades. It is therefore imperative that potential alternatives to conventional antibiotics are sought to aid management of these infections. Use of phospholipid vesicles in these conditions may be one such alternative. Here, the potential of a multi-lamellar liposome based upon host lamellar bodies, LMS-611, to act as an anti-virulence agent and antibiotic synergist is explored *in vitro* with clinical isolates of the respiratory pathogen *Pseudomonas aeruginosa.* LMS-611 was found to reduce accumulation of elastase, pyocyanin, exopolysaccharide and the siderophores pyoverdine and pyochelin. LMS-611 does not reduce biofilm accumulation or viability in these models but microscopic evidence suggests that it modifies biofilm architecture, leading to the formation of homogenous layers rather than differentiated three dimensional structures. Studies are also conducted upon micelles composed of monopalmitoylphosphatidic acid (MPPA), a lysophospholipid which occurs naturally in inflammatory exudates and has previously been reported to have anti-microbial effects. It is discovered that MPPA reduces accumulation of pyocyanin by some strains and it is suggested on the basis of gene expression, growth kinetics and phenotype microarrays that this may be the result of enhanced growth and catabolite repression. Hitherto unknown interactions of MPPA with non β-lactam antibiotics are also uncovered and possible reasons suggested for these. Paradoxically, it is found that MPPA increases both biofilm accumulation and swimming motility.

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Contents

CHAPTER 1

CYSTIC FIBROSIS AIRWAY DISEASE

SECTION 1.1

PATHOPHYSIOLOGICAL AND BIOCHEMICAL FOUNDATIONS OF CYSTIC FIBROSIS

Initially described in the 1930s by Dorothy H. Andersen, cystic fibrosis (CF), alternatively known as mucoviscidosis, is an autosomal recessive syndrome affecting multiple organ systems (Andersen, 1938). CF patients present with a characteristic tetrad of cardinal signs, namely, increased salt levels in sweat concomitant with malnutrition, pancreatic failure and unrelenting congestion of the airways with unusually viscous and dehydrated mucus which inevitably leads to repeated infections and declining respiratory function, infertility is also common in male sufferers (Davies, 2007; Mishra *et al*, 2005). Historically, CF patients usually succumbed to disease in early childhood, however, prognosis is improving with CF patients born in the UK at the turn of the millennium having a life expectancy of ~50 years which may increase further as novel treatments become available (Dodge *et al*, 2007). Availability of a wider range of antibiotics to manage infections, better nutrition and improved supportive interventions likely account for the rise in survivorship observed to date as no treatment targeting the underlying genetic defect is currently possible. The abnormalities observed in CF most likely owe to a dysregulated electrochemical potential at the apical membranes of epithelial cells ; defective secretion of chloride and other anions leads to increased uptake of sodium and water leading to an accumulation of thick, dehydrated luminal secretions and thus congestion in the affected organ systems, moreover, defective bicarbonate secretion results in suboptimal performance of pancreatic enzymes in the small intestine due to insufficiently high pH levels (Mall *et al* ,2004 ; Hug *et al*, 2003). Diagnosis of CF is most often made by detecting raised electrolyte levels in sweat samples (Davies *et al*, 2007). CF is the most common recessive genetic disorder of Caucasians with the greatest incidence, approximately1 in 2500, occurring in persons of Northwest European extraction; substantial albeit lower incidences occur in other ethnic groups (Buchanan *et al*, 2009). Approximately 60,000 persons globally suffer from CF and around 1 in 20 of the Caucasian populace possesses at least 1 CFTR mutation (Guggino & Stanton, 2006). Progress towards understanding the molecular basis of the disease began in 1989 when mutation of a gene found at position q31.2 on the long arm of chromosome 7 was identified as the cause (Riordan *et al*, 1989).

This gene incorporates 27 exons encoding a transcript which in total length is 6.5kb (Riordan *et al,* 1989). The product of this transcript is a protein of 1480 amino acid residues and known as the cystic fibrosis transmembrane conductance regulator (CFTR) which belongs to the ATP binding cassette (ABC) transporter superfamily and mediates cAMP dependent protein kinase (cAMP PKA) activated chloride (and probably bicarbonate) transport in human exocrine epithelial cells (Gadsby *et al*, 2006). CFTR also acts as a regulator of certain other ion channels, most notably ENaC, the epithelial sodium channel (König *et al*, 2001). Structurally, CFTR is composed of 2 equivalent sections each containing a transmembrane domain of 6 membrane spanning α-helices and a nucleotide binding domain located in the cytoplasm, the 2 halves are linked via a regulatory domain positioned centrally in the cytoplasm (figure 1) (Gadsby *et al*, 2006). More than 1,600 possible mutations can occur in the CFTR gene and some cause more severe disease than others, however, most CF cases can be attributed to quite a limited range of mutation types (Davies *et al*, 2007). Incidence of specific mutations is influenced by founding effects and human migration patterns and thus varies geographically, whilst ΔF508 is unquestionably the most common mutation overall with at least 70% of all CF sufferers possessing a copy, it is most prevalent in Nordic and Celtic nations which on a global scale have the greatest baseline incidence of CF, Onay and colleagues found the frequency of ΔF508 to decrease in a South Easterly gradient across Europe with the highest prevalence (90%) observed in Denmark and the lowest prevalence (18.8%) in Turkey (Onay *et al,* 1998). It has been hypothesised that certain infectious diseases may have provided a positive selective pressure to enable maintenance of mutant CFTR alleles via heterozygous individuals (Poolman & Galvani, 2007). CFTR mutations can be loosely assigned to five categories depending on their mechanism of dysfunction. Class I mutations can be subdivided into two groups, the first of these are responsible for the most severe variants of CF in which no functional protein at all is expressed and result from nonsense or framing errors that terminate transcription prematurely resulting in the formation of truncated proteins that are degraded by chaperones in the endoplasmic reticulum rather than being trafficked to the cell membrane (Zielenski & Tsui, 1995). Conversely, the remaining subgroup of class I mutations are associated with the mildest form of CF as they

result from an improper splicing which allows correct CFTR transcripts to be formed in insufficient quantities, hence, these mutations lead to a shortage rather than a complete absence of functional CFTR at the cell membrane (Rowntree & Harris, 2003). Class II mutations include the common ΔF508 type and result in inappropriate deletions or missense codons that usually allow proteins to be formed, however, the resultant proteins are misfolded and trafficking of them to the cell membrane is greatly diminished (Kalin *et al*, 1999). Class III mutations result in the synthesis of proteins that are trafficked to the cell membrane unhindered but fail to bind ATP or be stimulated by cAMP on arrival (Rowntree & Harris, 2003). Class IV mutations result in the development of CFTR which is effectively trafficked to the cell membrane, binds ATP and is stimulated by cAMP but nevertheless functions suboptimally as a chloride channel due to reduced amplitude of channel current and infrequent channel opening (Sheppard *et al*, 1993). Finally, class V mutations resulted in a protein that is truncated by less than 100 C-terminal amino acid residues which are necessary for the stability of the protein but not for its proper function, configuration or trafficking, thus, sufficient functional CFTR is initially expressed at the cell membrane but is degraded too rapidly (Haardt *et al*, 1999). As they permit partial expression of CFTR, mutations of classes IV and V are unsurprisingly associated with less severe disease presentations than those of earlier classes, excluding the minority subgroup of class I mutations that result in only minimal truncation of CFTR, however, compound mutations have also been encountered (Ahmed *et al*, 2003).

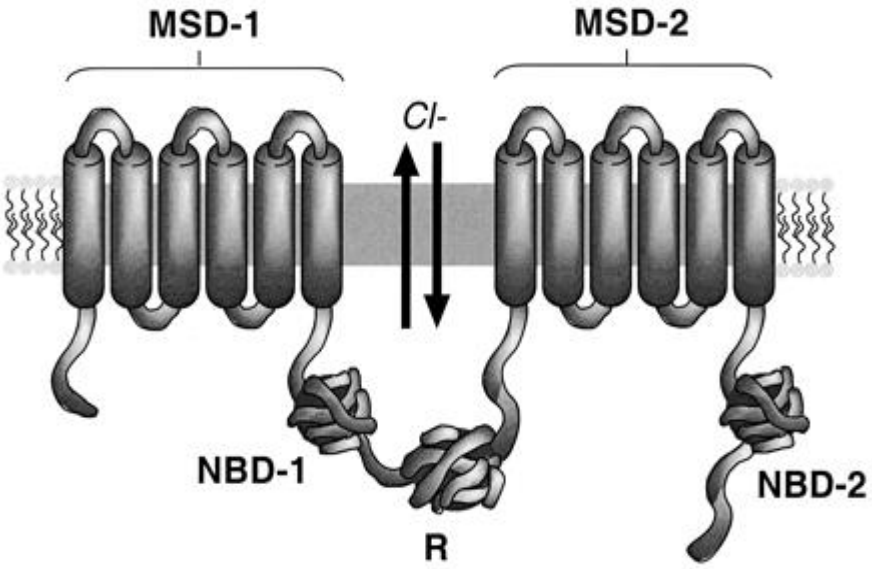

Figure 1

Structure of the CFTR protein (adapted from Lyczak *et al*, 2002)

ATP is bound and hydrolysed by the nuclear binding domains (NBDs) to activate bidirectional chloride transport. The channel opens when NBD-1 hydrolyses ATP and closes when ATP is hydrolysed by NBD-2. The class II ΔF508 mutation occurs via deletion of a critical phenylalanine residue located in the folds of NBD-1 which is vital for proper folding of the protein. Class III mutations also occur in the NBD folds and block ATP binding whereas most class IV mutations alter the membrane spanning domains (MSDs) to reduce the efficacy of channel function. Both class I and V mutations result in variable degrees of truncation. (Rowntree & Harris, 2003).

SECTION 1.2

IMPORTANCE OF AIRWAY INFECTIONS AND DEFECTIVE INNATE IMMUNITY IN CYSTIC FIBROSIS

Chronic and debilitating airway infections are the primary cause of morbidity and mortality in CF patients as a result of the deteriorating respiratory function they induce, usually remission though not cure of such infections is possible with radical antibiotic therapy (Lyczak *et al*, 2002). Virtually all CF sufferers develop intermittent infections of the airway in infancy which in the vast majority of cases give way to chronic infection later in life. The predisposition of CF patients for these infections is multifactorial and not fully understood, nevertheless, numerous defects of innate immunity in the CF host have been identified and studied in some detail. Respiratory epithelial cells are lubricated by a coating termed the airway surface

liquid (ASL) which is a bilayer comprised of a basal serous phase, the periciliary liquid layer, overlaid by mucus. The periciliary liquid layer is required for normal mucociliary escalation and has been reported to be diminished in the CF airway due to hyperabsorption of liquid by epithelial cells as a result of defective anion transport, the result being a stagnant collection of microbe laden mucus in the airways, concentration of molecules with proinflammatory signalling activity and ultimately infiltration of the airways by neutrophils leading to an excessive but nonetheless ineffective inflammatory response (Matsui *et al*, 1998 ; Machen 2006). Furthermore, the ASL in CF patients is deficient in surface active phospholipids including phosphatidylglycerol and phosphatidylcholine which are essential for lubrication of the airway, controlled viscosity and adherence of mucus and ultimately adequate mucociliary escalation (Puchelle *et al*, 2002). In addition, phospholipids have also been shown to modulate cell mediated inflammatory responses (Postle *et al*, 1999). In an alternative line of enquiry, ASL from CF patients demonstrated reduced bactericidal properties relative to that obtained from healthy individuals (Smith *et al*, 1996). A number of potential explanations have since been proposed for this finding including an observed deficiency in CF sufferers of the hydrophilic lung surfactant proteins A and D which demonstrate bactericidal action and the possibility that elevated salt levels in the CF airway antagonise defensins and other antimicrobial peptides (Postle *et al*, 1999 ; Zabner *et al*, 1998). Moreover, air exhaled by CF patients was found to contain lower levels of nitric oxide (NO) compared to control samples from healthy subjects and inducible NO synthase expression was later found to be lessened in both human and murine CF epithelial cell lines (Grasemann *et al*, 1997 ; Kelley & Drumm, 1998). Whether the effect of this reduced NO level on the pathology of CF airway disease (if any) is beneficial or detrimental is difficult to ascertain as this compound has both bactericidal and proinflammatory activities (Bals *et al*, 1999). Recent work has also shown that CFTR mutations can negatively impact the function of alveolar macrophages via several mechanisms including diminished phagolysosomal acidification, phagocytic uptake and release of secretory bodies, the overall consequence is reduced clearance of microorganisms and probably also necrotic polymorphonuclear cells (PMNs) and other debris (Deriy *et al*, 2009). In parallel with these discoveries, Painter and

colleagues found that hypochlorite production within the phagolysomes of neutrophils of a mutant CFTR genotype was impaired, perhaps having adverse effects on bacterial killing (Painter *et al*, 2006). Other authors have reported that PMN elastase, which is present in excess levels within the CF airway can degrade C3b complement receptors of certain bacterial species as well as CR1, an endogenous complement receptor of PMNs, the former event leads to inadequate complement mediated opsonisation whilst the latter prevents stimulation of bactericidal responses in cells successfully opsonised by complement fragments, this enzyme may also cleave IgG (Tosi *et al*, 1990 ; Eckle *et al*, 1990). Potentially, reinforcing these general immunodeficiencies are distinct features of the CF airway which may explain the unique propensity of various pathogens to produce disease there. It has been demonstrated that epithelial cells from CF sufferers display differential sialylation of apically exposed gangliosides relative to those of unaffected individuals and that one particular glycolipid (asialoGM1) which is overexpressed on CF epithelial cells contains a sequence (GalNacβ1-4Gal) which acts as a receptor for a myriad of bacteria known to be respiratory pathogens including many of those species most often encountered within the milieu of the CF airway (Krivan *et al*, 1988). Reception of various bacterial adhesins via these asialylated epithelial glycolipids has subsequently been shown to lead to increased intracellular calcium concentrations which induce protein kinase signalling cascades culminating in NF-κB controlled expression of the proinflammatory cytokine interleukin-8 (IL-8) (Rastogi *et al*, 2001). Pier and colleagues suggested that CFTR may in its own right act as a receptor for lipopolysaccharide (LPS) thus potentially mediating uptake of *Pseudomonas aeruginosa* and possibly other Gram negative CF pathogens by epithelial cells which may then apoptose or desquamate leading to clearance of organisms from the airway lumen whilst NF-κB is upregulated leading to an inflammatory response which eliminates residual bacteria (Pier *et al*, 1997 ; Schroeder *et al*, 2002). This is in direct contrast to the pathology of invasive enteric infections in which epithelial internalisation of bacteria is actually essential to the progress of disease, indeed, typhoid has been proposed as one of the diseases which may have exerted positive selective pressure for heterozygous CFTR mutations and transgenic murine models have shown that *Salmonella enterica* serovar Typhi

requires functional CFTR for epithelial invasion (Pier *et al,* 1998). Several criticisms of this hypothesis have arisen in the literature and it may be inferred from previous studies of cultured airway epithelium that the sequence of events concluding with apoptosis and sloughing of cells begins prior to bacterial binding and not as a result of it thus suggesting that organisms may preferentially bind to cells which are already dying (Lee *et al,* 1999). In addition, the fact that other workers have demonstrated that uptake and binding of *P. aeruginosa* occurs primarily at the basolateral membranes of epithelial cells as opposed to the apical surface where CFTR is located is not consistent with a role of CFTR in the internalisation of this organism (Pier *et al*, 1997 ; Fleiszig *et al*, 1997). Finally, challenge of the apical epithelial membrane with flagellin alone, even in the absence of the organism, is sufficient to upregulate NF-κB and likely to mediate cellular immune responses (Tseng *et al*, 2006). Elsewhere, other workers have found that CFTR mutations may actually increase epithelial uptake of *P. aeruginosa* and thus allow establishment of chronic intracellular infection which is in some ways analogous to that caused by certain strains of uropathogenic *Escherichia coli*, intracellular lifestyles that may include biofilm formation could afford the organism substantial protection from both immune responses and antimicrobial therapy (Darling *et al*, 2004 ; Garcia-Medina *et al*, 2005).

SECTION 1.3

MICROBIAL AETIOLOGY OF INFECTIONS AFFECTING THE CF AIRWAY

The spectrum of pathogens involved in respiratory tract infections occurring during CF is uniquely vast and inimitably diverse. Indeed the epidemiology and pathophysiology of such infections could be construed as a subspecialty in and of itself to which a number of microbiologists have dedicated their careers (Govan & Deretic, 1996). This is further emphasised by the fact that a number of bacterial species unheard of until recently were first discovered in CF sputum or bronchoalveolar lavage (BAL) samples, furthermore, many bacteria present in the CF airway may not be culturable and have been detected only by the use of

molecular techniques such as amplification of 16S rDNA via PCR followed by gene cloning and sequencing (Li Puma, 2010 ; Bittar *et al*, 2008). A further important consideration in the bacteriological diagnosis of infections occurring in the CF airway is specimen collection, in infants who cannot easily provide a sputum sample, pharyngeal swabs are used as a surrogate ; this may result in problems with accuracy of the diagnosis as the organisms sampled may differ from those in the airway, even sputum itself may be contaminated with benign organisms as it is impossible to collect with full aseptic ritual as the upper respiratory hosts an abundant microbiota, molecular methods or BAL although not standard may thus enable more accurate detection (Lyczak *et al*, 2002). Although it is impossible to give mention to all potential respiratory pathogens associated with CF, the most common ones of proven importance will be discussed briefly in this section. An especially surprising feature of infections arising in the CF airway is that professional human respiratory pathogens have not been conclusively incriminated in their pathology, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis,* by far the most important bacterial causes of respiratory tract infection in the general community, are rarely reported to cause disease in CF (del Campo *et al*, 2005 ; Poolman and Galvani, 2007). Most bacterial respiratory tract infections observed in CF patients are due to opportunistic pathogens, many of which are non- fermentative Gram negative bacilli of environmental origin, this group of organisms are highly problematic as they exhibit intrinsic resistance to a wide range of antimicrobial agents and are at best susceptible to only a select few drugs, at worst they may cause pan-resistant infections which are virtually untreatable (McGowan, 2006). Other opportunistic bacterial pathogens frequently associated with infection of the CF airway include *Staphylococcus aureus* and *Haemophilus influenzae,* both of which are common colonisers of the upper respiratory tract that occasionally cause disease if they are introduced further down the respiratory tree (Lyczak *et al*, 2002). The prevalence of specific pathogenic species has been repeatedly shown to correlate with patient age and in general *S. aureus* and *H. influenzae* predominately cause infections early in life and are then superseded by *P. aeruginosa* and possibly other Gram-negative opportunists as sufferers progress to adulthood (Figure 2). Although *P. aeruginosa* is now undoubtedly the overall most troublesome and common of CF associated pathogens,

this and other Gram-negative opportunistic organisms were not fully recognised as pathogens of the CF airway prior to the 1960s and *S. aureus* was traditionally viewed as the principal infectious hazard in CF patients, the reasons for this apparent shift in aetiology are not entirely clear but may relate to increased contact between patients due to the inauguration of specialist CF clinics since that time and the improved control of Staphylococcal infections afforded by the increasing range of antibiotics available by that period which in turn lead to increasing life expectancy, additionally, better methods of detection and identification of pathogenic traits may simply have enabled a clearer recognition of unfamiliar or previously dismissed organisms as pathogens (Lipuma, 2010). Amongst the more obscure non-fermenting Gram-negative bacilli associated with CF pulmonary disease, members of the *Burkholderia cepacia complex* (Bcc) warrant special mention as they have been associated with an especially poor prognosis despite institution of ostensibly appropriate therapy dictated by the antibiograms of isolates, moreover, these organisms often produce invasive infections characterised by fulminant septicaemia which terminate fatally in the vast majority of cases (Govan & Deretic, 1996). Of the other bacteria potentially linked with CF associated respiratory pathology, candidates include both aerobic and anaerobic members of the oropharyngeal flora as well as non-tuberculous Mycobacteria, the latter being associated, bizarrely, with CF patients with mild types of disease who have survived to middle age (Aitken *et al*, 1993 ; Ziedalski *et al*, 2006). Although members of the *Enterobacteriaceae*, in particular *Serratia marcescens* and *Klebsiella pneumoniae*, are not uncommonly isolated from the CF airway, there is insufficient evidence at present that they are anything more than transient colonisers (Coenye *et al*, 2002). Mycotic infections are also being increasingly reported within the context of the CF airway and many fungal pathogens, particularly *Aspergillus sp.*, are now known to cause respiratory disease in the CF population (Li Puma, 2010). Furthermore, a number of viruses may contribute to disease of the CF airway and these do not appear to differ substantially from the spectrum of agents causing viral respiratory tract infections in the wider population (van Ewijk *et al*, 2005).

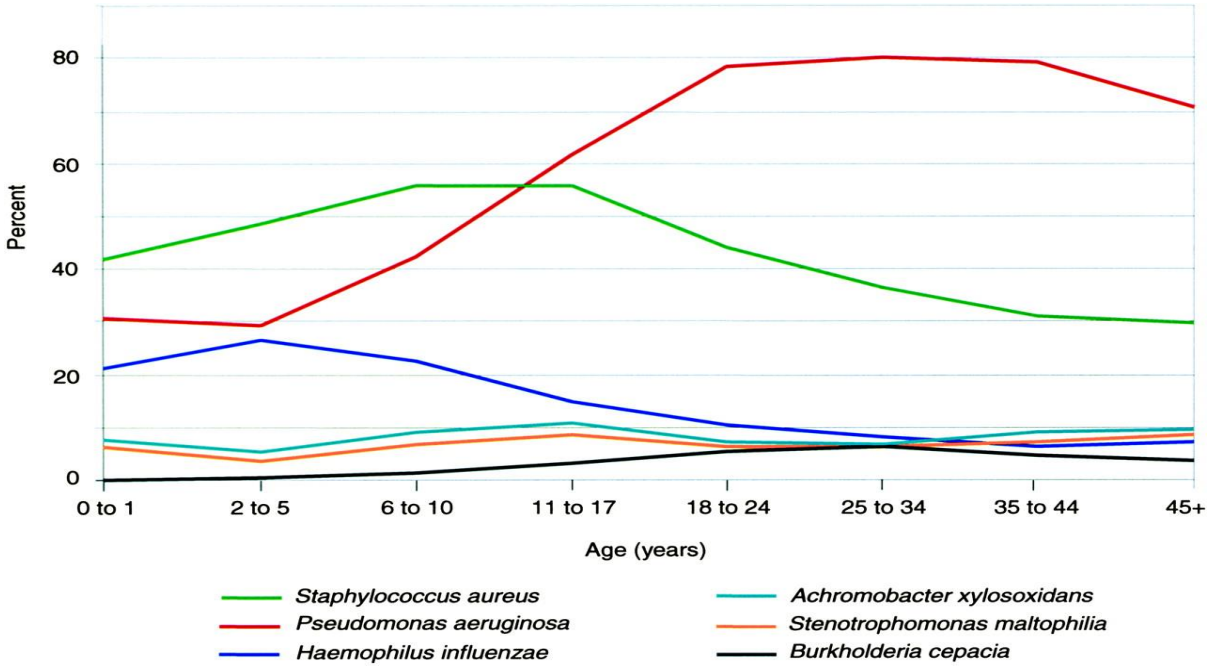

Figure 2

Prevalence of CF airway pathogens by age (adapted from US CF registry)

Infections due to *P. aeruginosa* have the greatest prevalence overall, acute infections by this organism occur in infancy and gradually give way to chronic infection in 80% of young adults, incidence declines in middle age, possibly due to mortality in this patient group. Infections due to *H. influenzae* peak in early childhood and decrease thereafter whereas those due to *S. aureus* increase rapidly throughout childhood from a high baseline (40%) in the first year of life, prevalence remains at its highest in adolescence and declines steadily afterwards. Infection with Bcc members rises steadily but remains low overall, after peaking in early adulthood, prevalence of this group of organism declines sharply, this may be attributed to the high mortality associated with these infections. Prevalence of infections caused by other non-fermentative Gram-negative bacteria including *S. maltophilia* and *A. xylosoxidans* varies erratically with age (Li Puma, 2010).

SECTION 1.4

NON-CF BRONCHIECTATIC CONDITIONS

Several other medical conditions may cause severe inflammation of the conducting airways similar to that observed in cystic fibrosis. These conditions are also associated with recalcitrant infections of the airway similar to those occurring in cystic fibrosis although the spectrum of pathogens involved differs in some regards. Amongst these conditions are chronic obstructive pulmonary disease (COPD) and

non CF bronchiectasis. COPD is a broad condition encompassing the airway diseases emphysema and chronic bronchitis, as these two clinical entities are now known to usually occur as twinned co-morbidities, with one often being present to a greater or lesser extent than the other, COPD has generally become the preferred diagnostic term. Clinical manifestations of COPD are similar to those of CF bronchiectasis but differ in causative factors, with CF being congenital and COPD usually being attributable to inhalation of tobacco smoke or other air pollutants in those who likely have a genetic predisposition to the disease (Punturieri *et al*, 2008 ; Mannino & Braman, 2008). COPD is a leading cause of morbidity and mortality worldwide (Rabe *et al*, 2007). COPD, like CF bronchiectasis, results in periodic exacerbations, which are associated with infections which may be bacterial and/or viral. The microbiology of COPD has not been as extensively studied as that of CF airway disease, however, in general, *P. aeruginosa* has a much lower albeit substantial incidence in COPD relative to CF whilst the converse is true for *H. influenzae*, *S. pneumoniae* and *Moraxella catarrhalis* (King *et al*, 2013). As with CF bronchiectasis, infections in COPD are often polymicrobial and the relative contribution of individual species or even distinct strains of a single species, are difficult to ascertain (King *et al*, 2013). With the advent of metagenomics it has become clear that the human airway, even in health, is not sterile as was previously assumed ; it does have a distinct resident microflora (Dickson *et al*, 2014). It has been suggested that infectious airway diseases such as those occurring in CF and COPD may be due to alterations in the make-up of this microflora and that even in acute lung infections (pneumonia) thought to be due to invasion of a 'sterile' site by a single causative pathogen (e.g. *Streptococcus pneumoniae*) that disease may be due to an overall increase in bacterial density with a co-incident decrease in bacterial diversity, in a manner reminiscent of other conditions such as mucosal candidiasis or *Clostridium difficile* associated colitis in which a disturbance of the normal microbiota enables a pathogen to gain a more prominent position at a non-sterile site (Dickson *et al*, 2014). Bronchiectasis may also have other causes including chronic or repeated infections of the airway, such as tuberculosis (McShane *et al*, 2013). Whilst the work presented here focuses primarily on CF related airway disease, it may also have applications in these other conditions.

SECTION 1.5

ROLE OF ANTIBIOTIC THERAPY AND SENSITIVITY TESTING

Although antibiotic therapy has undoubtedly revolutionised CF treatment and been a key driving factor in the increased life expectancy of these patients, information regarding optimal therapy is lacking. Indeed, conventional sensitivity testing of planktonically grown sputum isolates has been shown to be an inadequate predictor of clinical and bacteriological response to therapy and personalised antibiotic regimens tailored to sputum isolates based on the results of such tests does not generally lead to better outcomes when compared to blind treatment, clinical improvements are commonly encountered in spite of apparent resistance and conversely, treatment failures frequently occur despite apparent sensitivity *in vitro* (Hurley *et al*, 2012). Possible reasons for this are manifold. One explanation is that a great deal of genotypic and phenotypic variation occurs between isolates from a single patient and they may be infected with multiple distinct strains which may have acquired divergent genotypes through such means as hypermutation and recombination even if they have descended from a common founding population which originally infected the patient from an external source such as another patient or the environment (Oliver *et al*, 2004 : Mowat *et al*, 2011: Ashish *et al*, 2013 : Darch *et al*, 2015). Susceptibility testing in clinical laboratories relies on the sampling of distinct isolates from a given sputum sample as resolved by visible differences in colony morphology, therefore 2 ostensibly identical colonies may have different phenotypic and genotypic traits, including drug resistance (Foweraker *et al*, 2005). A second factor that probably contributes to discordances between clinical and bacteriological outcomes of therapy and sensitivity test results is the fact that CF airway infections are not monomicrobial but typically involve a myriad of interacting species whose individual and combined interactive contributions to airway pathology have not been ascertained. As a result it is probable that the relative contributions of some other less studied species to airway pathology has been grossly underestimated if not completely ignored. Antibiotic therapy targeting *P. aeruginosa* may serendipitously attack other co-infecting organisms whose pathogenic potential has

been overlooked, leading to clinical improvement even if the course of treatment has failed in its original objective of reducing *P. aeruginosa* density in sputum, likewise, therapy with agents that are inherently inactive against *P. aeruginosa* have occasionally improved lung function in patients heavily colonised with this organism who had an ongoing exacerbation of airway disease (Hauser *et al*, 2014 : Parkins *et al*, 2008). Yet another reason for the discrepancies often observed between *in vitro* sensitivity test results and the clinical response to treatment is that antibiotic therapy may influence virulence related attributes of *P. aeruginosa* such as quorum sensing, biofilm formation and exotoxin production at concentrations which are not inhibitory to growth, equally, some antibiotics which may be used in CF airway infections, such as macrolides, are known immunomodulators that may influence host inflammatory responses (Swatton *et al*, 2016 ; Steel *et al*, 2012). Therefore, the benefits of antibiotic treatment may not be solely due to direct bacteriostatic or bactericidal action. Organisms within the CF airway exist primarily in biofilm modes of growth and thereby display different (generally much higher) levels of resistance relative to their planktonic counterparts. A retrospective meta-analysis identified only 2 clinical trials with a low risk of bias comparing the efficacy of antibiotic therapies assigned by biofilm susceptibility testing with those assigned by standard susceptibility tests with planktonic organisms ; both trials found that susceptibility testing of CF *P. aeruginosa* isolates cultured as polystyrene bound biofilms in the microtitre plate format yielded different predictions for optimal anti-pseudomonal therapy relative to matched conventional planktonic susceptibility tests on the same isolates, however, no significant differences were found between the treatment arms in terms of either improved lung function or bacteriologic clearance (Waters & Ratjen, 2015). This calls into question the relevance of biofilm susceptibility testing of CF *P. aeruginosa* isolates in the microtitre plate format. Possible limitations of this test method that lead to discordant results are numerous. As with planktonic sensitivity tests, cultures are prepared from a limited sample number of sputum isolates, selected according to colonial morphotypes, which may not adequately mirror the phenotypic diversity of *P. aeruginosa* in the airway (Oliver *et al*, 2004). Moreover, polystyrene bound biofilms grown in standard broth media such as LB or MHB differ substantially from biofilms found in the airway which primarily grow on

amino-acids as colonies suspended in hypoxic mucus plugs containing DNA, mucin, protein, lipids and other cell debris which may inactivate or impede the penetration of antibiotics whilst inducing stress responses and hypermutability which further reduce antibiotic activity and support phenotypic diversification of the infecting organisms (Kirchner *et al*, 2012 ; Wright *et al*, 2013). Several different recipes for artificial sputum medium have been documented in the literature and it has been suggested that culture of sputum isolates in media such as these, possibly under conditions of varying mechanical shear or reduced oxygenation, may more accurately reflect the biofilm lifestyle naturally adopted by *P. aeruginosa* in the CF airway and enable more meaningful susceptibility testing to be performed with isolates from CF patients, enabling clinicians to make better informed choices of antibiotic therapy (Sriramulu *et al*, 2005 : Kirchner *et al*, 2012 : Fung *et al*, 2010). Although experiments in such media have arguably provided valuable insights into the pathobiology and population turnover of *P. aeruginosa* in CF airways, utility of media such as these in susceptibility testing has yet to be verified by clinical trials.

CHAPTER 2

PSEUDOMONAS AERUGINOSA AS A MODEL RESPIRATORY PATHOGEN

SECTION 2.1

SALIENT BACTERIOLOGICAL FEATURES AND ROLE IN CF

P. aeruginosa is a non-fermentative γ-proteobacterium widely distributed in nature particularly in soil, vegetation and aquatic environments. Organisms are motile by means of a polar flagellum and capable of metabolising diverse sources of carbon and nitrogen, the species is non-fastidious, growing at temperatures of 20 – 42 ˚C in almost any environment where moisture is in ample supply; although often considered an obligate aerobe, slow growth is possible in anoxic environments providing alternative electron acceptors are available (Ryan, 2004). *P. aeruginosa* grows readily on many common media to form large iridescent colonies which may secrete distinctive pigments that aid its preliminary identification (Ryan, 2004). The complete genome of the bacterium type strain PAO1 was first sequenced in 2000 and was at that time the largest bacterial genome sequenced (~6.3 million bp), moreover, it was found to be highly complex and contained an unusually high proportion of regulatory genes as well as genes necessary for catabolism and transport of organic compounds, reflecting its diverse repertoire of growth substrates and intrinsically high resistance to antimicrobials (Stover *et al*, 2000). Genomic analyses of other strains has since revealed that a great degree of heterogeneity exists between them and has lead to the concept of a 'core genome' which is relatively conserved throughout strains to govern fundamental cellular processes and an 'accessory genome' which is extremely variable and controls auxiliary functions (Kung *et al*, 2010). Although seldom a cause of serious disease in otherwise healthy and immunocompetent individuals, *P. aeruginosa* is a highly versatile opportunistic pathogen, being an exceedingly important cause of nosocomial infections including complicated urinary tract infections, sepsis and ventilator associated pneumonia (Ryan, 2004). Furthermore, this species has become the leading cause of infections in both burns patients and CF sufferers (Ryan, 2004). Within the context of the CF airway, *P. aeruginosa* typically causes transient infections beginning from a young age which resolve with appropriate antibiotic treatment, these infections generally involve several distinct non-mucoid strains and eventually give way to a chronic infection by a single strain which has mutated to a mucoid phenotype and persists despite therapeutic interventions (Govan & Deretic, 1996). The source of infection

was historically considered to lie in environmental reservoirs, however, recovery of several isolates with identical genotype and pyocin sensitivity profiles amongst separate patients attending a common CF clinic suggests interpatient spread is possible (Govan & Deretic, 1996). These strains are now termed 'epidemic clones' and evidence suggests they may have fitness adaptations which aid persistence and spread amongst patients, moreover, sporadic reports of spread to non CF contacts and even pets exist for these strains (McCallum *et al*, 2002 ; Mohan *et al*, 2008). Several factors have been proposed to account for the increased transmission of such strains including variations in the type IV pilus and the presence of prophage islands within the genome which may enhance fitness (Kus *et al*, 2004 ; Winstanley *et al*, 2009). The fact that such strains are often resistant to multiple drugs also implies that they are subject to positive selection as a result of antibiotic consumption in the CF population (Cheng *et al*, 1996). Some strains isolated at an unusually high frequency may not be truly epidemic but simply more widespread in nature (Romling *et al*, 1994). Chronic infection with *P. aeruginosa* demonstrably results in significantly higher mortality, declining respiratory function and retarded growth of patients as well as increased frequency of cough (Hauser *et al*, 2011). During the course of infection bacteria first settle in the oropharynx before being aspirated into the lower respiratory tree (Mainz *et al*, 2009). Here a number of virulence factors and CF specific phenotypic changes are deployed by *P. aeruginosa* in order to establish and maintain chronic infection, these will be discussed individually and in greater depth in section 3.2. The presence of antigenic bacterial products such as LPS, pilin and flagellin along with other bacterial components such as DNA and quorum sensing molecules upregulate host production of various inflammatory mediators including interleukins 1, 6 and 8, TNF-α, complement components and leukotriene-B_4 whilst down-regulating expression of anti-inflammatory cytokines such as IL-10 (Konstan & Berger, 1997 ; Hauser *et al*, 2011). Moreover, exotoxins of *P. aeruginosa*, including pyocyanin, may directly elicit inflammatory molecule production and release (Schwarzer *et al*, 2008). The massive infiltration of PMNs which ensues leads to deterioration in the integrity of the elastic extracellular matrix of the airways as a result of excessive release of PMN elastase, collagenase and reactive oxygen species (Hauser *et al*, 2011). Elastases from both *P. aeruginosa* and PMNs

themselves reduce ciliary movement and degrade antibacterial products such as lysozyme, transferrin and surfactant proteins causing reduced mucociliary clearance of bacteria (Hauser *et al*, 2011). Release of DNA from breakdown of neutrophil extracellular traps causes increased viscosity of respiratory secretions and eventually fibrosis and blockage of the airways occurs (Marcos *et al*, 2010). Ultimately bacterial burden increases due to the failure of the inflammatory response and may reach levels approaching 10^{11} CFU/ml in sputum (Aaron *et al*, 2004). A simplified schematic of the sequence of events occurring in infection of the CF airway due to *P. aeruginosa* is presented below (Figure 2.1).

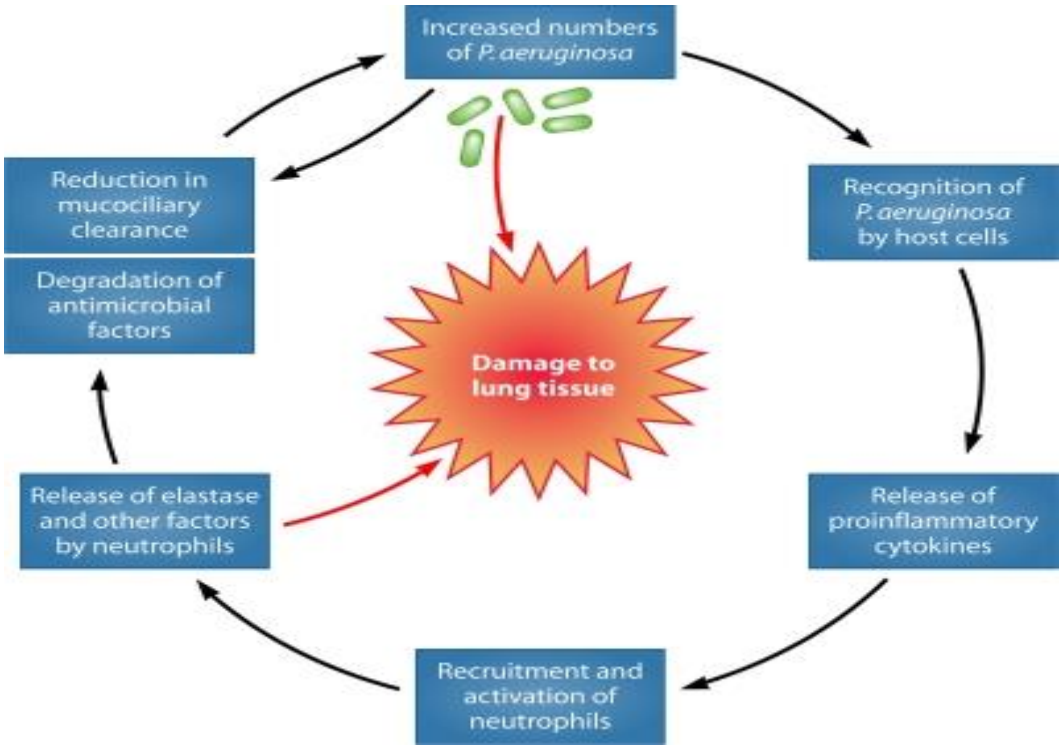

Figure 2.1
P.aeruginosa infection in CF – A vicious circle (adapted from Hauser *et al*, 2011)

Infection of the CF airway by *P. aeruginosa* essentially follows a positive feedback cycle in which organisms manipulate the host into launching excessive but counterproductive immune responses which paradoxically perpetuate infection.

SECTION 2.2

VIRULENCE MECHANISMS

In order to become established within the CF airway *P. aeruginosa* initially utilises a number of structures to co-ordinate both adhesion and motility. The type IV pilus has been shown to enable twitching motility on epithelial surfaces and is also involved in attachment of the organisms to receptors (asialoGM1, TLR-2) located there (Soong *et al*, 2004). The flagellae of organisms allow swimming motility in liquid environments and bind with TLR-5 in addition to TLR-2 and asialoGM1 (Adamo *et al*, 2004). LPS has also been reported to adhere to asialoGM1 (Gupta *et al*, 2004). A number of outer membrane proteins (OMPs) expressed by *P. aeruginosa* are reported to bind to respiratory mucins and are expressed more readily at low iron concentrations, more recently, microarray analyses have shown that strains grown in artificial mucus overexpress both siderophores and nitrate reductase, this likely indicates an adaptation on the part of the organism that allows it to sequester iron and utilise nitrate for respiration in the microaerophilic and iron replete milieu provided by sputum (Scharfman *et al*, 1996 ; Fung *et al*, 2010). Two key enzymes secreted by *P. aeruginosa*, elastase and alkaline protease, act to cleave a plethora of host bactericidal molecules including complement fragments, serum IgG, secretory IgA, transferrin and pulmonary surfactant proteins (Schultz & Miller, 1974 ; Döring *et al*, 1983 ; Hauser *et al*, 2011). Together with PMN elastase and other host derived proinflammatory mediators, these proteases gradually disrupt the collagenous matrix of the airway to impair its elasticity and correct functioning (Heck *et al*, 1986 ; Hauser *et al*, 2011). Moreover, production of exotoxin A may serve to damage host cells and liberate free iron whilst elastase cleaves transferrin (Scharfman *et al*, 1996 ; Hauser *et al*, 2011). Although isolates of this species cultured from CF sputum often produce relatively large amounts of exotoxin A, the insidious nature of airway damage associated with infection of the CF airway appears to be inconsistent with the pathologic features normally evoked by this especially aggressive virulence factor and it has been documented that some CF isolates with mutations in the *toxA*

gene express an unusual variant of exotoxin A which exhibits diminished ADP-ribosyltransferase activity whilst paradoxically maintaining a level of cytotoxic function which is at least equal to that of wild-type exotoxin A (Gallant *et al,* 2000). Recently, the role of pyocyanin as a virulence factor has come to light, in a murine model it induced airway fibrosis and abnormalities of secretory goblet cells whilst upregulating hyperinflammatory Th2 responses leading to influx of PMNs, macrophages and $CD4^+$ cells and further release of inflammatory mediators (Caldwell *et al,* 2009). Despite their undoubted importance in acute infections, whether and how other major *P. aeruginosa* exotoxins impact the progress of CF airway disease remains an open ended question but possible functions have been suggested for some of these. CF patients have been shown to have increased antibody titres to phospholipase C, this toxin may elicit release of proinflammatory mediators such as IL-8 and leukotriene B_4 in the CF airway in order to contribute to inflammation and may also exhibit direct cytotoxicity against epithelial and other host cell membranes (Konig *et al,* 1997). The onset of chronic infection usually coincides with the emergence of mucoid *P. aeruginosa* mutants in the CF airway, the mucoid phenotype is defined by overproduction of alginate, a β1-4 linked O-acetylated linear polymer of D-mannuronate and L-guluronate (Govan & Deretic, 1996 ; Douthit *et al,* 2005). Several different mutational events may trigger conversion to mucoidy in *P. aeruginosa,* the first and most extensively studied of these occur in the *algU mucABCD* operon ; *algU,* alternatively referred to as *algT* or *P. aeruginosa* σ^E, is a sigma factor which positively regulates *algD,* a promoter of alginate biosynthesis whereas the *mucABCD* genes negatively regulate *algU,* as a result, mutations in either *mucA, mucB,* or *mucD* have been shown to lead to mucoidy although mutations in *mucC* alone have not (Rowen & Deretic, 2000). Mutations in *mucA* are the most frequently reported amongst mucoid CF isolates (Boucher *et al,* 1997). Alginate is of paramount importance in the CF airway as it protects both sessile and planktonic bacteria from a myriad of insults from the immune system, it has been shown to impede phagocytosis by macrophages and neutrophils, interfere with neutrophil chemotaxis, quench hypochlorite and inhibit complement activation whilst stimulating oxidative burst in neutrophils which furthers leads to overwhelming inflammation (Meshulam *et* al, 1984 ; Krieg *et* al,

1988 ; Pedersen *et al*, 1990 ; Learn *et al*, 1987). Alginate may also impair lymphocyte transformation and the potency of alginate as a virulence factor has been reported to depend on the ratio of its two uronic acid monomers and its degree of acetylation (Mai *et al*, 1993). Mucoidy is infrequent amongst *P. aeruginosa* clinical isolates originating in pathologic settings other than CF and is thus probably selected for over time by factors unique to bronchiectatic airways, although a minority of CF patients present with a mucoid isolate at initial bacteriological assessment with no known history of prior infection with other *P. aeruginosa* strains (McAvoy *et al*, 1989 ; Govan & Deretic, 1996). The length of time taken for mucoid strains to manifest in sputum is variable but generally occurs after a mean period of 3 years in which the individual suffers repeated infections with non-mucoid strains (Mahenthiralingam *et al*, 1994). The presence of reactive oxygen species from neutrophil infiltration of the airways stimulates conversion to mucoidy (Mathee *et al*, 1999). Yet another unique virulence determinant employed by *P. aeruginosa* in the CF airway is the ability to produce modified LPS. Whilst the lipid A of non CF isolates typically displays penta-acylation and lacks aminoarabinose residues, that deriving from CF isolates often incorporates aminoarabinose and has one or even two additional acyl substitutions due to mutation of such genes as *pagL* which encode lipid A deacylases (Ernst *et al*, 1999 ; Ernst *et al*, 2006). This appears to be significant as inclusion of aminoarabinose residues may confer some degree of resistance to host antimicrobial peptides and certain antibiotics whereas excessive acylation may prevent recognition of epitopes via TLR-4 and thus aid evasion of immune responses, the fact that LPS extracted from a late CF isolate exhibited reduced inflammatory effects relative to that obtained from an earlier isolate originating in the same patient may lend credence to this view (Ernst *et al*, 1999 ; Cigana *et al*, 2009). Furthermore, deletions and insertions occurring in the *rfb* locus which lead to either the loss or abbreviation of O antigen chains are frequent amongst CF isolates, these alterations in O antigen profile may hinder recognition of organisms by the immune system and may also explain the remarkable rarity of bacteraemic complications due to CF related *P. aeruginosa* infections as intact O antigen is essential for resistance of Gram negative bacteria to serum mediated killing (Evans *et al*, 1994). The role played by the type III secretion system (T3SS)

of *P. aeruginosa* in CF has not been extensively studied. The T3SS is a highly conserved and crucial virulence determinant of many Gram negative pathogens and is known to be of paramount importance in numerous infections, including many pseudomonal infections occurring outwith the context of CF (Hauser, 2010). In *P. aeruginosa*, the T3SS injects 4 principal effector proteins (designated Exo S, T, U and Y) which all have important cytotoxic functions (Veesenmeyer *et al*, 2010). Dacheux and colleagues noted that a CF isolate (CHA) of *P. aeruginosa* induced cell rounding and death in J774 macrophages, HeLa cells and B-lymphocytes whereas an isogenic variant of this strain (CHA-D1) lacking the *exsA* transcriptional activator of the T3SS did not, unless complemented (Dacheux *et al*, 2001). The CHA strain employed in the aforementioned study, however, lacked the single most potent *P. aeruginosa* T3SS effector ExoU, thus implying that this protein is not indispensable for the cytotoxic effect the authors observed (Dacheux *et al*, 2001). A further report found that repeated recovery of T3SS positive isolates was associated with significantly more rapid respiratory decline but also indicated that the prevalence of such isolates decreased rapidly with advancing patient age and that overall only 12 % of *P. aeruginosa* isolates from CF patients had an active T3SS compared to at least 75% of *P. aeruginosa* isolates from various acute infections, additionally, none of the T3SS positive CF isolates expressed the most toxic T3SS effector ExoU (Jain *et al*, 2004). The age related decline of T3SS expression in these isolates may be explained by the fact that many T3SS components are immunogenic and that antibodies raised to one of these components, the translocator PcrV, and possibly also to others, can result in sterilising immunity at least in lung infections of non CF individuals (Sawa *et al*, 1999). In conclusion, the T3SS may carry out tasks necessary for establishment of CF related respiratory tract infection in the first instance but nonetheless becomes surplus to requirement in the CF airway and is probably later selected against by host adaptive immune responses (Hauser *et al*, 2011). A similar situation has also been reported regarding proinflammatory virulence factors which function to co-ordinate motility and adhesion of *P. aeruginosa*. Type IV pili and flagella which, like LPS, are present in ordinary form during early infection to enable binding of organisms to epithelia and incite inflammation, are lost or at least modified in more advanced stages of disease (Hauser *et al*, 2011). Late CF isolates have often incurred mutations

in either the *pilB* gene which encodes pilin, the *pilQ* gene which is involved in the assembly of complete pili at the outer membrane and/or *rpoN*, a gene encoding a sigma factor required for regulation of pilus biogenesis (Pasloske *et al*, 1988 ; Smith *et al*, 2006 ; Chang *et al*, 2007). Moreover, mutations in *rpoN*, as well as in other regulatory genes such as *vfr* and *fleQ*, can hinder manufacture of flagellae in these isolates and decreased expression of the flagellin biosynthesis gene itself, *fliC*, can also occur (Smith *et al*, 2006 ; Jyot *et al*, 2007). The reasons behind loss of these virulence determinants remain elusive but a likely hypothesis is that motility and possibly excess inflammation enabled by them is necessary for the initial establishment but not for the maintenance of infection ; loss of these virulence factors may serve evasion of immune responses they would otherwise provoke via interaction with TLRs and, in the case of flagellin loss, may also serve to disrupt phagocytosis (Zhang *et al*, 2005 ; Mahenthiralingam & Speert, 1995). In recent years, it has been reported that *P. aeruginosa* rhamnolipids are important virulence factors required for infection of the CF airway. Zulianello and colleagues have shown that fluorescently labelled rhamnolipids did not directly affect epithelial cell viability but could bind via their alkyl chains to cell surfaces (being included firstly at the apical and later basolateral membranes of cells) and induce loss of ciliation, disrupt the normal apical localisation of ezrin and break fibril connections of the tight junction (TJ) belt ; these effects coincided with a disruption in cell polarity and migration of bacteria to the basolateral surface of the epithelium, it is yet to be determined whether alterations in the TJ connections themselves or rather disruption of the TJ lipid composition is responsible for these effects (Zulianello *et al*, 2006). This disruption of transepithelial resistance could be induced by purified rhamnolipid extracts alone and thus appears to be independent of other *P. aeruginosa* virulence attributes including elastase which had been shown to disrupt transepithelial resistance in previous work (Zulianello *et al*, 2006 ; Azghani *et al*, 2000). In addition, paracellular invasion of epithelial cell isolates in a cell polarity dependent fashion had been observed by other authors, taken together with the results of Zulianello and co-workers, this may imply that depolarisation of cells is necessary for bacterial infiltration of epithelial cells (Hirakata *et al*, 2000 ; Kazmierczak *et al*, 2004). Rhamnolipids have also been reported to serve other functions in *P.*

aeruginosa virulence including disruption of the phagocytic and killing actions of macrophages by reducing uptake of particles and inhibiting phagosome-lysosome fusion, moreover, rhamnolipids induce necrotic killing of PMNs and impair their migration (Jensen *et al*, 2007). Finally, rhamnolipids can exert antimicrobial activities against numerous other bacterial and fungal species, including some which are involved in CF related respiratory tract infections, this could conceivably aid persistence of *P. aeruginosa* in the airways by allowing it to compete more efficiently with other microorganisms which may be co-present (Haba *et al*, 2003). During the progress of CF associated airway infections, modifications in rhamnolipids emerge, those extracted from early, non mucoid isolates are greater in haemolytic and possibly other cytotoxic actions relative to those from late mucoid isolates, this could imply that the action of rhamnolipids is most critical in the initial setup of infection and becomes less crucial once an infecting strain is firmly established (McClure & Schiller, 1992). Yet another unique property of *P. aeruginosa* strains infecting the CF airway is the unusually frequent mutation of genes encoding proteins required for the proofreading of DNA (*mutS, mutL* and *uvrD*) and increase strain mutability from 20 to 1,000 fold (Mena *et al*, 2008 ; Hauser *et al*, 2011). However, the relevance of this phenomenon in conversion to mucoidy and certain other CF related phenotypes is debatable as this adaptation usually emerges only in the latter stages of the progression of CF associated respiratory disease (Feliziani *et al*, 2010). In summary, the deployment of virulence factors by *P. aeruginosa* in the CF airway is a dynamic process governed by the condition of the airway observed at different stages of infection. Many virulence determinants discussed above are subject to control by quorum sensing (discussed in section 3.5) and may be linked to biofilm modes of growth (discussed in section 3.4).

SECTION 2.3

INTRINSIC AND ACQUIRED DRUG RESISTANCE MECHANISMS

Aside from its extreme versatility as a pathogen and high levels of metabolic complexity, the broad array of antimicrobial resistance mechanisms it possesses is the most intriguing feature of *P. aeruginosa*. In hindsight, this extreme adaptability

does not come as a surprise giving the unusually large and diverse genome of the organism and the fact that its primary reservoir is in soil and freshwater where it could conceivably have endured fierce competition from antibiotic producing saprophytes as well as other toxic insults such as the presence of heavy metals, throughout its evolutionary past (Lambert, 2002 ; Knapp *et al*, 2011). As with most Gram negative bacteria, the organism is intrinsically resistant to many antibiotics which are active against Gram positive species (penicillins G & V, isoxazolylpenicillins, glycopeptides, bacitracin, macrolides, lincosamides, streptogramins, fusidanes, novobiocin, rifamycins, oxazolidinones etc) but whose large and /or hydrophobic structures do not allow them to negotiate the relatively impermeable barrier presented by the outer membrane (OM) of Gram negative bacteria, thus rendering them unable to reach their biochemical targets in these organisms (Nikaido & Nakae, 1979). Confounding this problem, *P. aeruginosa* is also innately resistant to many antibiotics (folate antagonists, aminopenicillins, tetracyclines, chloramphenicol, most cephalosporins and earlier aminoglycosides such as streptomycin and kanamycin) which can traverse the OM of other Gram negative pathogens including most genera of the *Enterobacteriaceae* and are thus generally effective against these other organisms, this may owe to species specific attributes of the OM, for instance, it has been noted that more porins in the OM of *P. aeruginosa* than in that of *E. coli* are in a closed configuration and this is consistent with the observation that the latter is around 100 fold more permeable to various hydrophilic solutes (Angus *et al*, 1982 ; Yoshimura & Nikaido, 1982 ; Yoshimura *et al*, 1983). As a result, the range of therapeutic agents available to treat pseudomonal infections is heavily restricted in the first instance. Furthermore, acquired mechanisms of resistance mediated by mutational events or receipt of mobile genetic elements have lead *P. aeruginosa* to become resistant to the precious few agents against which it is not inherently resistant. This is highly problematic and pan-resistant strains now exist which may cause practically untreatable disease. Broadly speaking, drug resistance mechanisms can be assigned to four categories (Table 1). Antimicrobial resistance observed in *P. aeruginosa* and indeed Gram negative genera as a group, whether intrinsic or acquired, is often due to an impermeability of the OM, the protective efficacy of which is further enhanced by the presence of multi-

drug efflux pumps (Livermore, 2001). As most antimicrobials act at cytoplasmic target sites or the cell wall, all of which are internalised by the OM in the case of a Gram negative species, these molecules generally rely on porins for their conveyance into the cells of these organisms and mimic the natural nutrient substrates of these channels in order to do so. The most abundant porin in *P. aeruginosa* is oprF and there is evidence that this and potentially also another major porin (oprC) act as significant albeit relatively inefficient channels for fluoroquinolones and anionic β-lactam antibiotics such as ceftazidime, piperacillin and aztreonam (Hancock, 1998 ; Satake *et al*, 1990). Other porins may also be involved in uptake of these agents and the role of individual porins has been disputed (Perez *et al*, 1997 ; Yoshihara & Nakae, 1989). Reduced sensitivity, to some of these agents is often conferred by mutations which result in either reduced expression levels or altered configuration of porins identified as necessary for influx of the drug, clinically relevant resistance, however, usually requires reinforcement via a second mechanism, often efflux and/or β-lactamase expression (Hancock, 1998). A further example of resistance occurring due to alterations in porin profile is the occurrence of high level imipenem resistance in strains lacking oprD expression, this porin serves as a conduit for the carbapenems which are unique amongst the β-lactams in that they possess a zwitterionic structure (Satake *et al*, 1990). This mutational resistance is readily selected for during therapy and may emerge spontaneously in around 17% of *P. aeruginosa* infections in CF patients receiving imipenem therapy, full resistance to meropenem is not achieved via this mechanism suggesting that this, unlike imipenem, can utilise other means of uptake in addition to oprD and should probably be the carbapenem of choice in pseudomonal infections for now (Lambert, 2002). Finally, deficiencies in both oprF and oprD have been reported to result in reduced fluoroquinolone susceptibility (Woodruff & Hancock, 1988 ; Ishii & Nakae, 1996). Efflux pumps may export a very diverse repertoire of noxious substrates including not only miscellaneous classes of antibiotic which can be completely unrelated in both chemical structure and mode of action but also a myriad of biocides, this is especially worrisome as it means that exposure to a single substrate, possibly a common disinfectant, could upregulate resistance to a wide variety of distinct agents in the organism before it even encounters them (Chuanchuen *et al*, 2001). Bioinformatic analyses have

revealed that multi-drug efflux pumps can be grouped into a number of families depending on their encoding sequences (Saier, 1998). The most common multi-drug efflux pumps amongst Gram negative pathogens belong to the group termed resistance nodulation division (RND) which includes the Mex pumps encoded in the genome of *P. aeruginosa* (Zgurskaya & Nikaido, 1999 ; Lambert, 2002). RND pumps are comprised of a transporter/entrance domain located in the cytoplasmic membrane and powered via the proton motive force, an OM channel/exit and finally a periplasm spanning domain which is centrally located to connect the other two parts (Zgurskaya & Nikaido, 1999). The basic structure of such pumps is outlined in figure 4. Often times, multi-drug resistance pumps do not in and of themselves afford degrees of resistance that reach clinical significance, however, expression of such pumps may allow members of a bacterial population to survive and replicate for longer in the presence of an antibiotic thus buying time for the emergence of mutants with more robust mechanisms of resistance which do render drugs clinically ineffective (Cohen *et al*, 1989). In *P. aeruginosa*, there are no less than four chromosomally encoded Mex pumps, with the sole exception of MexAB-OprM these are not constitutively expressed at a high enough level to confer intrinsic drug resistance and become functional only following mutation in their cognate regulators (Lomovskaya *et al*, 2001). Expression of these pumps appears to be greater at times of stress and likely carries a relatively high fitness cost which includes impaired virulence, nevertheless, mutants expressing them can be stable enough to cause infection and may have incurred compensatory mutations which bring their fitness back in line with that of the wild-type (Sanchez *et al*, 2002 ; Webber & Piddock, 2003). Collectively, the Mex pumps can confer some level of resistance to all currently available classes of drug exhibiting useful anti-pseudomonal action apart from the polymyxins and each has a specific repertoire of substrates which it can eject, however, there is a great deal of overlap between the substrate profiles of each individual Mex pump and therefore a degree of functional redundancy exists between them (Lambert, 2002). Table 1 gives details of the substrate specificity of each Mex system. A further important mechanism of drug resistance utilised by *P. aeruginosa* relies on the enzymatic inactivation of antibiotics before they can reach their targets. The most prevalent example of this is chromosomal encoded ampC β-lactamase, this

enzyme acts by hydrolysing the active structure (β-lactam ring) of most β-lactam antibiotics excluding the carbapenems (Bratu *et al*, 2007). It is released into the periplasm where it acts to neutralise incoming drugs but is only expressed to a sufficiently active level in depressed mutant cells which have underwent mutations in the cognate regulatory gene *ampR*, this has been a frequent occurrence in CF patients who have received repeated or prolonged courses of ceftazidime therapy (Lambert, 2002). For total resistance to be achieved in ampC producers, the co-presence of another mechanism (usually Mex pump expression or reduction in OM permeability) is sometimes required (Bratu *et al*, 2007). Even more worrisome is the fact that the organism can acquire further β-lactamases from other species, depending on the precise type, these enzymes will hydrolyse different categories of drugs to varying degrees, amongst such enzymes, metallo-β-lactamases such as IMP and VIM are the most concerning as they can hydrolyse carbapenems as well as all of the other β-lactams, excluding aztreonam, which is cleavable by ampC in any case, moreover, the class I and III integrons which encode them often carry resistance determinants affecting many non β-lactam agents (Walsh *et al*, 2005). Enzymatic inactivation, alongside efflux, is also the primary means of aminoglycoside resistance in *P. aeruginosa*, the enzymes involved in this process are encoded by genes received on plasmids or other mobile elements and depend on the use of cytosolic co-factors (co-enzyme A or ATP) in order to phosphorylate, acetylate or adenylate the aminoglycoside molecules which hence become ineffective at binding their targets on the 30S ribosomal subunit (Lambert, 2002). As with β-lactamases, aminoglycoside modifying enzymes differ in their substrate specificity, amikacin is affected by a lesser range of these than is either gentamicin or tobramycin, however, some variants will inactivate this and multiple other aminoglycosides, shockingly, a variant of these enzymes was recently identified that inactivates fluoroquinolones, a synthetic class of antibacterials that are completely unrelated to aminoglycosides (Maurice *et al*, 2008). Yet another type of drug resistance mechanism that is often found in *P. aeruginosa* involves adaptation of drug target sites so that they can no longer bind the drugs. The most frequent example of this is conferred by mutations in the *gyrA*/*B* genes or *parC/E* genes which encode DNA gyrase and topoisomerase IV, respectively, both of which normally bind fluoroquinolones to create a toxic nuclease

complex which cuts DNA and initiates bacterial killing (Lomovskaya *et al*, 2001 ; Drlica & Zhao, 1997). Additionally, alterations in the penicillin binding proteins (PBPs) of *P. aeruginosa* has been reported to result in varying levels of resistance to β-lactams due to a reduced binding affinity although this mechanism is controversial (Bratu *et al*, 2007). PBPs are transpeptidases which catalyse the cross linkage of peptidoglycan in the cell wall, binding of β-lactams to these proteins results in formation of weak cell walls which buckle under osmotic pressure and may also induce cellular autolytic enzymes to be produced (Kitano & Tomasz, 1979). Furthermore, acquisition of 16S rRNA methylases has recently been reported to confer high level resistance to virtually all aminoglycosides in *P. aeruginosa* through preventing the misreading of RNA that aminoglycosides induce, these enzymes are acquired through lateral exchange of genes such as *rmtA* which encode them and show homology with enzymes employed by actinomycetes to survive the action of their own aminoglycoside metabolites (Yokoyama *et al*, 2003). Modifications to the aminoglycoside binding sites of the 30S ribosomal subunit itself reportedly results in resistance to streptomycin though not to the newer aminoglycosides including gentamicin, tobramycin and amikacin which have multiple binding mechanisms (Lambert, 2002). Further examples of target modification include OM alterations that reduce sensitivity to aminoglycosides and polymyxins ; aminoglycosides initially bind to the LPS of bacteria where they induce their own cellular uptake whereas polymyxins act directly as detergents which disrupt the integrity of the OM itself, overexpression of the oprH porin has been shown to block access of both aminoglycosides and polymyxins to LPS and thus result in resistance to both of these antibiotic classes (Gilleland *et al*, 1989 ; Lambert, 2002). Various mutations which act to alter the usual components of the LPS itself may also confer resistance to polymyxins (Schurek *et al*, 2009). In addition to this expansive repertoire of classical antibiotic resistance mechanisms utilised by *P. aeruginosa*, a number of unorthodox mechanisms also exist which, many of which are largely unique to CF isolates and linked with biofilm formation and quorum sensing and will thus be considered separately in sections 3.4 and 3.5.

Resistance Mechanisms	Examples	Drugs Affected
cell impermeability	loss of porins	ß-lactams, fluoroquinolones
	OMP overexpression	polymyxins, aminoglycosides
enzymatic inactivation of drug	ß-lactamases	ß-lactams
	aminoglycoside modifying enzymes	aminoglycosides (some also affect fluoroquinolones)
altered drug target	DNA gyrase	fluoroquinolones
	PBPs	ß-lactams
	LPS	polymyxins, aminoglycosides
	16S rRNA	aminoglycosides
multidrug efflux systems	MexAB-OprM MexEF-OprN MexCD-OprJ	ß-lactams, fluoroquinolones
	MexXY-OprM	β-lactams, fluoroquinolones, aminoglycosides

Table 1

Drug Resistance Mechanisms of *P. aeruginosa*

P. aeruginosa possesses several mechanisms of resistance which can be grouped into 4 broad categories. Mex pumps extrude diverse anti-pseudomonal drugs and have over-lapping substrate specificities, in addition they may extrude various other antibiotics such as tetracyclines and chloramphenicol as well as various biocides including dyes and detergents (Masuda *et al*, 2000).

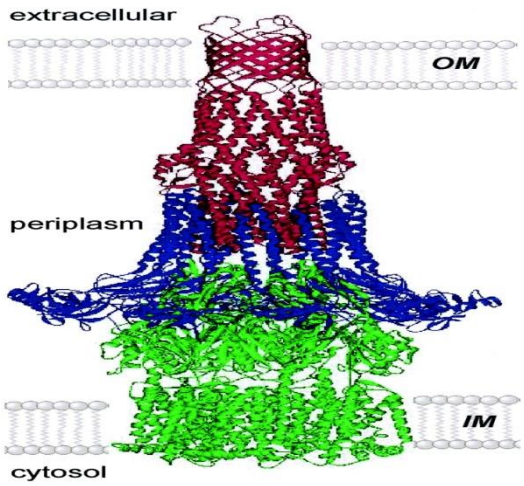

Figure 2.2

Basic Domain Structure of an RND pump (adapted from Piddock, 2006)

Mex-Opr and other RND multidrug efflux systems of Gram negative bacteria are composed of 3 domains. They possess a transporter/entrance domain anchored in the cytoplasmic membrane, this takes up substrates and is powered via the proton motive force, here this domain is shown in green. In addition, they have an exit domain located in the outer membrane, this is shown here in maroon. Finally, they possess a periplasmic spanning domain which acts as a conduit for substrates and connects the other 2 domains, here this is shown in blue.

SECTION 2.4

SIGNIFICANCE OF BIOFILMS

Many bacterial and fungal species, especially during times of stress, congregate as dense monomicrobial or polymicrobial communites encased within an extracellular matrix largely composed of exopolysaccharides, proteins and nucleic acids which derive largely from the member cells. These settlements are termed biofilms and are erected where moisture is present at the interface of liquid, solid or semi-solid surfaces which can be either biotic or abiotic. It is now estimated that biofilm

formation occurs in >65% of all bacterial diseases and is known to be a prominent feature not only of CF associated airway infections but also other entities such as UTIs and infections relating to prosthetic medical devices (Costerton *et al*, 1999). The biofilm mode of growth is highly problematic as sessile organisms can be 100-1000 fold more tolerant to antimicrobics relative to those in the planktonic state and are also somewhat shielded from immune responses (Hoyle & Costerton, 1991). Biofilm formation occurs in a stepwise fashion, although the exact details of each stage are species dependent, they can be grouped loosely into a chronological series of five for most organisms (Figure 5). The first stage involves reversible attachment of planktonic bacteria to a surface, this is followed by fixed attachment and the formation of microcolonies, the next stage involves increased exopolymer production and growth of attached organisms which overlaps with a fourth stage during which this process continues and the biofilm becomes structurally differentiated and mature, the fifth stage is a dispersal event during which organisms exist the biofilm, sometimes adsorbing elsewhere to form a new biofilm. Biofilms vary widely in architecture, some develop as flat uniform layers whilst others are heterogenous in structure with distinctive 'toadstool' or 'hill' shaped microcolonies and flapping 'streamer' elements (Lawrence *et al*, 1991 ; Costerton *et al*, 1999). Shear force and type of flow (laminar or turbulent) in the environmental surroundings also influence the structure, microrheology and strength of biofilms (Rogers *et al*, 2008). Regional variations in gene expression and physiological state occur amongst bacteria occupying different sections of the biofilm (Chiang *et al*, 2012). Furthermore, the close proximity of bacterial cells in a matrix containing considerable levels of microbial DNA allows for a higher frequency of lateral genetic transfer via both conjugative and transformative processes, the genes involved may in some cases be associated with antibiotic resistance or increased stabilisation of the biofilm (Molin & Tolker-Nielsen, 2003 ; Król *et al*, 2011). Hollow aqueous channels are incorporated throughout the biofilm to enable access of water and nutrients to the constituent cells (Costerton, 1999). There are a number of pieces of evidence suggesting that biofilm formation plays a part in the establishment of recalcitrant infections of the CF airway by *P. aeruginosa*, which incidentally is a model organism for biofilm development in Gram negative bacteria. Amongst these are the

previously demonstrated presence of distinctive *P. aeruginosa* microcolonies in samples of patients' sputa and also of lung tissue either from freshly excised sections or whole organs viewed *post mortem* (Singh *et al*, 2000 ; Worlitzsch *et al*, 2002 ; Lam *et al*, 1980). It is believed that the relatively anoxic milieu provided by the abnormally dehydrated and viscous mucus layer of the CF airway together with the presence of excess DNA and actin from necrotic PMNs facilitates the initial formation of *P. aeruginosa* biofilms (Worlitzsch *et al*, 2002 ; Boucher, 2004). Moreover, another group has shown that the ratio of two key *P. aeruginosa* quorum sensing molecules in CF sputum samples was closer to that normally secreted by sessile culturess *in vitro* as opposed to planktonic ones (Singh *et al*, 2000). In addition to this, macrolides, known to modulate both quorum sensing and alginate biosynthesis pathways *in vitro*, have helped eradicate *P. aeruginosa* from a murine model of chronic lung infection despite the fact that this species is intrinsically resistant to this class of antibiotics (Hoffman *et al*, 2007). Studies of *P. aeruginosa* biofilms have usually been carried out *ex vivo* on abiotic (glass, plastics etc) surfaces both in static formats such as microtitre plates and in flow cells which more realistically mimic *in vivo* conditions as the mobile broth medium can supply varying conditions of shear stress (Moreau-Marquis *et al*, 2008). Although static *in vitro* models have the advantage of being amenable to high-throughput and flow cell systems enable study of biofilms in a dynamic system, both have several drawbacks relating to the fact that they cannot incorporate various substrates and conditions unique to the CF airway (or other pathological systems), it has therefore been proposed that some studies of biofilm associated infection may be carried out with more realistic results on polarised human airway epithelial cells (Moreau-Marquis *et al*, 2008). Formation of *P. aeruginosa* biofilms is a dynamic, multifactorial phenomenon easily influenced by environmental surroundings and to a large extent governed by quorum sensing. It was hitherto thought that flagellae were necessary to convey individual bacteria to the substratum on which biofilm formation occurs and that on arrival, twitching motility mediated by type IV pili was required for adhesion and microcolony development (O'Toole & Kolter, 1998). This notion was later disputed when it was realised that PAO1 mutants lacking functional flagella (Δ*fliM*) and/or type IV pili (Δ*pilA*) could form biofilms on glass surfaces in flow cell systems

and to wells of microtitre plates (Klausen *et al*, 2003). In the aforementioned study, however, mutant strains formed biofilm in citrate based minimal medium but not in a minimal medium based on glucose with casamino acids, moreover biofilms formed by the (Δ*fliM*) and (Δ*pilA*) mutants matured as 'hill like' and 'towering' structures, respectively, whilst that formed by the wild-type was flat and homogenous, accordingly, the authors inferred that the necessity of motility determinants for biofilm development is dependent on the carbon sources available and that motility, especially type IV pilus mediated twitching motility, whilst not always essential for initial attachment and microcolony formation, is vital for horizontal expansion and dispersion in biofilm architecture (Klausen *et al*, 2003). This seems consistent with the findings of Sauer and Camper who found that *pilA* was not expressed at high levels until the later stages of biofilm formation (Sauer & Camper, 2001). Klausen's group also noted that the previous study of O'Toole & Kolter which found motility to be necessary for attachment and microcolony formation used strain PA14 as opposed to PAO1, thus suggesting the likelihood of strain dependent variations in the requirement of motility for biofilm formation (O'Toole & Kolter, 1998 ; Klausen *et al*, 2003). During and after microcolony formation, components of the biofilm matrix are produced and assembled. Amongst these matrix constituents is DNA, in the case of the CF airway, this was originally thought to derive mostly from necrotic PMNs and lysed bacteria but it is nevertheless known that substantial quantities of DNA are released in microbodies secreted by intact bacteria (Muto & Goto, 1986 ; Kadurugamuwa & Beveridge, 1995). Whitchurch and colleagues have performed flow cell studies to test the effect of DNaseI on biofilm formation by *P. aeruginosa* strain PAO1 in which they determined that this enzyme did not affect the viability of individual bacteria but could prevent the initial establishment of biofilm and disperse young (12-60 hours old) biofilms whilst having minimal disruptive effect on biofilms that had matured for 84 hours (Whitchurch *et al*, 2002). Later evidence suggested that DNA was in fact necessary for integrity in mature biofilms as DNaseI pretreatments of these biofilms were found to result in enhanced disruption on subsequent challenge with sodium dodecyl sulphate (Allesen-Holm *et al*, 2006). Moreover, Whitchurch and co-workers employed a carbazole colorimetric assay to show that DNA and not alginate was the predominant matrix component in these

early biofilms, this may suggest a particularly vital role of DNA in early biofilm development whilst indicating that other components may become more prevalent as the biofilm matures and achieves stability towards DNaseI or alternatively that increased expression of proteolytic enzymes protecting the biofilm from DNaseI activity occurs during maturation (Whitchurch *et al*, 2002). Evidence has been presented to suggest that the the previously mentioned type IV pili of some species, including *P. aeruginosa*, bind to DNA and may thus anchor the organisms in the biofilm matrix (van Schaik *et al*, 2005). Evidence obtained via PCR and Southern blots implies that the DNA content within biofilm of *P. aeruginosa* is likely derived from a whole genome source (Steinberger & Holden, 2005 ; Allesen-Holm *et al*, 2006). It has been found that some *P. aeruginosa* isolates release DNA on lysis via a prophage mediated mechanism that is inducible by quinolone compounds both in the form of antibiotics such as ciprofloxacin and as the endogenous quinolone signalling molecules of *P. aeruginosa* (Froshauer *et al*, 1996 ; Webb *et al*, 2003). Additionally, it has been shown that these quinolone signalling molecules are required for release of DNA in the form of vesicles (Mashburn & Whiteley, 2005). Section 3.5 of this review will discuss the impact of these and other quorum sensing molecules on biofilm formation in greater detail. It has been shown that in heterogeneous *P. aeruginosa* biofilms, extracellular DNA is concentrated in the stalk region of the toadstool shaped structures (Allesen-Holm *et al*, 2006). Previous studies have shown that bacteria located within the basal stalk region are also less motile than those in the apical cap region and some authors have surmised that this may indicate that motile forms expressing type IV pili (which bind to DNA) migrate to the top of the stalks and use them as a pedestal on which to attach via type IV pili and form the caps (Klausen *et al*, 2003). Host DNA and actin are abundant in the CF airway and it has been shown that DNA-actin filaments can be incorporated into the matrix of *P. aeruginosa* biofilm (Walker *et al*, 2005). In artificially generated media mimicking CF sputa, it was found that biofilm formation by *P. aeruginosa* demands the presence of certain amino-acids (Sriramulu *et al*, 2005). This may be due to the fact that aromatic amino-acids are needed for synthesis of the PQS that conducts DNA release from *P. aeruginosa* cells and this hypothesis is supported by the finding that greater amino-acid levels in the sputum correlates with worse disease outcomes in

CF (Palmer *et al*, 2005 ; Thomas *et al*, 2000). Amongst the exopolysaccharides secreted by *P. aeruginosa*, alginate is known to contribute to the maturation of biofilm matrices but appears to be dispensible for biofilm formation per se (Stapper *et al*, 2004). More recent works have found that alginate is not at all important in biofilm formation by laboratory strains PAO1 and PA14 which although generally non-mucoid can produce alginate, this of course does not categorically rule out a role for alginate in the development of biofilms by mucoid strains in the CF airway (Wozniak *et al*, 2003). Aside from alginate, the production and chemistry of which has already been detailed in section 3.2 of this review, at least two other exopolysaccharides secreted by *P. aeruginosa* are known to contribute to biofilm formation. These polymers are termed Pel and Psl and unlike alginate are branching rather than linear structures, the former is composed largely of glucose and the latter of mannose although additional constituents are also present (Friedman & Kolter, 2004a ; Friedman & Kolter 2004b). Pel, as the name insinuates, is important for the formation of pellicle biofilms suspended on the surface of liquid cultures and may interact with cup fimbriae during the process of biofilm formation (Friedman & Kolter ; 2004a). Cup fimbriae are bacterial appendages whose expression is dependent on up-regulation of the intracellular second messenger cyclic di-GMP via components of the *wsp* chemosensory system and have previously been shown to be required in the adhesion of *P. aeruginosa* cells to substrata and to each other (Vallet *et al*, 2001 ; Kuchma *et al*, 2005). Pel has been demonstrated to be the most significant exopolymer in biofilm formation by PA14, which is devoid of the *psl* operon, the role of Pel in biofilm development by PAO1, which possesses both the *psl* and *pel* operons, was not demonstrated and Psl was revealed to be the primary polysaccharide incorporated into biofilms of this strain (Colvin *et al*, 2011a,b). Using a Δ*pslAB* mutant of PAO1, Ma and colleagues found Psl to be necessary for both the maintenance and continued growth of PAO1 biofilm (Ma *et al*, 2006). In contrast, Pel appears to be important for the continued growth of PA14 biofilms but is not vital to the maintenance of pre-established biofilms of this strain (Colvin *et al*, 2011a,b). This would not conflict with the earlier finding that Pel is essential primarily in the latter stages of biofilm development whilst Psl is required early on in biofilm development in strains with the capacity to produce both of these

polysaccharide types (Friedman & Kolter, 2004b ; Vasseur *et al*, 2005). There is also evidence that Pel has adhesive properties and may compensate for the loss of other adhesins such as the type IV pilus (Vasseur *et al*, 2005). Transcription of the *pel* and *psl* operons depends on up-regulation of cyclic di-GMP via the *wsp* system, which has been shown to be required for optimal biofilm development in microtitre dish and flow cell assays (Hickman *et al*, 2005). Amongst proteins attached to bacterial cell surfaces, the importance of the fucose binding lectin, Lec B, in binding to the biofilm matrix, has been demonstrated in *P. aeruginosa* (Loris *et al*, 2003 ; Tielker *et al*, 2005). A similar situation exists with the galactose binding lectin, LecA (Diggle *et al*, 2006b). There is evidence to suggest that rhamnolipids use their surfactant properties to help co-ordinate structure in biofilms of *P. aeruginosa*, these aid in the maintenance of fluid channels throughout the biofilm and may help prevent 'invading' bacteria from joining the structure (Espinosa-Urgel, 2003). Rhamnolipids also contribute to dispersion of biofilms (Kaplan *et al*, 2010). Other factors leading to dispersal include prophage mediated bacterial lysis and liberation of endogenous nitric oxide (Webb *et al*, 2003 ; Barraud *et al*, 2006). Research pursuing explanations for biofilm specific antibiotic resistance has increased over the last 20 years or so. *P. aeruginosa* cells growing within biofilms may or may not express some of the classical antibiotic resistance mechanisms described above in section 3.3, but, regardless, they have a greater tolerance to antimicrobial agents than do drug sensitive planktonic populations of this species. The first and most obvious explanation proposed to explain biofilm specific resilience to antimicrobial agents held that the biofilm matric impeded penetration of drug molecules. Conflicting data have emerged from this line of inquiry. A number of studies have found that the *P. aeruginosa* biofilm matrix may delay penetration of β-lactams and aminoglycosides (Kumon *et al*, 1994 ; Shigeta *et al*, 1997). The effects of biofilm matrix on the penetration of fluoroquinolones has differed between studies, some papers report that penetration of these agents was impeded whilst others report their rapid and complete penetrative power (Suci *et al*, 1994 ; Shigeta *et al*, 1997). Caution should be exercised in interpretation of these studies as it is possible that defects of penetration could be due to overlooked factors other than sequestration of drugs by the biofilm matrix. One study showed that Pel polysaccharide reduced the antibacterial efficacy

of aminoglycoside antibiotics against *P. aeruginosa* strain PAO1 biofilms whilst in a previous study, Pel polysaccharide producing PA14 biofilms were actually more susceptible to aminoglycosides than those formed by an isogenic mutant of PA14 that did not produce Pel, fluoroquinolone sensitivity was indifferent to the presence of Pel in these PA14 biofilms (Colvin *et al*, 2011 a,b ; Khan *et al*, 2010). Several studies have indicated that alginate production is linked to reduced aminoglycoside sensitivity. Alginate was shown to bind tobramycin and increase MICs of this drug towards mucoid *P. aeruginosa* in one study (Nichols *et al*, 1988). Hatch and Schiller report that a 2% suspension of alginate extracted from mucoid *P. aeruginosa* allowed passage of carbenicillin but impeded access of both gentamicin and tobramycin, an effect that was reduced on application of alginate lyase (Hatch & Schiller, 1998). In another study, fluoroquinolones were shown to stimulate conversion of non-mucoid *P. aeruginosa* strains to mucoid variants which displayed greater resistance to these drugs than the non-mucoid parent (Pina & Mattingly, 1997). Extracellular DNA has also been shown to contribute to biofilm related antibiotic resistance in one study. The authors mention that extracellular DNA can have bactericidal properties via chelating LPS associated cations which are essential to OM stability in bacteria, they then demonstrate that organisms may thus adapt to produce a substituted LPS which in addition to withstanding the effects of DNA, will not efficiently bind many antimicrobial agents that require normal LPS for self-promoted uptake, this includes aminoglycosides, and cationic antimicrobial peptides – either polymyxin drugs or host defense peptides (Mulcahy *et al*, 2008). In addition to biofilm matrix constituents, several other factors may induce biofilm linked antimicrobial resistance. One of these is the biofilm specific expression of *ndvB*, a gene which encodes periplasmic glucans which can bind antibiotics such as aminoglycosides and fluoroquinolones before they reach their cytoplasmic targets (Mah *et al*, 2003). Yet another example is the finding by Zhang and Mah that expression of a putative novel efflux pump is linked to the biofilm phenotype and may co-operate with the previously mentioned periplasmic glucans to increase antibiotic resistance of sessile *P. aeruginosa* populations (Zhang & Mah, 2008). The fact that biofilms in the CF airway grow in a relatively anoxic atmosphere may have implications for the efficacy of aminoglycosides, these drugs achieve maximal effect only against aerobically

respiring organisms, anaerobically respiring bacteria are resistant to the extent that these agents are incorporated into many different media used for the selective cultivation of obligate anaerobes such as species of *Clostridium* and *Bacteroides* (Tack & Sabath, 1985). The CF airway may act as a set up for *P. aeruginosa* strains which form small colony variants (SCVs), these subpopulations of organisms can not only exhibit enhanced powers of adhesion and virulence but also of antibiotic resistance, this is due to a form of phase variation and the genetic control mechanisms for this phenomena are not yet fully elucidated, emergence of these variants is linked to consumption of anti-pseudomonal medication (Drenkard & Ausubel, 2002). Possibly the single most important reason for the failure of conventional antimicrobial therapy to fully eradicate biofilm related infections is the emergence of bacterial subpopulations termed persister cells. The concept of persister cells dates back as far as the introduction antibiotic therapy itself in the 1940s when Bigger noted that cultures of staphylococci could never be completely sterilised by addition of penicillin, even at very high concentrations of the drug, dormant but viable bacteria would remain and these could resume growth but only upon transfer to fresh media lacking the antibiotic, treatment of this new subculture with penicillin would again decimate but not entirely wipe out the bacterial population (Lewis, 2010). As a result, persistence is not an example of true resistance but merely of tolerance to antimicrobial agents as drug MICs of the bacterial population do not rise in the persister cells relative to the population at large, hence persisters cannot grow in the presence of antibiotics although they do survive their action (Keren *et al*, 2004). Furthermore, persistence is not a heritable fixed genotypic property but rather a fleeting epigenetic trait that is conceptually analogous to numerous other bacterial behaviours such as sporulation by Gram positive bacilli (Lewis, 2008). Persister subpopulations have been observed in all pathogenic bacterial species studied to date in this regard (Mulcahy *et al*, 2010). A particularly interesting feature of persistence is that it is not a unidirectional adaptation ; it allows the majority of a population to reproduce at optimal rates under normal conditions whilst ensuring that extinction does not occur upon antibiotic exposures, therefore, persistence may provide an ideal evolutionary 'safety net' in environments where antibiotic encounters are a plausible albeit improbable risk (Gardner *et al*, 2007). The

number of persisters resident within a population has been shown to vary according to growth phase. Shah and colleagues employed both FACS and microarray analyses to show that persisters were 20 fold less abundant in an *E. coli* population during exponential growth relative to when this organism was in the stationary growth phase (Shah *et al*, 2006). Elsewhere, it has been suggested that around 1% of cells in stationary phase cultures are persisters and that this distribution is similar for biofilms, which grow slowly (Lewis, 2008). Whilst bacteriostatic antibiotics such as the tetracyclines simply block the functions of their targets, bactericidal types (including virtually all anti-pseudomonal drugs) usually pervert the function of their targets leading to the formation of toxic molecules which are responsible for the actual lethal events (Lewis, 2008). Hence, persistence mechanisms are protective as they act by suspending the downstream cellular processes which are otherwise initiated by the target molecules of bactericidal antibiotics, in contrast, true drug resistance mechanisms operate by simply preventing binding of the drug to its target in the first instance (Lewis, 2008). Whilst persistence seems to be a universal epigenetic function, its incidence within a population may be increased by a number of mutations. Most experience with persister genetics has come from work on *E. coli*. Genes that are likely to be involved include those encoding the toxin components of toxin/antitoxin operons, global transcription regulators and those mediating metabolism of glycerol or nucleotides (Mulcahy *et al*, 2010). The unifying aspect of these mutations is that they can block fundamental metabolic activities such as biosynthesis of ATP or proteins, furthermore, many mechanisms controlling persister emergence are functionally superfluous (Mulcahy *et al*, 2010). To date, no homologues of these *E. coli* persister genes have been identified in major biofilm forming pathogens of the CF airway, nevertheless, candidate persister genes in *P. aeruginosa* include *dinG* which encodes a predicted DNA helicase, the *pilH* type IV pilus response regulator and two other proteins (PA3589 and PA5002) of unknown function (De Groote *et al*, 2009a,b). A number of workers have shown that antibiotic exposure can affect the number of persisters in a bacterial population. Dörr and colleagues have shown that ciprofloxacin may increase persister formation rates in *E. coli* by triggering overexpression of toxin/antitoxin modules as part of the SOS response (Dörr *et al*, 2010). Moreover, a study by Mulcahy and co-workers assessed

paired *P. aeruginosa* isolates obtained from CF patients at early and late intervals during infection. When challenged with 3 antibiotics (carbenicillin, tobramycin and ofloxacin), 10 of 14 late stage isolates exhibited distinguished biphasic kill curves relative to their early counterparts, this likely indicates increased formation of persisters as infection of the CF airway progressed (Mulcahy *et al*, 2010). Although persistence of biofilm cells is not necessarily greater than that of stationary phase planktonic cells, existence within the context of a biofilm such as that found in the CF airway gives persister cell subpopulations a unique double barrelled advantage. Theoretically, slow growing persister cells should be eliminated almost effortlessly by the host immune response after antimicrobial therapy has cleared the drug susceptible majority of the bacterial population ; it has been proposed that this does not occur in biofilms as the exopolysaccharide matrix impedes opsonophagocytosis long enough for the surviving persisters to resume growth and replenish the biofilm (Lewis, 2010). It has been suggested that in mucoid biofilms, alginate disrupts phagocytosis of the bacteria by macrophages whilst rhamnolipids result in rapid necrosis of incoming PMNs (Leid *et al*, 2005 ; Alhede *et al*, 2009).

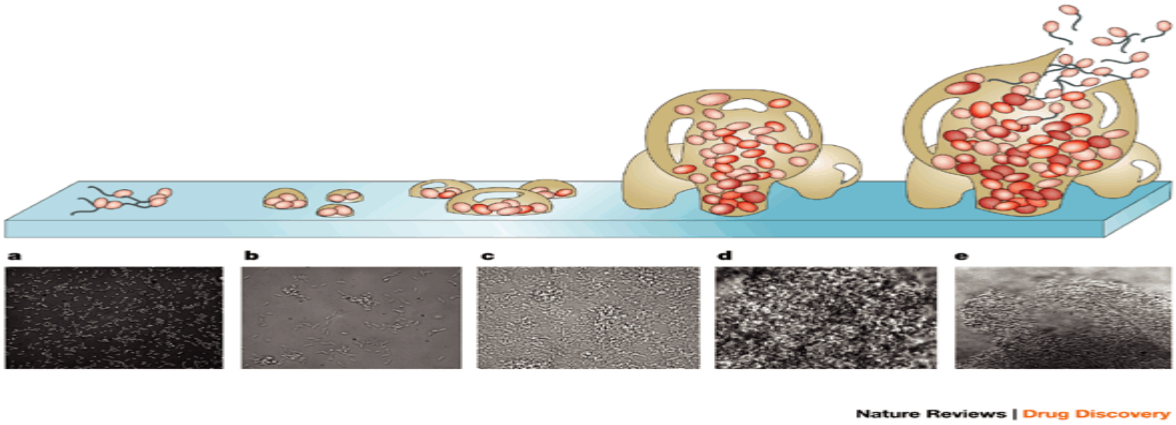

Figure 2.3
The 5 Stages of Biofilm Development (adapted from Davies, 2003)

Bacterial biofilm formation, regardless of the species involved, generally occurs in 5 broadly defined stages. The first stage involves adhesion of organisms to a substratum, the second stage involves irreversible attachment and formation of microcolonies, the next stage involves exopolymer secretion whilst the fourth stage involves structural differentiation. Dispersion occurs in the fifth and final stage. Here, artist's impressions of these stages are depicted and designated alphabetically (a through to e). Also shown are real photomicrographs depicting each corresponding stage of *P. aeruginosa* biofilm formation.

SECTION 2.5

ROLE OF QUORUM SENSING

Following the illustrious experiments of Tomasz and Mosser who found that pneumococcal transformation was not a random event and of Nealson and co-workers who elucidated the control mechanisms of bioluminescence in the marine bacterium *Vibrio fischeri*, it gradually became apparent that bacteria were social organisms which could communicate via means of secretion and reception of signalling molecules on reaching sufficient cell densities (Tomasz & Mosser, 1966 ; Nealson *et al*, 1970). This phenomenon is now termed quorum sensing (QS). Cells activated by the secreted autoinducer molecules then proceed to secrete their own signals, thus initiating a positive feedback loop which allows population members to act in concert via synchronised gene expression (Ng & Bassler, 2009). Two main

classes of signalling molecule operate in Gram negative bacteria, namely acyl homoserine lactones (AHLs) and members of the autoinducer-2 (AI2) group, the latter class also functions in Gram positive species (Winans, 2002). *P. aeruginosa* is amongst the most intensely studied species in relation to QS. It possesses at least 3 distinct but interconnected QS systems. The first of these, the *las* system, was described in the early 1990s on discovery of the *lasR* transcriptional regulator which aside from activating the elastase encoding *lasB* gene controls expression of *lasI*, a synthase gene which encodes the AHL signalling molecule 3-oxo-C_{12}-HSL (Passador *et al*, 1993 ; Pearson *et al*, 1994). 3-oxo-C_{12}-HSL induces expression of *lasI*, thus regulating its own release via positive feedback (Seed *et al*, 1995). Intriguingly, the MexAB-OprM efflux pump acts as a secretory device for 3-oxo-C_{12}-HSL (Pearson *et al*, 1999). The second QS system of *P. aeruginosa* is the *rhl* system, which incorporates the RhlR transcription factor and its cognate RhlI synthase which manufactures C_4-HSL, a further AHL signalling molecule (Pearson *et al*, 1995). Many functions are conducted by the *rhl* system, some of which are also subject to modulation by the *las* system, including biofilm formation and a myriad of virulence factors (Juhas *et al*, 2005). Elements involved in activation of the *rhl* system include not only *rhlI* itself but additionally components of the *las* system ; it is thus evident that the *rhl* and *las* systems can not only be auto-regulated but may also cross-regulate each other (Juhas *et al*, 2005). Although this is consistent with the original view that QS systems of *P. aeruginosa* form a hierarchical assembly lead by the *las* system, work by Medina and colleagues has shown that this is not always the case as they found that *rhlR* transcription may occur independently of *las* components under specific environmental conditions (Latifi *et al*, 1996 ; Medina *et al*, 2003). Both the LasR and RhlR proteins have been grouped within the LuxR family of transcription regulators which bind promoters via palindromic DNA stretches known, in the case of *P. aeruginosa*, as Las boxes (Whiteley *et al*, 1999). The base sequence of these regions was initially thought to determine whether a promoter required either C_4-HSL-RhlR, 3-oxo-C_{12}-HSL-LasR or both for activation (Whiteley & Greenberg, 2001). However, a later paper states that although RhlR acts like other LuxR regulators and binds at Las boxes, LasR does not and its binding sites do not share any single consensus sequence (Schuster *et al*, 2004). More recently, a third QS

system was identified in *P. aeruginosa*. The operons *phnAB* and *pqsABCDE* enable synthesis of 4-hydroxy-2-alkylquinolines (HAQs), one type of which is HHQ or 4-hydroxy-2-heptylquinoline (Déziel *et al*, 2004). Both *phnAB* and *pqsABCDE* are under positive regulation by MvfR, a virulence linked transcription factor (Xiao *et al*, 2006). HHQ facilitates binding of MvfR to the *pqsABCDE* promoter thus positively regulating its own production (Xiao *et al*, 2006). Following secretion, HHQ enters neighbouring bacteria where it is converted into *Pseudomonas* quinolone signal (PQS) by PqsH, a LasR regulated putative monooxygenase (Gallagher *et al*, 2002 ; Diggle *et al*, 2006a). In addition to stimulating biofilm formation on addition to cultures, PQS regulates many virulence genes (Juhas *et al*, 2005). It is now appreciated that PQS is partially redundant for many of the functions it co-ordinates can be delegated to its precursor HHQ (Xiao *et al*, 2006). Some authors have suggested that *las* independent activation of the *rhl* system may involve the PQS system and that certain other LasR mediated functions such as biosynthesis of PQS and 3-oxo-C_{12}-HSL may be taken over by the *rhl* system, moreover, PQS can overcome the population density dependent nature of QS (Diggle *et al*, 2003 ; Dekimpe & Déziel, 2009). Hence, the originally proposed QS hierarchy may still apply if PQS is considered as a central link between the *las* and *rhl* systems (Diggle *et al*, 2003 ; Dekimpe & Déziel, 2009). *P. aeruginosa* is somewhat unusual in that it does not have the capacity to express the universal autoinducer-2 type QS molecules, it can, however, detect and respond to these molecules when they are produced by other bacterial species, an event which may occur in the milieus of CF sputum or polymicrobial biofilms (Duan *et al*, 2003). Table 2 summarises the many functions of the *P. aeruginosa* QS systems. In addition to the 3 formal QS systems discussed above, *P. aeruginosa* possesses at least 64 two component signal transduction systems, some of which play roles in virulence and environmental adaptations of the organism (Rodrigue *et al*, 2000). Many of these interact with the QS hierarchy of *P. aeruginosa* at various points, it would not be feasible to mention all of these in due detail in a brief review but some of those influencing QS are listed below (Figure 6). Much evidence has accrued to support the roles of QS molecules specifically in the framework of biofilm development. In flow cell studies, *P. aeruginosa lasI* mutants have been shown to form scant, 2 dimensional biofilms that were easily dispersible

by sodium dodecyl sulphate, by comparison, their wild-type counterparts were 3 dimensional, structurally differentiated and stable to dispersal with detergent (Davies *et al*, 1998). In a similar experiment, wild-type as well as *lasI* mutant biofilms formed scant, unstructured biofilms (Heydorn *et al*, 2002). The discrepancies in these results could perhaps be accounted for by the fact that the former group utilised a glucose based minimal medium whilst the latter used a citrate based minimal medium (Davies *et al*, 1998 ; Heydorn *et al*, 2002). In other studies employing dilute Luria Bertani medium, both wildtype and *lasI* mutant strains grew as structured biofilms but discernible microscopic differences were nevertheless present between them (Purevdorj *et al*, 2002). *rhlI* mutants have been shown shown to form biofilms that are devoid of the usual toadstool cap structures, furthermore, chemical inhibition of either AHLs (Patriquin *et al*, 2008) or PQS (Yang *et al*, 2009) has also been demonstrated to have this effect. Rhamnolipids are known to form under the influence of QS and function in the development of cap structures via mediation of bacterial motility in the late stages of biofilm maturation (Pamp & Tolker-Nielsen, 2007). There is also evidence that extracellular DNA is required for formation of these biofilm caps and that its release depends heavily on the PQS system (Allesen-Holm *et al*, 2006). As QS molecules are expressed primarily in the stalk structures of biofilms, (de Kievit *et al*, 2001 ; Yang *et al*, 2009) bacteria located there may secrete large amounts of DNA to act as a ligand for incoming organisms with motility mediated by rhamnolipids, flagellae and type IV pili (Pamp & Tolker-Nielsen, 2007).

las system	*rhl* system	PQS system
PQS system	PQS system	*rhl* system
rhl system	rhamnolipids	biofilm formation
biofilm formation	alkaline protease	elastase
alkaline protease	pyocyanin	pyocyanin
elastase	lipase	lectins A and B
lipase	lectins A and B	
hydrogen cyanide	hydrogen cyanide	
Xcp secretion	Xcp secretion	
exotoxin A	chitinase	
neuraminidase	RpoS	
Pvds-reg. endoprotease	exoenzyme S	
catalase	swarming motility	
superoxide dismutase	twitching motility	
aminopeptidase	biofilm formation	
swimming motility		
swarming		
twitching motility		

Table 2
A summary of *P. aeruginosa* QS functions (adapted from Juhas *et al*, 2005)

Many virulence factors, metabolic pathways and behaviours such as biofilm formation are influenced by the 3 major QS systems in *P. aeruginosa*. There is a substantial degree of cross regulation between the 3 systems and a certain level of functional redundancy is enabled by this. Notably, all 3 systems can contribute to biofilm formation.

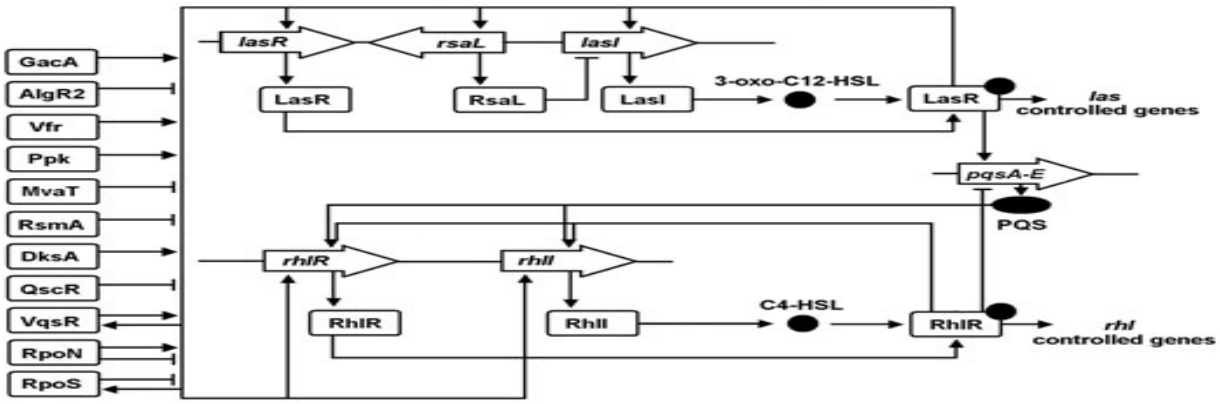

Figure 2.4
The Hierarchy of *P. aeruginosa* QS systems (adapted from Juhas *et al*, 2005)

P. aeruginosa QS systems are hierarchically linked to each other with the *las* system on top being linked to the *rhl* system on the bottom via the PQS system. Numerous other regulatory systems, including a multitude of two component signal transduction pathways also link into the QS hierarchy, some better defined examples of these are listed on the left of the schematic.

CHAPTER 3

LAMELLASOMES™ ; POTENTIAL IN CYSTIC FIBROSIS AIRWAY DISEASE

SECTION 3.1

LAMELLASOMES™ - AN INTRODUCTION

Lamellar bodies are membrane bound organelles comprising many phospholipid layers, typically measuring 100-2400nm in diameter, they are present in a wide variety of cell types including epidermal cells, various epithelial cells and type II pneumocytes of the airways where their function to store and secrete phospholipid surfactants for tissue lubrication (Schmitz & Muller, 1991). Several pieces of evidence exist to suggest that lamellar bodies are deficient within the CF respiratory tract. Puchelle and co-workers have reported that there is a paucity of surfactant phospholipids in the CF airway (Puchelle *et al*, 2002). CFTR knockout mice have demonstrably far fewer lamellar bodies in their tissues, a defect that is reversible via *in utero* replacement of the CFTR gene (Larson *et al*, 2000). Furthermore, a clear reduction in both the number and size of lamellar bodies has been observed in electron microscopic studies of tissue biopsied from CF airways (Appendix B). Lamellar bodies contain a mixture of different phospholipids including phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol as well as cholesterol and surfactant proteins (Griese, 1999 ; Lamellar Biomedical, personal correspondence). Lamellar bodies function in mucociliary transport of inhaled debris which includes microorganisms and also in maintaining airway elasticity, both of which are features jeopardised in the CF airway (Griese, 1999). As a result, exogenous lamellar body mimetics administered as inhaled therapeutics could conceivably improve these functions in CF patients. Lamellar Biomedical's LMS-611 is one such lamellar body mimetic. LMS-611 is a formulation of multi-lamellar liposomes (lamellasomes) whose lipid content mimics that of naturally occurring lamellar bodies in both type and ratio, incorporating 55 % phosphatidylcholine, 19.4 % sphingomyelin, 10.2 % phosphatidylethanolamine, 4.1 % phosphatidylserine, 3.1 % phosphatidylinositol and 8.5 % cholesterol (Lamellar biomedical. These lamellasomes have a median diameter of 6.3µm, a zeta potential of -35mV ± 10mV and are dispensed in normal saline (0.9% NaCl) solution to give a white suspension with total lipid content of 19.6mg lipid/20mg lamellasomes/ml and viscosity of $1.5 \times 10^{-3} \pm 0.2$ PaS (Lamellar Biomedical, personal correspondence). Acute toxicity studies show that LMS-611 is tolerated well in dogs at doses as high

as 19mg/kg when given via the inhalational route, it also reaches its anatomical target site in the periciliary liquid layer of the airway where it remains for at least 6 hours (Lamellar Biomedical, personal correspondence). Preliminary *in vitro* studies have shown LMS-611 to be capable of consistently reducing the viscoelasticity of CF sputum by 37-48%, an effect owing to its surface tension altering properties on biological gels including mucin, DNA and alginate. Lamellasomes carry a negative charge and thus may repel other negatively charged biopolymers, likewise, lamellasomes could plausibly sequester many positively charged molecules, including such inflammatory mediators as LPS, IL-8 and elastase (Lamellar Biomedical, personal correspondence). In a male Durkin-Hartley guinea-pig model, LMS-611 resulted in 41% faster clearance of implanted carboxymethylcellulose gel from the trachea than did a control of liposome L4395 (Appendix B). In another experiment, Balb/c mice were given either 50µl of LMS-611 (20mg/ml) or vehicle control (normal saline), 2 hours later, they were challenged with 1 x 10^6 cfu of *P. aeruginosa* (strain LES65B), LMS-611 or control treatments were then repeated every 24 hours for a total of 72 hours and the progress of infection was followed by sampling of the lungs, blood, and nasopharynx 5 hours after each dose was administered (Appendix B). On day 3 of this study, cfu counts obtained from treated mice were 1.5 log (92%) lower than those from control mice, on follow up at day 7 no difference of bacterial load was apparent between the two groups, however, treated mice were found to have higher airway macrophage counts relative to controls, in spite of this, proinflammatory cytokine levels were not significantly higher in the treated group (Appendix B). This animal model has previously been validated in the literature although not in regard to LMS-611 (Carter *et al*, 2010). In summary, LMS-611 shows potential as an inhaled therapy for infections of the respiratory tract in CF patients as it may not only enhance airway clearance but also exhibits both anti-inflammatory and indirect anti-infective action. Several *in vitro* studies were thus undertaken in an attempt to investigate the effects of LMS-611 alone and with established antimicrobials on biofilm forming strains of *P. aeruginosa*.

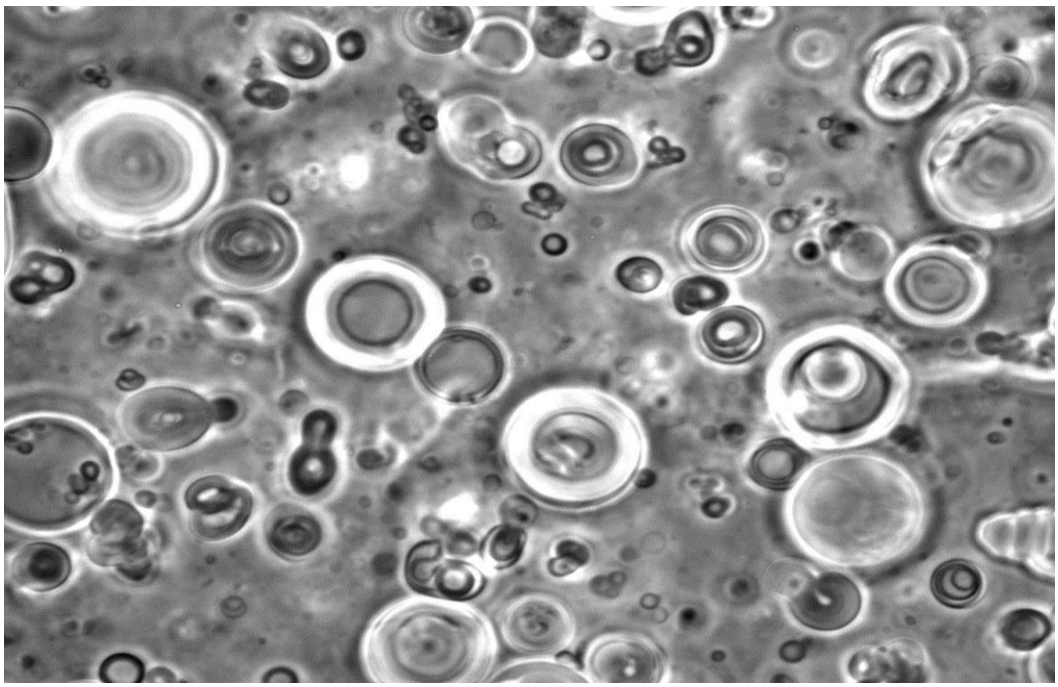

Figure 3.1.1

Confocal brightfield micrograph of LMS-611 formulation. 100 x magnification of LMS-611 formulation viewed using a Nikon TE2000 inverted confocal microscope and captured with a Hamamatsu digital camera. Note the concentric ring structure of the lamellar body like vesicles.

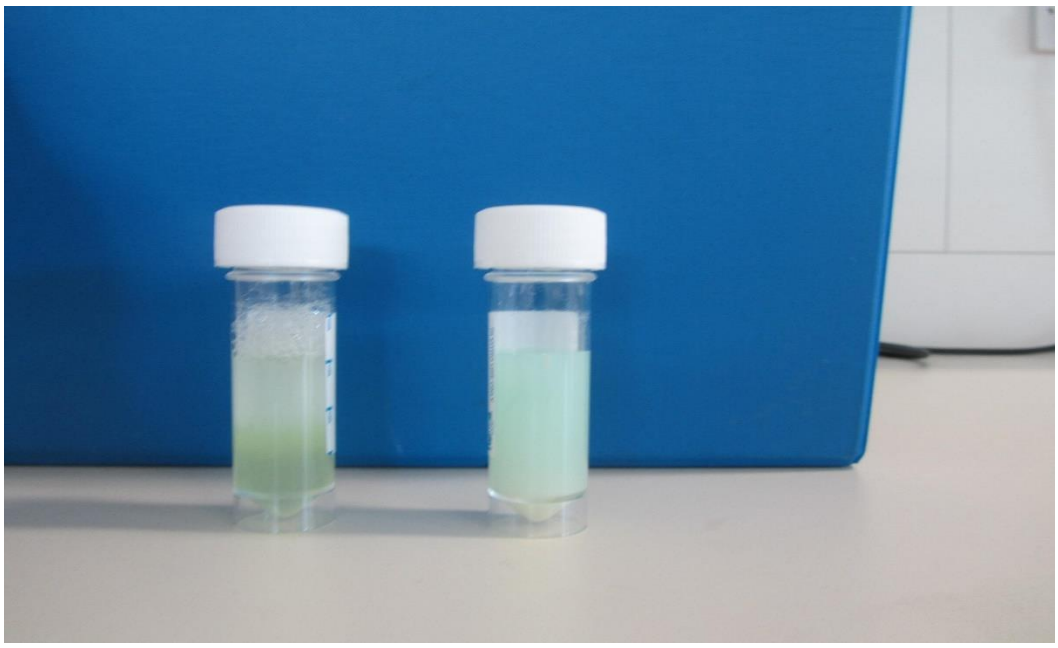

Figure 3.1.2

Surfactant action of 10 mg/ml LMS-611 on pellicle biofilm.Pellicle biofilm formed by *P. aeruginosa* strain PAO1 treated with 10 mg/ml LMS-611 (right) or 0.9% NaCl vehicle (left). Biofilm treated with LMS-611 appears to lose buoyancy, foaming properties (note lack of bubbles) and be partially dispersed with cells sedimenting on the bottom of the tube, in addition, it is readily miscible with an equal volume of saline applied on top. Biofilm treated with 0.9% NaCl control vehicle retains buoyancy and is not immediately miscible with a further volume of saline layered on top – two separate layers, green and colourless, are clearly distinguishable in the control sample.

SECTION 3.2

MATERIALS AND METHODS

SECTION 3.2.1

EFFECTS OF LMS-611 ON BIOFILM BIOMASS – CRYSTAL VIOLET ASSAYS

In order to determine the effects of LMS-611 alone and in combination with antibiotics active against *P. aeruginosa* upon total biomass of biofilms formed by type strain PAO1 and sputum isolate J1385, an adaptation of the popular crystal violet method was employed. This assay has been used previously found extensive use in high throughput screening of potential anti-biofilm agents. Strains were grown

from frozen Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) held at -20 ˚C by inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain single isolated colonies for transfer to 3-5 ml of LB in universals which were incubated overnight at 37 ˚C/250 rpm to obtain stationary phase cultures for adjustment with LB to an A_{600} value of 0.18 ± 0.02, approximating a 1.0 McFarland Standard or 3.0 x 10^8 cfu/ml. These adjusted cultures were then further diluted 1:30 with fresh LB to obtain starter inocula containing approximately 1 x 10^7 cfu/ml. 180 µl of these starter inocula were pipetted into 40 inner wells of an Innovotech MBEC device (Innovotech, Edmonton, Alberta, Canada) and outer wells were filled with an equal volume of sterile water in order to minimise evaporation during incubation. Peg studded device lids were attached to allow biofilm formation on submerged pegs as per the manufacturer's recommendations (Ceri *et al*, 2001) and the devices were sealed with parafilm to prevent evaporation. Devices were then incubated statically at 37 ˚C for 48 hours to allow biofilm formation on pegs. Pegs were then rinsed twice in fresh plates containing 200 µl of PBS per well before being transferred to challenge plates containing either 10 mg/ml LMS-611 or 0.9% NaCl vehicle – 20 replicates were used for each condition. Challenge plates were sealed with parafilm and incubated statically for 20 hours at 37 ˚C before the peg studded lid was transferred to a rinse plate containing 200 µl of PBS per well for 1 minute. Pegs were then stained for 5 minutes in a 96 well plate containing 200 µl per well of filtered 0.5 % aqueous crystal violet. Excess stain was removed by rinsing as previously, for 1 minute, in a fresh PBS rinse plate before lids were dried and transferred to a plate containing 200 µl of 30 % acetic acid per well for 15 minutes, to leach stain from biofilms. A_{570} was read immediately thereafter. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.2

EFFECT OF LMS-611 ON PLANKTONIC GROWTH & ANTIBIOTIC SENSITIVITY – RESAZURIN MIC ASSAY

Strains were grown as before from frozen Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) vials held at -20 ˚C by inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm to maintain culture aeration and planktonic state until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain isolated colonies. Sensitivity testing was then carried out using a modified broth microdilution method which incorporates a colorimetric/fluorometric indicator dye, due to the opacity of LMS-611 (LiPuma *et al*, 2009). Sterile cotton swabs were then used to emulsify 5 colonies per strain into 5 ml of 1 x MHBII, which was then adjusted to an A_{600} value of 0.1 ± 0.02, approximating a 0.5 McFarland Standard or 1 x 10^8 cfu/ml. This culture was then diluted 1:150 in 2 x MHBII to obtain an inoculum with a cell density of approximately 1 x 10^6 cfu/ml. 96 well plates with 3 inner rows containing 100 µl of 20 mg/ml LMS-611 and antibiotic at twice the desired final concentration along with the remaining 3 inner rows containing 100 µl 0.9% NaCl vehicle and antibiotic at twice the desired final concentration were topped up with one volume of the aforementioned inoculum to give a final cell density approximating 5 x 10^5 cfu/ml. Antibiotics tested were ceftazidime, ciprofloxacin, colistin and tobramycin and were ordered from Sigma-Aldrich, Poole, UK except ceftazidime which was supplied by Melford Laboratories, Ipswich, UK. Outer wells were filled with 200 µl of sterile water and plates were sealed with breathe-easy membranes (Sigma-Aldrich, Poole, UK) before incubation for 20 hours at 37 ˚C. Following incubation, 20 µl of filter sterilised 0.02 % resazurin sodium in PBS was added to each well and plates were resealed and returned to incubation at 37 ˚C for a further 1-2 hours before fluorescence emitted at a wavelength of 590 nm was measured upon excitation at a wavelength of 540 nm. Each test condition was replicated at least 6 times and results were analysed using one way ANOVA and Tukey's paired comparison test for significance at the $\alpha = 0.05$ level.

SECTION 3.2.3

EFFECT OF LMS-611 ON BIOFILM GROWTH & ANTIBIOTIC SENSITIVITY – RESAZURIN MIC ASSAY

Viability of biofilms was quantified using a variation of the method published by Li Puma and colleagues (Li Puma *et al*, 2009). Strains were grown as before from frozen Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) held at -20 ˚C by inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain isolated colonies. Single colonies were then transferred to 3-5 ml volumes of LB and grown overnight at 37 ˚C with shaking at 250 rpm to obtain stationary phase cultures. These were diluted in fresh LB to an A_{600} value of 0.18 ± 0.02, approximating a 1.0 McFarland Standard or 3.0 x 10^8 cfu/ml. These adjusted cultures were then further diluted 1:30 with fresh LB to obtain starter inocula containing approximately 1 x 10^7 cfu/ml. 180 µl of these starter inocula were pipetted into the 60 inner wells of 96 well plates. Outer wells were filled with an equal volume of sterile water, lids were attached and the plate edges were sealed with parafilm to minimise evaporation. Plates were then incubated statically at 37 ˚C for 24 hours to allow formation of biofilm on the well surfaces. Thereafter plates were opened and culture from the inner wells was aspirated with a multichannel pipette. These wells were then rinsed twice with 180 µl volumes of sterile PBS to remove loosely adherent planktonic cells. Serial dilutions of ceftazidime, ciprofloxacin, colistin and tobramycin at twice the desired concentrations were prepared in 100 µl volumes of 2x MHBII in a separate 96 well microtitre plate. Volumes were completed to 200 µl using either 20 mg/ml LMS-611 or 0.9 % NaCl vehicle. Controls were prepared in the same manner with the omission of antibiotics and enough dilutions were prepared to allow each condition to be tested in triplicate. The dilutions were then transferred to the inner wells of the aforementioned plate, containing rinsed biofilms. Plates were then resealed with lids and parafilm before being incubated statically at 37 ˚C for 18-20 hours. Plates were then removed from

incubation and the inner wells were then aspirated as before and rinsed twice using sterile PBS in 200 µl volumes so that only surface bound biofilm remained. A 0.04 % solution of resazurin sodium in PBS was prepared and sterilised using a syringe driven Millex 0.22 µm filter unit (Millipore, Cork, Republic of Ireland). This was diluted 1:10 with MHBII and transferred to the freshly rinsed biofilms. The plates were then resealed as before and incubated statically at 37 ˚C for 16 hours to allow surviving biofilm cells to reduce the resazurin indicator. Results were analysed for significance at the α = 0.05 level via one way ANOVA after reading F_{590} values.

SECTION 3.2.4

QUANTITATIVE VIABILITY OF BIOFILM CELLS & VESICLE/CELL INTERACTIONS BY CONFOCAL FLUORESCENCE MICROSCOPY

Strains were grown as before from frozen Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) vials held at -20 ˚C by inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain isolated colonies. Single colonies were then transferred to 3-5 ml volumes of LB and grown overnight at 37 ˚C with shaking at 250 rpm to obtain stationary phase cultures. These were diluted in fresh LB to an A_{600} value of 0.18 ± 0.02, approximating a 1.0 McFarland Standard or 3.0 x 10^8 cfu/ml. These adjusted cultures were then further diluted 1:30 with fresh LB to obtain starter inocula containing approximately 1 x 10^7 cfu/ml. 2 ml volumes of turbidity adjusted inocula were then pipetted into Ibidi microdishes (Thistle Scientific, Uddingston, UK) which were subsequently locked and swirled briefly before undergoing 24 hours of static incubation at 37 ˚C to allow biofilm growth on their transparent interiors. Microdishes were then removed from incubation, their contents were carefully aspirated by pipetting and the interiors were rinsed twice with 2 ml volumes of sterile PBS. Microdishes were then refilled with 2ml volumes

of MHBII containing either 10 mg/ml LMS-611 only, 0.9 % NaCl vehicle only, 10 mg/ml LMS-611 and 20 µg/ml tobramycin (5x the predetermined biofilm MIC) or 0.9 % NaCl vehicle and 20 µg/ml tobramycin before being resealed and returned to static incubation at 37 ˚C for 18 hours. Aspiration and rinsing were then repeated as before and biofilms were stained by applying 150 µl of composite Baclight LIVE/DEAD stain (Life Technologies, Paisley, UK), prepared by mixing 1.5 µl each of components A and B into 997 µl of sterile distilled water as previously reported (Smith & Hunter, 2008). Microdishes were then incubated in darkness at room temperature for 15 minutes to allow stain uptake by biofilms. Microdishes were then viewed at 100x magnification with oil immersion, using FITC and TRITC filters, on a Nikon TE 2000 inverted microscope. At least 2 images per microdish were captured using a Hammamatsu digital camera and the IP-Lab software. 6 image fields from 3 replicate microdishes were imaged for each condition tested, giving a total of at least 18 image replicates per condition. Mean numbers of green and red pixels in each image were tallied using ImageJ software to give estimates of the number of cells surviving each treatment and of the overall area of substratum covered by biofilm in each test group. Moreover, to investigate the possibility that lamellasomes fuse with bacteria, potentially delivering a passenger drug payload directly into the periplasms of biofilm associated cells, a fluorescent tracer study was carried out. Stationary phase cultures were in LB were diluted to an A_{600} value of 0.05 using fresh LB, 2 ml of this were added to each of 6 Ibidi microdishes which were then locked and swirled briefly before being incubated statically at 37 ˚C for 24 hours to allow biofilm formation. Resultant biofilms were then rinsed twice in sterile PBS. 100 µg of the red lipophilic dye, FM 4-64 (Life Technologies, Paisley, UK) was dissolved in 10 ml of 0.9 % NaCl vehicle, 5 ml of which was then used to reconstitute a 100 mg cake of LMS-611 formulation. After 2 hours of shaking periodically the resulting suspension was divided equally between 5 sterile 1.5 ml microcentrifuge tubes and spun down at 13,000 rpm for 30 minutes at 4 ˚C. Supernatants were then discarded and pellets were resuspended in 1ml of fresh 0.9 % NaCl vehicle before centrifugation was repeated as before. Finally, supernatants were again discarded and pellets were each resuspended in 1 ml of fresh 0.9 % NaCl vehicle to obtain a 20 mg/ml stock suspension of FM 4-64 loaded LMS-611. This

was mixed in a 1:1 ratio with MHBII before being added in 2 ml volumes to 3 of the 6 biofilms prepared previously. Controls were prepared by mixing the remainder of the FM 4-64 loading solution in a 1:1 ration with MHBII and adding in 2 ml volumes to the remaining 3 biofilms. Microdishes were then sealed and incubated statically and in darkness for 18 hours at 37 ˚C, they were then aspirated and rinsed as before and viewed at 100x magnification with oil immersion, using a TRITC filter, on a Nikon TE 2000 inverted microscope. At least 2 images per microdish were captured using a Hammamatsu digital camera and the IP-Lab software. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.5

EFFECTS OF LMS-611 ON BIOFILM STRUCURE – FIELD EMISSION SCANNING ELECTRON MICROSCOPY

Strains were grown as before from frozen Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) vials held at -20 ˚C by inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain isolated colonies. Single colonies were then transferred to 3-5 ml volumes of LB and grown overnight at 37 ˚C with shaking at 250 rpm to obtain stationary phase cultures. These were diluted in fresh LB to an A_{600} value of 0.18 ± 0.02, approximating a 1.0 McFarland Standard or 3.0 x 10^8 cfu/ml. These adjusted cultures were then further diluted 1:30 with fresh LB to obtain starter inocula containing approximately 1 x 10^7 cfu/ml. Round 13mm glass coverslips were placed into the inner wells of a 24 well plate and the lid was attached. Coverslips were then sterilised by placing the 24 well plate into a UV crosslinker oven and running on full power for 30 minutes. Biofilms were then grown and fixed using a modified version of the method previously reported by Harrison and colleagues (Harrison *et al*, 2005). 1ml of turbidity adjusted inoculum was then pipetted into each well containing coverslips and the remaining outer wells

were filled with an equal volume of sterile water to minimise evaporation. The 24 well plates were then sealed around the perimeter with parafilm and incubated statically at 37 ˚C for 48 hours to allow biofilm formation. Biofilm coated coverslips were then transferred using flamed forceps to fresh 24 well plates with inner wells containing 1ml volumes of either 10 mg/ml LMS-611 or 0.9 % NaCl vehicle in MHBII. Outer wells were filled as before to prevent evaporation, the plate edges were sealed with parafilm and the plates were incubated statically for 18 hours at 37 ˚C. After Incubation, coverslips were transferred using forceps to fresh 24 well plates with wells containing 1 ml of fixative :- 2.5 % electron microscopy grade glutaraldehyde in 0.1 M cacodylate buffer. Fixation was allowed to continue for 2 hours at room temperature before coverslips were sequentially rinsed for 10 minutes in cacodylate buffer and 18.2 MOh water and then air dried for 120 hours. Coverslips were then mounted on stalks, sputter-coated in gold and imaged under FE-SEM.

SECTION 3.2.6

ASSAYS FOR SECRETED VIRULENCE FACTORS

Strains were grown from frozen held at -20 ˚C b Microbank™ advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) y inoculating 3-5 ml of LB with a single bead and growing at 37 ˚C with agitation at 250 rpm until turbidity appeared. The resultant broth cultures were then streaked onto LB agar plates which were then incubated statically overnight at 37 ˚C to obtain isolated colonies. Single colonies were then transferred to 3-5 ml volumes of LB and grown overnight at 37 ˚C with shaking at 250 rpm to obtain stationary phase cultures. These were diluted to an A_{600} value of 0.1 ± 0.02 in fresh LB and then further diluted 1:300 with more LB to obtain a cell density of around 1 x 10^5 cfu/ml. 1.8 ml of this turbidity adjusted culture was added to each of 8 inner wells of a 24 well plate which were then topped up to 2 ml by adding 200 µl of 100 mg/ml LMS-611 suspension, giving a final concentration of 10 mg/ml LMS-611. A control plate was prepared in an identical format, substituting 0.9% NaCl vehicle for 100 mg/ml LMS-611 suspension. All outer wells were filled with 2 ml of sterile water and plates were sealed around the edges with parafilm to

minimise evaporation. They were then incubated statically at 37 ˚C for 48 hours. This allowed formation of pellicle biofilm under shear conditions and enabled secretion of virulence associated exoproducts. Each sample was then disrupted by shearing with a 1 ml syringe and 200 µl samples were withdrawn from each well and transferred to a 96 well plate. 20 µl of 0.04 % resazurin solution was then added to each well and quickly mixed in by pipetting. The 96 well plate was then incubated statically at 37 ˚C for 15 minutes before absorbances were read at wavelengths of 570 and 600 nm. Percentage growth was then estimated using the following equation, originally devised for proprietary (alamarBlue®) resazurin (Thermo Fisher Scientific ; O'Brien *et al*, 2000)

$$\frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

Where: O1 = molar extinction coefficient (80586 L mol^{-1} cm^{-1}) of oxidized resazurin at 570nm

O2 = molar extinction coefficient (117216 L mol^{-1} cm^{-1}) of oxidized resazurin at 600nm

P1 = absorbance of positive growth control well (bacteria + resazurin and saline vehicle) at 570nm

P2 = absorbance of growth control well (bacteria + resazurin and saline vehicle) at 600nm

A1 = absorbance of test well at 570nm

A2 = absorbance of test well at 600nm

The remaining 1.8 ml samples of culture were retained for assay of virulence associated exoproducts. 1.6 ml of each was spun down at 4 ˚C/13,300 rpm to remove, as far as possible, cells, lamellasomes and debris. The supernatants were then passed through a 0.22 µm filter unit to further remove unwanted matter. The resulting filtrates (8 replicates per strain and condition) were used to assay for pyocyanin, the fluorescent siderophores pyoverdine and pyochelin, elastase and total protease. For each sample, a growth correction was made to account for the greater

number of bacteria presented in pre-filtered LMS-611 treated cultures relative to the saline vehicle treated controls. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.6.1

PYOCYANIN, PYOVERDINE & PYOCHELIN ASSAYS

Pyocyanin was quantified via the method previously reported by Fothergill and colleagues (Fothergill *et al*, 2007) Pyocyanin production was measured by reading A_{695} of filtrates after 150 µl volumes had been transferred to a 96 well plate. The same samples were then re-read at an excitation wavelength of 400 nm and emission wavelength of 460 nm to determine pyoverdine levels followed by excitation at 350 nm and emission at 430 nm to quantify pyochelin as previously reported by Harrison and colleagues (Harrison *et al*, 2014). Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.6.2

ELASTASE ASSAY

In order to measure elastase activity, the method previously reported by Jiricny and colleagues was used (Jiricny *et al*, 2014). 27.5 mg of elastin congo red granules were dispensed into the bottoms of 20 ml universal tubes along with 1.8 ml of elastase buffer and 200 µl of filtered supernatant. The tubes were then incubated for 4 hours at 37 ˚C with shaking at 250 rpm. Thereafter, 1 ml aliquots were transferred to microcentrifuge tubes and spun down at 4 ˚C/13,300 rpm to remove granules. 150 µl volumes of the resulting supernatant were then transferred to a 96 well plate and read at A_{495}. A negative control is made by using a tube with LB in place of culture

supernatant. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.6.3

TOTAL PROTEASE ASSAY

To measure total protease activity, a method previously reported by Jiricny and colleagues was used (Jiricny *et al*, 2014). 250 µl of 2 % filter sterilised azocasein solution in protease assay buffer was added to 2 ml microcentrifuge tubes containing 150 µl of filtered culture supernatant and vortexed for 5 seconds before being incubated statically at 37 ˚C for 45 minutes to allow substrate digestion. Reactions were then stopped by precipitating out undigested substrate via the addition of 1.2 ml of 10 % trichloroacetic acid per tube for 15 minutes at room temperature and then centrifuging at 13,300 rpm for a further 10 minutes. 900 µl volumes of the resulting supernatant were then transferred to fresh 2 ml microcentrifuge tubes containing 750 µl of 1M NaOH. 150 µl volumes were then transferred to 96 well plates and A_{440} was read. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.6.4

ALGINATE ASSAY

Alginate was assayed by adaptation of a method that has previously been reported in the literature (May & Chakrabarty, 1994). Borate stock solution was prepared by adding 6.865 g of H_3BO_3 to 45 ml of 1M KOH and completing the volume to 100 ml with distilled water. This was then diluted 1:10 with concentrated H_2SO_4 to obtain borate working solution. 600 µl samples of working solution in microcentrifuge tubes were then chilled on ice before 70 µl of the remaining unfiltered and

uncentrifuged portions of culture were added. Samples were then votexed for 4 seconds before being returned to the icebox. 20 µl of 0.1 % carbazole solution in absolute ethanol was then added to each tube and vortexing was repeated. Samples were then immediately transferred to a waterbath set to 55.2 ˚C and allowed to incubate for 25 minutes to enable development of colour changes. Uronic acid monomers in the sample were then quantified by transferring 150 µl volumes to a 96 well plate and reading A_{530}. Four replicates samples were used for each assay condition. A triplicate series of halving dilutions of sodium alginate from *Macrocystis pyrifera* was prepared in distilled water with standard concentrations beginning at 625 µg/ml and ranging down to 19.5 µg/ml. These were then assayed for uronic acid in the same manner as the test samples in order to obtain A_{530} readings from which to generate a standard curve. Alginate concentrations in test sample were read from the curve and corrected for differences in cell density observed in each sample. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 3.2.7

RESULTS

SECTION 3.2.7.1

It was a matter of interest to determine whether or not LMS-611 could reduce formation of or disrupt biofilm, as this could provide clues as to its mechanism of action. In order to test this hypothesis, a simple crystal violet binding assay was employed which works upon the principle that absorbed crystal violet stain is directly proportional to biofilm biomass present upon polystyrene well surfaces. LMS-611 appeared to increase biofilm formation by all test strains.

EFFECTS OF LMS-611 ON BIOFILM BIOMASS – CRYSTAL VIOLET ASSAYS

LMS-611 significantly increased net PAO1 biofilm formation by 65 % relative to negative controls (Figure 3.2.1) and also significantly increased J1385 biofilm formation by 45 % relative to negative controls (Figure 3.2.2).

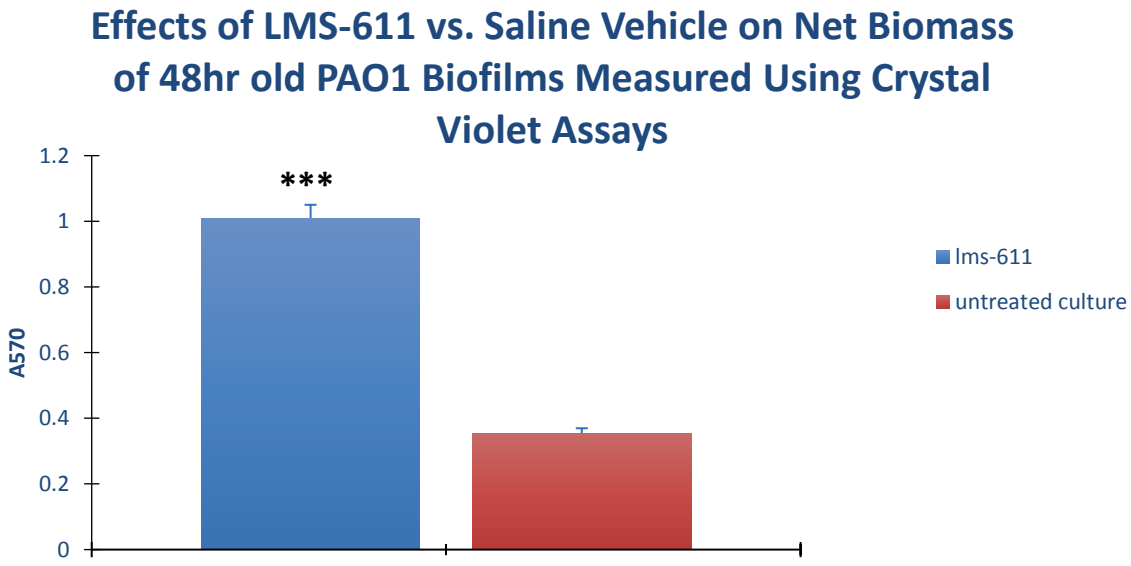

Figure 3.2.1

Effects of 10 mg/ml LMS-611 +/- on Total Biomass of 48 hour old PAO1 Biofilms As Measured by Absorbance of Leached Crystal Violet at 570nm and Analysed by one way ANOVA at $\alpha = 0.05$, $n = 3$. Groups differing significantly from the saline vehicle control are denoted by *** ($p < 0.05$).

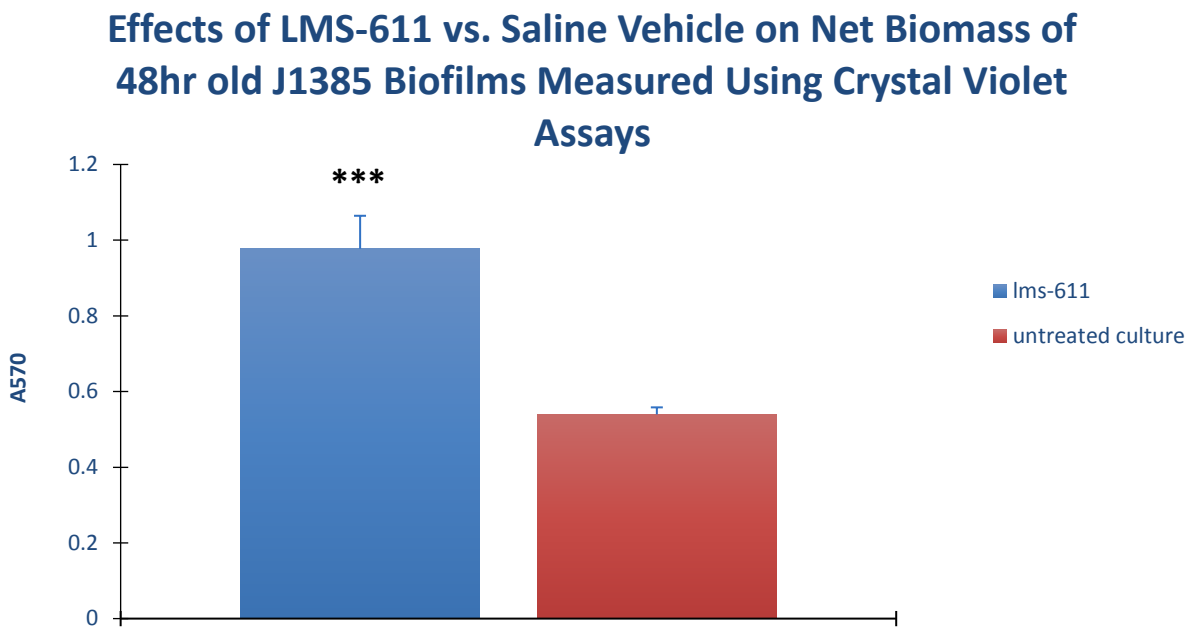

Figure 3.2.2

Effects of 10 mg/ml LMS-611 +/- on Total Biomass of 48 hour old J1385 Biofilms. As Measured by Absorbance of Leached Crystal Violet at 570nm and Analysed by one way ANOVA at $\alpha = 0.05$, $n = 3$. Groups differing significantly from the saline vehicle control are denoted by *** ($p < 0.05$).

SECTION 3.2.7.2

It was deemed to be of interest to determine whether LMS-611 could enhance the potency of relevant antibiotics against *P. aeruginosa* strains grown planktonically, due to the therapeutic potential this could imply. To test this hypothesis, a resazurin based metabolic assay was used to determine MICs of drugs in the presence and absence of LMS-611. It was found that LMS-611 vary altered the planktonic drug MIC depending on the strain-drug combination tested.

EFFECT OF LMS-611 ON PLANKTONIC GROWTH & ANTIBIOTIC SENSITIVITY – RESAZURIN MIC ASSAY

LMS-611 reduced planktonic ceftazidime MIC values of strains PAO1 and J1532 by 2 fold from 2 to 1 µg/ml, this difference was found to be significant for J1532 ($p < 0.05$) but not PAO1 ($p > 0.05$) . Conversely, LMS-611 did not alter the ceftazidime MIC for strain J1385, which remained at 2 µg/ml regardless of the presence of LMS-611. LMS-611 increased ciprofloxacin MIC by 2 fold from 0.125 to 0.25 µg/ml for strain PAO1 and from 0.0625 to 0.125 µg/ml for strain J1385 and this MIC doubling was significant for both of these strains ($p < 0.05$), the ciprofloxacin MIC of J1532 remained unaffected by LMS-611 and was determined to be 0.125 µg/ml. LMS-611 did not alter colistin MIC values for strains PAO1 and J1385 which remained at 1 and 0.5 µg/ml, respectively, regardless of whether or not LMS-611 was present. LMS-611significantly reduced colistin MIC by one doubling dilution, from 0.25 to 0.125 µg/ml, for strain J1532 ($p < 0.05$). LMS-611 significantly increased the tobramycin MIC of strain PAO1 from 0.5 to 1 µg/ml but had no effect on the tobramycin MIC values of the other test strains. In the absence of antibiotics, LMS-611 increased growth of all strains, but this was statistically significant only for strain J1532 ($p < 0.05$).

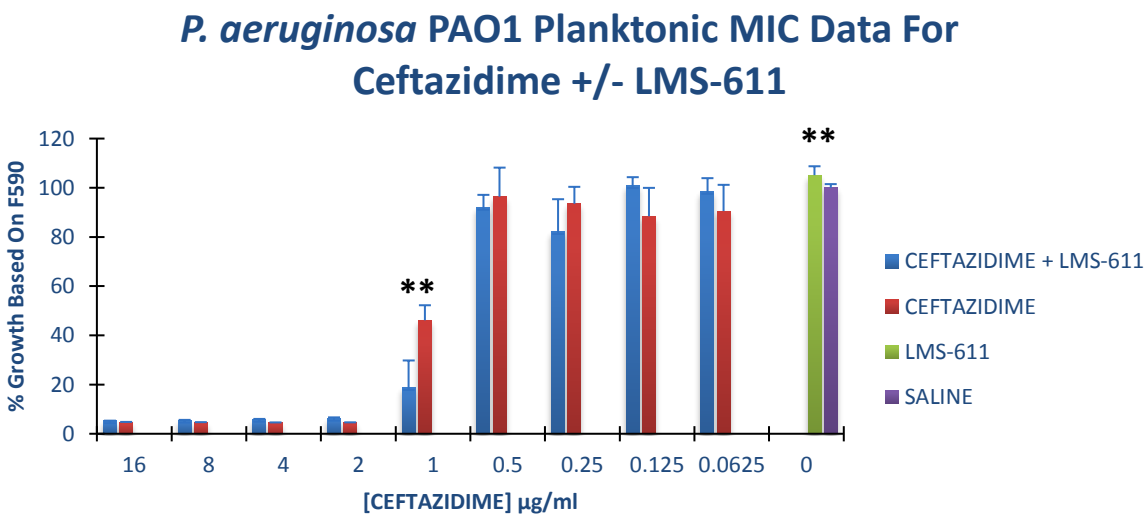

Figure 3.3.1

Effects of 10 mg/ml LMS-611 on growth and ceftazidime MIC of strain PAO1.The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented ($n = 6$) + S.E.M. Results were analysed by one way ANOVA at $\alpha = 0.05$. (**= not significant, *** = significant).

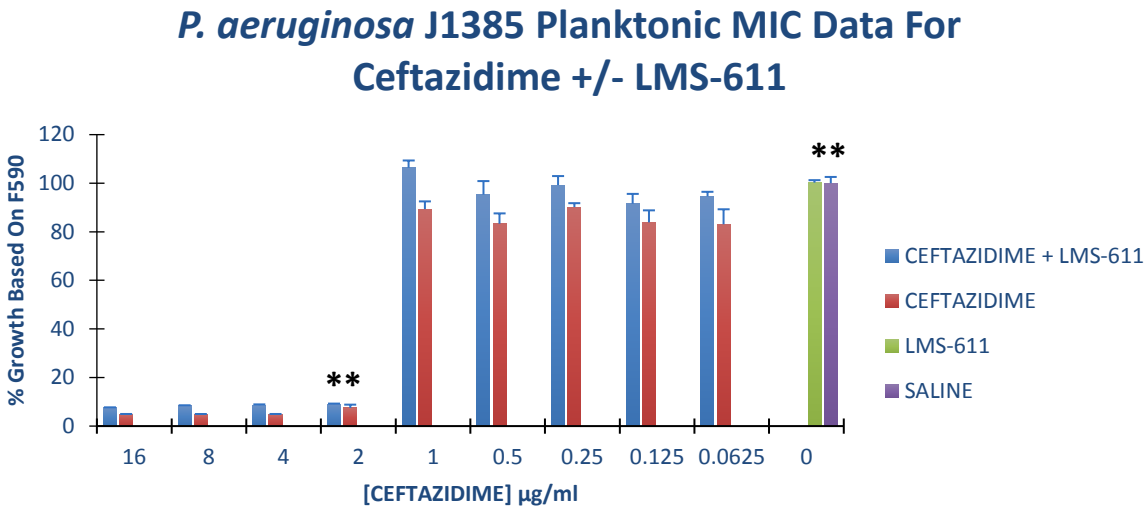

Figure 3.3.2

Effects of 10 mg/ml LMS-611 on growth and ceftazidime MIC of strain J1385.The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

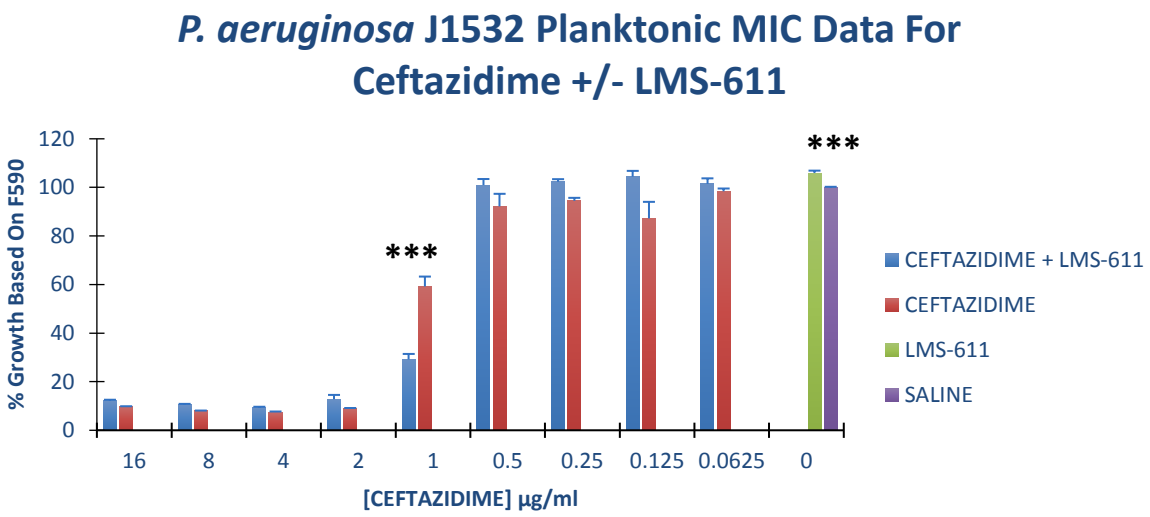

Figure 3.3.3

Effects of 10 mg/ml LMS-611 on growth and ceftazidime MIC of strain J1532. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

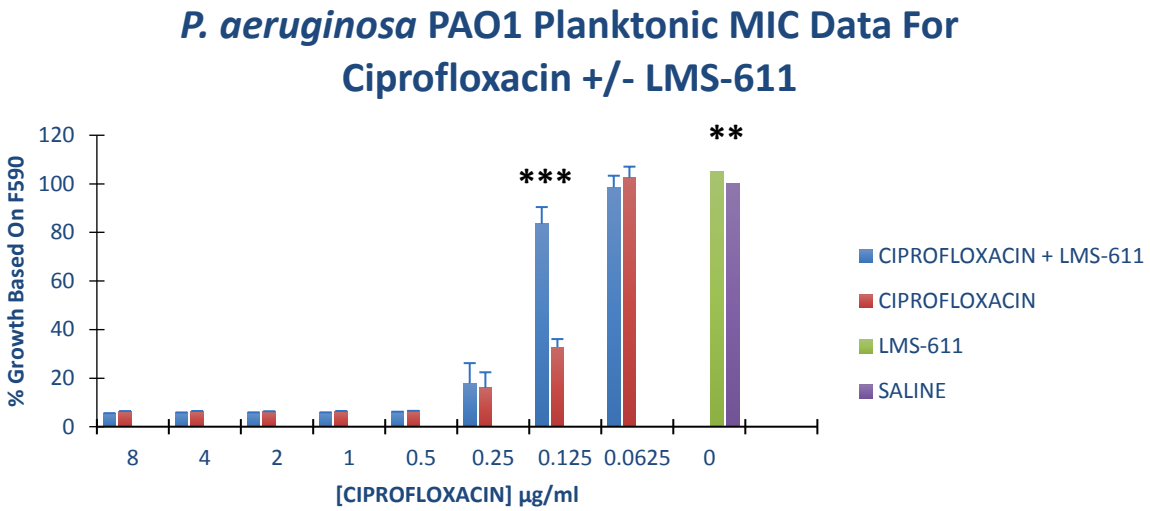

Figure 3.3.4

Effects of 10 mg/ml LMS-611 on growth and ciprofloxacin MIC of strain PAO1. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

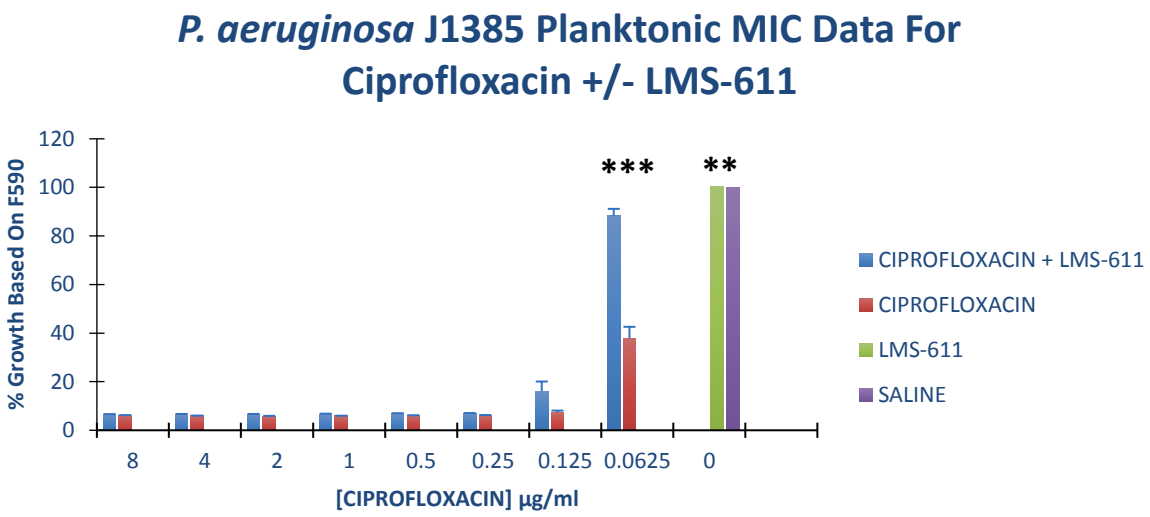

Figure 3.3.5

Effects of 10 mg/ml LMS-611 on growth and ciprofloxacin MIC of strain J1385. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

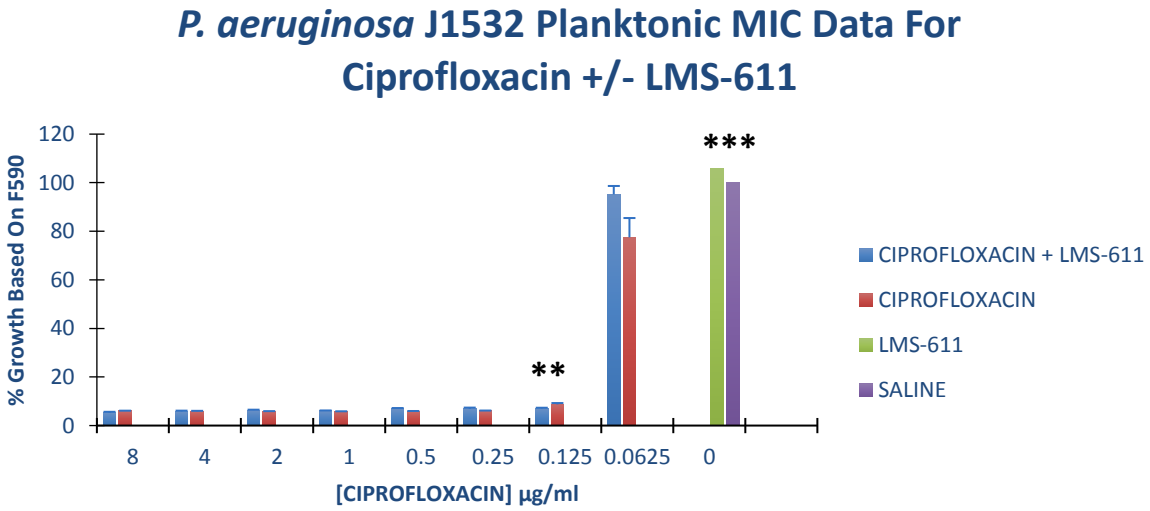

Figure 3.3.6

Effects of 10 mg/ml LMS-611 on growth and ciprofloxacin MIC of strain J1532. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

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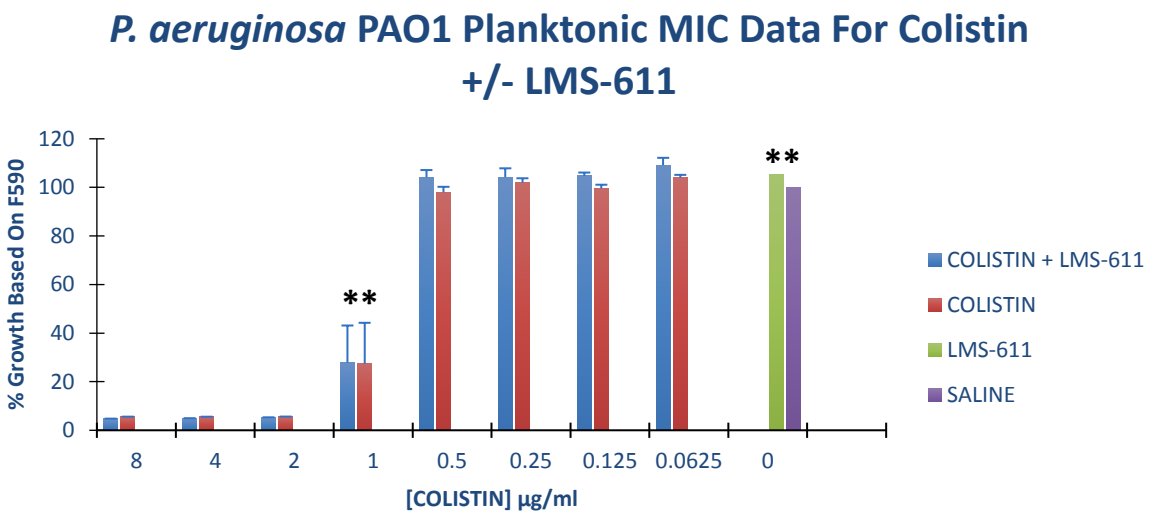

Figure 3.3.7

Effects of 10 mg/ml LMS-611 on growth and colistin MIC of strain PAO1. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

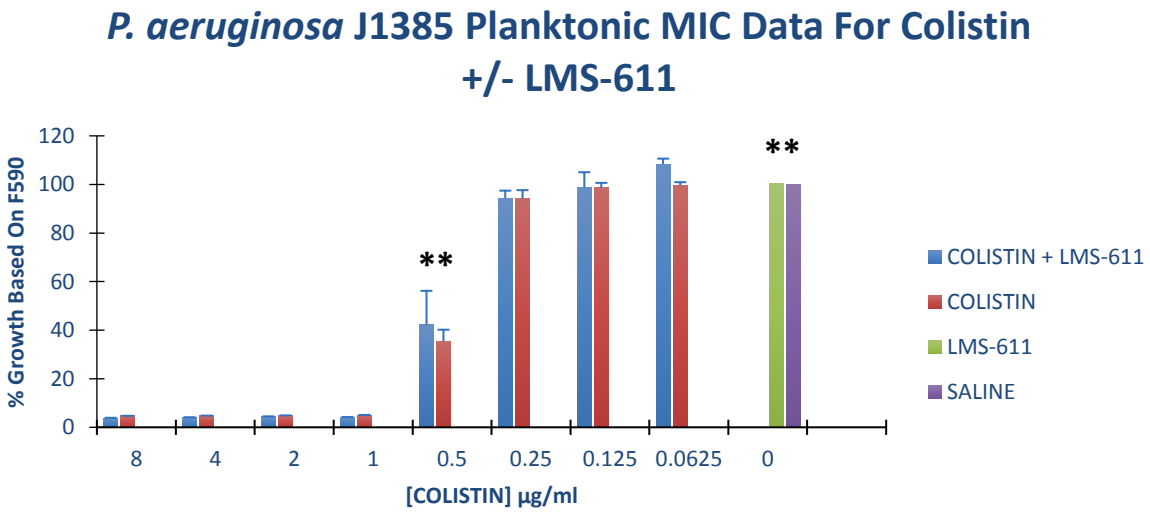

Figure 3.3.8

Effects of 10 mg/ml LMS-611 on growth and colistin MIC of strain J1385. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

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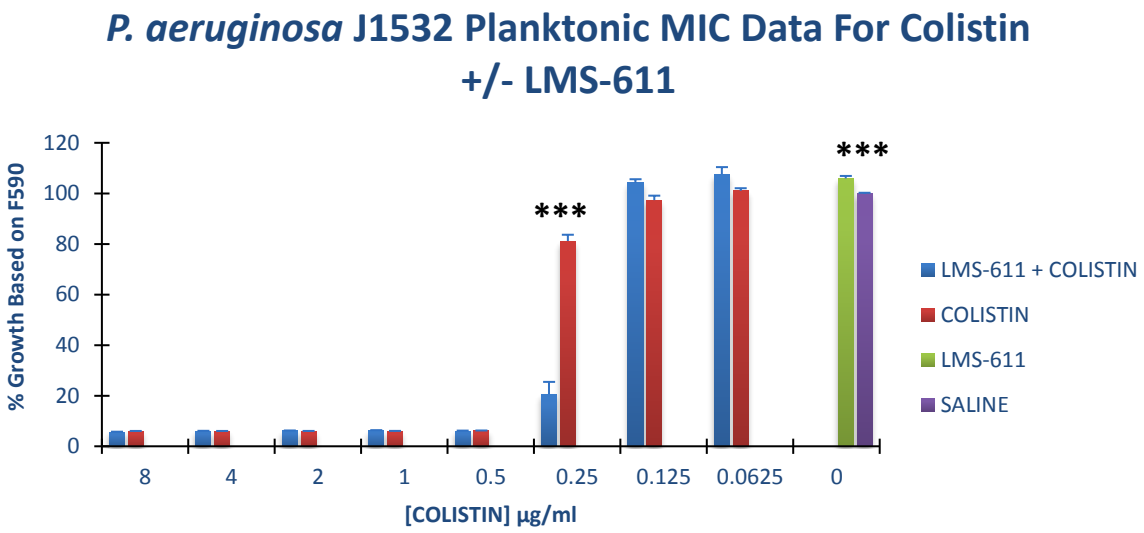

Figure 3.3.9

Effects of 10 mg/ml LMS-611 on growth and colistin MIC of strain J1532. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

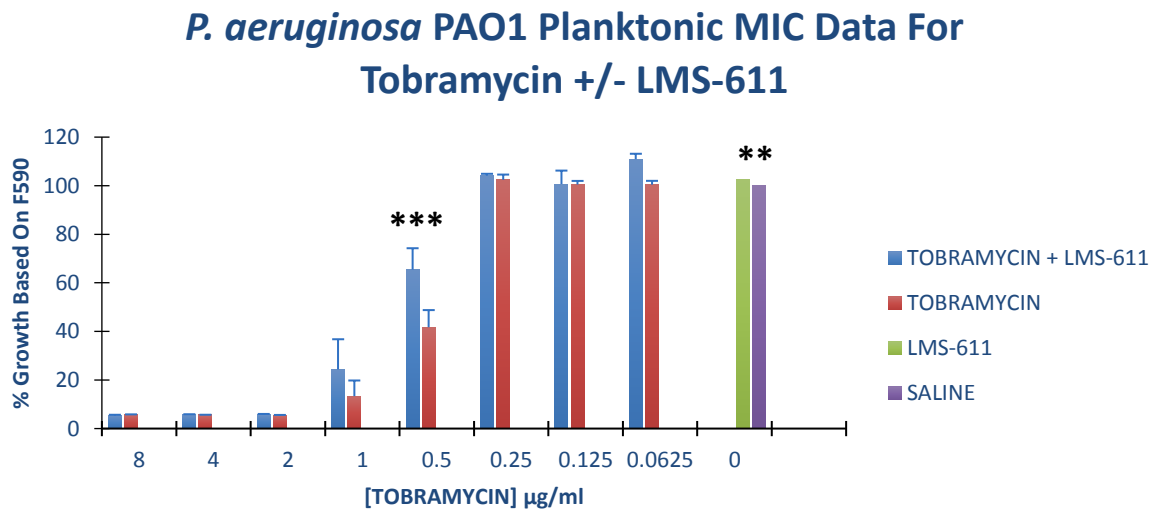

Figure 3.3.10

Effects of 10 mg/ml LMS-611 on growth and tobramycin MIC of strain PAO1. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

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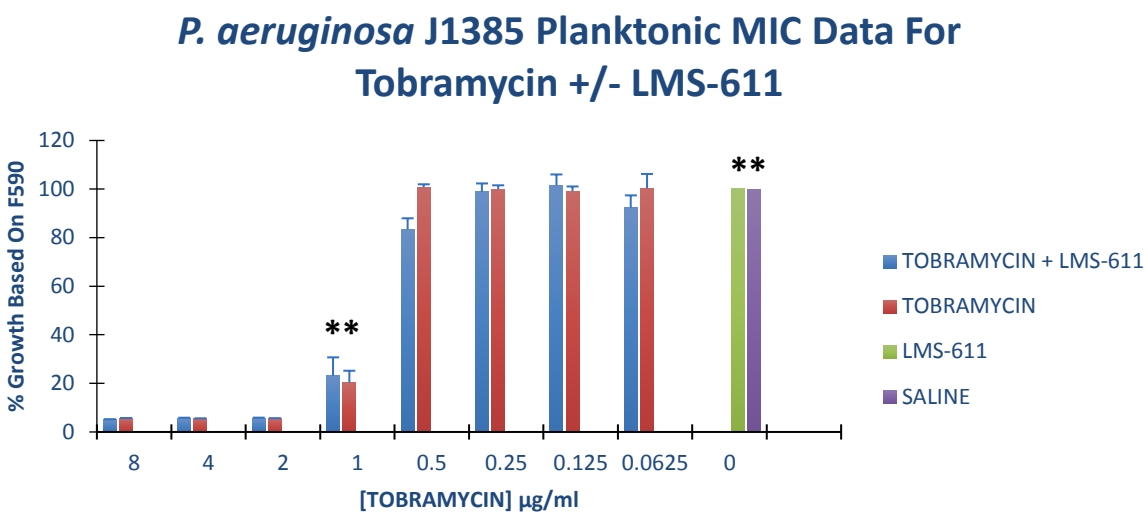


Figure 3.3.11

Effects of 10 mg/ml LMS-611 on growth and tobramycin MIC of strain J1385. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05. (**= not significant, *** = significant).

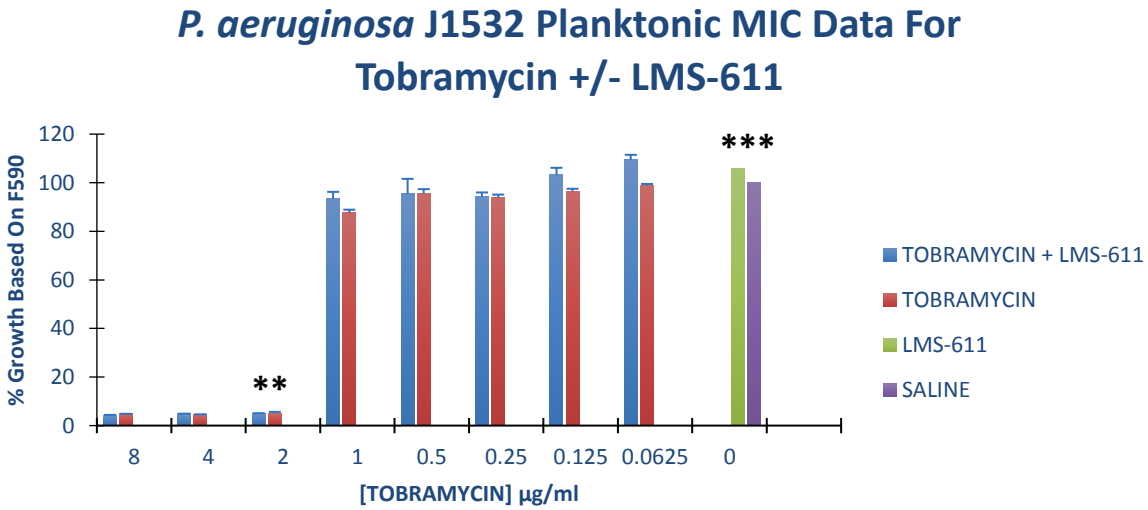


Figure 3.3.12

Effects of 10 mg/ml LMS-611 on growth and tobramycin MIC of strain J1532. The planktonic MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented (n = 6) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

SECTION 3.2.7.3

It was deemed to be of interest to determine whether LMS-611 could enhance the potency of relevant antibiotics against *P. aeruginosa* biofilms, due to the therapeutic potential this could imply. To test this hypothesis, a resazurin based metabolic assay was used to determine MICs of drugs in the presence and absence of LMS-611. It was found that LMS-611 did not reduce biofilm MIC for any drug-strain combination tested and sometimes even appeared to increase MIC.

EFFECT OF LMS-611 ON BIOFILM GROWTH & ANTIBIOTIC SENSITIVITY – RESAZURIN MIC ASSAYS

Both PAO1 and J1385 biofilms were resistant to ceftazidime at the highest concentration tested and no effect of LMS-611 on MIC could be discerned (Figures 3.4.1 – 3.4.2). LMS-611 significantly increased the ciprofloxacin MIC of PAO1 biofilms from a value of 8 to > 64 µg/ml ($p < 0.05$) although no sharply defined endpoint was clear in this assay (Figure 3.4.3). LMS-611 significantly increased biofilm growth of J1385 at the ciprofloxacin MIC of 8 µg/ml (Figure 3.4.4) although this was not enough to cause a change in MIC by a single doubling dilution ($p < 0.05$). LMS-611 increased the colistin MIC of PAO1 biofilms (Figure 3.4.5) by 4 fold from 32 to 128 µg/ml ($p = 0.00$). The colistin MIC of J1385 biofilms was doubled from 8 to 16 µg/ml ($p > 0.05$) in the presence of LMS-611 although this was not significant (Figure 3.4.6). LMS-611 increased the tobramycin MIC values of biofilms by 2 fold from 4 to 8 µg/ml in the case of both PAO1 ($p > 0.05$) and J1385 ($p > 0.05$) but this was not significant for either strain (Figures 3.4.7 & 3.4.8). LMS-611 by itself increased the growth of both PAO1 ($p = 0.00$) and J1385 biofilms ($p > 0.05$), this difference was significant only in the case of PAO1 (Figures 3.4.1 – 3.4.8).

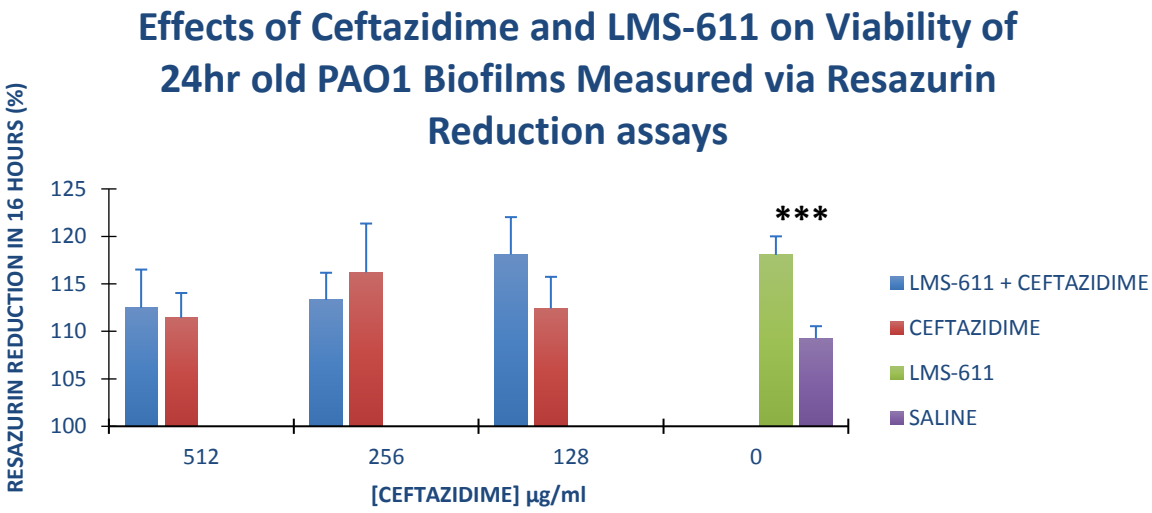


Figure 3.4.1

Effects of 10 mg/ml LMS-611 on growth and ceftazidime MIC of strain PAO1. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

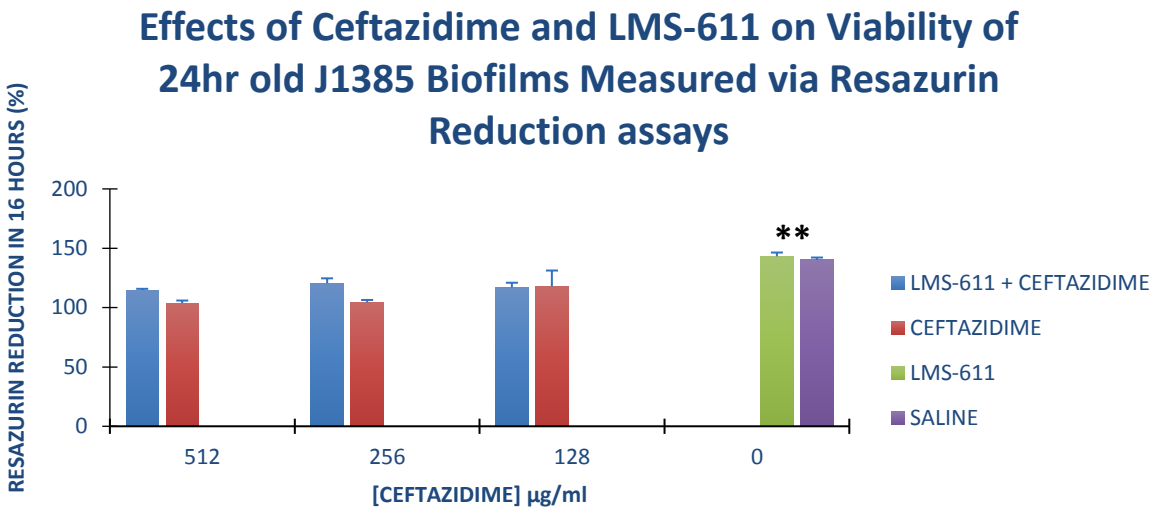


Figure 3.4.2

Effects of 10 mg/ml LMS-611 on growth and ceftazidime MIC of strain J1385. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

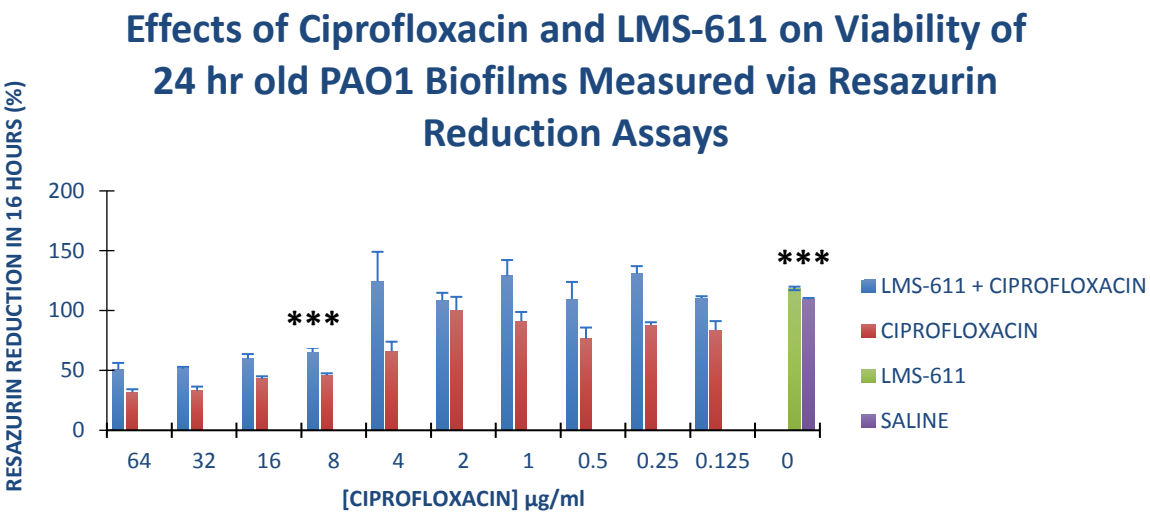


Figure 3.4.3

Effects of 10 mg/ml LMS-611 on growth and ciprofloxacin MIC of strain PAO1. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

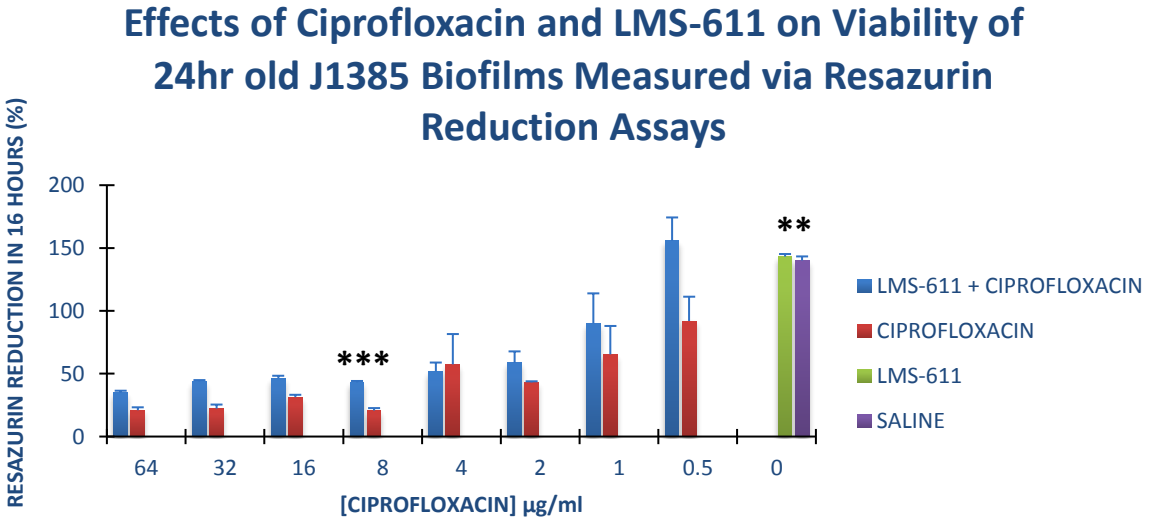


Figure 3.4.4

Effects of 10 mg/ml LMS-611 on growth and ciprofloxacin MIC of strain J1385. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

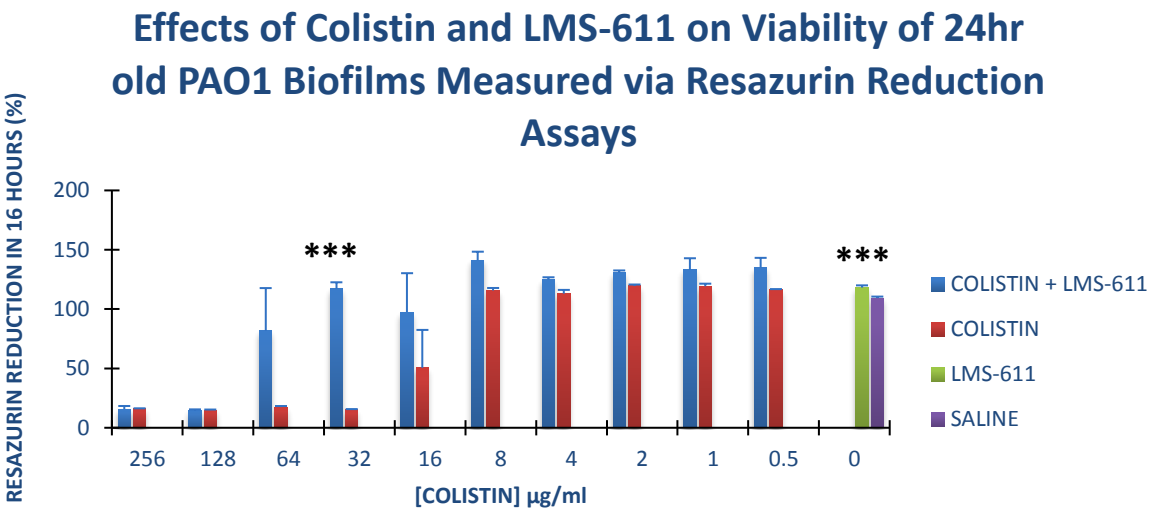


Figure 3.4.5

Effects of 10 mg/ml LMS-611 on growth and colistin MIC of strain PAO1. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

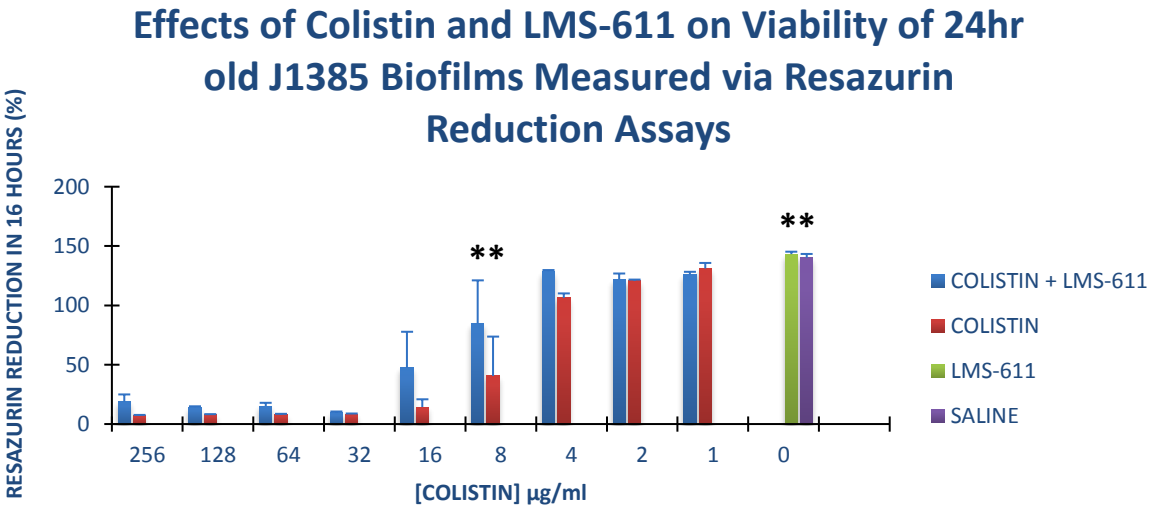


Figure 3.4.6

Effects of 10 mg/ml LMS-611 on growth and colistin MIC of strain J1385. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

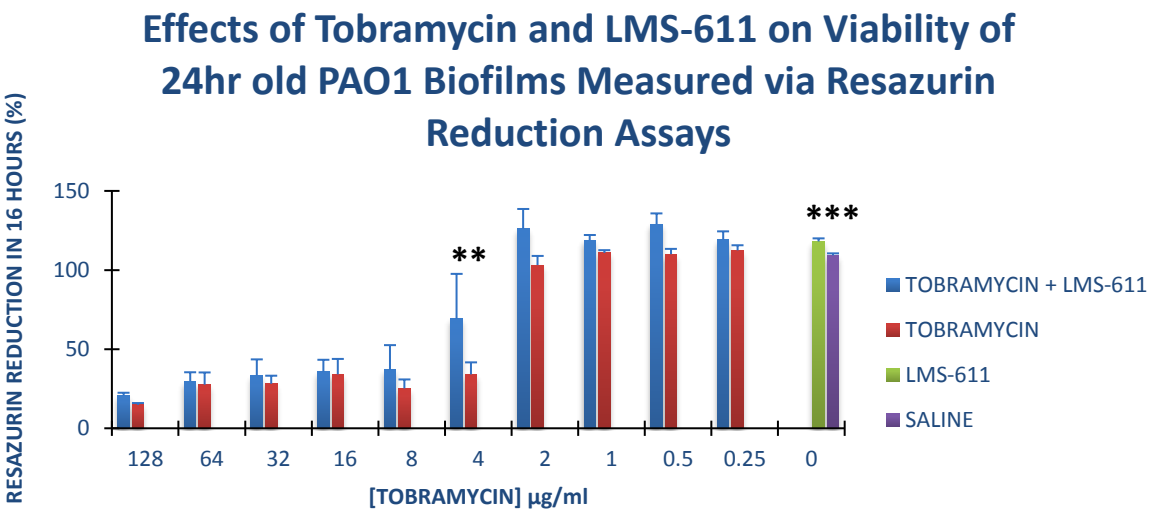


Figure 3.4.7

Effects of 10 mg/ml LMS-611 on growth and tobramycin MIC of strain PAO1. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

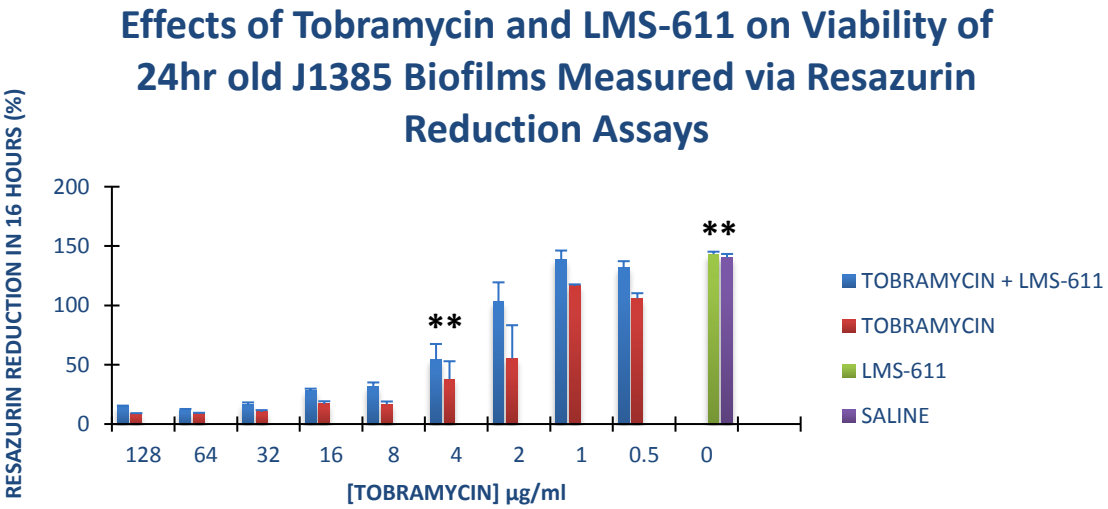


Figure 3.4.8

Effects of 10 mg/ml LMS-611 on growth and tobramycin MIC of strain J1385. The biofilm MIC was defined as the lowest antibiotic concentration at which growth was inhibited by ≥ 50 % relative to the control treated with saline only. Mean results are presented for drug treated (n = 3) and control wells (n = 26) + S.E.M. Results were analysed by one way ANOVA at α = 0.05 (**= not significant, *** = significant).

SECTION 3.2.7.4

In order to quantify and visualise the proportion of biofilm cells surviving treatment with LMS-611 alone, tobramycin alone or a fixed concentration combination of both, fluorescent microscopy experiments with a differential viability dye were employed. Likewise, total fluorescing area (proportional to net biofilm coverage) was also quantified in these experiments. The results indicated that LMS-611 does not enhance either the killing or detachment of biofilm cells by tobramycin and may actually increase horizontal biofilm coverage. A further experiment using fluorescently labelled LMS-611 was carried out to establish whether or not LMS-611 vesicles can fuse with biofilm cells – this revealed that LMS-611 vesicles did not fuse with the biofilm associated cells as the fluorescent tracer remained localised to the vesicles.

QUANTITATIVE VIABILITY OF BIOFILM CELLS & VESICLE/CELL INTERACTIONS BY CONFOCAL FLUORESCENCE MICROSCOPY

No significant differences were evident in the numbers of live and dead cells in biofilms treated with saline vehicle, LMS-611, tobramycin or the combination of LMS-611 and tobramycin (Figures 3.5.1 & 3.5.2) The area of biofilm coverage was significantly greater (relative to saline treated negative controls) in samples treated with LMS-611 alone and significantly less in samples treated with tobramycin alone, whereas the combination had no significant effect on the area of biofilm coverage (Figure 3.5.3). Fluorescently labelled LMS-611 vesicles did not appear to fuse with biofilm cells (Figure 3.5.4).

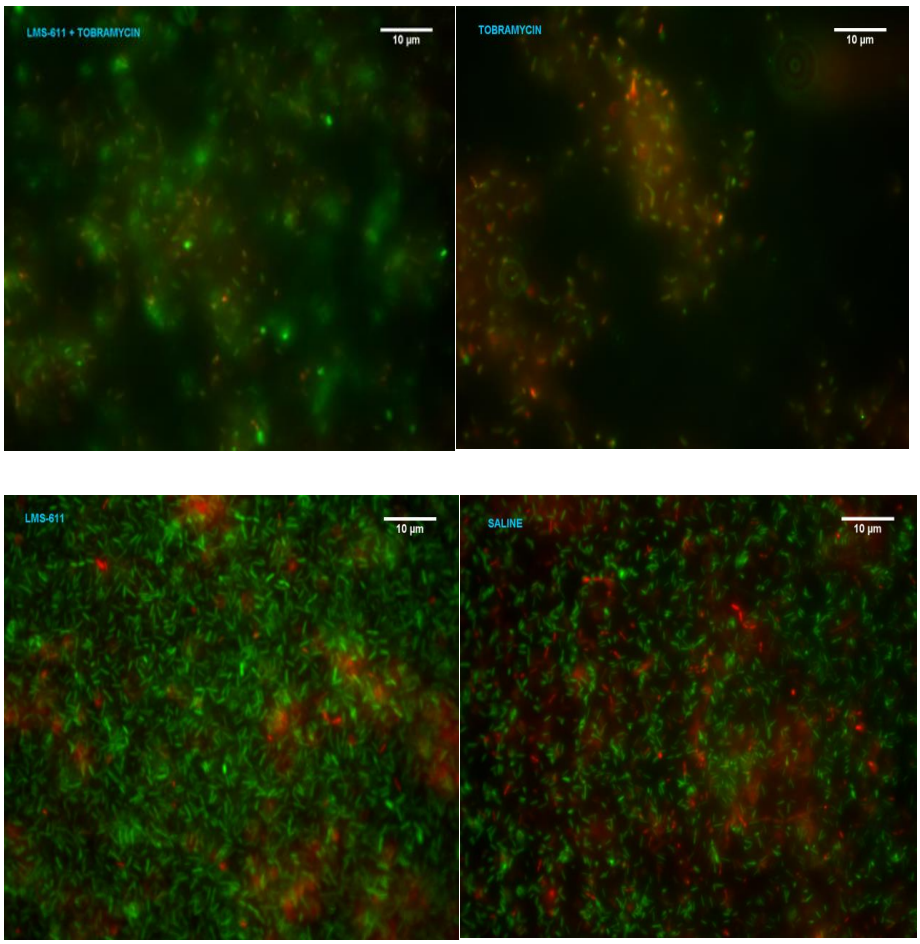


Figure 3.5.1

Activities of LMS-611 with or without tobramycin on 24 hour old PAO1 biofilms, compared to tobramycin and saline controls – representative fluorescent images stained with Baclight LIVE/DEAD, a dual component (syto-9 and propidium iodide) fluorophore. Syto-9 permeates all cells whereas propidium iodide enters only damaged ones, thus live cells appear green whilst dead cells have their green colouration masked by the red propidium iodide.

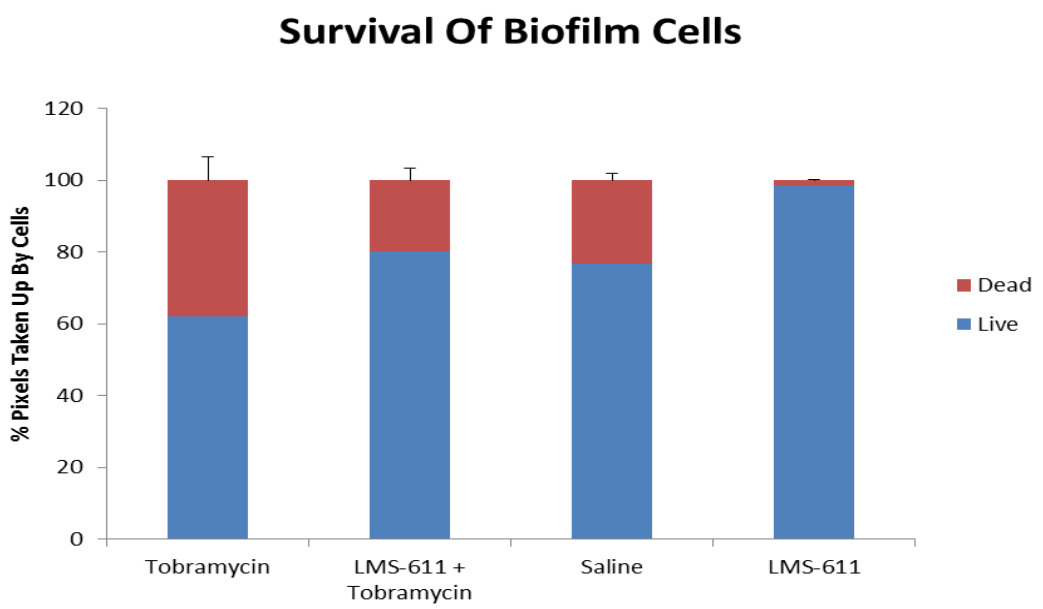


Figure 3.5.2

Activities of LMS-611 with or without tobramycin on 24 hour old PAO1 biofilms, compared to tobramycin and saline controls – percentages of live vs. dead cells were measured as a function of the total number of green (live) versus red (dead) pixels per image. Results were analysed by one way ANOVA for significance at $\alpha = 0.05$, groups differing significantly from the saline vehicle control are denoted by ***.

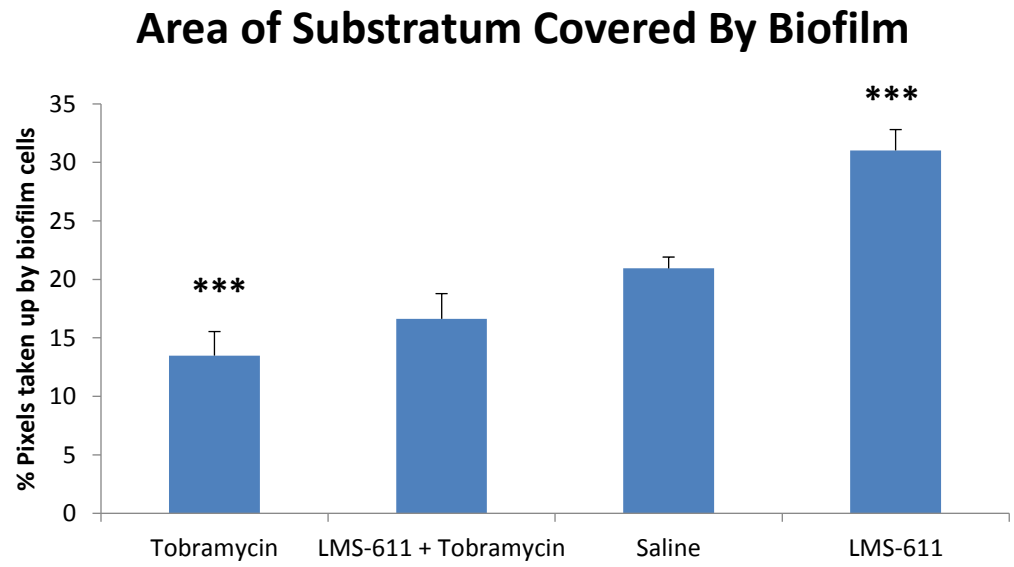


Figure 3.5.3

Activities of LMS-611 with or without tobramycin on 24 hour old PAO1 biofilms, compared to tobramycin and saline controls – percentage of area covered measured as a function of the total number of fluorescent (biofilm) versus dark field (blank) pixels per image. Results were analysed by one way ANOVA for significance at $\alpha = 0.05$, groups differing significantly from the saline vehicle control are denoted by ***.

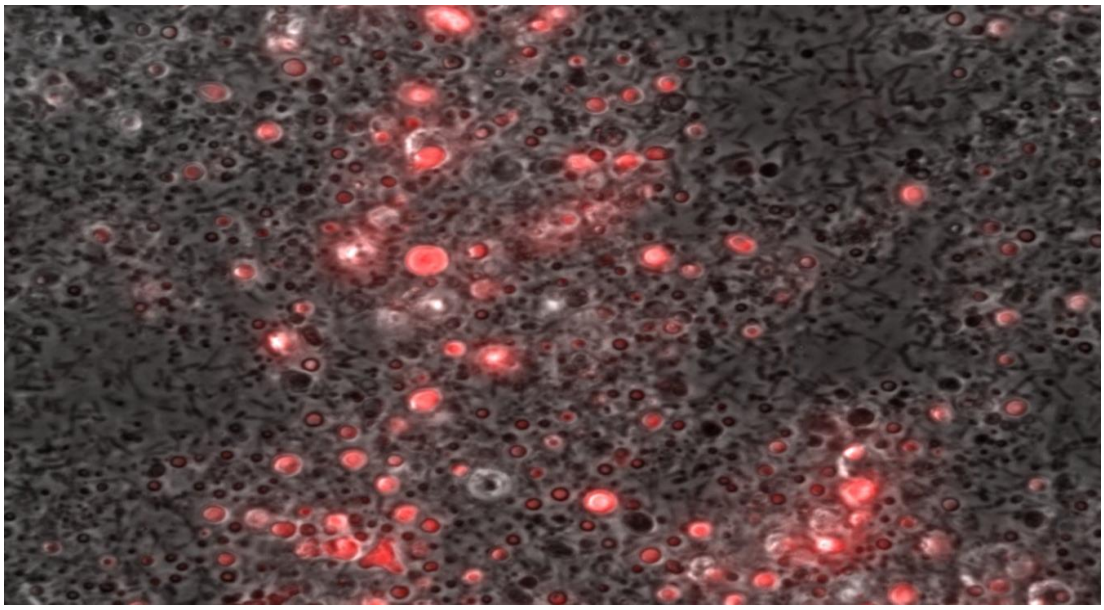


Figure 3.5.4

Fluorescently labelled LMS-611 interaction with PAO1 biofilm cells ; Lamellasomes loaded with the lipophilic fluorophore FM 4-64 do not appear to fuse with biofilm cells, red fluorescence clearly remains localised within the vesicles.

SECTION 3.2.7.5

It was deemed to be of interest to establish whether or not LMS-611 could influence the structure of *P. aeruginosa* biofilms. To answer this question, LMS-611 treated and control biofilms of 2 strains, cultured and fixed on glass coverslips were examined by field emission scanning electron microscopy. These experiments indicated that LMS-611 caused biofilms of both strains to grow as tightly compacted uniform monolayers devoid of the usual structural differentiation.

EFFECTS OF LMS-611 ON BIOFILM STRUCURE – FIELD EMISSION SCANNING ELECTRON MICROSCOPY

LMS-611 treatment caused biofilms formed by both PAO1 and J1385 to appear as compact monolayers lacking structural differentiation. Features such as 'mushroom caps' and fluid channels were evident in controls but not in LMS-611 treated biofilms.

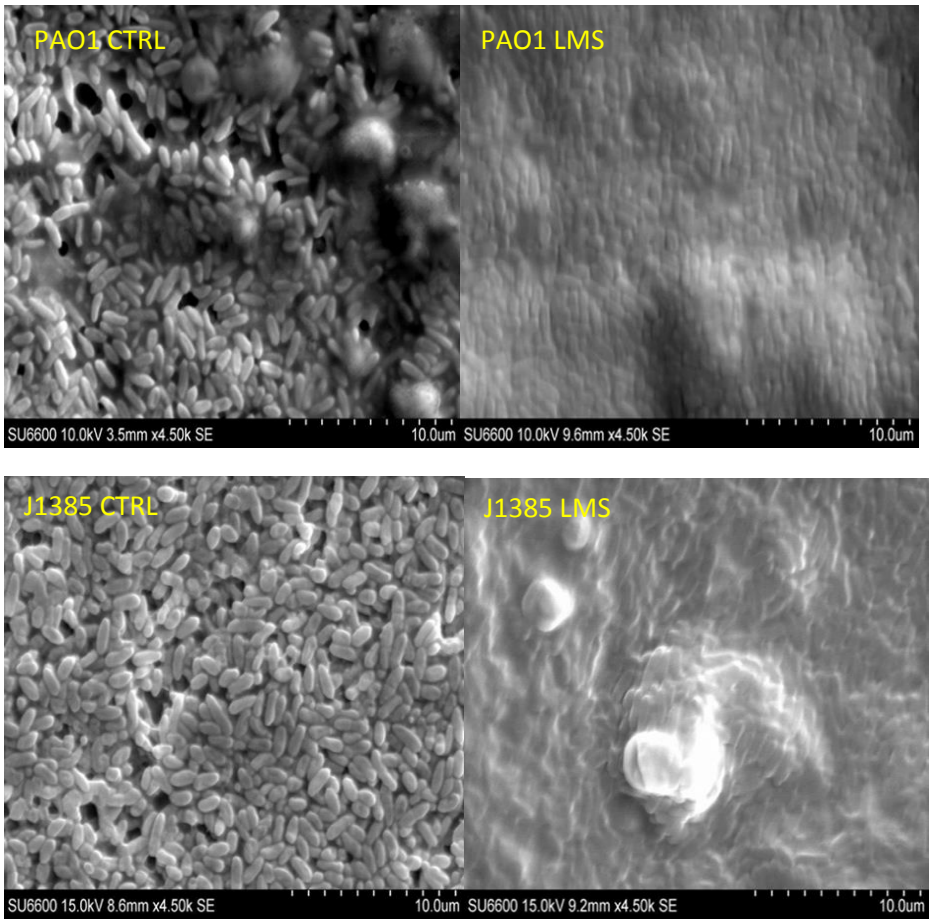


Figure 3.6.1

Field emission scanning electron micrographs at 4,500 X magnification. Shown clockwise from top left to bottom left – PAO1 biofilm treated with 0.9 % NaCl vehicle, PAO1 biofilm treated with 10 mg/ml LMS-611, J1385 biofilm treated with 10 mg/ml LMS-611 and J1385 biofilm treated with 0.9 % NaCl vehicle.

SECTION 3.2.7.6

It was of interest to determine whether or not LMS-611 could interfere with the production of virulence related exoproducts in *P. aeruginosa* biofilm, as this could provide a potential explanation for the enhanced clearance of this organism previously observed in a rat model of lung infection (Appendix B). Therefore pyocyanin was assayed by absorbance, the fluorescent siderophores pyochelin and pyoverdine by fluorescence, alginate by a carbazole colorimetric assay, elastase by enzymatic assay with congo-red conjugated elastin and total exoprotease by enzymatic assay using azocasein, a promiscuous dye conjugated substrate. LMS-611 markedly reduced accumulation of all of these exoproducts in pellicle biofilm cultures of all 3 strains tested, confirming the ability of LMS-611 to reduce production of these virulence factors.

ASSAYS FOR SECRETED VIRULENCE FACTORS

LMS-611 (10 mg/ml) and saline treated (control) pellicle biofilm cultures grown statically for 48 hours in LB were quantitatively assayed for the presence of various secreted virulence factors. 3 *P. aeruginosa* strains were employed in these experiments and significant differences ($\alpha = 0.05$) were noted in the quantity of each virulence factor secreted by each strain in LMS-611 treated versus control cultures.

Virulence Factor	change in PAO1 cultures ($p < 0.05$)	change in J1385 cultures ($p < 0.05$)	change in J1532 cultures ($p < 0.05$)
Pyocyanin ($n = 8$)	- 27 %	- 15 %	- 60 %
Pyoverdine ($n = 8$)	- 77 %	- 94 %	- 79 %
Pyochelin ($n = 8$)	- 38 %	- 52 %	- 21 %
Elastase ($n = 8$)	- 25 %	- 42 %	- 43 %
Total Exoprotease ($n = 8$)	- 35 %	- 61 %	- 64 %
Alginate ($n = 4$)	NT	- 61 %	- 88 %

Table 3
% change in virulence factors induced by 10 mg/ml LMS-611.

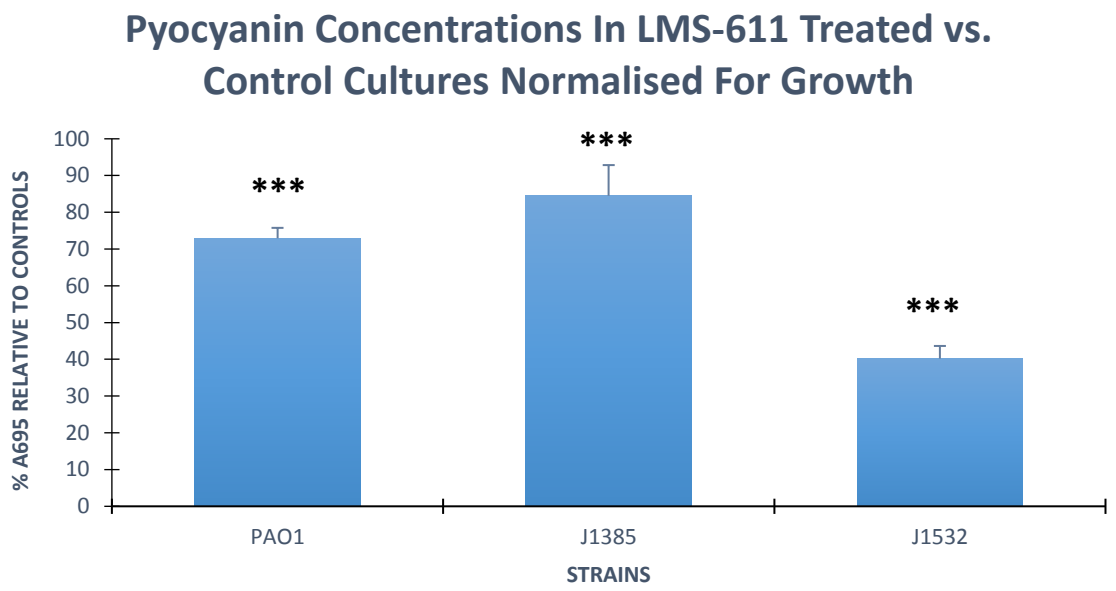


Figure 3.7.1

Effect of LMS-611 on mean pyocyanin accumulation in pellicle biofilm cultures (n = 8). Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

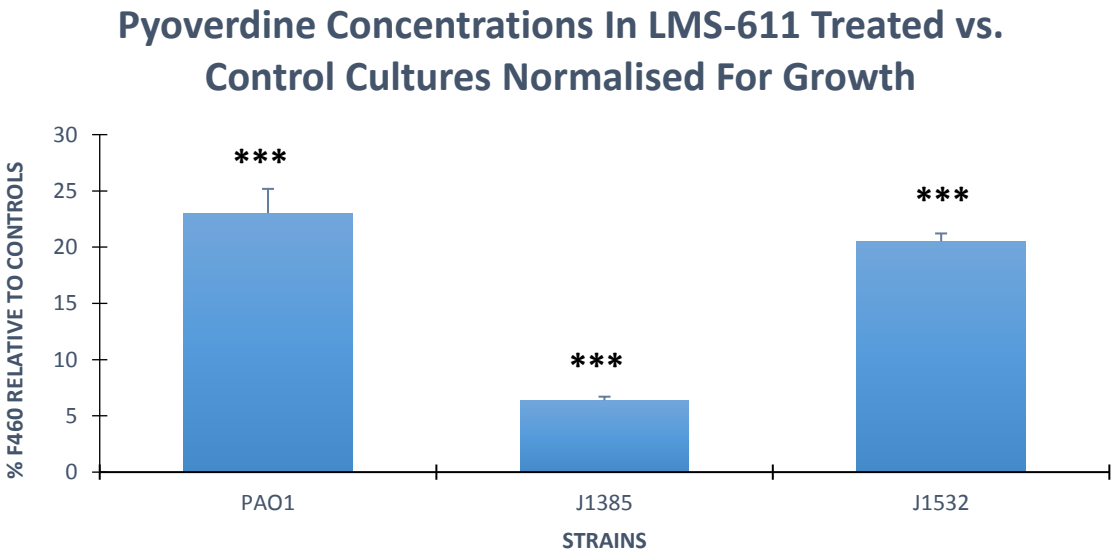


Figure 3.7.2

Effect of LMS-611 on mean pyoverdine accumulation in pellicle biofilm cultures (n = 8). Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

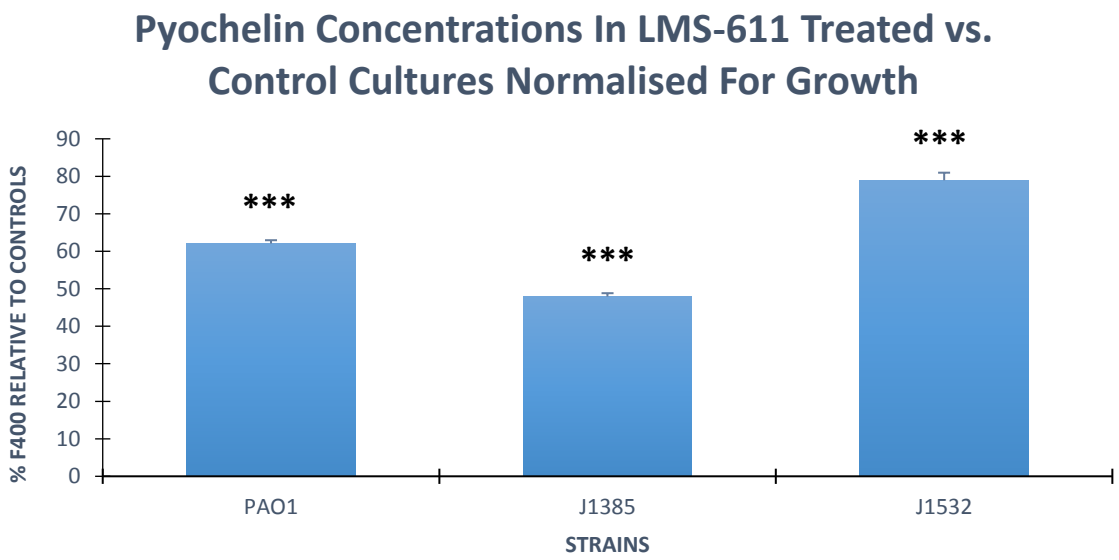


Figure 3.7.3

Effect of LMS-611 on mean pyochelin accumulation in pellicle biofilm cultures (n = 8). Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

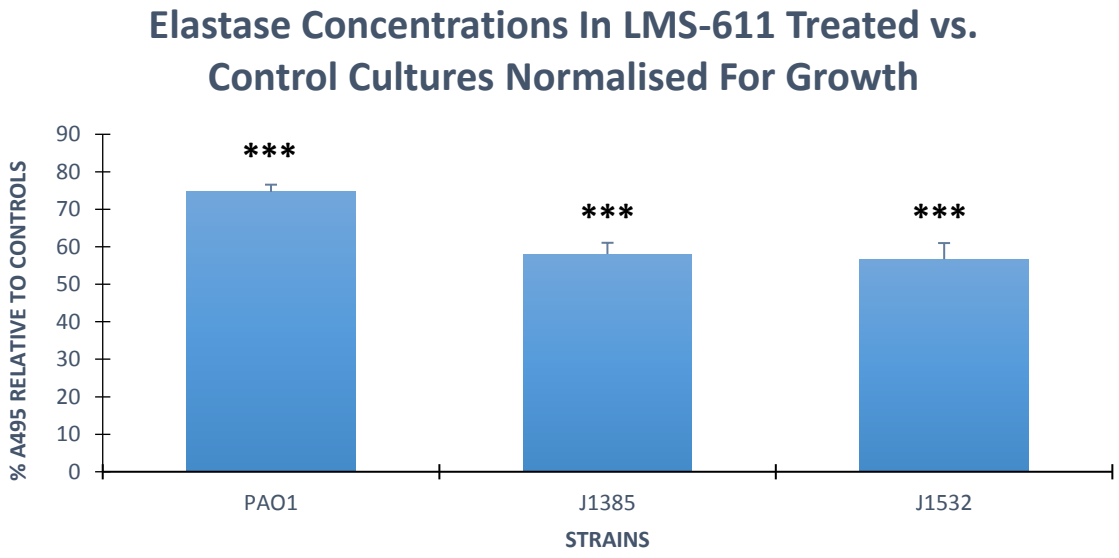


Figure 3.7.4

Effect of LMS-611 on mean elastase accumulation in pellicle biofilm cultures (n = 8). Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

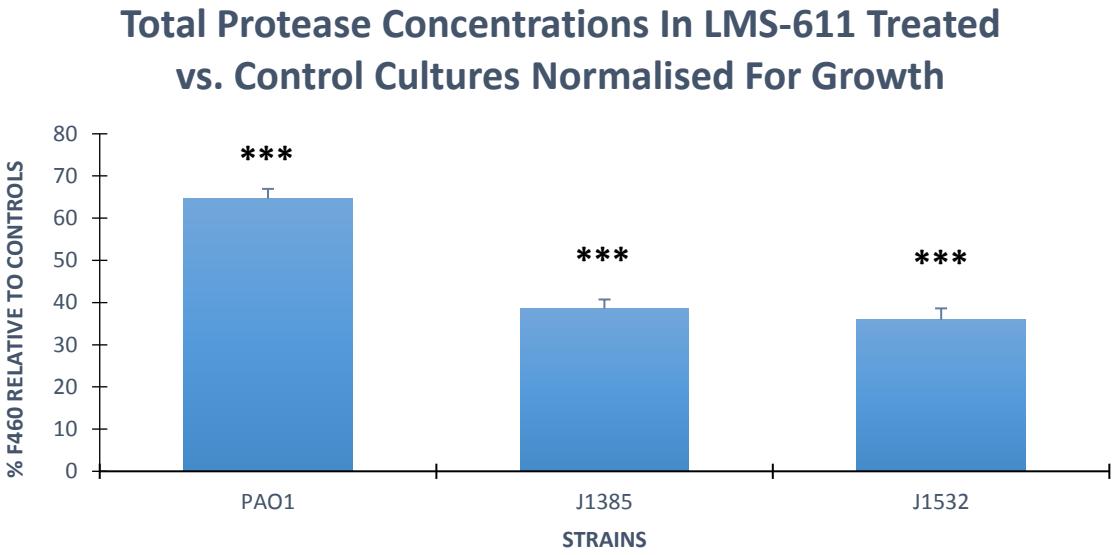


Figure 3.7.5

Effect of LMS-611 on mean total exorotease accumulation in pellicle biofilm cultures (n = 8) . Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

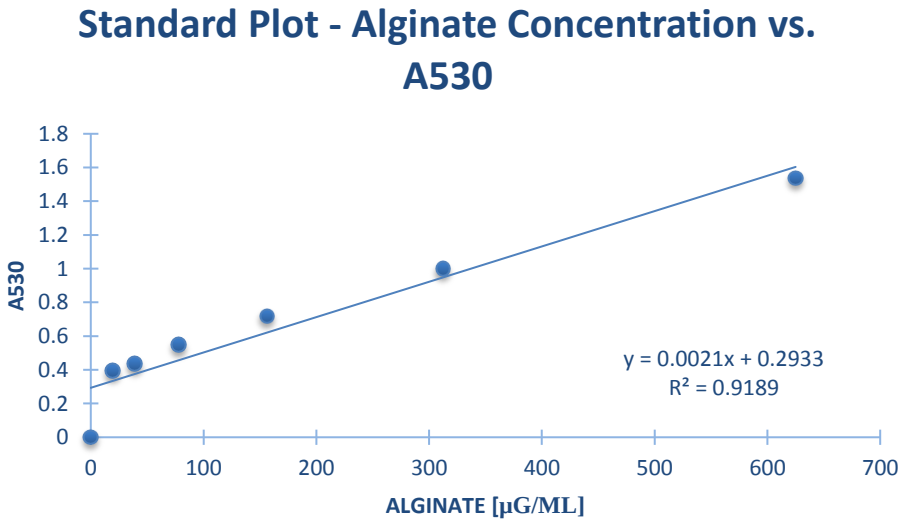


Figure 3.7.6

Standard Calibration Curve for Determination of Alginate Concentration. Triplicate alginate standards prepared at known concentrations of 19.5, 39.1, 78.125, 156.25, 312.5 and 625 µg/ml were subjected to carbazole assays and mean A_{530} readings were plotted against concentration (n = 3).

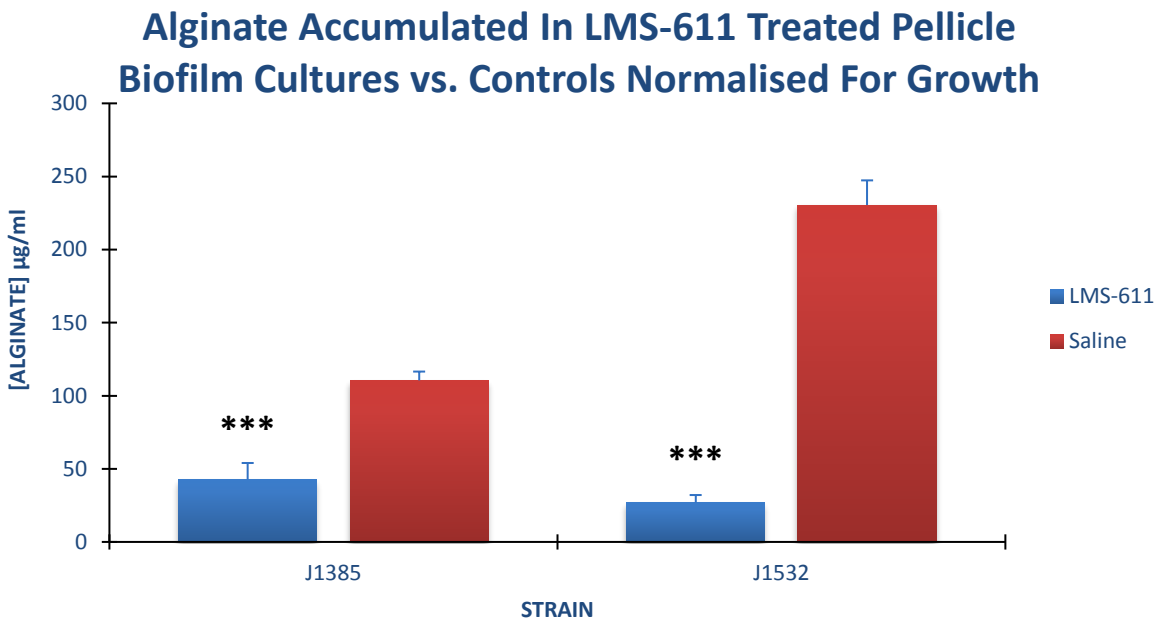


Figure 3.7.7

Effect of LMS-611 on mean alginate accumulation in pellicle biofilm cultures (n = 4). Results were analysed by one way ANOVA for significance at α = 0.05, groups differing significantly from the saline vehicle control are denoted by ***.

SECTION 3.2.8

DISCUSSION

In accordance with the results described above from crystal violet staining assays, confocal fluorescence microscopy and scanning electron microscopy, LMS-611 does not appear to reduce overall biofilm formation per se and actually seems to have increased biofilm coverage on the horizontal plane based on these results. Although LMS-611 has not been shown to reduce the overall area of biofilm coverage, the possibility that LMS-611 modifies biofilm density and structural differentiation cannot be excluded and actually seems to be supported by the results obtained via scanning electron microscopy (Figure 3.6.1), biofilms treated with LMS-611 appear as a homogenous monolayer and lack the architectural features clearly seen in their respective controls such as water channels and matrix caps. Observations from pellicle biofilm described in Figure 3.1.2 also lend credence to this argument ;

control biofilms maintain buoyancy and the ability to exclude a layer of saline applied over them whereas LMS-611 treated biofilms lose buoyancy and are freely miscible with saline and the constituent cells appear to have sedimented at the bottom of the tube as pellet like clumps (Figure 3.1.2). It is also possible that the surfactant action exhibited by LMS-611 upon biofilms makes them more permeable to hydrophobic stains such as crystal violet or that lamellasomes capable of binding such stains remain trapped in the biofilm matrix in spite of rinsing, thus potentially confounding the results of these stain based assays. Moreover, although this crystal violet method is in common use and widely reported throughout the literature, some workers have criticised its performance with biofilms of *P. aeruginosa*, noting that the matrices of these tend to contain a higher relative water content than those found in biofilms of most other commonly studied organisms such as *Staphylococcus sp.* (Peeters *et al*, 2008). The ability of LMS-611 to reduce accumulation of biofilm alginate (Figure 3.7.7) may partially explain the lack of architectural heterogeneity observed in biofilms treated with this formulation. Alginate is not essential for initial surface attachment and biofilm formation but can have roles in the structural maturation of biofilms, at least for some strains, however, this assay whilst sensitive is sensitive to interference from neutral sugars and its results must be interpreted with caution as it is likely that some of the material reacting in this assay, particularly in the case of non-mucoid strains, is not alginate but other sugar containing matrix compounds such as eDNA, which, nevertheless also contribute to biofilm structuring (Wozniak *et al*, 2003 ; Whitchurch *et al*, 2002). Alginate is also known to be an important virulence determinant of *P. aeruginosa*, with a wide range of roles in the pathology of infected brochiectatic airways; it impedes phagocytosis of the organism, quenches hypochlorite, inhibits complement activation and PMN chemotaxis and stimulates respiratory burst, all contributing to inflammation (Meshulam *et* al, 1984 ; Krieg *et* al, 1988 ; Pedersen *et al*, 1990 ; Learn *et al*, 1987). Rhamnolipids are biosurfactants known to be important in the structural differention of biofilms, particularly in regards to formation of water channels and biofilm dispersal (Espinosa-Urgel, 2003). The fact that LMS-611 treated biofilms lack water channels (Figure 3.6.1) and do not foam when shaken (Figure 3.1.2) may suggest that LMS-611 reduces rhamnolipid production. Other investigators have shown that *rhl* is

downregulated in LMS-611 treated cultures, further suggesting that this may be the case (Lamellar Biomedical, personal correspondence). Rhamnolipids, like alginate, are known to disrupt phagocytosis and retard macrophage chemotaxis, to induce necrosis in neutrophils and disrupt epithelial junctions making them yet another important virulence factor deployed by *P. aeruginosa* in CF airways (Zulianello *et al*, 2006 ; Jensen *et al*, 2007). The results obtained in this work also show that LMS-611 is capable of reducing the accumulation of several other crucial virulence associated exoproducts from this organism. It reduces accumulation of elastase and other proteases (Figures 3.7.4 and 3.7.5). These enzymes have been demonstrated to cleave a plethora of bactericidal defence factors of the airway, including complement fragments, transferrin, pulmonary surfactant proteins, secretory IgA and serum IgG (Schultz & Miller, 1974 ; Döring *et al*, 1983 ; Hauser *et al*, 2011). Furthermore, these proteases directly degrade the collagenous airway matrix contributing to inflammation and can also inhibit ciliary beat (Heck *et al*, 1986 ; Hauser *et al*, 2011). LMS-611 also reduces levels of pyocyanin (Figure 3.7.1), an exotoxin which induces abnormalities in airway goblet cells, upregulates inflammatory Th2 responses and induces neutrophil apoptosis (Caldwell *et al*, 2009). Finally, LMS-611 substantially reduces accumulation of the fluorescent siderophore molecules pyoverdine and pyochelin (Figures 3.7.2 and 3.7.3). The importance of this finding is not clear ; whilst there is little doubt that these are important virulence factors within the context of acute infections such as sepsis, their role in chronic airway infection is less obvious (Harrison *et al*, 2014). Nevertheless, iron is known to play important roles in biofilm formation and a diverse array of chelators including EDTA, lactoferrin, gallium and desferrioxamine are all known to limit or disrupt *P. aeruginosa* biofilm formation, often causing the organism to revert to a planktonic, motile state and become considerably more susceptible to antibiotics such as the aminoglycosides (Moreau-Marquis *et al*, 2009 ; Singh *et al*, 2002). It is possible in some cases that the observed reduction in individual virulence factors, especially those with complementary cationic charges or hydrophobic character, may have been due, at least in part, to physicochemical sequestration or inactivation of these preformed molecules by the LMS-611 vesicles rather than to an effect at the metabolic level, this possibility was not tested but analogous phenomena have been reported

elsewhere for other lipid bodies in the literature and it has also been suggested that virulence blocking therapies acting in this way may be less likely to encourage resistance than those possessing actual metabolic targets within the pathogen (Henry *et al*, 2014; Allen *et al*, 2014). Somewhat disappointingly, no evidence was found in these experiments to suggest that LMS-611 enhances activity of key antibiotics against *P. aeruginosa* biofilms grown *in vitro* (Figures 3.4.1 – 3.4.8) and in some cases, antagonistic interactions were even evident. The reasons for this remain open to conjecture and there are a number of potential explanations. A subpopulation of the biofilm cells are likely 'persisters' or at least remaining in the stationary phase of growth, cells such as these, even when in a planktonic state, are more tolerant to antimicrobials than actively dividing cells and are not necessarily less tolerant to bactericidal agents than biofilm cells themselves have been found to be (Spoering and Lewis, 2001). As LMS-611 appears to increase growth and metabolism of organisms, it is possible that outgrowth of biofilm cells surviving antibiotic action occurred more readily in LMS-611 treated biofilms. In the specific instances of the polycationic antibiotics colistin and tobramycin, it is also possible that the high concentrations of anionic LMS-611 used in these experiments partially competed with negatively charged drug binding sites on bacterial membranes, thereby antagonising these drugs, which must be electrostatically drawn to anionic sites in the lipid A of Gram-negative organisms before self-promoted uptake and lethal effect commences (Vaara, 1992). This explanation, however, would not explain the observed antagonism with ciprofloxacin, an anionic compound which enters bacterial cells via porins (Hancock and Brinkman, 2002). In the case of planktonic cultures, interactions of LMS-611 and antibiotics were mostly indifferent (Figures 3.3.1 – 3.3.12), displaying neither synergistic nor antagonistic effects. This lack of effect may have been due to the fact that all strains were highly sensitive to all antibiotics tested and the drugs may simply have been performing maximally at baseline. There were a few exceptions noted to this general trend of indifferent interactions. LMS-611 appeared to cause significant twofold reductions in the planktonic MIC of both ceftazidime and colistin from 2 to 1 µg/ml and from 0.5 to 0.25 µg/ml, respectively, in strain J1532 (Figures 3.3.3 and 3.3.9). The fact that this was the only constitutively mucoid strain tested and that this synergistic effect was absent when the same

combinations were tested on other strains, including J1385, the isogenic non mucoid progenitor of J1532, may imply that synergy was due to reduction in the mucoid capsule produced by this strain, which may otherwise have retarded the penetration of these drugs. The other exception was that LMS-611 appeared to cause significant twofold increases in planktonic ciprofloxacin MIC from 0.125 to 0.25 µg/ml for strain PAO1 and from 0.0625 to 0.125 µg/ml for strain J1385. Since this effect was seen only at these very low concentrations of ciprofloxacin, it seems possible that a subpopulation of surviving organisms, perhaps with first step point mutations conferring low level ciprofloxacin resistance, survived exposure to this low drug concentration and then grew more rapidly in the presence of LMS-611 than in the wells treated with saline as a control, as LMS-611 has been shown to increase metabolic activity and planktonic growth, it is likely, given more time, that growth in the control wells would have come to equal that in the LMS-611 treated wells. In contrast to most other clinically employed classes of antibacterial for which lateral gene transfers rather than *de novo* mutation are the most important driving factors of resistance, most resistance to fluoroquinolones such as ciprofloxacin occurs via cumulative acquisition of sequential point mutations which individually occur at relatively high frequencies and are then selected by subinhibitory drug levels leading to incrementally greater resistance levels (Sanders, 2001). No evidence was found to suggest that lamellasomes labelled with a fluorescent probe can fuse with bacterial cells (Figure 3.5.4) as has been previously reported for some other liposomal formulations (Beaulac *et al*, 1996 ; Nicolosi *et al*, 2010). Although little to no antibiotic potentiating effect of LMS-611 has been identified in these *ex vivo* studies, this does not rule out the possibility that LMS-611 could enhance antibiotic efficacy in the context of bronchiectatic airways where other factors such as the host immune response and bacterial virulence come into play, indeed, the marked surfactant action of LMS-611 together with its effects on biofilm structure and bacterial virulence noted in these experiments suggest that this may well be a potential outcome and that future work should endeavour to test this. The relevance of *in vitro* antibiotic susceptibility testing for respiratory isolates from CF airways or even the results of therapeutic studies undertaken *in vivo* with animal models and defined strains must be interpreted with caution ; naturally occurring CF airway infections are not

monomicrobial entities and typically involve not only multiple phenotypically distinct strains of *P. aeruginosa* but also many other species of both aerobic and anaerobic bacteria and even fungi whose clinical significance is not always known and likely underappreciated, indeed, some of these organisms are uncultivable and have only recently been identified in CF sputum samples using molecular techniques (Li Puma, 2010 ; Bittar *et al,* 2008). It is possible, regardless of whether or not any antibiotic enhancing effects of LMS-611 are ever realised *in vivo,* that LMS-611, given its capacity to suppress bacterial virulence traits, may have potential as a standalone therapeutic targeting these. Due to increasing problems posed by the inevitable evolution of multi-drug resistant organisms and the paucity of new antibiotics with conventional bactericidal or bacteriostatic modes of action together with the increasing realisation that antimicrobial agents have deleterious impacts on the ecology of human microbiomes that may be linked to pathology in some cases, therapies which act as modulators of bacterial virulence and/or host immunity are receiving increasing attention as potential long term alternatives to classical antimicrobial chemotherapeutics that do not impose as great a selection pressure for resistance development (Rasko and Sperandio, 2010).

CHAPTER 4

THE PHENOTYPIC RESPONSE TO MPPA

SECTION 4.1

INTRODUCTION

Monopalmitoylphosphatic acid (MPPA), better known as lysophosphatidic acid, is a bioactive lysophospholipid that occurs naturally in human serum and inflammatory exudates, including those of the airways, at concentrations which range from approximately 2 to 20 μM (Ediger & Toews, 2001). Krogfelt and colleagues found that MPPA greatly enhances the antibacterial action of various β-lactam antibiotics including ampicillin, piperacillin and ceftazidime against *P. aeruginosa* grown planktonically in LB with agitation, as MPPA binds cations in a manner analogous to membrane permeabilising chelators such as EDTA and hexametaphosphate, it was hypothesised that there may be a similar mechanism underlying the observed synergistic interaction of MPPA with these β-lactam compounds (Krogfelt *et al*, 2000). No synergistic interactions were observed with the quinolone nalidixic acid or with the aminoglycoside gentamicin, implying that antibiotic synergy with MPPA may be class specific (Krogfelt *et al*, 2000). MPPA was also found to slow the growth of mucoid CF sputum isolates by itself in these experiments (Krogfelt *et al*, 2000). Later it was shown that MPPA could also lessen biofilm formation and reduce the accumulation of alginate, pyoverdine and Las proteases in statically grown *P. aeruginosa* LB cultures at concentrations as low as 40 to 80 μM, no definitive mechanism of action was identified for these observed effects but it was found that they occurred independently of both homoserine lactone mediated quorum sensing and the stationary phase sigma factor *rpoS* and that reduced transcription of *lasB* had direct involvement in the reduced accumulation of a catalytically inactive form of proelastase which could be activated via proteolytic cleavage by exogenous elastase (Laux *et al*, 2002). As a result of its impressive abilities to both potentiate antibiotic activity and limit production of multiple virulence factors, it was suggested that MPPA might be of therapeutic value in the management of recalcitrant *P. aeruginosa* infections, for example, as an inhaled formulation in cystic fibrosis airway disease or as a topical preparation for application to infected burns (Krogfelt *et al*, Laux *et al*, 2002). The aims of this chapter are to determine the effects of MPPA upon the growth rates of a collection of 14 *P.aeruginosa* isolates, some of which are

sequenced strains. These include the historical PAO1 type strain, the widely studied virulent PA14 wound isolate, C1426/C1433 and J1385/J1532 - 2 pairs of isogenic nonmucoid/mucoid CF sputum isolates, isolate 28, an extremely mucoid CF sputum isolate and 7 non-mucoid CF sputum isolates that have previously been identified as belonging to the highly transmissible and virulent Liverpool Epidemic Strain (LES). The interactions of MPPA with several distinct classes of antibiotic not previously reported (fluoroquinolones, tetracyclines, macrolides, chloramphenicol, polymyxins and trimethoprim) was also studied with these 14 strains, in addition to interactions with β-lactams, aminoglycosides and the non-fluorinated quinolone nalidixic acid, which had previously been tested with PAO1 by Krogfelt and colleagues (Krogfelt *et al*, 2000). The hitherto untested effects of MPPA on pyocyanin production in shaken and static cultures were also tested for 13 and 6 strains, respectively. Levels of elastase and total protease were also assayed under static conditions for the latter subset of 6 strains. Effects of MPPA on the accumulation of pyoverdine and pyochelin in shaken cultures were assayed for 13 strains ; effects of MPPA on pyochelin production have not previously been reported and for pyoverdine have only been tested in static culture. Effects of MPPA on the motility of *P. aeruginosa* have not previously been reported and these were determined for strain PA14 in assays of swimming, swarming and twitching motility. Effects of MPPA upon biofilm formation over 24 hours upon the well surfaces of a microtitre plate were also determined for 7 strains using the crystal violet staining method. For a subset of 4 of these strains, effects of MPPA upon biofilm architecture were also observed with field emission scanning electron microscopy. Finally, BioLog™ phenotype microarrays assessing carbon utilisation (PM-1 and PM-2A) and chemical sensitivity (PM-15 and PM-17) were used to quantify these phenotypes in the absence and presence of MPPA.

SECTION 4.2

MATERIALS AND METHODS

SECTION 4.2.1

PREPARATION OF MHB CONTAINING MPPA

12.5 mg of powdered MPPA (Avanti Polar Lipids) was accurately weighed out and added to a glass round bottomed flask that had previously been cleaned sequentially with boiling water and 70 % ethanol before being dried in an oven. 5 ml of chloroform was then added and swirled in order to both suspend the lipid and kill microbial contaminants. The chloroform was then removed by rotary evaporation at 250 rpm in a water bath set to 60˚C to obtain a dry, uniform lipid film. Any excess traces of chloroform were then removed briefly by applying a nitrogen stream. The mouth of the round bottomed flask was then sealed with cling film and it was transferred to a UV cross-linker oven set to full power for 45 minutes in order to kill any surviving contaminants and further ensure sterility. A 10 ml aliquot of sterile PBS in a 50 ml Falcon tube was then heated in a water bath set to 60˚C - above the phase transition temperature of MPPA. This preheated PBS was immediately added to the round bottomed flask containing the sterilised lipid film upon removal from the water bath, the flask was then swirled to dissolve the MPPA and obtain a clear solution of micellar MPPA. This micellar MPPA solution was then added to a 50 ml Falcon tube containing 40 ml of MHB containing 78 µM Ca^{2+} and 225 µM Mg^{2+} (Oxoid). Control MHB lacking MPPA was prepared using preheated PBS vehicle in place of the micellar MPPA solution. This MHB contained 250 µg/ml and was used for the growth curve measurements, assays of virulence factors and assays of biofilm formation reported in this chapter. For the antibiotic sensitivity MIC microdilution assays reported in this chapter, twice the initial concentration of MPPA was used in order to account for the 2 fold dilution of the media in these tests. For agar based motility assays, half the volume of PBS was used so as not to reduce the agar viscosity too much.

SECTION 4.2.2

GROWTH CURVES

Isolates were stored at – 80˚C on Microbank™ storage beads. Cultures of 13 isolates were set up by inoculating universals tubes containing 5 ml of LB with microbank beads and incubating overnight -or until turbid – at 37˚C with shaking at 250 rpm. These overnight cultures were then used to streak LB agar plates which were then incubated statically overnight at 37˚C in order to obtain isolated colonies for setting up pure cultures. Single colonies of each strain were inoculated into universal tubes containing 5 ml MHB and incubated overnight at 37˚C with shaking at 250 rpm. MHB with and without the addition of 250 µg/ml MPPA was prepared as described and decanted into 10 ml aliquots in sterile universal tubes in matched MPPA and control pairs for each strain. These were then each inoculated with 25 µl of overnight MHB culture and shaken to mix. A multi-channel pipette was then used to add 200 µl volumes of each culture to the wells of a 96 well plate in such an array as to produce triplicate results for each strain in both the absence and presence of MPPA, in other words, 3 wells per test condition. Uninoculated controls of MHB with and without MPPA were also included. 0.03 % resazurin metabolic indicator solution was prepared by adding 15 mg of resasurin sodium salt to 50 ml of sterile PBS, shaking to dissolve and then passing the solution through a 0.2 µm pore size filter using a disposable plastic 50 ml syringe. 20 µl of 0.03 % resazurin metabolic indicator solution was then added to all wells of the aforementioned 96 well plate before it was sealed with a breathe easy membrane and transferred to a Biotek kinetic plate reader with temperature preset to 37˚C and platform set to shake continuously at full speed. Absorbances at 570 and 600 nm wavelengths were then recorded by the instrument every 15 minutes for 24 hours and saved as a Microsoft Excel spreadsheet. The experiment was repeated on 3 occasions and the mean results were recorded and used to calculate the percentage reduction of resazurin using the following equation (O'Brien *et al,* 2000 ; Alamar Blue Technical Datasheet)

$$\frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where: O1 = molar extinction coefficient (80586 L mol^{-1} cm^{-1}) of oxidized resazurin at 570nm

O2 = molar extinction coefficient (117216 L mol^{-1} cm^{-1}) of oxidized resazurin at 600nm

R1 = molar extinction coefficient (155677 L mol^{-1} cm^{-1}) of reduced resazurin at 570nm

R2 = molar extinction coefficient (14652 L mol^{-1} cm^{-1}) of reduced resazurin at 600nm

N1 = absorbance of negative control well at 570nm

N2 = absorbance of negative control well at 600nm

Resazurin reduction values obtained over 24 hours were then plotted to obtain growth curves and doubling times were extrapolated from the regions signifying mid-exponential growth phase. It was deemed necessary to use the percentage reduction of this metabolic indicator dye as a surrogate measure of growth given that MPPA has the tendency to form precipitates with cations in bacteriological broth media which would interfere with standard OD600 measurements.

SECTION 4.2.3

ANTIBIOTIC SENSITIVITY - MIC DETERMINATIONS BY MICRODILUTION

Antibiotics used were supplied in the form of powders as follows : ceftazidime (as hydrate), chloramphenicol, ciprofloxacin, colistin, erythromycin, nalidixic acid (as sodium salt), tetracycline, tobramycin and trimethoprim (Sigma-Aldrich). Stock solutions of ceftazidime, colistin, nalidixic acid, tetracycline and tobramycin were prepared at a concentration of 1.024 mg/ml in distilled water and filter sterilised. Stock solutions of chloramphenicol and erythromycin were prepared at concentrations of 25 mg/ml in 100 % ethanol. Ciprofloxacin stock solution was prepared at a concentration of 1 mg/ml in 0.05 N hydrochloric acid. Trimethoprim stock solution was prepared at a final concentration of 20 mg/ml in a 15 % solution

of acetic acid. The wells in 3 inner rows of a 96 plate were filled with 100 µl of MHB prepared with or without 500 µg/ml MPPA as described – 3 rows with MPPA and 3 without per plate. Antibiotics were then diluted across the rows from left to right to obtain a series of 9 doubling dilutions in columns 2 to 10 of the microtitre plate containing twice the desired concentration of antibiotic and drug free controls with and without MPPA in column 11. Inocula of each isolate were prepared by culturing a single colony of each overnight in 5 ml of MHB within a universal tube in an incubator set to 37˚C and 250 rpm. These overnight cultures were then diluted with fresh MHB to an A_{600} value of 0.1 ± 0.02, then further diluted in a 1:150 ratio with more MHB to obtain an inoculum containing ~ 1 x 10^6 cfu/ml, 100 µl of inoculum were then used to complete the volumes of the microtitre plate wells to 200 µl – an inoculum of ~ 5 x 10^5 cfu/ml (CLSI, 2014). One plate was used per test, perimeter wells were avoided due to the possibility of discrepant 'edge effects' caused by evaporation and uneven aeration, these were filled with an equal volume of sterile MHB to minimise evaporation, then plate lids were attached and they were sealed with parafilm. Plates were then stacked and secured with autoclave tape before being secured in a shaking incubator set to 37 ˚ C and 250 rpm with the aid of oblong metal clamps. Plates were removed from incubation after 18 hours and opened before 20 µl of 0.03 % resazurin metabolic indicator solution was added to each well using a multichannel pipette as standard turbidity based MICs would have been subject to interference from MPPA precipitates (Lipuma *et al*, 2009). Plates were then resealed and returned to incubation under the previously stated conditions for a further 2 hours. They were then removed from incubation and fluorescent emissions at a wavelength of 590 nm were read after excitation at a wavelength of 540 nm in a plate reader. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA. The MIC was defined as the lowest antibiotic concentration at which fluorescence was less than 50 % of that in the drug free control, this had previously been found to correspond with turbidity based MIC values recorded in the absence of MPPA and a separate uninoculated plate filled in the same format was read to ensure that neither MPPA nor the test drugs themselves had substantially reduced the metabolic indicator dye, thus confounding the results (Li Puma *et al*, 2009). Although broth microdilution MIC protocols are ordinarily carried out under

static incubation conditions, we found that shaking was necessary to ensure growth of some slow growing isolates belonging to the LES strain, at least at this relatively low inoculum.

SECTION 4.2.4

QUANTIFYING VIRULENCE RELATED EXOPRODUCTS IN AGITATED CULTURES

Overnight cultures were prepared by inoculating a single colony of each test isolate into a 5ml aliquot of MHB in a universal tube and incubating overnight at 37˚C and 250 rpm. These cultures were then diluted with fresh MHB to an A_{600} value of 0.2 ± 0.02 before being further diluted in a 1:30 ratio with MHB with or without the addition of 250 µg/ml MPPA, prepared as previously described to give a starting inoculum of ~ 1 x 10^7 cfu/ml (section). Triplicate wells of a 96 well plate for each isolate and test condition (i.e. +/- MPPA) were then filled with 180 µl of inoculum. Perimeter wells were avoided to avoid the possibility of discrepant 'edge effects' occurring due to evaporation and uneven aeration, these and any other unused wells were filled with an equal volume of sterile MHB to minimise evaporation, then plate lids were attached and they were sealed with parafilm. Plates were then stacked and secured with autoclave tape before being secured in a shaking incubator set to 37 ˚ C and 250 rpm with the aid of oblong metal clamps. Plates were then removed from incubation after 24 hours and immediately assessed for production of pyocyanin, pyoverdine and pyochelin. Pyocyanin was measured by recording the A_{695} value of each well (Fothergill *et al*, 2010). Pyoverdine was quantified by measuring F_{460} emission values after excitation with light at a wavelength of 400 nm whilst pyochelin was quantified by measuring F_{430} emission values after excitation with light at a wavelength of 350 nm (Harrison *et al*, 2014). Plates were then opened before 20 µl of 0.03 % resazurin metabolic indicator solution was added to each well using a multichannel pipette. Plates were resealed and returned to incubation under the previously stated conditions for a further hour after which F_{590} emissions were measured following excitation with light at a wavelength of 540 nm in order to quantify resazurin reduction as a surrogate marker for growth in each well.

Percentage growth in triplicate MPPA treated wells versus matched control wells was determined by dividing the F_{590} reading of the MPPA treated well by that of its matched control and multiplying by 100 (Kirchner *et al*, 2012). Pyocyanin, pyoverdine and pyochelin levels quantified in each well were then normalised to percentage growth as determined by resazurin reduction in the same well. Assays were repeated 3 times and normalised results were processed as bar charts displaying the mean of 9 observations plus standard error of the mean. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 4.2.5

QUANTIFYING VIRULENCE RELATED EXOPRODUCTS IN BIOFILM CULTURES

Production of some virulence related exoproducts of *P. aeruginosa* including pyocyanin and elastase is known to be increased in stationary and biofilm modes of growth relative to planktonic growth, higher cation levels are also known to support protease production and biofilm formation (Fothergill *et al*, 2010 ; Laux *et al*, 2002). Therefore a subset of 6 isolates, all of which were defined strains, production of pyocyanin, elastase and total protease was determined in surface bound biofilm cultures in MHB that had been supplemented to contain a final Ca^{2+} content of 594 µM vs 78 µM prior to supplementation and a final Mg^{2+} content of 509 µM vs 225 µM prior to supplementation. Overnight cultures were prepared by inoculating a single colony of each test isolate into a 5ml aliquot of cation supplemented MHB in a universal tube and incubating overnight at 37˚C and 250 rpm. These cultures were then diluted with fresh cation supplemented MHB to an A_{600} value of 0.2 ± 0.02 before being further diluted in a 1:30 ratio with cation supplemented MHB to obtain a starting inoculum of ~ 1 x 10^7 cfu/ml (section). Inoculum was then added to the 8 inner wells of a 24 well plate in 1.8 ml aliquots and outer wells were filled with an equal volume of sterile water to minimise evaporation. Lids were then attached to plates and they were sealed with parafilm before being incubated statically at 37˚C

for 24 hours to allow bacterial attachment and biofilm formation on the polystyrene well surfaces. After 24 hours, plates were removed from incubation and all liquid culture was removed by pipetting, leaving only preformed biofilms on the wells. Wells were then refilled in quadruplicate with 2 ml volumes of cation supplemented MHB prepared with or without the addition of 250 µg/ml MPPA as described. 3 plates were set up per experiment to give a total of 12 wells per test condition. Lids were then reattached, plates were resealed with parafilm and incubated statically at 37˚C for a further 48 hours to enable production of virulence associated exoproducts. After incubation, plates were re-opened and well contents were mixed by pipetting and scraping the well surfaces with the pipette tip to resuspend the biofilm as far as possible, visible biofilm was disrupted by repeated shearing using a sterile 1 ml disposable syringe. Control and MPPA treated samples were then pooled in triplicate and transferred to 50 ml Falcon tubes to give a total 4 tubes containing ~ 6ml of MPPA treated sample and 4 tubes containing ~ 6ml of control sample. A 200 µl aliquot of each pooled, homogenised culture was then taken from each of the tubes and transferred to the wells of a 96 well plate, 20 µl of 0.03 % resazurin metabolic indicator solution was then added to each well of the 96 well plate before it was incubated at 37 ˚C with shaking at 250 rpm for an hour. F_{590} emissions were read from each well after excitation with light at a wavelength of 540 nm. Percentage growth in quadruplicate samples from MPPA treated tubes versus matched control wells was determined by dividing the F_{590} reading of the MPPA treated sample by that of its matched control and multiplying by 100. The remaining contents in each tube (~ 5.8 ml) were centrifuged for 15 minutes at 4200 rpm to remove the cells and the resulting supernatant was sterilised by passing it through a 0.2 µM pore size membrane filter with the aid of a disposable 5 ml syringe. This supernatant was retained for the assays of pyocyanin, elastase and total exoprotease described below and results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 4.2.6

PYOCYANIN ASSAY – BIOFILM SUPERNATANTS

Pyocyanin was extracted and assayed based on a method previously reported in the literature (Essar *et al*, 1990 ; Jiricny *et al*, 2014). 5 ml samples of each quadruplicate supernatant were transferred to 50 ml Falcon tubes to which 3 ml of chloroform had previously been added. The tubes were then capped and vortexed on full power for 5 seconds before being centrifuged at 4200 rpm for 10 minutes to allow formation of separate aqueous and organic phases. 2 ml of the organic phase (containing oxidised blue pyocyanin) was siphoned off by pipetting and transferred to a separate 15 ml Falcon tube. 1 ml of 0.2 N hydrochloric acid was then added to the chloroform extract, the tube was capped and vortexed for 5 seconds on full power. The tube was then centrifuged at 4200 rpm for 10 minutes to obtain distinct organic and acidified aqueous phases. 150 µl of the acidic aqueous phase (containing reduced red pyocyanin) was then transferred to a 96 well plate and A_{520} was measured. The A_{520} value was then multiplied by 17.072 (the molar extinction coefficient of pyocyanin) to reveal the extracted pyocyanin concentration in µg/ml, this value was then multiplied by 0.66 to correct for the fact that only 2/3 of the organic phase had been sampled. 0.2 N hydrochloric acid was used as a negative control. Pyocyanin concentrations were normalised to account for any differences in percentage growth, as measured by fluorescence of reduced resazurin, that had been observed in the cultures prior to centrifugation and filtration and (section). Results were plotted on bar charts as quadruplicate means plus standard error. Statistical significance was analysed by one way ANOVA at $\alpha = 0.05$.

SECTION 4.2.7

ELASTASE ASSAY – BIOFILM SUPERNATANTS

Elastase activity was measured using a method previously reported in the literature that uses a granular elastin-dye conjugate, elastin congo-red (Ohman *et al*, 1980 ; Jiricny *et al*, 2014). This assay exploits the fact that liberation of soluble congo-red dye is directly proportional to enzymatic cleavage of the insoluble elastin congo-red

conjugate. In order to perform this assay, 25.7 mg samples of elastin congo-red granules were weighed out and dispensed into the conical base of a 20 ml universal tube. One tube was used per test supernatant. The granules were then suspended by adding 1.8 ml of elastase buffer and 200 µl of test supernatant. Tubes were then capped and transferred to a shaking incubator set at 37˚C and 250 rpm for 4 hours. Thereafter, 1 ml aliquots were transferred into 1.5 ml microcentrifuge tubes and spun down at 4 ˚C/13,300 rpm to remove granules. 150 µl volumes of the resulting supernatant were then transferred to a 96 well plate and read at A_{495}. A negative control is made by using a tube treated with MHB in place of culture supernatant. Elastase concentrations were normalised to account for any differences in percentage growth, as measured by fluorescence of reduced resazurin, that had been observed in the cultures prior to centrifugation and filtration. Results were plotted on bar charts as quadruplicate means plus standard error. Statistical significance was analysed by one way ANOVA at $\alpha = 0.05$.

SECTION 4.2.8

TOTAL EXOPROTEASE ASSAY – BIOFILM SUPERNATANTS

Total exoprotease activity in supernatants was quantified using a method previously described in the literature that uses a casein conjugated to an azo-dye as a promiscuous protease substrate (Jiricny *et al*, 2014). This assay exploits the fact that liberation of soluble orange azo-dye is directly proportional to enzymatic cleavage of the insoluble azocasein conjugate. To measure total exoprotease activity, 250 µl of 2 % filter sterilised azocasein solution in total exoprotease assay buffer was added to 2 ml microcentrifuge tubes containing 150 µl of filtered culture supernatant and vortexed for 5 seconds before being incubated statically at 37 ˚C for 45 minutes to allow substrate digestion. Reactions were then stopped by precipitating out any undigested substrate by adding 1.2 ml of 10 % trichloroacetic acid per tube prior to incubating for 15 minutes at room temperature and then centrifuging at 13,300 rpm for a further 10 minutes. 900 µl volumes of the resulting supernatant were then transferred to fresh 2 ml microcentrifuge tubes containing 750 µl of 1M NaOH to

neutralise the trichloracetic acid. 150 µl volumes of the centrifuged supernatant were then transferred to 96 well plates and A_{440} was read. A negative control is made by using a tube treated with MHB in place of culture supernatant. Total exoprotease concentrations were normalised to account for any differences in percentage growth, as measured by fluorescence of reduced resazurin, that had been observed in the cultures prior to centrifugation and filtration and (section). Results were plotted on bar charts as quadruplicate means plus standard error. Statistical significance was analysed by one way ANOVA tests at $\alpha = 0.05$.

SECTION 4.2.9

BIOFILM FORMATION ASSAYS

It has previously been reported that concentrations of MPPA as low as 80 µM can reduce biofilm formation in static cultures (Laux *et al*, 2002). In order to confirm whether or not MPPA could reduce biofilm formation, a simple microtitre plate assay was used, based on modifications of that proposed previously (O'Toole, 2011). Each test isolate was inoculated into MHB prepared with and without 250 µg/ml MPPA at a cell density of ~ 1.0 x 10^7 cfu/ml. 180 µl of this culture was then pipetted into the wells of a 96 well plate in such a manner as to produce 6 replicate wells for each strain and test condition. The plate lid was then attached and sealed with parafilm to minimise evaporation before the plate was incubated statically at 37˚C for 24 hours. After incubation, culture was siphoned out of the wells using a multichannel pipette and the wells were rinsed twice with 200 µl volumes of sterile PBS so that only biofilm would remain on the well surfaces. 180 µl volumes of filtered 0.1 % crystal violet solution was then added to each well and left in place for 5 minutes to stain the biofilms. PBS rinsing was then repeated once after the crystal violet had been pipetted off and 200 µl of 30 % acetic acid was added to each well and left for 15 minutes to allow leaching of biofilm absorbed crystal violet. After this time, the acid was transferred to a fresh 96 well plate and absorbance of leached crystal violet, which is proportional to biofilm formation, was read at 570 nm in a plate reader. The

assay was repeated on 2 occasions to give 12 replicates per condition. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 4.2.10

FIELD EMISSION SCANING ELECTRON MICROSCOPY OF MPPA TREATED AND CONTROL BIOFILMS

MPPA has previously been reported to reduce biofilm formation in static *P. aeruginosa* cultures at concentrations of as low as 80 µM (Laux *et al*, 2002). The effects of MPPA on the architecture of biofilms have not been reported in the literature. It was therefore considered to be of interest to study these using field emission scanning electron microscopy using a subset of 4 strains. The protocol used to grow and fix biofilms and prepare them for scanning electron microscopy was an adaptation of that previously described by Harrison and colleagues, differing in that glass coverslips were used instead of the Calgary biofilm device (Harrison *et al*, 2005). Single colonies of each strain were transferred to 5 ml volumes of MHB and grown overnight at 37 ˚C with shaking at 250 rpm to obtain stationary phase cultures. These were diluted in fresh MHB to an A_{600} value of 0.2 ± 0.02. These adjusted cultures were then further diluted 1:30 with fresh MHB, prepared with or without the addition of 250 µg/ml MPPA, to obtain starter inocula of each strain containing approximately 1 x 10^7 cfu/ml. Round 13mm glass coverslips were placed into the inner wells of a 24 well plate and the lid was attached. Coverslips were then sterilised by placing the 24 well plate into a UV crosslinker oven and running on full power for 30 minutes. 1ml of turbidity adjusted inoculum was then pipetted into each well containing coverslips and the remaining outer wells were filled with an equal volume of sterile water to minimise evaporation. The 24 well plates were then sealed around the perimeter with parafilm and incubated statically at 37 ˚C for 24 hours to allow biofilm formation. After Incubation, coverslips were transferred using forceps to fresh 24 well plates with wells containing 1 ml of fixative :- 2.5 % electron microscopy grade glutaraldehyde in 0.1 M cacodylate buffer. Fixation was allowed

to continue for 2 hours at room temperature before coverslips were sequentially rinsed for 10 minutes in cacodylate buffer and 18.2 MOh water and then air dried for 120 hours. Coverslips were then mounted on stalks, sputter-coated in gold and imaged under FE-SEM.

SECTION 4.2.11

SWIMMING MOTILITY IN THE ABSENCE AND PRESENCE OF MPPA

Swimming motility was assayed using adaptations of a method previously reported in the literature (Chow *et al*, 2011). 25 mg of MPPA was dissolved in 10 ml of sterile PBS as previously described (section). Molten MH agar (0.5 % agar content) was decanted in 45 ml volumes into 4 separate 50 ml polypropylene tubes half of which were then topped up with 5 ml of micellar MPPA solution and the other half with preheated PBS, capped tightly and swirled vigorously to mix. 3 petri dishes each of agar (30 ml) with or without the addition of 250 µg/ml MPPA were then poured and allowed to air dry for 2 hours prior to use. 3 separate overnight cultures of PA14 in MHB were diluted with fresh MHB to an A_{600} value of 0.4. Thereafter, 1 µl volumes of diluted culture were then pipetted up and aliquoted into the centre of the agar medium in each plate in such a way as to have an MPPA containing and matched control plate prepared from each of the 3 cultures. Lids were attached and the plates were incubated statically at 37 ˚ C for 20 hours. After this time, diameters of swim zones were measured with a ruler and the triplicate means for each group were calculated and graphed with standard error values. Statistical significance was analysed by one way ANOVA at $\alpha = 0.05$.

SECTION 4.2.12

TWITCHING MOTILITY IN THE ABSENCE AND PRESENCE OF MPPA

Assays for twitching motility were carried out by preparing MH agar plates as in the swimming assays but incorporating twice as much (1 %) agar. Single isolated PA14 colonies from an LB streak plate were then picked and stab inoculated through the agar layer and onto the polystyrene base of the agar plate using a sterile toothpick. After 20 hours of incubation, the plate was flooded with 0.1 % crystal violet for 5 minutes and this was then poured off to reveal the area over which organisms had travelled via twitching, which was then measured with a ruler. Means for each group were calculated and graphed with standard error values. Statistical significance was analysed by one way ANOVA at $\alpha = 0.05$.

SECTION 4.2.13

BIOLOG PHENOTYPE MICROARRAYS

Biolog plates PM-1 and PM-2 (carbon utilisation assays), PM-15 and PM-17 (chemical sensitivity assays) were set up as per the manufacturer's instructions with one modification ; MPPA dissolved in PBS was added to the inoculating fluids to obtain a final concentration of 250 µg/ml, not exceeding 10 % of the total volume. Controls were prepared using an equal volume of sterile PBS without MPPA. Growth in plates was recorded over 48 hours in an Omnilog plate reader with dedicated software. Triplicate results were then averaged and plotted to allow observance of growth differences between MPPA treated and matched control wells.

SECTION 4.3

RESULTS

SECTION 4.3.1

Since MPPA has previously been demonstrated to slow the growth of *P. aeruginosa in vitro*, including that of type strain PAO1 and mucoid cystic fibrosis sputum isolates (Krogfelt *et al*, 2000), an attempt was made to corroborate these earlier findings and expand this testing to a wider variety of clinical sputum isolates to determine whether or not MPPA generally slows growth in this species, with potential clinical applications. To achieve this goal, growth kinetics were monitored over 24 hours with a fluorescence based resazurin reduction assay – owing to the opacity of MPPA precipitates which precluded simple absorbance measurements. Unexpectedly, MPPA was found to prolong the lag phase of growth for a subset of tested sputum isolates but to shorten lag phase for other isolates tested, moreover, MPPA generally reduced or did not affect generation times for all test strains, including PAO1, indicating an overall tendency for MPPA to enhance rather than delay growth.

GROWTH CURVES

The effect of 250 μg/ml MPPA on the reduction of resazurin by 13 *P. aeruginosa* isolates grown planktonically in MHB was monitored at 15 minute intervals for 24 hours in a kinetic plate reader set to shake continuously and maintain a temperature of 37˚C for the duration of the experiment. Resazurin, a blue dye, is reduced through the metabolic activities of growing cells to a pink intermediate product, resorufin, whose increasing concentration is directly proportional to growth rate. Eventually, resorufin is further reduced to an undetectable colourless product, dihydroresorufin. As a result, maximum peaks in the growth curves presented in this section do not correspond to the beginning of stationary phase as in the case of a conventional turbidity based growth curve but rather to the time point at which resorufin concentration peaked and then began to decline during late exponential phase.

Stationary phase therefore corresponds, approximately, with the levelling off the curve after an initial period of decline. For each of the 13 isolates, generation times in the presence and absence of 250 μg/ml MPPA were extrapolated, to the nearest 0.25 hours, from the upward sloping regions of the curve corresponding to mid-exponential phase, prior to the decline in resorufin levels beginning at the peak of the curve. The effect of 250 μg/ml MPPA on the time taken to exit lag phase was also noted for each isolate.

In the case of acute isolate 36, MPPA had no apparent effect on the length of time taken to transition from lag to exponential growth phases with exponential growth commencing after approximately 5 hours regardless of whether or not MPPA was present, however, during exponential phase, MPPA was noted to enhance growth rate and halve the generation time from 1 to 0.5 hours (Figure 4.1). Length of lag phase was similarly unaffected for C1426, with exponential growth beginning after 6 hours in both the absence and presence of MPPA and yet again the generation time was halved from 1.5 to 0.75 hours, indicating an increased growth rate (Figure 4.2). Neither growth rate nor time taken to exit lag phase (~ 6 hours) were altered by MPPA in the case of C1433, the isogenic mucoid derivative of C1426 (Figure 4.3). For J1385, no differences were noted in time taken to reach exponential phase and this commenced after 5 hours regardless of whether or not MPPA was present, the generation time, nevertheless, was reduced from 1.25 to 0.5 hours (Figure 4.4). Likewise, MPPA had no effect on time taken to exit lag phase (~ 6 hours) by J1532, the isogenic mucoid derivative of J1385 although MPPA did halve the generation time from 1.5 to 0.75 hours (Figure 4.5). In the case of LES400, exponential growth commenced after 4.75 hours regardless of whether or not MPPA was present, however, MPPA quartered the generation time from 2.0 to 0.5 hours for this strain (Figure 4.6). For strain LES431, MPPA did not alter the time taken to transition from lag to exponential phase (~ 7 hours) but halved the generation time from 1 to 0.5 hours after this time had elapsed (Figure 4.7). No effects of MPPA on either time taken to transition from lag to exponential phase (~ 9 hours) or on generation time could be discerned for mucoid isolate 28 (Figure 4.8). In the case of reference strain

PAO1, lag phase was unaffected by the addition of MPPA and ended after around 5 hours of incubation, the generation time during exponential growth was nevertheless halved by MPPA from 1.5 to 0.75 hours for this strain (Figure 4.9). For strain PA14, no effects of MPPA were apparent on either time taken to enter exponential growth phase (~ 6 hours) or generation time (Figure 4.10). In the case of stable isolate 1, MPPA extended the duration of the lag phase from approximately 6 to 10 hours and increased generation time from 1.75 to 2.25 hours during exponential growth (Figure 4.11). For stable isolate 2, lag phase duration was also extended from around 8 to 12.5 hours but generation time was reduced thereafter from 1.25 to 0.5 hours once in exponential phase (Figure 4.12). Similarly, MPPA extended the duration of the lag phase for S38 from approximately 7 to 12 hours but halved generation time during exponential phase from 1.5 to 0.75 hours (Figure 4.13).

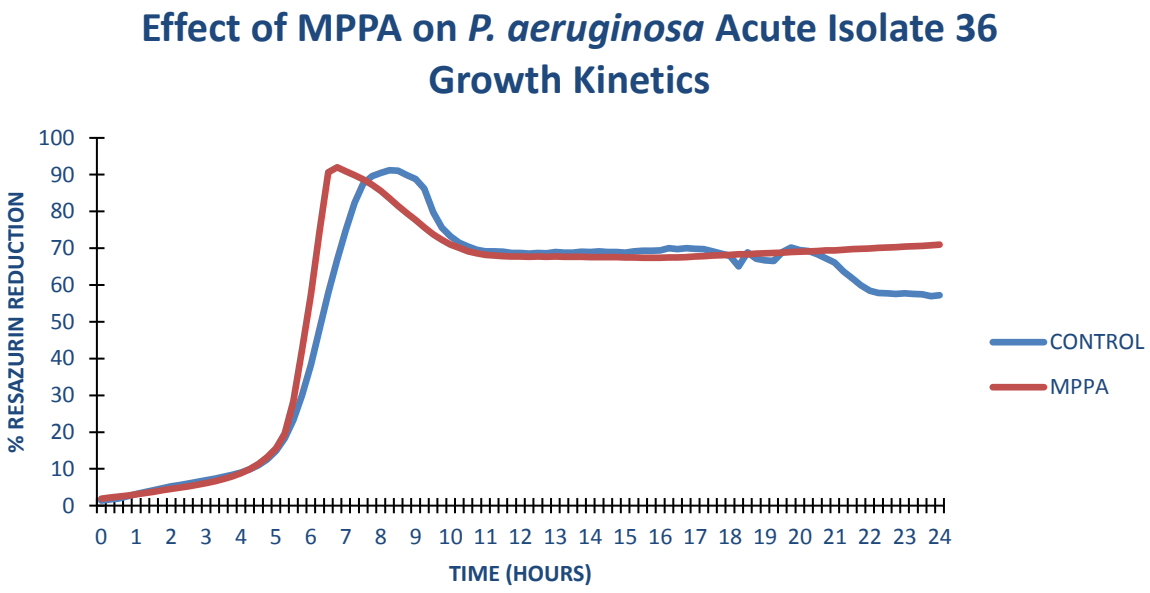


Figure 4.1
Growth curves of non-mucoid acute isolate 36 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

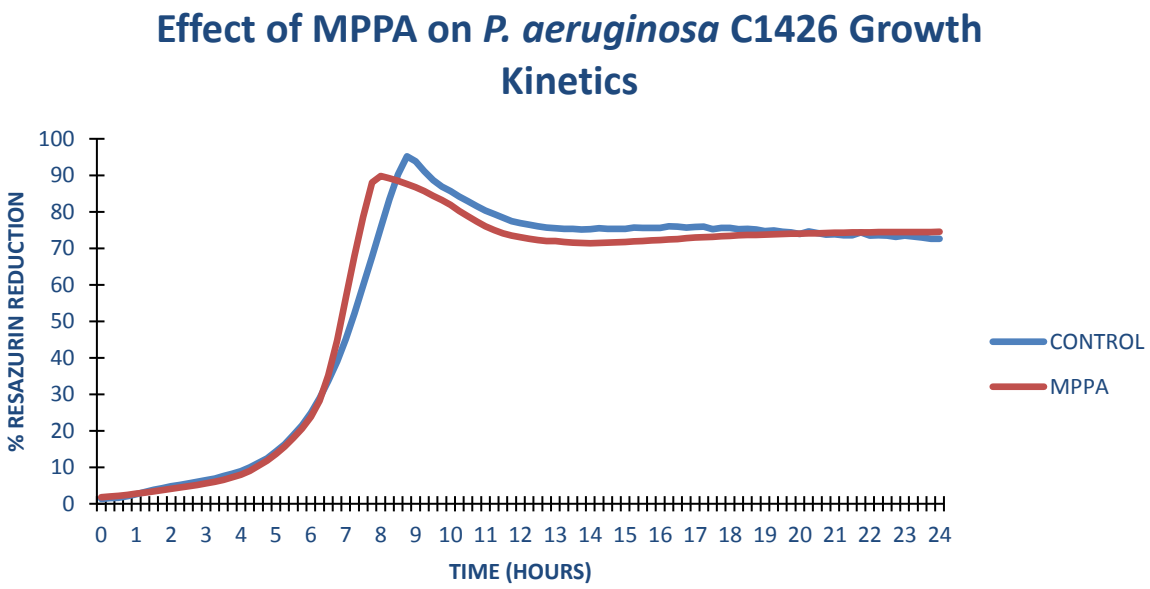


Figure 4.2

Growth curves of non-mucoid isolate C1426 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

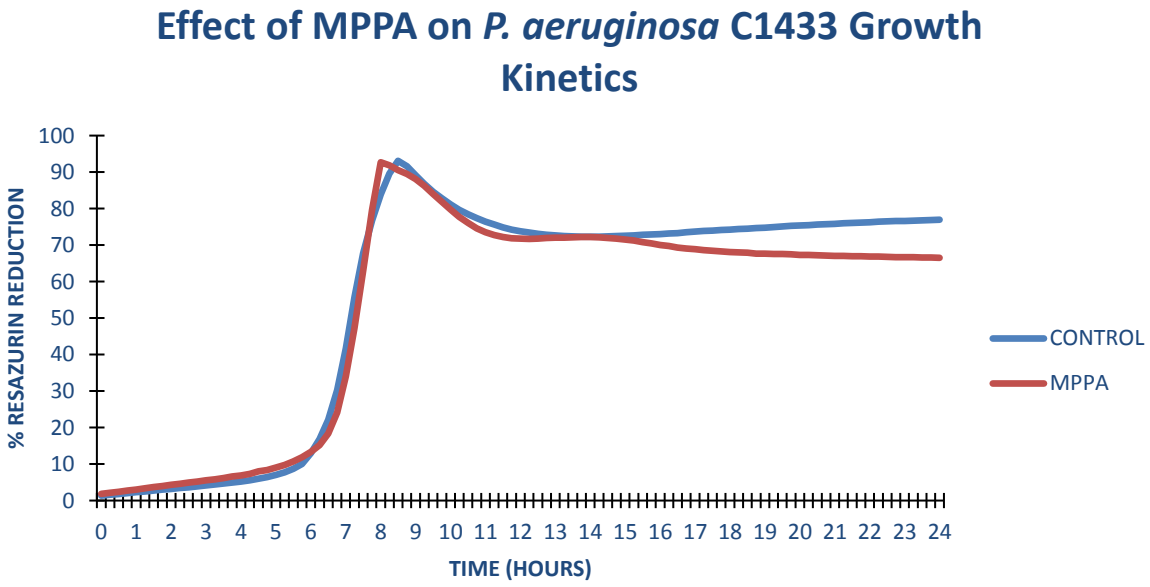


Figure 4.3

Growth curves of mucoid isolate C1433 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

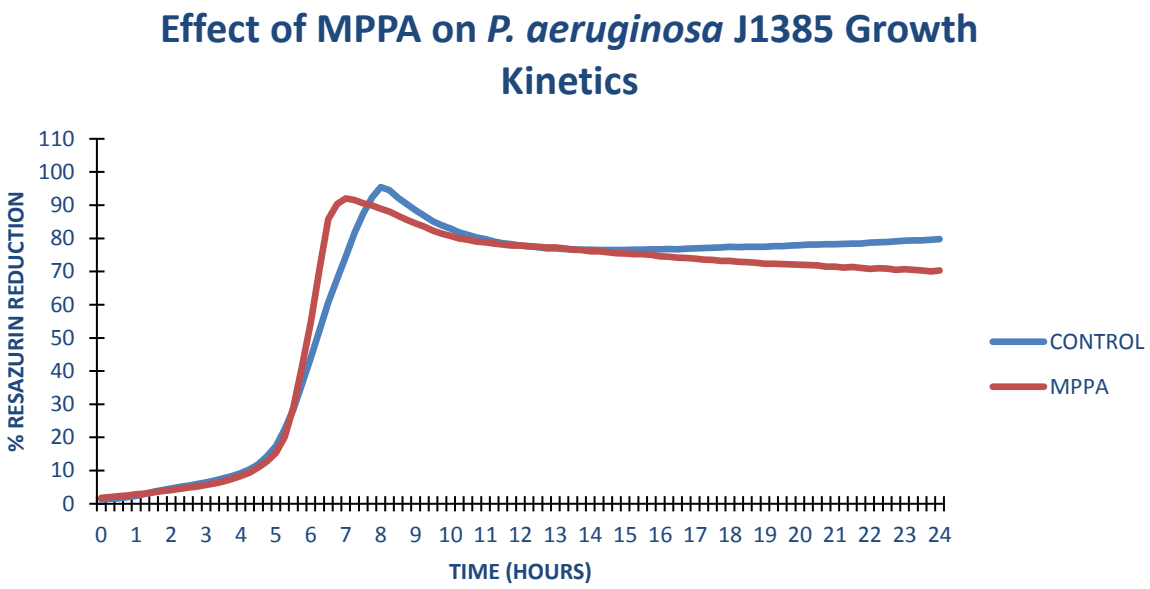


Figure 4.4

Growth curves of non-mucoid isolate J1385 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

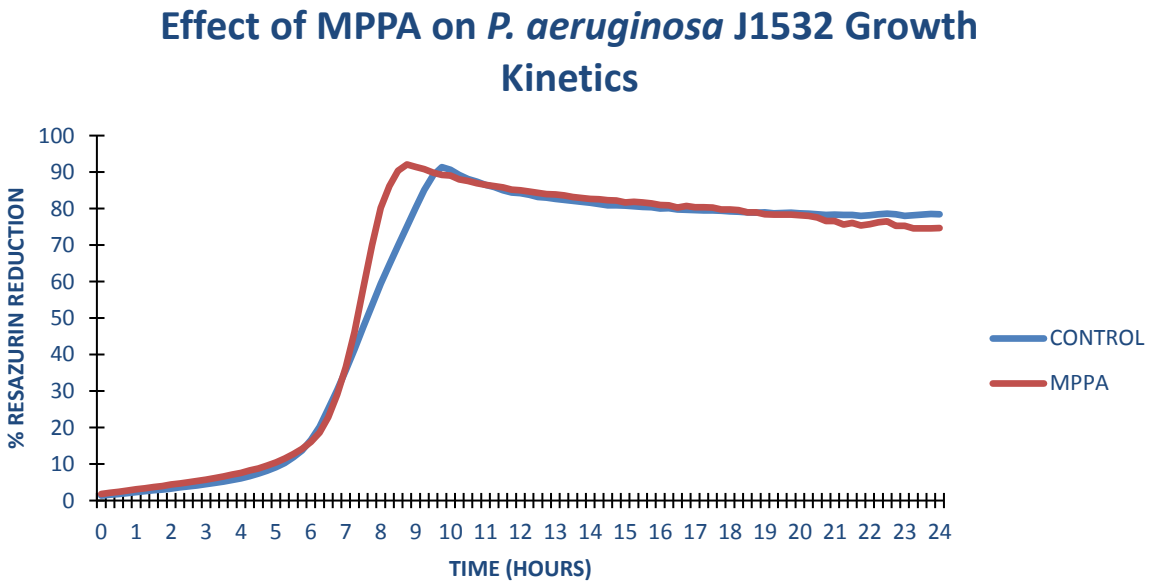


Figure 4.5

Growth curves of mucoid isolate J1532 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

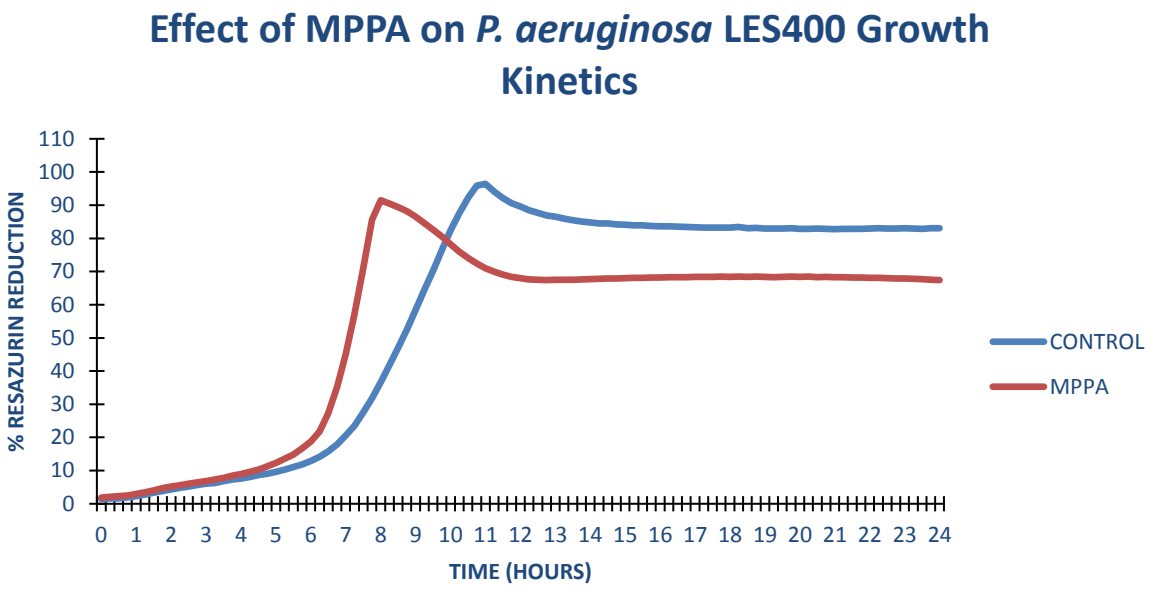


Figure 4.6

Growth curves of non-mucoid isolate LES400 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

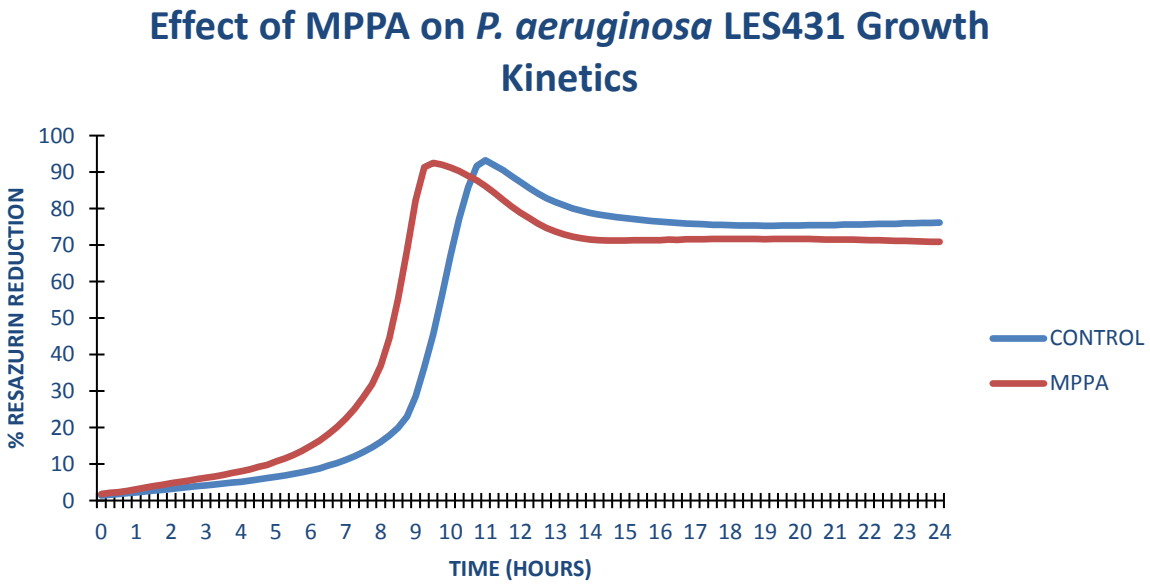


Figure 4.7

Growth curves of non-mucoid isolate LES431 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

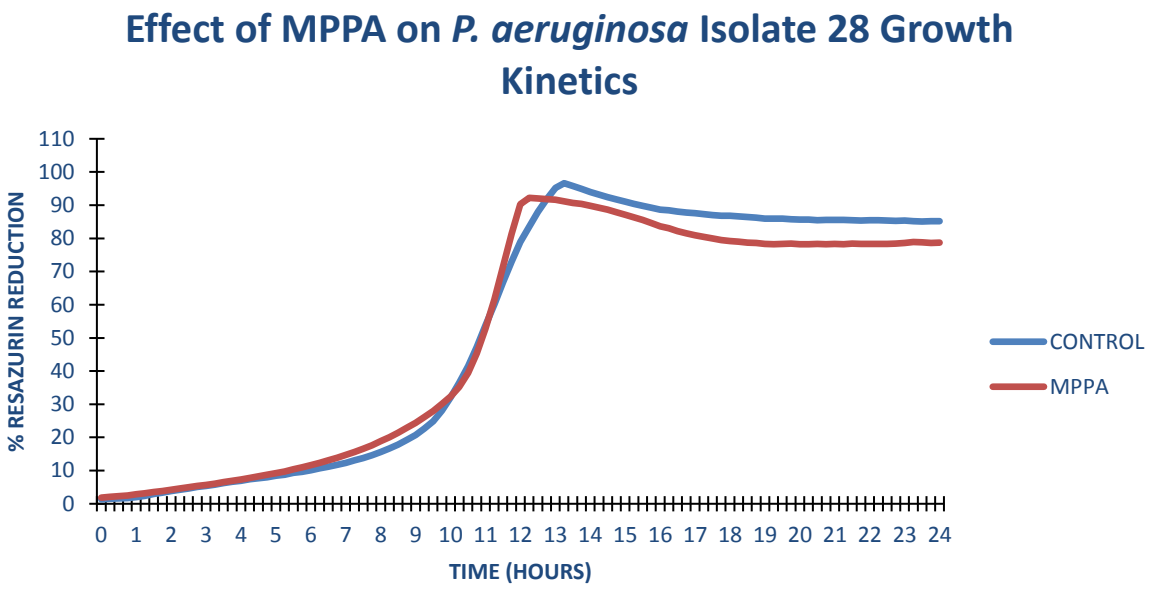


Figure 4.8

Growth curves of mucoid isolate 28 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

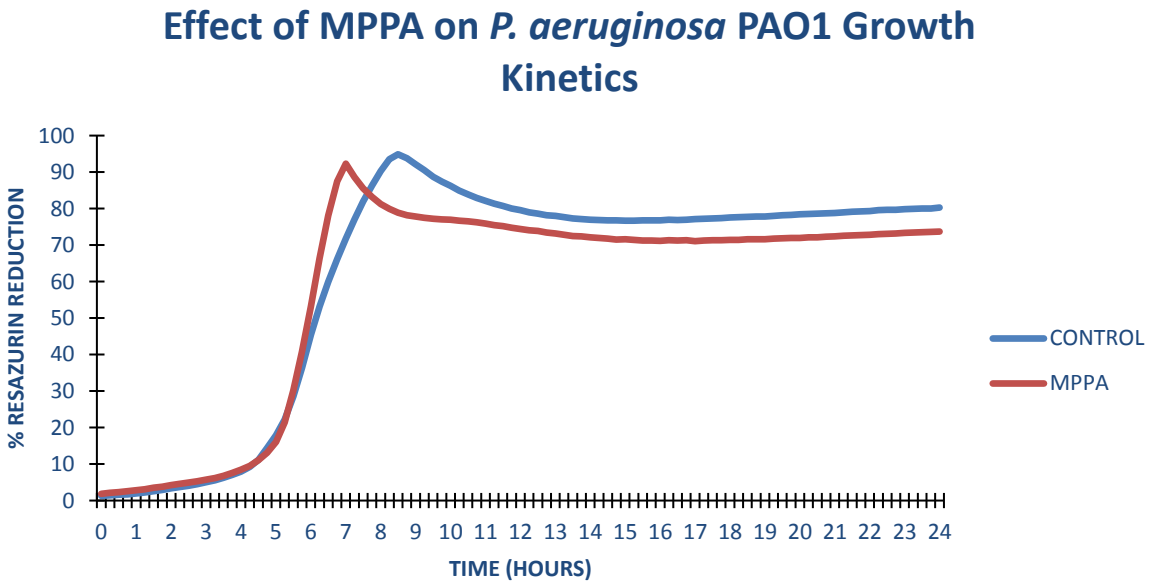


Figure 4.9

Growth curves of non-mucoid isolate PAO1 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

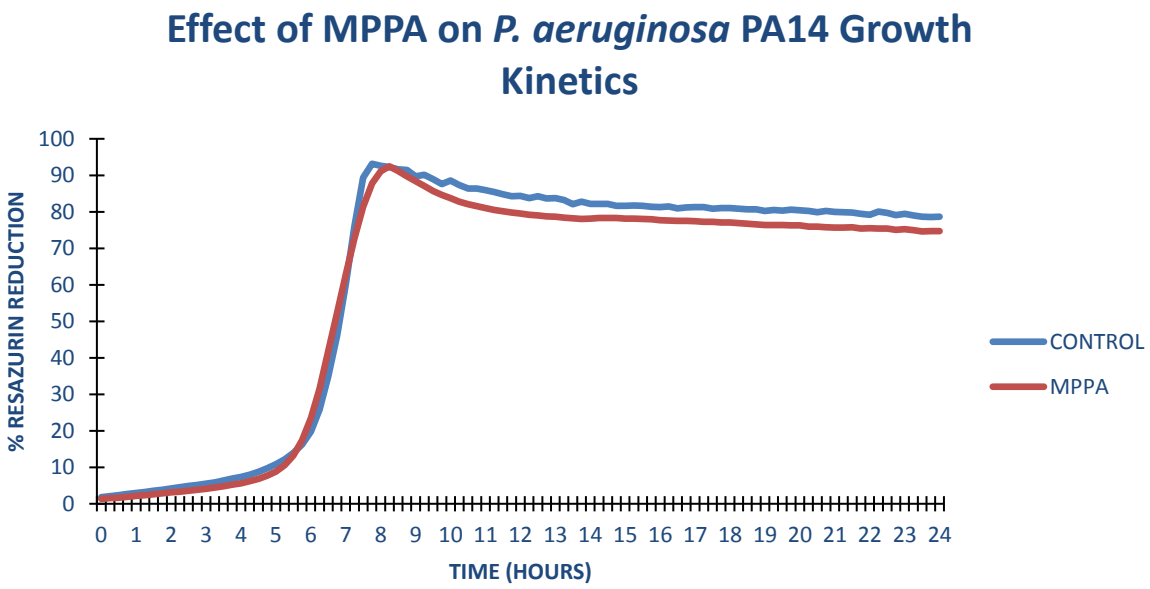


Figure 4.10

Growth curves of non-mucoid isolate PA14 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

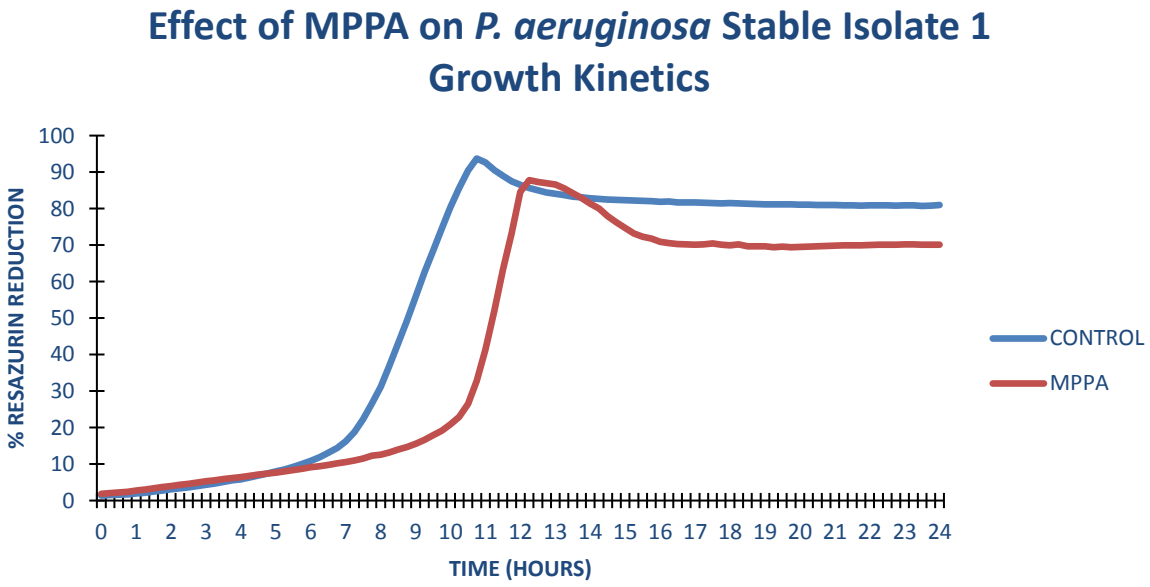


Figure 4.11

Growth curves of non-mucoid stable isolate 1 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

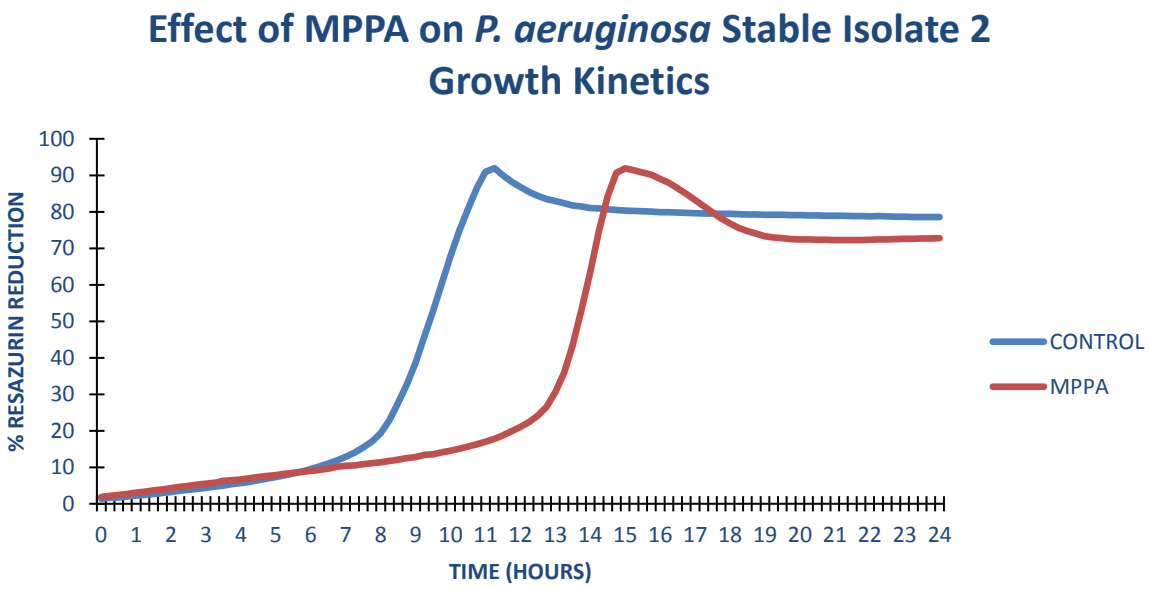


Figure 4.12

Growth curves of non-mucoid stable isolate 2 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

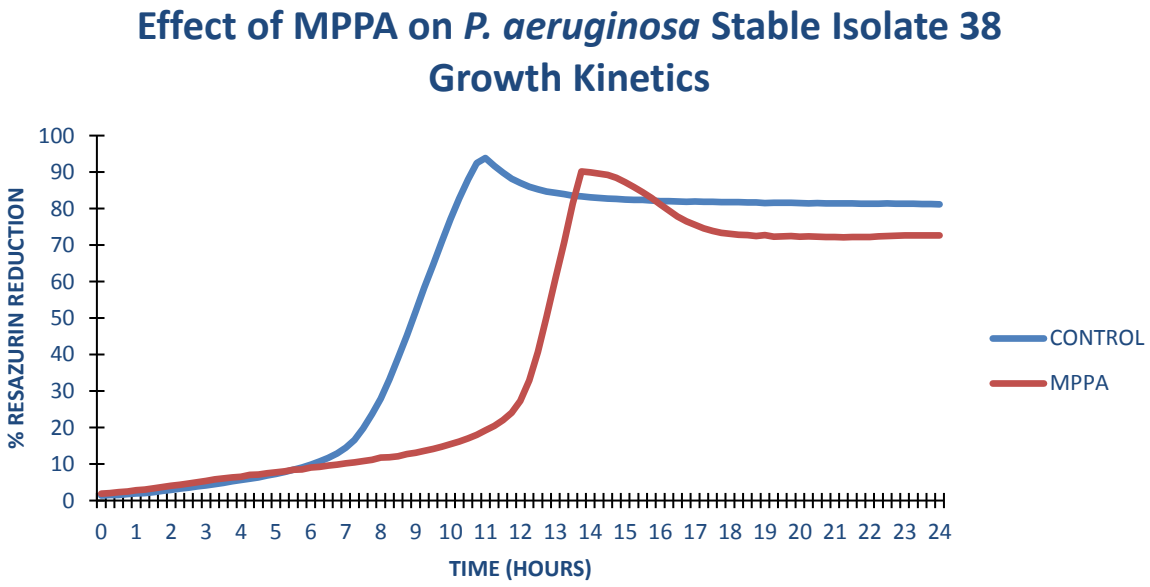


Figure 4.13 ;

Growth curves of non-mucoid stable isolate 38 cultured +/- 250 µg/ml MPPA as measured via % reduction in resazurin. Measurements presented are the mean of 3 biological replicates. Error bars omitted for clarity.

SECTION 4.3.2

As MPPA had previously been demonstrated to greatly enhance β-lactam activity against *P. aeruginosa* with suggested therapeutic potential (Krogfelt *et al*, 2000), it was of interest to test its ability to enhance the effect of not only β-lactams but also other chemically distinct classes of antibiotic with differing mechanisms of action and cell entry in *P. aeruginosa*. This was achieved using a simple resazurin aided MIC microplate assay. The results of this assay were intriguing in that they could suggest a broader therapeutic potential of MPPA as a synergist for many different antibiotics and also give clues as to the possible mechanisms of action exhibited by MPPA. These assays revealed that MPPA often attained additive or synergistic interactions with anionic or neutrally charged molecules which enter cells via porins but was profoundly antagonistic at higher concentrations with cationic agents with self promoted uptake mechanisms (polymyxins and aminoglycosides). This may indicate that MPPA could have a membrane disrupting effect, potentially with binding sites and reactive stress responses overlapping with those of other membrane disruptors.

EFFECTS OF MPPA UPON THE *IN VITRO* SUSCEPTIBILITIES OF *P. AERUGINOSA* ISOLATES TO VARIOUS ANTIBIOTIC CLASSES

Sensitivity of 14 *P. aeruginosa* isolates to a range of different antibiotics from distinct chemically and functionally distinct classes was assayed with and without 250 μg/ml MPPA in MHB via a modified broth microdilution method exploiting resazurin as a metabolic incubator, this was deemed necessary due to the background turbidity generated by MPPA precipitates. The MIC was defined as the lowest antibiotic concentration at which the resazurin indicator did not exhibit a blue to pink colour change and was reduced by less than 50 % relative to matched uninoculated control wells with and without 250 μg/ml MPPA. This had previously been found to correspond well with MICs obtained in MPPA free wells by visual inspection of turbidity after an 18 hour incubation. Representative antibiotic compounds included a β-lactam (ceftazidime), an aminoglycoside (tobramycin), a polymyxin (colistin), a fluoroquinolone (ciprofloxacin), a non-fluorinated quinolone (nalidixic acid), a

tetracycline (tetracycline), a phenylpropanoid (chloramphenicol), a macrolide (erythromycin) and a diaminopyrimidine (trimethoprim).

In the case of ceftazidime, synergy with MPPA was evident in all tested isolates as a 2 to 32 fold reduction in MIC depending on the specific isolate (Table 4.1). For tobramycin, antagonism with MPPA was evident in all isolates as a 4 to 32 fold rise in MIC depending on the specific isolate (Table 4.1). MPPA also antagonised colistin activity against all isolates. In order to further probe the nature of this antagonism and determine if it was due to a heritable change in the organisms, strains PAO1 and PA14 were preincubated with 250 μg/ml MPPA and allowed to grow to stationary phase overnight, cells were then pelleted and resuspended in PBS thrice to remove residual MPPA and then finally OD adjusted and used as starter inocula for MIC testing with tobramycin and colistin as before ; no difference in MIC was evident. MPPA was also tested at a much lower concentration reflecting the maximum concentration likely to occur naturally *in vivo* (~ 8.7 μg/ml or 20 μM) with both tobramycin and colistin and at this MPPA concentration, the MIC for both drugs was reduced 2 fold. Interactions of MPPA with ciprofloxacin varied qualitatively between isolates ; synergy, evident as a 2 to 8 fold reduction in MIC was observed for 9 of 14 isolates, antagonism, evident as a 2 fold rise in MIC was observed for 1 of 14 isolates and no change in MIC could be discerned for 3 of 14 isolates . Interactions between MPPA and nalidixic acid also varied qualitatively by isolate ; synergy, evident as a 2 to 4 fold reduction in MIC was evident with 7 of 14 isolates and no change in MIC was evident for 6 of 14 isolates, no instances of antagonism were observed with this combination. The combination of MPPA and tetracycline interacted synergistically against 11 of 14 isolates as evidenced by a 4 to 16 fold reduction in MIC depending on the specific isolate, no difference in MIC could be discerned for 2 of 14 isolates tested with this combination, no instance of antagonism was observed. MPPA interacted synergistically with chloramphenicol against 12 of 14 isolates as evidenced by a 2 to 8 fold reduction in MIC depending on the specific isolate, no difference in MIC could be discerned for 1 of 14 isolates and no instance of antagonism was noted. In the case of erythromycin, synergy with MPPA was occurred for 7 of 14 isolates as evidenced by a 2 to 8 fold decrease in MIC depending on the individual isolate, interactions were indifferent for 5 of 14

isolates with no discernible change in MIC whilst antagonism was observed for 1 of 14 isolates as a 2 fold increase in MIC. The combination of MPPA and trimethoprim interacted synergistically against 11 of 14 isolates as evidenced by a 2 to 8 fold reduction in MIC depending on the specific isolate, no difference in MIC could be found for 2 of 14 isolates and no instance of antagonism was observed. Effects of MPPA on the MIC of test drugs could not be assessed for 1 of 14 isolates (A38) as MPPA alone completely inhibited growth of this isolate, viable plate counts obtained in triplicate from these wells in fact revealed that MPPA was not merely inhibitory but profoundly bactericidal resulting in ~ 4 log reduction in viable organisms relative to the initial inoculum of ~ 5 x 10^5 cfu/ml.

DRUG	CAZ		CHL		CIP		COL		ERY		NAL		TET		TOB		TMP	
MPPA [250 µg/ml]	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
A36	2	0.5	16	4	0.06	0.03	0.5	32	128	64	64	32	4	0.25	0.06	2	64	16
C1426	2	0.5	32	8	0.06	0.03	0.5	32	128	128	256	128	32	4	0.5	16	32	4
C1433	8	1	32	4	0.03	0.03	0.5	32	128	32	64	32	32	8	0.25	8	32	16
J1385	2	0.5	16	8	0.06	0.03	0.5	32	64	64	64	64	16	4	0.5	8	32	16
J1532	2	1	8	4	0.06	0.125	0.5	32	16	16	128	128	8	8	0.5	8	16	16
LES400	64	8	4	0.5	0.125	0.125	0.5	32	32	64	128	128	2	0.5	4	32	64	16
LES431	256	64	1	0.25	1	0.25	0.5	32	128	32	1024	256	1	0.25	0.5	8	4	1
MUC28	32	1	0.5	0.5	0.5	0.125	0.25	32	16	16	256	128	0.5	0.5	0.25	8	64	64
PAO1	2	0.5	8	4	0.06	0.03	0.5	32	64	16	64	64	8	2	0.25	2	16	8
PA14	0.5	0.25	8	4	0.06	0.03	0.25	32	64	32	64	64	16	4	0.25	1	32	8
S1	128	16	8	2	4	0.5	0.5	32	1024	128	256	128	4	0.5	64	512	64	16
S2	128	16	8	2	2	2	0.5	32	1024	1024	1024	1024	16	4	64	512	32	8
S38	256	16	8	4	4	1	0.5	32	512	64	512	128	4	0.5	64	512	64	32
MPPA [8.65 µg/ml]	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
PA14	NT	NT	NT	NT	NT	NT	0.5	0.25	NT	NT	NT	NT	NT	NT	0.25	0.125	NT	NT

Table 4.1

Antibiotic MICs of *P. aeruginosa* isolates grown planktonically in MHB +/- MPPA at a concentration of 250 µg/ml. For some drugs, strain PA14 was also tested at a lower MPPA concentration of 8.65 µg/ml (20 µM).

SECTION 4.3.3

It was deemed to be of interest to ascertain whether MPPA could affect the ability of *P. aeruginosa* strains growing planktonically to secrete various virulence associated exoproducts of relevance to the pathology of infection as this could potentially signify therapeutic potential. Pyocyanin, pyoverdine and pyochelin were each assayed. Pyocyanin was first assayed by turbidity corrected absorbance whilst the fluorescent siderophores pyoverdine and pyochelin were assayed in the same samples by measuring fluorescent emmissions at their respective λ max values . The results of these assays were of further intrigue as transcriptomic analyses, presented in chapter 5, have indicated a downregulation of many genes involved in iron uptake and in the uptake of aromatic amino-acid precursors of phenazine biosynthesis pathways. Results of all 3 assays varied widely between strains, with MPPA appearing to be either increase or decrease accumulation of the same exoproduct, depending on the planktonically grown test strain. It was therefore impossible to derive any broad conclusions from these data.

EFFECTS OF MPPA ON PRODUCTION OF VIRULENCE ASSOCIATED EXOPRODUCTS BY *P. AERUGINOSA* ISOLATES IN AGITATED MHB CULTURES OVER 24 HOURS

Shaken MHB cultures of 13 *P. aeruginosa* strains in 96 well format were assayed in triplicate for accumulation of the redox active exotoxin pyocyanin as well as the fluorescent siderophores pyochelin and pyoverdine in the absence and presence of 250 μg/ml MPPA. Pyocyanin was measured as a function of absorbance values recorded at 695 nm immediately after incubation whilst the same plates were assayed for pyochelin by measuring fluorescent emissions at 430 nm after excitation at a wavelength of 350 nm and pyoverdine by measuring fluorescent emissions at 460 nm after excitation at a wavelength of 400 nm. The resultant values were then normalised for growth differences as assessed by a resazurin reduction endpoint test performed immediately afterwards on the same plates.

SECTION 4.3.3.1

PYOCYANIN ACCUMULATION IN AGITATED CULTURES

MPPA reduced pyocyanin accumulation by 9 of 13 test isolates (Figure 4.14). Pyocyanin accumulation was reduced by 19 % in MPPA treated C1426 cultures although this difference was not significant ($p > 0.05$). In J1385 cultures, MPPA significantly reduced pyocyanin accumulation by 11.95 % ($p < 0.05$). MPPA reduced pyocyanin accumulation by 0.4 % in J1532 cultures, a difference that was not significant ($p > 0.05$). In the cases of LES400, LES431 and MUC28, MPPA significantly reduced pyocyanin accumulation by 62 % , 43 % and 53 % , respectively ($p < 0.05$). MPPA also reduced pyocyanin accumulation in PAO1 cultures by 4 % although this decrease was not significant ($p > 0.05$). In PA14 cultures MPPA significantly reduced pyocyanin accumulation by 16 % ($p < 0.05$). MPPA reduced pyocyanin accumulation by 7 % in S2 cultures, this decrease was not significant ($p > 0.05$). Conversely, MPPA increased pyocyanin accumulation for 4 of 13 test isolates. Pyocyanin accumulation was significantly increased by 29 % in MPPA treated A36 cultures ($p < 0.05$). Pyocyanin production by C1433 appeared to increase by 3 % in response to MPPA although this increase did not reach significance ($p > 0.05$). Pyocyanin accumulation by S1 increased by 58.31 % in the presence of MPPA, this increase was significant ($p < 0.05$). Finally, pyocyanin production in S38 cultures was significantly increased in the presence of MPPA ($p < 0.05$).

SECTION 4.3.3.2

PYOCHELIN ACCUMULATION IN AGITATED CULTURES

MPPA reduced pyochelin production by 7 of 13 test isolates (Figure 4.15). Pyochelin accumulation was reduced by 3 % in MPPA treated C1433 cultures although this difference was not significant ($p = 0.594$). In LES400 and LES431 cultures, MPPA significantly reduced pyochelin accumulation by 74 % ($p < 0.05$) and 78 % ($p <$

0.05), respectively. MPPA significantly reduced pyochelin accumulation by 53 % in cultures of MUC28 ($p < 0.05$). In PAO1 cultures, the level of pyochelin accumulation was significantly reduced by 25 % ($p < 0.05$) as a result of MPPA activity. Pyochelin accumulation in cultures of S2 and S38 was also significantly reduced by 46 % ($p < 0.05$) and 24 % ($p < 0.05$), respectively. Conversely, MPPA increased pyochelin production by 6 of 13 isolates. In the case of A36, MPPA was found to increase pyochelin production significantly by 18 % ($p < 0.05$). C1426 also increased pyochelin production significantly by 63 % in the presence of MPPA ($p < 0.05$). Pyochelin production by J1385 was increased by 4 % in the presence of MPPA though this was not significant ($p > 0.05$). J1532 also increased pyochelin production significantly by 19 % in response to MPPA ($p < 0.05$). Pyochelin production by PA14 underwent a significant increase of 35 % in the MPPA treated cultures ($p < 0.05$). Finally, MPPA increased pyochelin production by 3 % in S1 cultures although this did not reach significance ($p > 0.05$).

SECTION 4.3.3.3

PYOVERDINE ACCUMULATION IN AGITATED CULTURES

MPPA decreased pyoverdine production by 5 of 13 test isolates (Figure 4.16). Pyoverdine production by LES400 decreased significantly by 77 % in response to MPPA ($p < 0.05$). Pyoverdine accumulation by MUC28 also decreased significantly by 57 % in the presence of MPPA ($p < 0.05$). PAO1 also significantly reduced pyoverdine production by 43 % in response to MPPA ($p < 0.05$). Likewise, S2 and S38 significantly decreased pyoverdine production by 26 % ($p < 0.05$) and 18 % respectively ($p < 0.05$), respectively. The remaining 8 of 13 isolates all increased pyoverdine production in response to MPPA. A36 increased pyoverdine production by 11 % in response to MPPA although this difference was not found to be significant ($p > 0.05$). C1426 and C1433 both significantly increased pyoverdine production in MPPA treated cultures by 66 % ($p < 0.05$) and 19 % ($p < 0.05$), respectively. J1385 increased pyoverdine production by 5 % in response to MPPA although this difference was not significant ($p > 0.05$). J1532 increased pyoverdine

production by 54 % in MPPA treated cultures and this was significant ($p < 0.05$). LES431 and PA14 both significantly increased pyoverdine production by 591 % ($p < 0.05$) and 3039 % ($p < 0.05$), respectively, in the presence of MPPA. Finally, S1 increased pyoverdine accumulation by 5 % in response to MPPA, although this did not reach significance ($p > 0.05$).

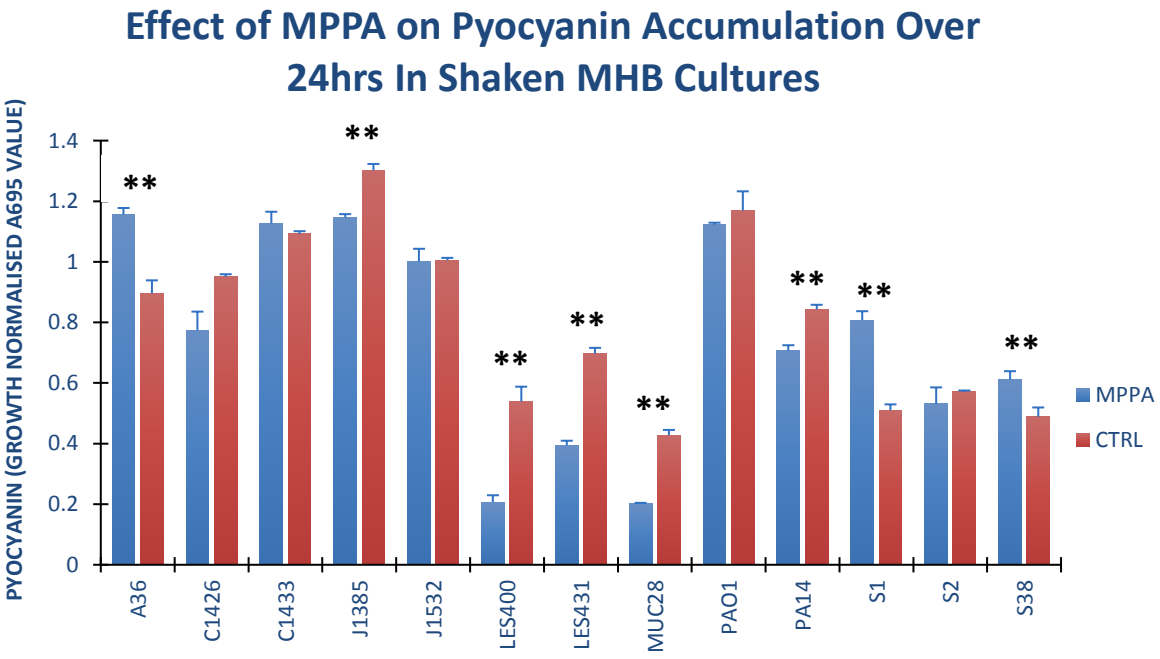


Figure 4.14

Mean absorbances of pyocyanin at 695 nm + S.E.M, normalised for growth differences in MPPA treated vs. control cultures ($n = 9$). Statistically significant results denoted ** ($\alpha = 0.05$).

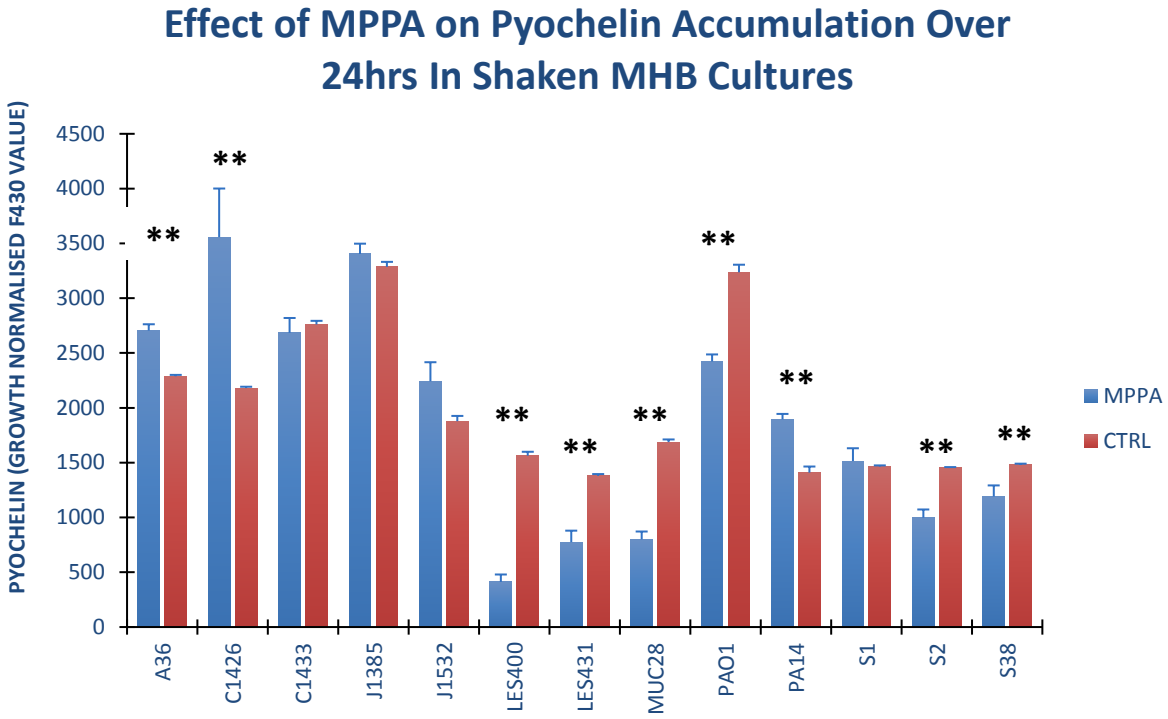
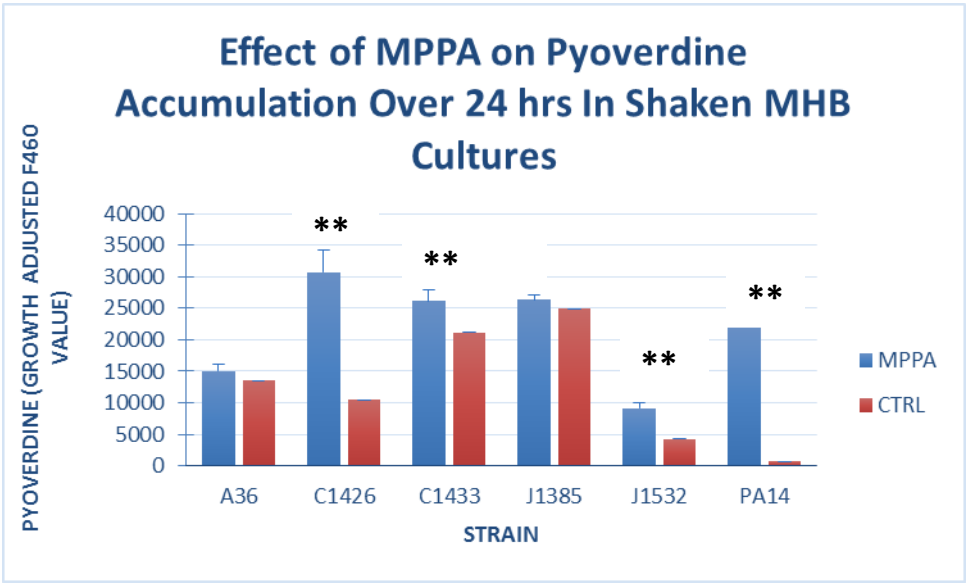


Figure 4.15

Mean relative fluorescence units emitted by pyochelin at 430 nm + S.E.M, normalised for growth differences in MPPA treated vs. control cultures (n = 9). Statistically significant results denoted ** (α = 0.05).



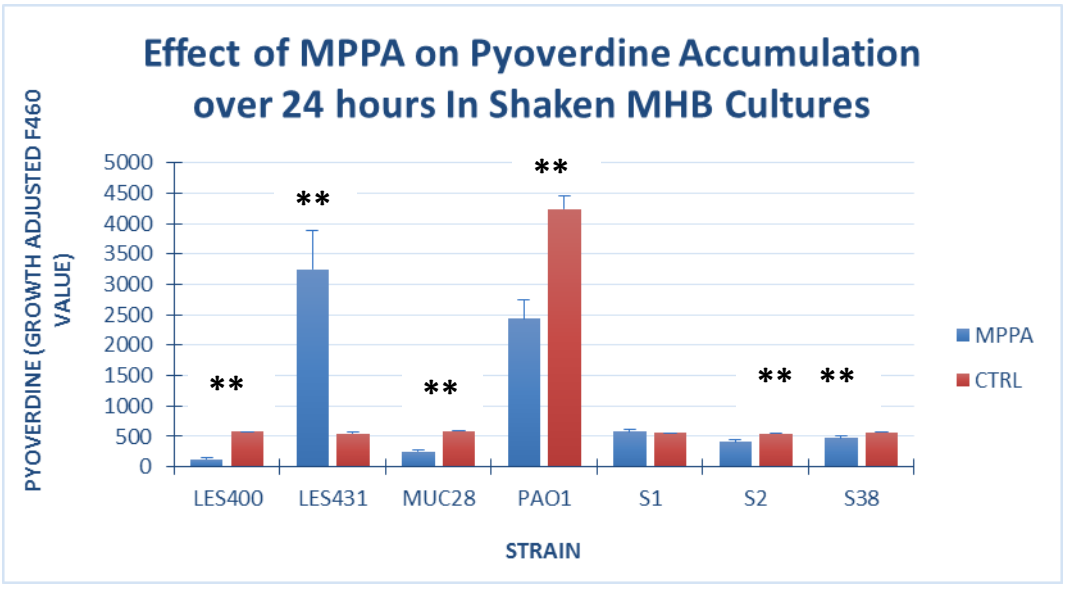


Figure 4.16
Mean relative fluorescence units emitted by pyoverdine at 460 nm + S.E.M, normalised for growth differences in MPPA treated vs. control cultures (n = 9). Statistically significant results denoted **(α = 0.05).

SECTION 4.4

It was deemed to be of interest to ascertain whether MPPA could affect the ability of *P. aeruginosa* strains growing as biofilms to secrete various virulence associated

exoproducts of relevance to the pathology of biofilm mediated infection as this could potentially signify therapeutic potential. A selection of biochemical assays were used to test this. Pyocyanin was assayed via solvent extraction and colorimetric analysis, whilst elastase and total exoprotease were each assayed enzymatically with the use of dye conjugated substrates. These assays revealed that MPPA has the potential to reduce production of these virulence factors by biofilm cells.

EFFECTS OF MPPA ON PRODUCTION OF VIRULENCE ASSOCIATED EXOPRODUCTS BY *P. AERUGINOSA* ISOLATES IN BIOFILM MHBII CULTURES OVER 48 HOURS

Biofilm MHB II cultures of 6 *P. aeruginosa* strains in 24 well format were assayed in triplicate for accumulation of the redox active exotoxin pyocyanin, elastase and total protease in both the absence and presence of 250 μg/ml MPPA. Purified pyocyanin was sequentially extracted from centrifuged culture supernatants using chloroform and hydrochloric acid before being quantified in μg/ml via reading absorbance at 520 nm in acidic solution and multiplying the resulting value by the extinction coefficient, correcting for the sampled volume. Elastase activity was measured by assaying at 495 nm the absorbance of congo red dye liberated by an elastin conjugate upon degradation by enzyme in supernatants from MPPA treated and control cultures in buffer over a 4 hour period, absorbance corresponding positively with elastase activity of the sample. Total protease activity was measured by assaying at 440 nm the absorbance of azo dye liberated by a casein conjugate upon degradation by enzymes in supernatants from MPPA treated and control cultures in buffer over a period of 45 minutes. Results were analysed for significance at the $\alpha = 0.05$ level via one way ANOVA.

SECTION 4.4.1

PYOCYANIN PRODUCTION IN STATIC BIOFILM CULTURES

MPPA decreased pyocyanin production by 5 of 6 isolates in static biofilm culture but did not have any effect on pyocyanin production by the other remaining isolate (Figure 4.17). MPPA significantly reduced ($p < 0.05$) production of pyocyanin by 22 % , 39 % , 28 % , 32 % and 36 % for isolates C1426, C1433, J1532, PAO1 and PA14, respectively. Pyocyanin production by J1385 remained completely unchanged relative to the MPPA free control under these conditions ($p > 0.05$).

SECTION 4.4.2

TOTAL EXOPROTEASE ACTIVITY IN STATIC BIOFILM CULTURE

MPPA significantly decreased ($p < 0.05$) total exoprotease activity of all 6 isolates tested in static biofilm culture (Figure 4.18). Detectable protease activity in MPPA treated cultures was reduced by 38 %, 41 % , 33 % , 58 % , 47 % and 32 %, respectively, for isolates C1426, C1433, J1385, J1532, PAO1 and PA14.

SECTION 4.4.3

ELASTASE ACTIVITY IN STATIC BIOFILM CULTURES

MPPA significantly decreased ($p < 0.05$) accumulation of active elastase by all 6 isolates tested in static biofilm culture (Figure 4.19). Detectable elastase activity in MPPA treated cultures was reduced by 52 % , 52 % , 50 % , 52 % , 50 % and 51 % for isolates C1426, C1433, J1385, J1532, PAO1 and PA14, respectively.

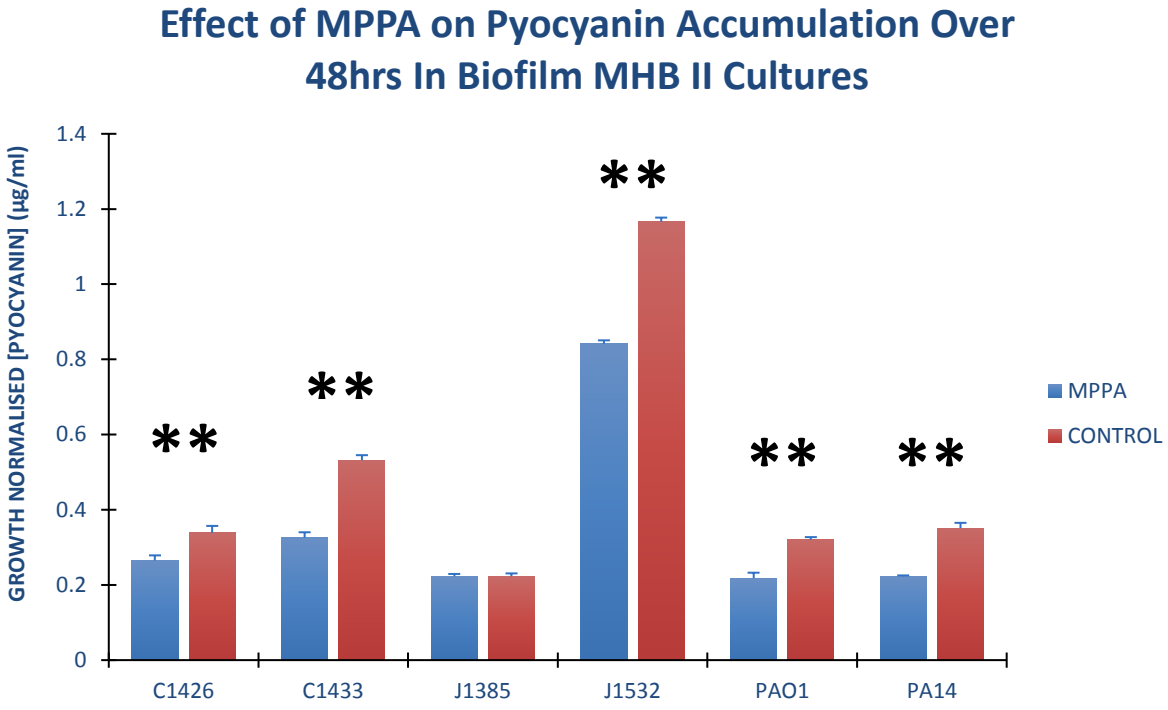


Figure 4.17

Mean pyocyanin concentrations accumulated over 48 hrs in static biofilm cultures, normalised for growth differences in MPPA treated vs. control cultures (n = 4). Statistically significant results denoted ** (α = 0.05).

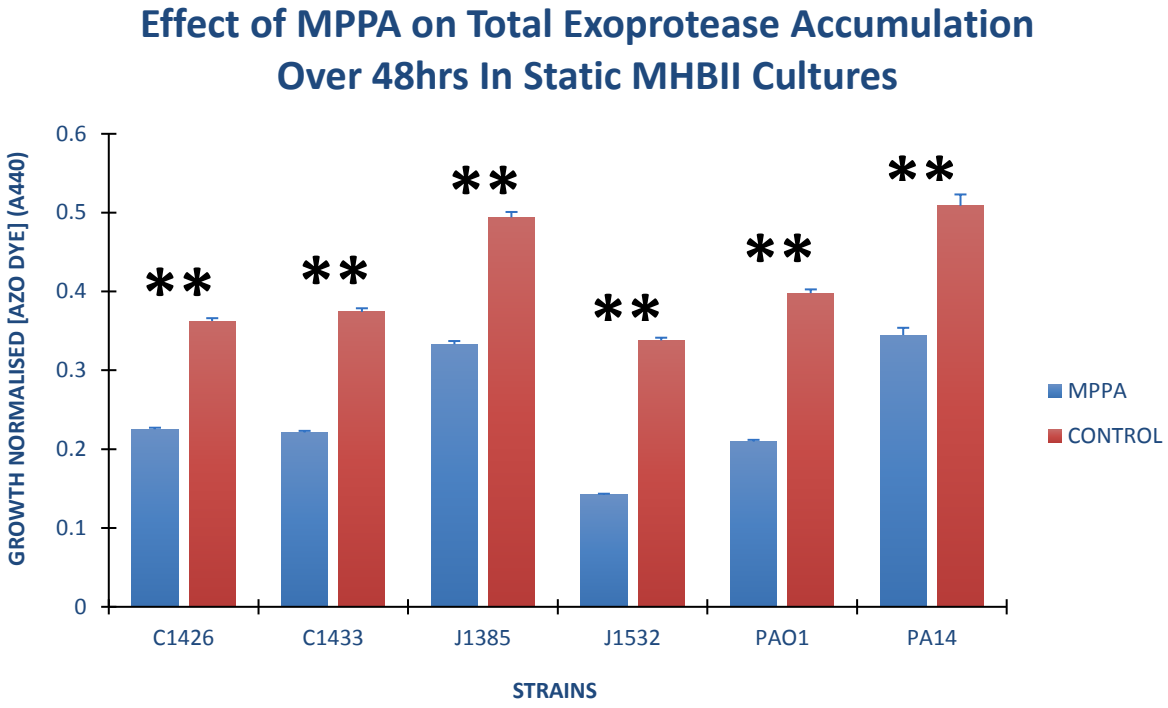


Figure 4.18

Mean total exoprotease activity detected after 48 hrs in static biofilm cultures, normalised for growth differences in MPPA treated vs. control cultures (n = 4). Statistically significant results denoted ** (α = 0.05).

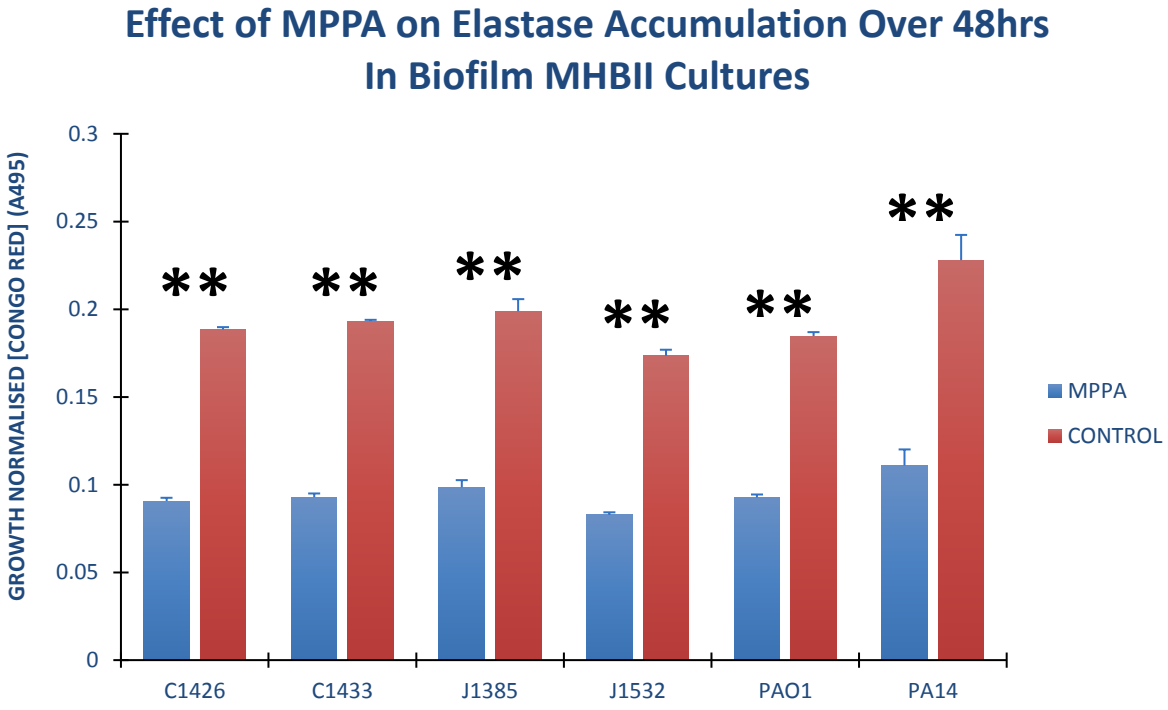


Figure 4.19
Mean elastase activity detected after 48 hrs in static biofilm cultures, normalised for growth differences in MPPA treated vs. control cultures (n = 4). Statistically significant results denoted ** (α = 0.05).

SECTION 4.5

In order to establish whether or not MPPA influences motility in strain PA14, agar stab inoculation assays for twitching (1.0 % agar) and swimming motility (0.5 % agar) were employed. This was deemed to be of interest as the transcriptomic data presented in chapter 5 had revealed substantially enriched expression of many genes governing motility and chemotaxis. These phenotyping assays confirm that MPPA enhances swimming but not twitching motility, significantly, in PA14.

EFFECTS OF MPPA ON *P. AERUGINOSA* PA14 MOTILITY

Assays of swimming and twitching motility were carried out in triplicate. PA14 was selected as a model strain in these experiments given that RNA sequencing was also carried out using this strain.

SECTION 4.5.1

SWIMMING MOTILITY

Swimming motility was quantified by measuring the diameter through which visible growth had swam from a central inoculation site in semi solid MHA (0.5 % agar) during a 20 hour incubation with and without the addition of 250 μg/ml MPPA (Figures 4.20.1 – 4.20.2). Assays were carried out using 3 separate overnight cultures to provide triplicate biological replicates and mean measurements were then recorded. In the presence of MPPA, the mean distance over which organisms had visibly swam from the inoculation site was reduced by 66 % relative to controls ($p < 0.05$).

SECTION 4.5.2

TWITCHING MOTILITY

Twitching motility was quantified by measuring the diameter over which organisms had spread from a central inoculation site over the base of a polystyrene Petri dish containing MHA (1.0 % agar) during a 20 hour incubation with and without the addition of 150 μg/ml MPPA. Assays were carried out using 3 separate isolated colonies from streak plates to provide triplicate biological replicates and the mean measurements were then recorded (Figure 4.20.1). In the presence of MPPA, the mean twitching distance was increased by 10 % relative to controls, an insignificant difference ($p > 0.05$).

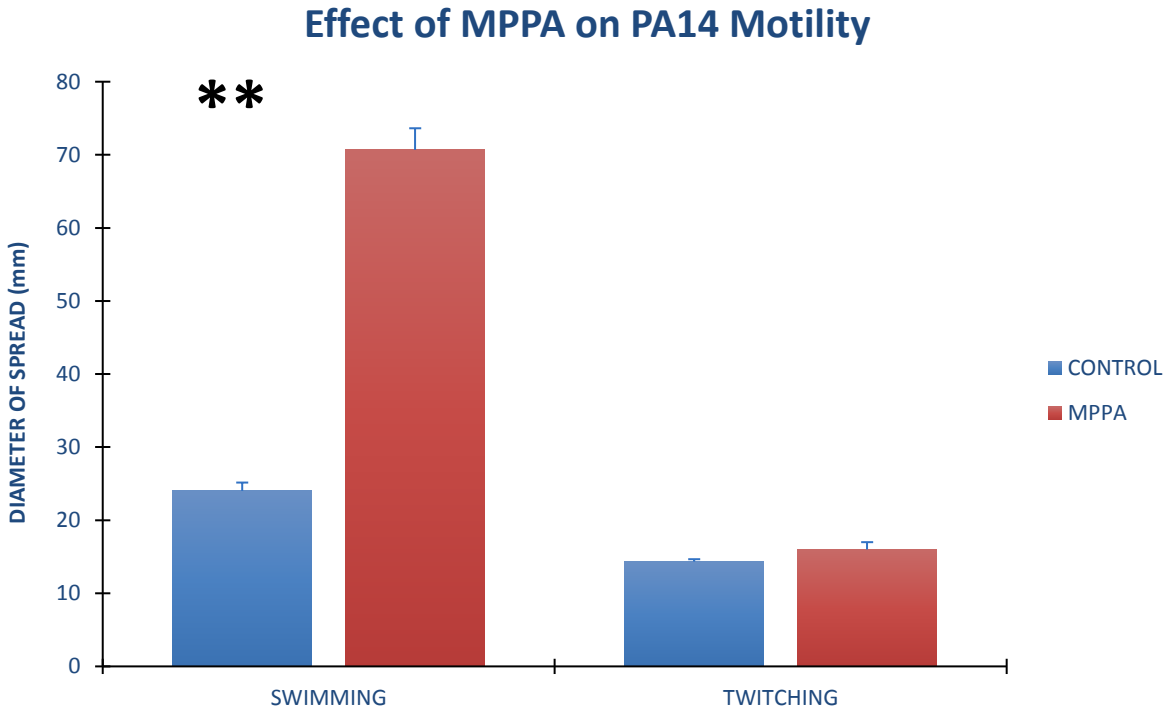


Figure 4.20.1

Mean distance travelled after 20 hours in MPPA treated vs. control cultures (n = 3). Statistically significant results denoted ** (α = 0.05).

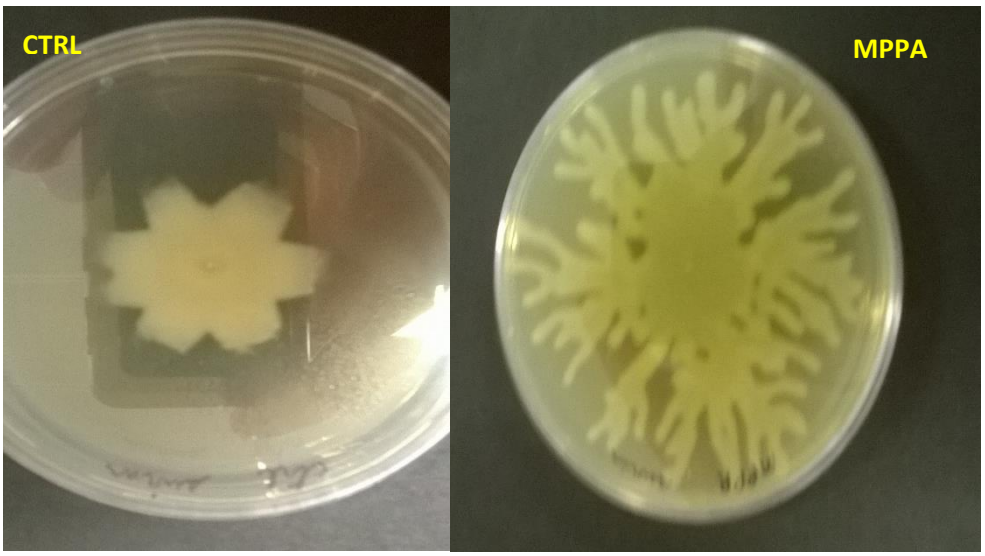


Figure 4.20.2
Swimming motility after 20 hours in MPPA treated vs. control cultures.

SECTION 4.6

It was of interest to note whether MPPA affected the extent and architectural features of biofilm formation by a test panel of *P. aeruginosa* isolates. This was tested by 2 complementary methods. A crystal violet binding assay was employed to quantitate overall levels of biofilm formation in the presence and absence of MPPA whilst scanning electron microscopy was used to visualise biofilm structure in finer detail. This revealed that MPPA increased biofilm formation by 5 of 6 test isolates whilst reducing biofilm formation by a single strain, PA14. Moreover, SEM showed that MPPA appeared to induce the formation of appendages in biofilms of a single strain, J1532.

EFFECTS OF MPPA ON *P. AERUGINOSA* BIOFILM FORMATION OVER 24 HOURS

MPPA increased biofilm formation on the wells of a 96 well plate by 6 of 7 assayed isolates (Figure 4.21) : A36, C1426, C1433, J1385, J1532 and PAO1 and reduced

biofilm formation by a single isolate. In the case of isolate A36, MPPA increased biofilm formation by 9.71 % although this increase was not significant ($p > 0.05$). For isolates C1426, C1433, J1385, J1532 and PAO1, MPPA significantly increased the observed levels of biofilm formation by 28 % , 27 % , 18 % , 23 % and 23 % , respectively ($p < 0.05$). In the case of PA14, biofilm formation was reduced by 7 % in the presence of MPPA, although this was not significant ($p > 0.05$). Scanning electron micrographs of biofilms grown on glass coverslips in the presence of MPPA showed little discernible difference with matched negative controls with the exception that MPPA clearly increased matrix production in J1532 biofilms (Figures 4.22.1 – 4.25.2).

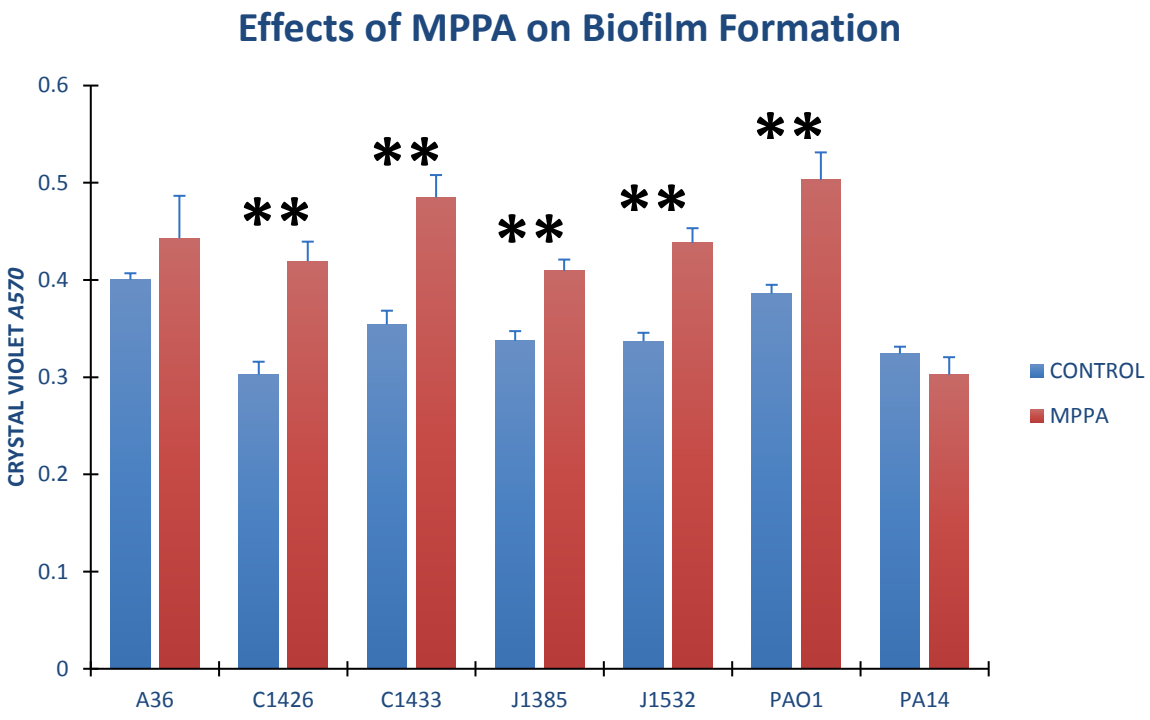


Figure 4.21
Mean biofilm formation quantified by crystal violet absorbance at a 570 nm wavelength ($n = 12$). Statistically significant results denoted ** ($\alpha = 0.05$).

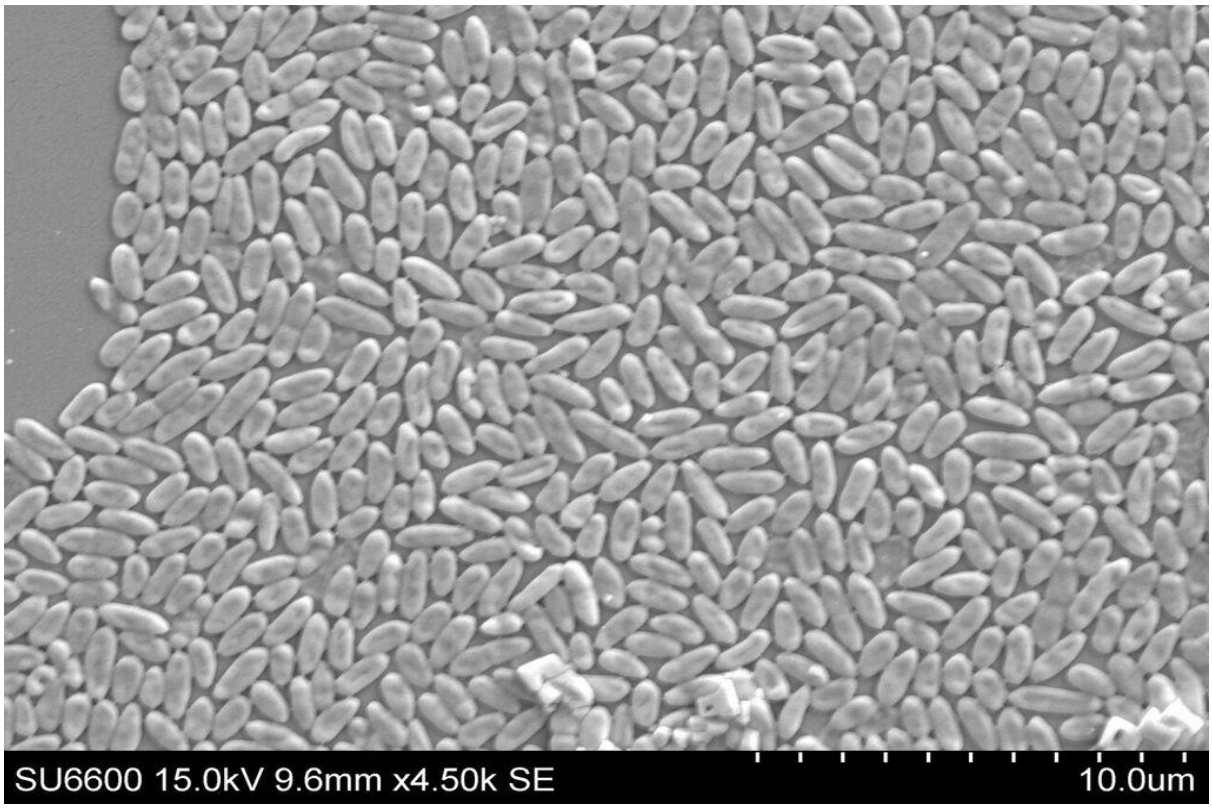


Figure 4.22.1
FE-SEM micrograph of J1385 control biofilm treated only with PBS.

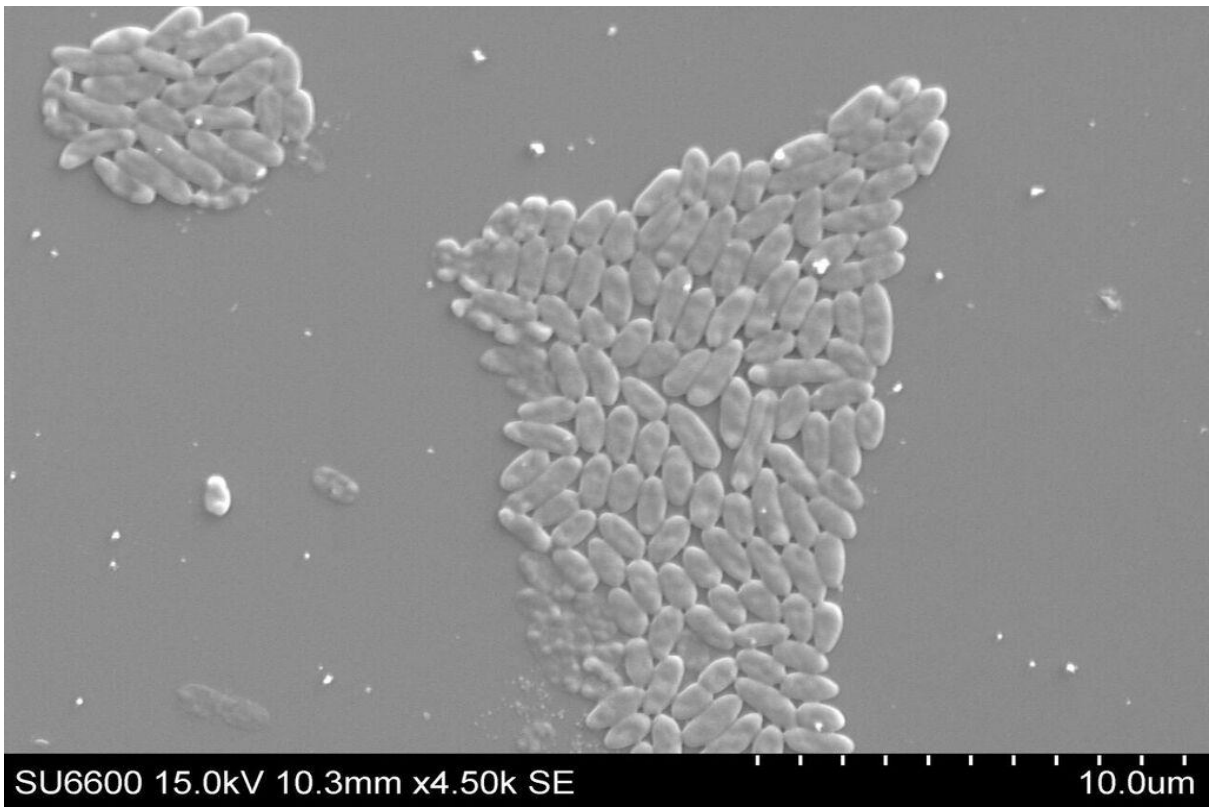


Figure 4.22.2
FE-SEM micrograph of J1385 biofilm treated with MPPA.

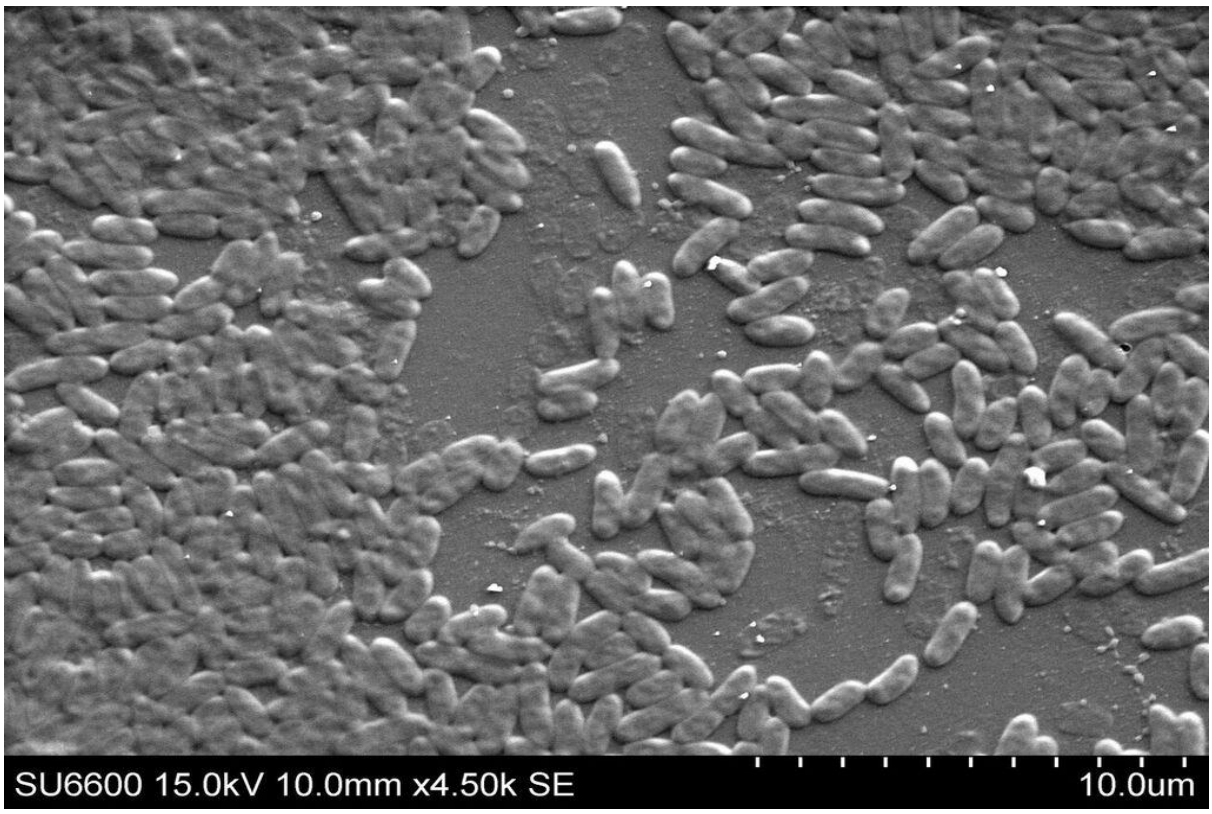


Figure 4.23.1
FE-SEM micrograph of J1532 control biofilm treated only with PBS.

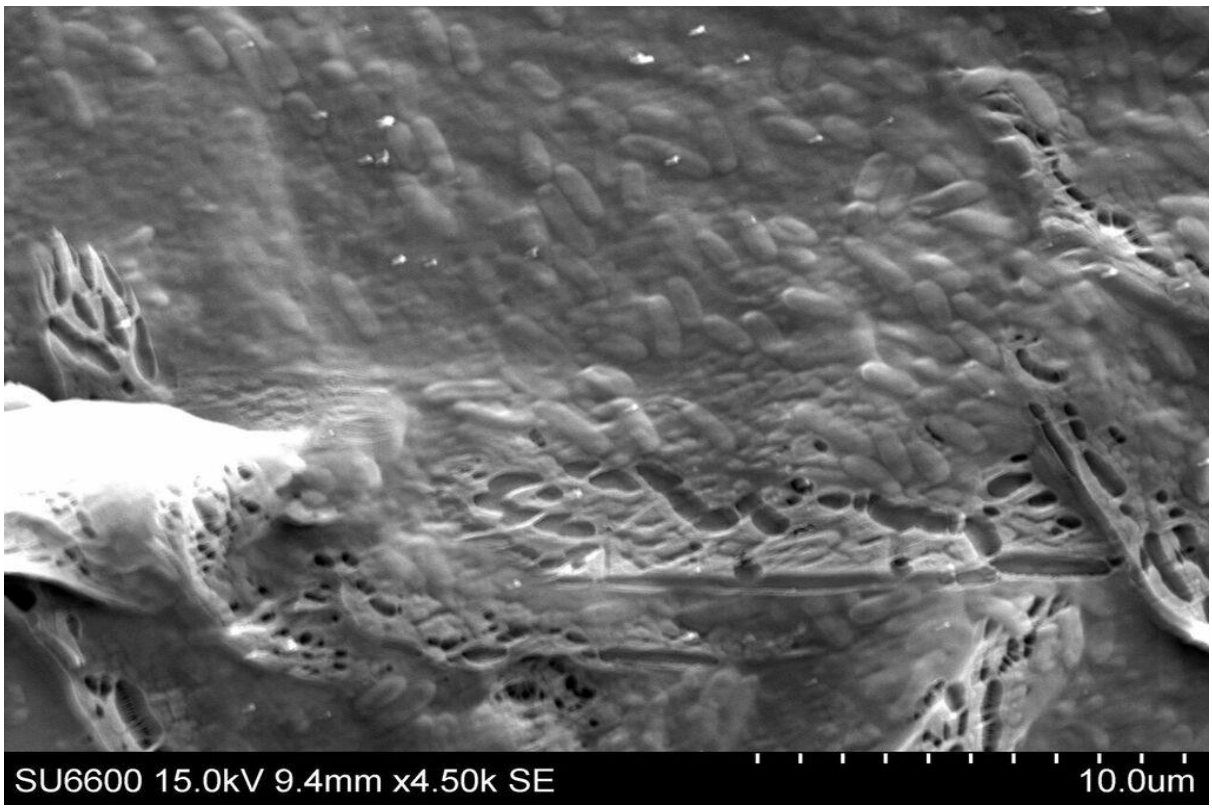


Figure 4.23.2
FE-SEM micrograph of J1532 biofilm treated with MPPA

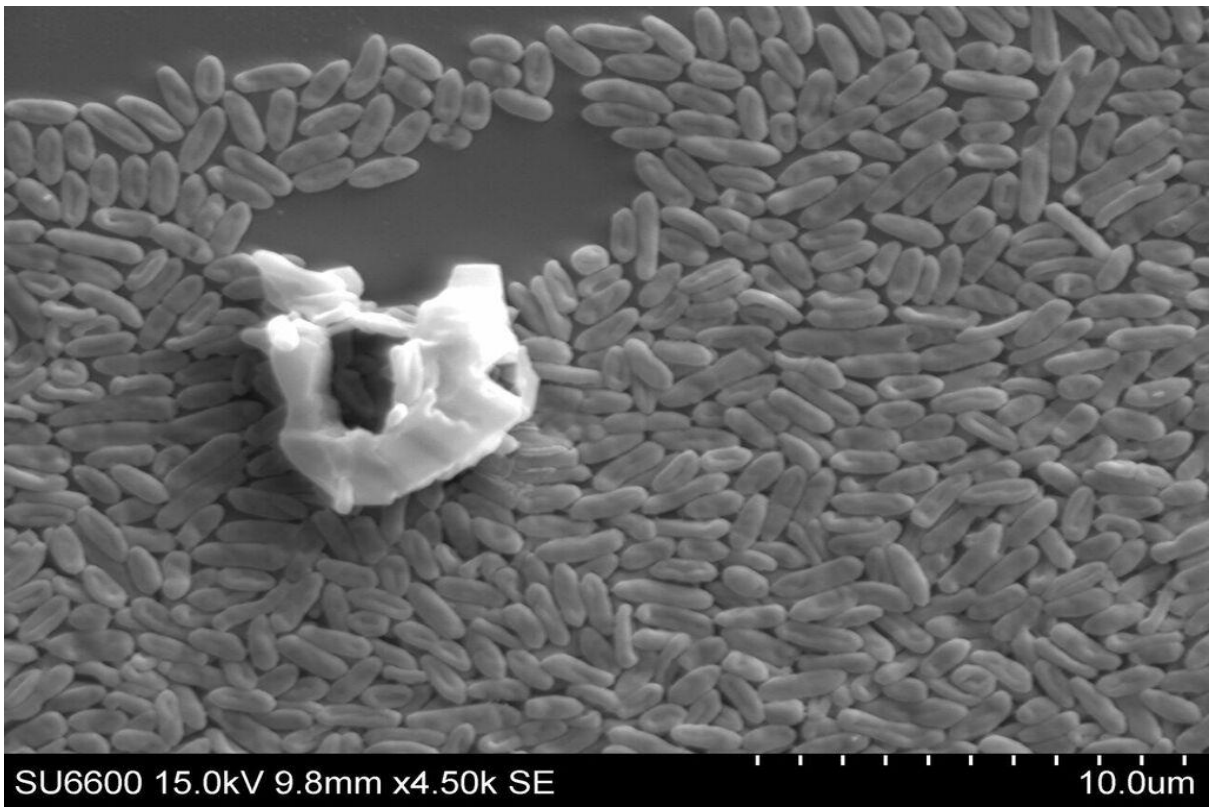


Figure 4.24.1
FE-SEM micrograph of PAO1 control biofilm treated only with PBS

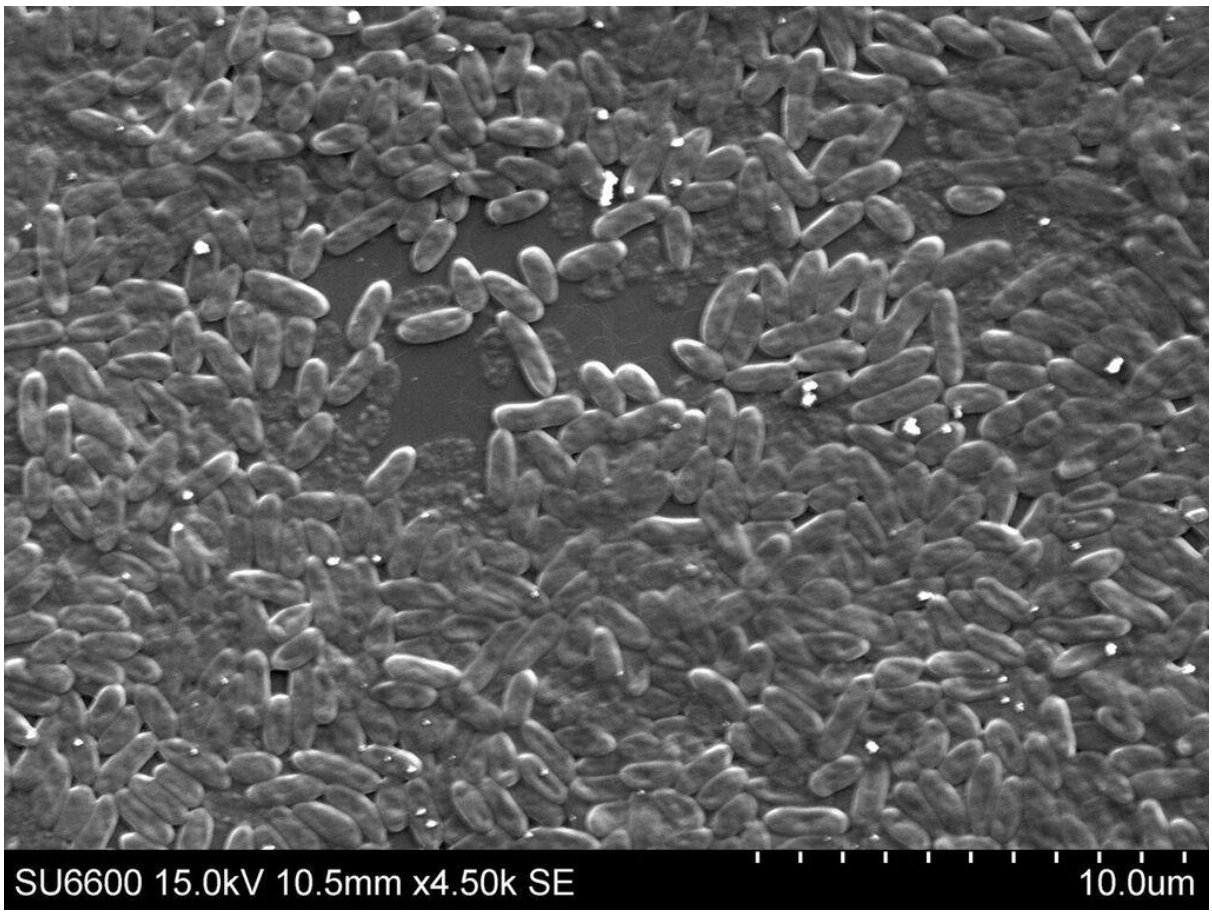


Figure 4.24.2
FE-SEM micrograph of PAO1 biofilm treated with MPPA.

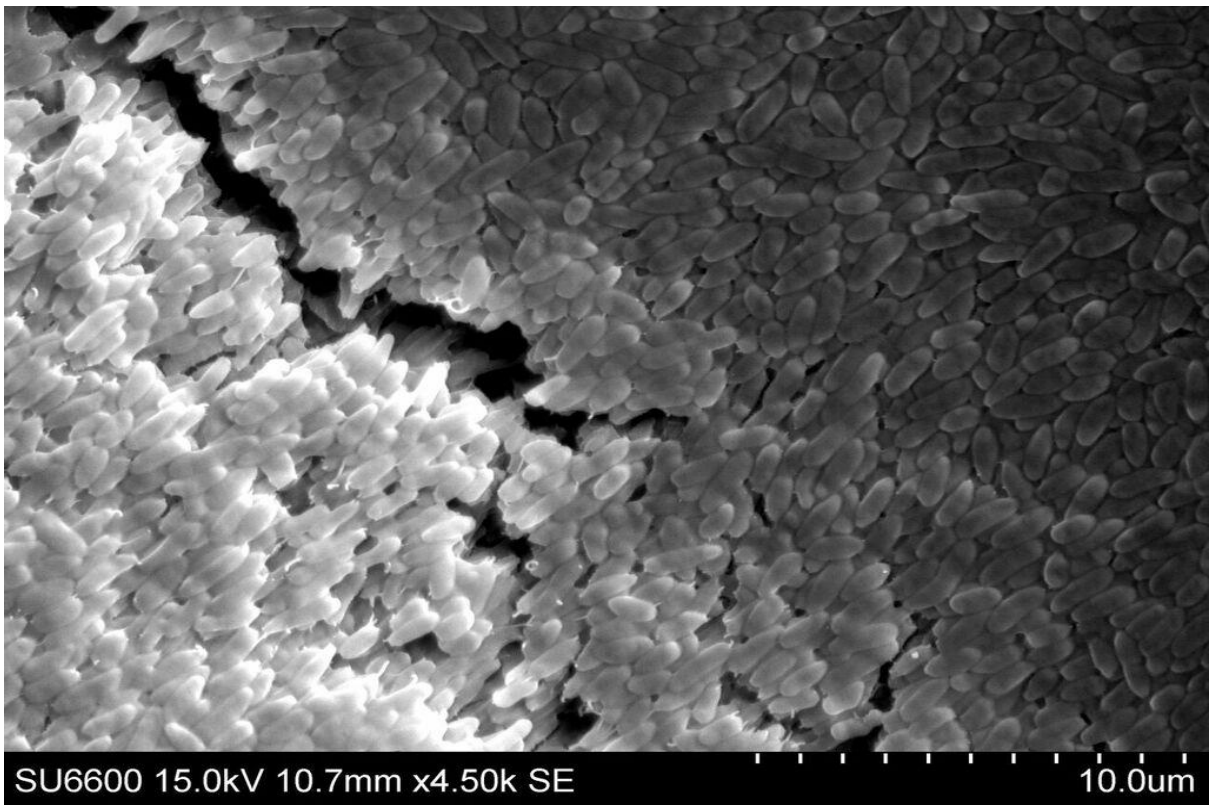


Figure 4.25.1
FE-SEM micrograph of PA14 control biofilm treated only with PBS.

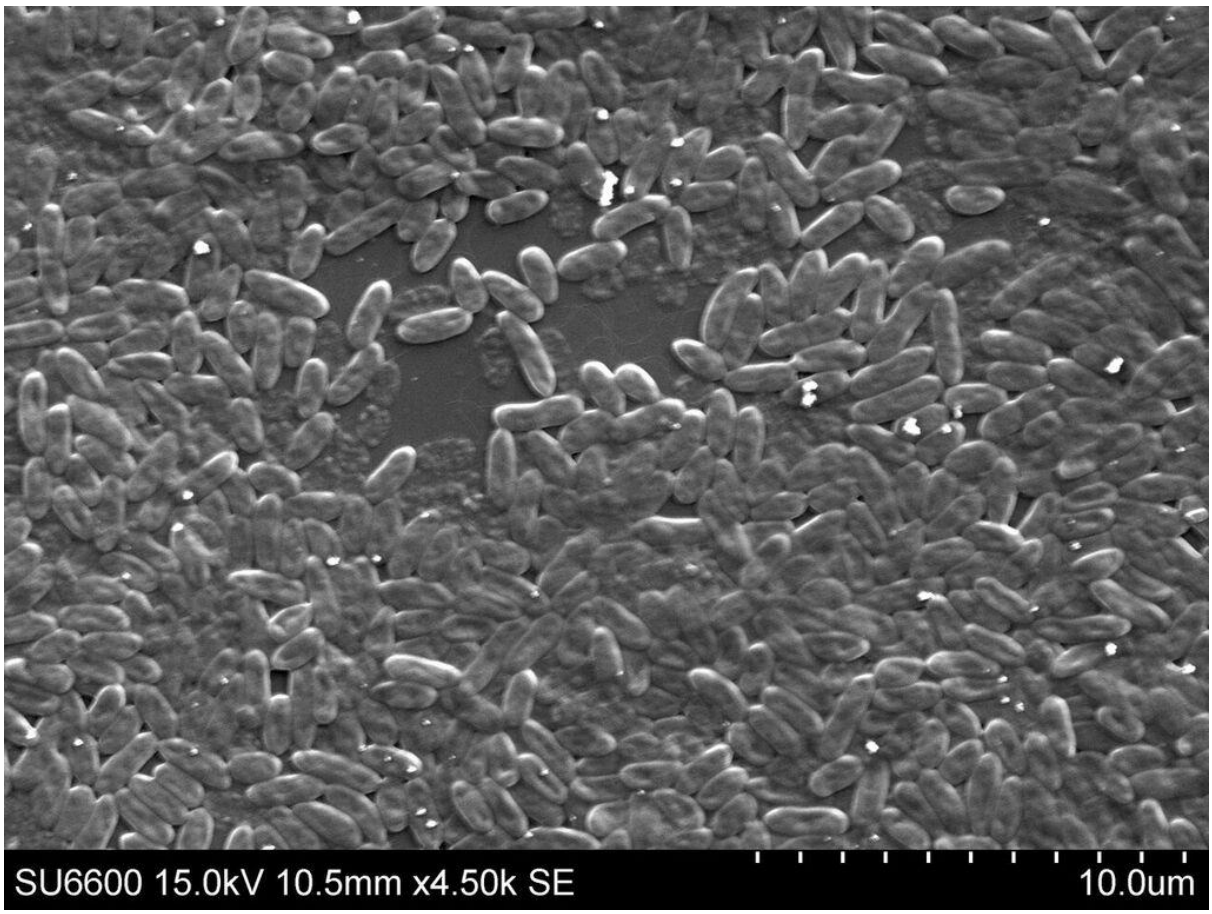


Figure 4.25.2
FE-SEM micrograph of PA14 biofilm treated with MPPA.

SECTION 4.7

BIOLOG PHENOTYPE MICROARRAYS

SECTION 4.7.1

It was of interest to determine whether *P. aeruginosa* strain PA14 could utilise MPPA as a sole or preferable carbon source. In order to answer this question, PA14 was grown in the wells of BioLog carbon utilisation phenotype microarrays with and without MPPA supplementation. These experiments revealed that PA14 could in fact use MPPA as a sole carbon source and lend credence to the hypothesis that the downregulation of sugar and amino-acid metabolism genes observed in chapter 5 may result from carbon catabolite repression induced by MPPA.

PM1-2 PLATES – CARBON SOURCE UTILISATION

Strain PA14 was cultured in triplicate in PM-1 and PM-2 BioLog plates which comprise 96 separate wells containing individual lone carbon sources, with and without the addition of 250 µg/ml MPPA. In negative control wells (A1) and wells containing certain carbon sources - as detailed by the plate maps in Figures 4.26 and 4.27, growth only occurred in the series in which MPPA had been added. In the case of certain other carbon sources, growth was evident in the MPPA free control series but was significantly greater in corresponding wells supplemented with 250 µg/ml MPPA. In no case did MPPA significantly reduce growth.

SECTION 4.7.2

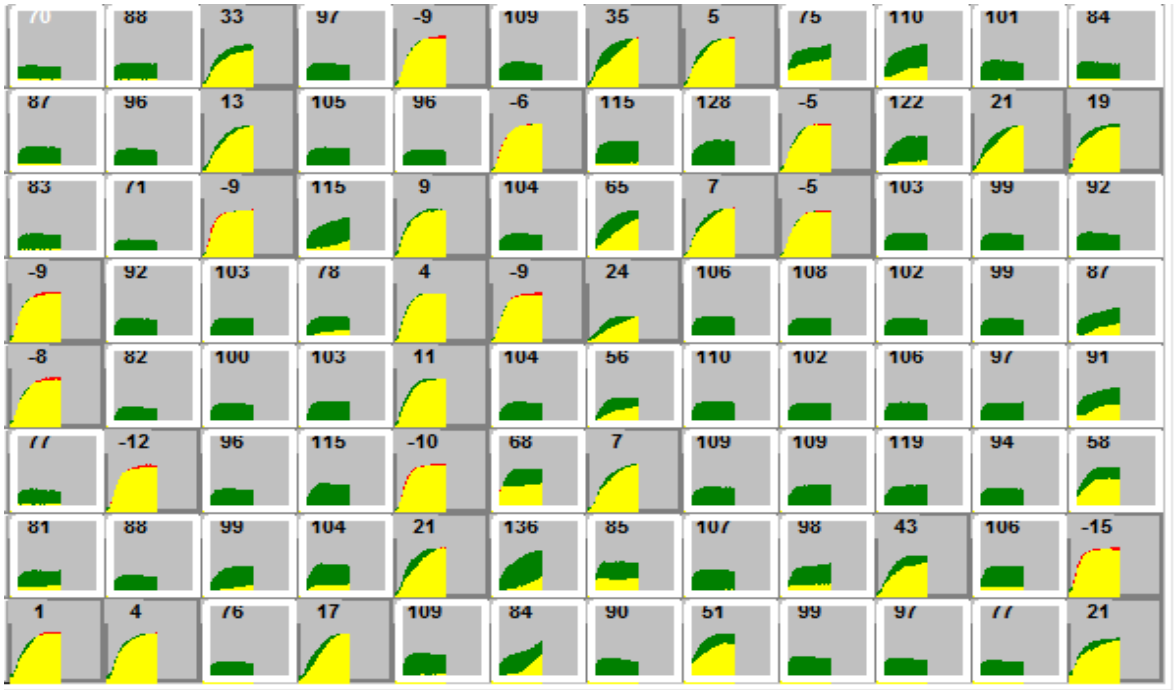
It was deemed to be of interest to elucidate whether or not MPPA could enhance or antagonise the inhibitory effects of a library of other compounds upon the growth of

P. aeruginosa strain PA14. The reasons for this were twofold - potential synergists of MPPA could firstly be of use therapeutically, moreover, it was thought that interactions of MPPA with other compounds with known mechanisms of action may have yielded clues as to the mode by which MPPA itself affects *P. aeruginosa.* Through the use of MPPA supplemented BioLog chemical sensitivity phenotype microarrays, it was found that MPPA interacted most significantly with many agents known to be membrane stressors, implying that MPPA itself might, as hypothesised previously, act as a membrane disrupting agent, which can variably compete or synergise with other agents having a similar mode of action.

PM15 and 17 PLATES – CHEMICAL SENSITIVITIES

Strain PA14 was cultured in triplicate in PM-15 and PM-17 BioLog plates which comprise 96 separate wells containing individual chemical challenge agents in series of quadruplicate doubling dilutions, with and without the addition of 250 µg/ml MPPA. In most cases MPPA had no significant effect on chemical sensitivities but in a minority of wells that contained chemicals that limited or inhibited PA14 growth at the concentration present - as detailed by the plate maps in Figures 4.28 and 4.29, growth was significantly increased in the presence of MPPA. In only a single instance did MPPA significantly potentiate the effects of a toxic agent against PA14 – in well A12 of the PM-17 plates.

(a)



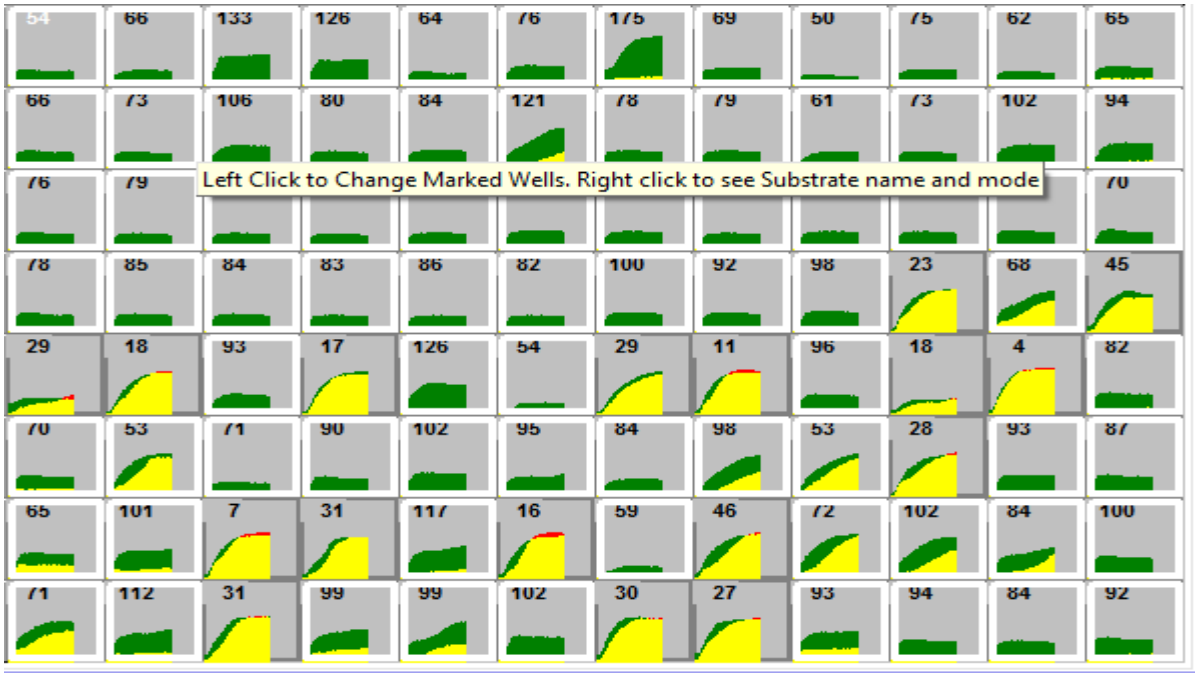
(b)

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

Figure 4.26

Biolog PM-1 plate – carbon source utilisation assay results for PA14 (a) Green and red areas on growth curves represent increases and reductions in growth, respectively, observed in MPPA treated wells relative to matched control wells, and indicated by numbers. Yellow areas represent growth overlap between MPPA containing wells and matched controls. Significant differences are highlighted with white squares. (b) Table indicating conditions under examination in each well of Biolog plate.

(a)



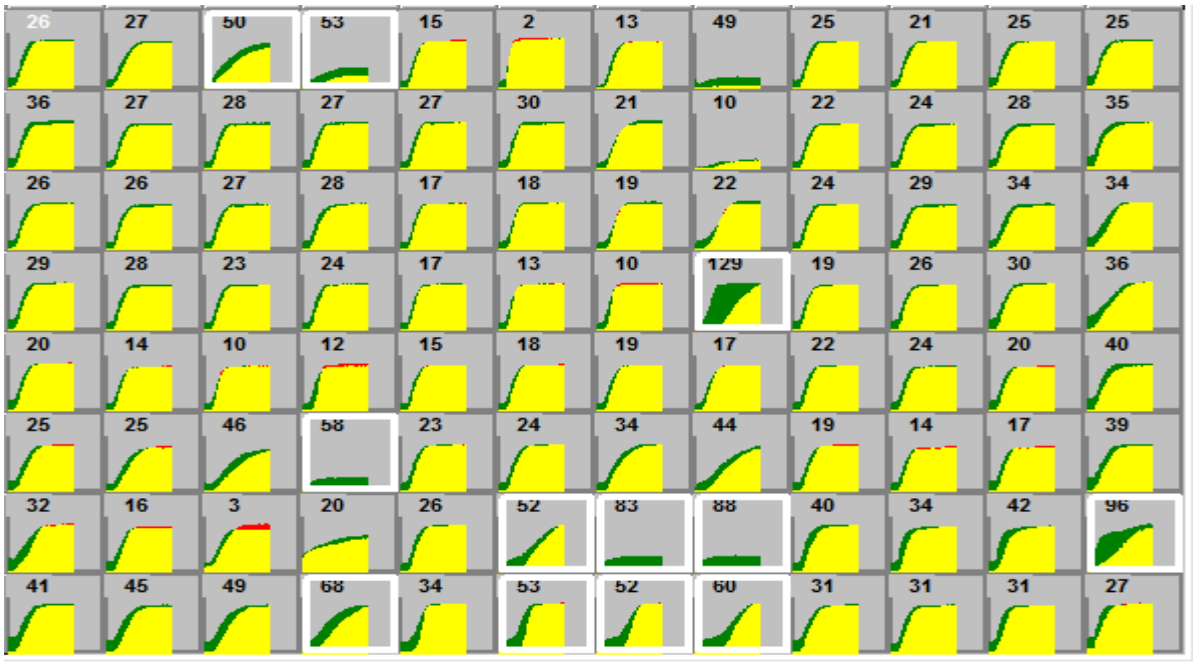
(b)

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 a-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 a-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

Figure 4.27

Biolog PM-2 plate – carbon source utilisation assay results for PA14 (a) Green and red areas on growth curves represent increases and reductions in growth, respectively, observed in MPPA treated wells relative to matched control wells, and indicated by numbers. Yellow areas represent growth overlap between MPPA containing wells and matched controls. Significant differences are highlighted with white squares.(b) Table indicating conditions under examination in each well of Biolog plate.

(a)



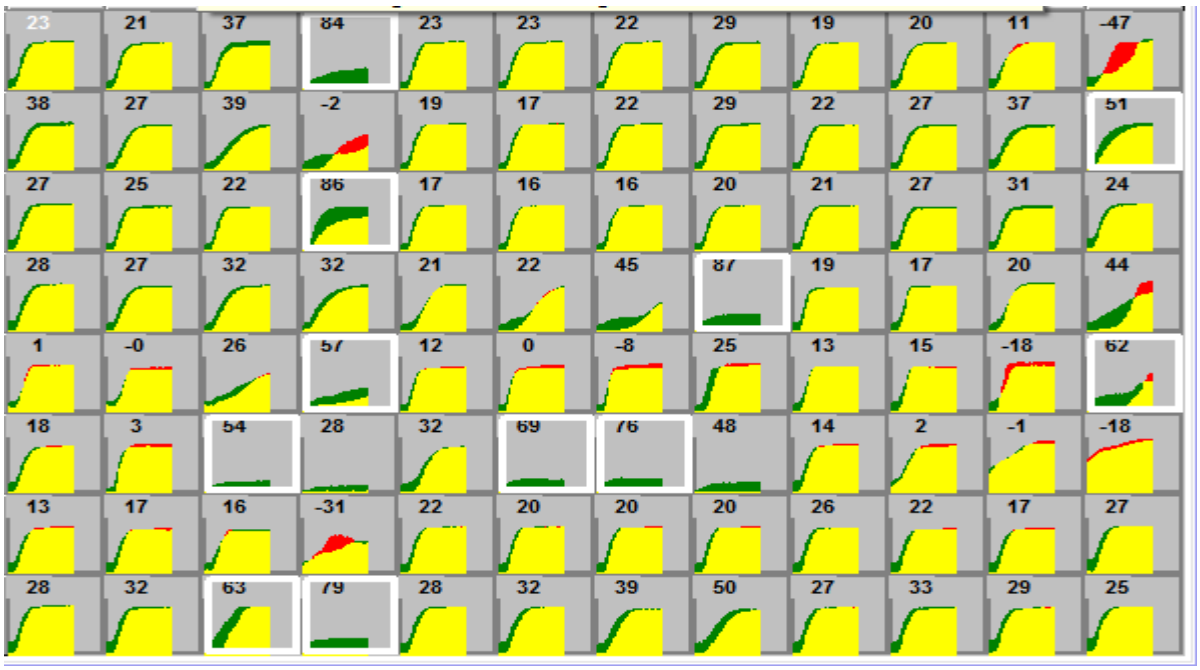
(b)

A1 Procaine 1	A2 Procaine 2	A3 Procaine 3	A4 Procaine 4	A5 Guanidine hydrochloride 1	A6 Guanidine hydrochloride 2	A7 Guanidine hydrochloride 3	A8 Guanidine hydrochloride 4	A9 Cefmetazole 1	A10 Cefmetazole 2	A11 Cefmetazole 3	A12 Cefmetazole 4
B1 D-Cycloserine 1	B2 D-Cycloserine 2	B3 D-Cycloserine 3	B4 D-Cycloserine 4	B5 EDTA 1	B6 EDTA 2	B7 EDTA 3	B8 EDTA 4	B9 5,7-Dichloro- 8-hydroxy-quinaldine 1	B10 5,7-Dichloro- 8-hydroxy-quinaldine 2	B11 5,7-Dichloro- 8-hydroxy-quinaldine 3	B12 5,7-Dichloro- 8-hydroxy-quinaldine 4
C1 5,7-Dichloro-8-hydroxyquinoline 1	C2 5,7-Dichloro-8-hydroxyquinoline 2	C3 5,7-Dichloro-8-hydroxyquinoline 3	C4 5,7-Dichloro-8-hydroxyquinoline 4	C5 Fusidic acid 1	C6 Fusidic acid 2	C7 Fusidic acid 3	C8 Fusidic acid 4	C9 1,10-Phenanthroline 1	C10 1,10-Phenanthroline 2	C11 1,10-Phenanthroline 3	C12 1,10-Phenanthroline 4
D1 Phleomycin 1	D2 Phleomycin 2	D3 Phleomycin 3	D4 Phleomycin 4	D5 Domiphen bromide 1	D6 Domiphen bromide 2	D7 Domiphen bromide 3	D8 Domiphen bromide 4	D9 Nordihydroguaia retic acid 1	D10 Nordihydroguaia retic acid 2	D11 Nordihydroguaia retic acid 3	D12 Nordihydroguaia retic acid 4
E1 Alexidine 1	E2 Alexidine 2	E3 Alexidine 3	E4 Alexidine 4	E5 5-Nitro-2-furaldehyde semicarbazone 1	E6 5-Nitro-2-furaldehyde semicarbazone 2	E7 5-Nitro-2-furaldehyde semicarbazone 3	E8 5-Nitro-2-furaldehyde semicarbazone 4	E9 Methyl viologen 1	E10 Methyl viologen 2	E11 Methyl viologen 3	E12 Methyl viologen 4
F1 3, 4-Dimethoxy-benzyl alcohol 1	F2 3, 4-Dimethoxy-benzyl alcohol 2	F3 3, 4-Dimethoxy-benzyl alcohol 3	F4 3, 4-Dimethoxy-benzyl alcohol 4	F5 Oleandomycin 1	F6 Oleandomycin 2	F7 Oleandomycin 3	F8 Oleandomycin 4	F9 Puromycin 1	F10 Puromycin 2	F11 Puromycin 3	F12 Puromycin 4
G1 CCCP 1	G2 CCCP 2	G3 CCCP 3	G4 CCCP 4	G5 Sodium azide 1	G6 Sodium azide 2	G7 Sodium azide 3	G8 Sodium azide 4	G9 Menadione 1	G10 Menadione 2	G11 Menadione 3	G12 Menadione 4
H1 2-Nitroimidazole 1	H2 2-Nitroimidazole 2	H3 2-Nitroimidazole 3	H4 2-Nitroimidazole 4	H5 Hydroxyurea 1	H6 Hydroxyurea 2	H7 Hydroxyurea 3	H8 Hydroxyurea 4	H9 Zinc chloride 1	H10 Zinc chloride 2	H11 Zinc chloride 3	H12 Zinc chloride 4

Figure 4.28

Biolog PM-15 plate – chemical sensitivity assay results for PA14 (a) Green and red areas on growth curves represent increases and reductions in growth, respectively, observed in MPPA treated wells relative to matched control wells, and indicated by numbers. Yellow areas represent growth overlap between MPPA containing wells and matched controls. Significant differences are highlighted with white squares.(b) Table indicating conditions under examination in each well of Biolog plate.

(a)



(b)

A1 D-Serine 1	A2 D-Serine 2	A3 D-Serine 3	A4 D-Serine 4	A5 β-Chloro-L-alanine hydrochloride 1	A6 β-Chloro-L-alanine hydrochloride 2	A7 β-Chloro-L-alanine hydrochloride 3	A8 β-Chloro-L-alanine hydrochloride 4	A9 Thiosalicylic acid 1	A10 Thiosalicylic acid 2	A11 Thiosalicylic acid 3	A12 Thiosalicylic acid 4
B1 Sodium salicylate 1	B2 Sodium salicylate 2	B3 Sodium salicylate 3	B4 Sodium salicylate 4	B5 Hygromycin B 1	B6 Hygromycin B 2	B7 Hygromycin B 3	B8 Hygromycin B 4	B9 Ethionamide 1	B10 Ethionamide 2	B11 Ethionamide 3	B12 Ethionamide 4
C1 4-Aminopyridine 1	C2 4-Aminopyridine 2	C3 4-Aminopyridine 3	C4 4-Aminopyridine 4	C5 Sulfachloro-pyridazine 1	C6 Sulfachloro-pyridazine 2	C7 Sulfachloro-pyridazine 3	C8 Sulfachloro-pyridazine 4	C9 Sulfamono-methoxine 1	C10 Sulfamono-methoxine 2	C11 Sulfamono-methoxine 3	C12 Sulfamono-methoxine 4
D1 Oxycarboxin 1	D2 Oxycarboxin 2	D3 Oxycarboxin 3	D4 Oxycarboxin 4	D5 3-Amino-1,2,4-triazole 1	D6 3-Amino-1,2,4-triazole 2	D7 3-Amino-1,2,4-triazole 3	D8 3-Amino-1,2,4-triazole 4	D9 Chlorpromazine 1	D10 Chlorpromazine 2	D11 Chlorpromazine 3	D12 Chlorpromazine 4
E1 Niaproof 1	E2 Niaproof 2	E3 Niaproof 3	E4 Niaproof 4	E5 Compound 48/80 1	E6 Compound 48/80 2	E7 Compound 48/80 3	E8 Compound 48/80 4	E9 Sodium tungstate 1	E10 Sodium tungstate 2	E11 Sodium tungstate 3	E12 Sodium tungstate 4
F1 Lithium chloride 1	F2 Lithium chloride 2	F3 Lithium chloride 3	F4 Lithium chloride 4	F5 DL-Methionine hydroxamate 1	F6 DL-Methionine hydroxamate 2	F7 DL-Methionine hydroxamate 3	F8 DL-Methionine hydroxamate 4	F9 Tannic acid 1	F10 Tannic acid 2	F11 Tannic acid 3	F12 Tannic acid 4
G1 Chlorambucil 1	G2 Chlorambucil 2	G3 Chlorambucil 3	G4 Chlorambucil 4	G5 Cefamandole nafate 1	G6 Cefamandole nafate 2	G7 Cefamandole nafate 3	G8 Cefamandole nafate 4	G9 Cefoperazone 1	G10 Cefoperazone 2	G11 Cefoperazone 3	G12 Cefoperazone 4
H1 Cefsulodin 1	H2 Cefsulodin 2	H3 Cefsulodin 3	H4 Cefsulodin 4	H5 Caffeine 1	H6 Caffeine 2	H7 Caffeine 3	H8 Caffeine 4	H9 Phenylarsine oxide 1	H10 Phenylarsine oxide 2	H11 Phenylarsine oxide 3	H12 Phenylarsine oxide 4

Figure 4.29

Biolog PM-17 plate – chemical sensitivity assay results for PA14 (a) Green and red areas on growth curves represent increases and reductions in growth, respectively, observed in MPPA treated wells relative to matched control wells, and indicated by numbers. Yellow areas represent growth overlap between MPPA containing wells and matched controls. Significant differences are highlighted with white squares.(b) Table indicating conditions under examination in each well of Biolog plate.

SECTION 4.8

DISCUSSION

SECTION 4.8.1

GROWTH CURVES

It was observed that MPPA at a concentration of 250 μg/ml markedly increased the growth rate of 8 of the 14 *P. aeruginosa* isolates (A36, C1426, J1385, J1532, LES400, LES431, S2 & S38) tested in MHB with agitation, generation times being reduced by 2 to 4 fold depending on the specific strain (Figures 4.1, 4.2, 4.4, 4.5, 4.6, 4.7, 4.12 & 4.13). Excepting S2 and S38 for which MPPA extended lag phase by approximately 4.5 and 5.0 hours, respectively, no effect on the time taken to transition from lag to exponential growth phases was evident for any of these 8 strains. Of these 8 strains only J1532 is mucoid. No effects on the growth curves of 4 isolates - PAO1, PA14 or the mucoid isolates C1433 and MUC28 were apparent (Figures 4.9, 4.10, 4.3 & 4.8). These findings were highly unexpected given that a much lower solubilised MPPA concentration (85 ± 7.8 μg/ml) had previously been shown elsewhere to reduce the growth of strain PAO1 in shaken LB cultures by 24 % over an 18 hour incubation period and that even more profound reductions in growth rate (~ 2 to 5 log reduction in final viable counts after 18 hours growth) were also recorded for 6 CF isolates tested by this group (Krogfelt *et al*, 2000). The possibility that the differences observed were due to use of a different growth medium and/or to the nature of the test isolates themselves cannot be ruled out as MHB was used in place of LB in the current work and PAO1 (which exhibited the least discrepancy) was the only strain common to both experiments. Nevertheless the fact that the MHB used in the current experiments had a substantially lower net content of Ca^{2+} and Mg^{2+} ions at 78 and 225 μM respectively relative to the LB used in the original study which had 263 μM Ca^{2+} and 225 μM Mg^{2+} lead to the expectation that any growth inhibitory effects of MPPA would be more prominent in the low cation MHB than in LB as MPPA had been shown to exert its effects via cation chelation and these are reversible where the action of MPPA is overwhelmed by sufficiently high cation

concentrations (Krogfelt *et al*, 2000). Based on the results of phenotype microarrays, it has been observed that *P. aeruginosa* can utilise MPPA, or more likely its breakdown products (glycerol and fatty acids) as favoured carbon sources (Figures 4.26 & 4.27). This provides a probable explanation for the increased growth rates we have observed in response to MPPA especially given the fact that a higher concentration of solubilised lipid (250 μg/ml) was used in our experiment relative to the 85 μg/ml used by the other investigators (Krogfelt *et al*, 2000). Curiously, all strains exhibiting enhanced growth in response to MPPA originated as CF sputum isolates. This might be of significance as MPPA occurs naturally in inflammatory exudates of the airway (Ediger *et al*, 2001) and strains adapted to CF airways have shown enhanced capacity to use glycerol as a carbon source (Daniels *et al*, 2014). It has previously been shown that mucoid strains are generally more susceptible to MPPA, possibly as a result of possessing stunted O-antigen chains on the outer membrane LPS (Krogfelt *et al*, 2000). Of the 3 mucoid isolates studied (C1433, MUC28 and J1532), only J1532, the least mucoid of the 3, increased growth in response to MPPA. It is therefore possible that the growth stimulating effects of MPPA were counterbalanced by increased membrane damage in both C1433 and MUC28 meaning that no net change in growth rate was detected though this is purely speculative. A similar effect may also be involved for strains PAO1 and PA14. MPPA was shown to slow the growth of a single isolate (S1), extending its lag phase by approximately 4 hours and increasing the generation time thereafter from approximately 1.75 to 2.25 hours (Figure 4.11). For a single isolate (A38) growth was completely inhibited by addition of 250 μg/ml MPPA and therefore no curve was plotted. It might be of interest to note that isolate A38 and all other isolates (S1, S2 and S38) exhibiting slowed or inhibited growth, either in the form of extended lag phases, longer generation times or both, in response to MPPA, were all LES isolates displaying high level tobramycin resistance and were in fact the only tobramycin resistant isolates included in this experiment (Figures 4.11, 4.12 & 4.13 ; Table 4.1). Conversely, tobramycin sensitive LES isolates (A36, LES400 and LES431) did not show any slowing of growth rates in response to MPPA and were actually amongst the isolates whose growth was most stimulated by this lipid, the generation time of LES400 being quartered by MPPA (Figures 4.1, 4.6 & 4.7 ; Table 4.1). This finding

may indicate an inverse relationship between sensitivity to tobramycin and MPPA in these isolates, this hypothesis is further reinforced by the finding that MPPA strongly antagonises the activity of tobramycin (Table 4.1) ; this may suggest competition between these 2 molecules for overlapping or identical binding sites on the outer membrane.

SECTION 4.8.2

EFFECTS OF MPPA ON THE SENSITIVITY OF *P. AERUGINOSA* ISOLATES TO VARIOUS ANTIBIOTICS

SECTION 4.8.2.1

CEFTAZIDIME

As would have been expected based on the previously reported findings of Krogfelt and colleagues (Krogfelt *et al*, 2000), MPPA consistently potentiated the activity of ceftazidime, a 3rd generation cephalosporin widely used in the treatment of pseudomonal infections. Synergy was evident as a 2 to 32 fold drop in MIC in the presence of MPPA for 13 of 14 tested isolates, the degree of MIC change being dependent on the specific isolate. MIC values for the remaining isolate, A38, could not be compared as MPPA alone was completely inhibitory to growth at the concentration employed (250 μg/ml). Based on the MIC sensitivity interpretative breakpoint of 8 μg/ml as published by the clinical and laboratory standards institute in 2014 for *P. aeruginosa* and *Acinetobacter spp.* (CLSI, 2014), 6 isolates (LES400, LES431, MUC28, S1, S2 and S38) would be considered resistant to ceftazidime with observed MIC values > 16 μg/ml, 1 isolate (A38) would have been considered intermediately sensitive with a ceftazidime MIC of 16 μg/ml and the remaining isolates (C1426, C1433, J1385, J1532, PAO1 and PA14) would all have been considered susceptible to ceftazidime, with MIC values ≤ 8 μg/ml (Table 4.1). Addition of MPPA reduced the ceftazidime MIC values for the ceftazidime resistant isolates LES400 and MUC28, respectively, from 64 μg/ml to 8 μg/ml and from 32

μg/ml to 1 μg/ml, indicating a shift from resistance to below the sensitivity breakpoint. For isolates S1 and S2, MPPA was observed to reduce the ceftazidime MIC by 8 fold, from 128 μg/ml to 16 μg/ml and for isolate S38, MPPA was observed to reduce the ceftazidime MIC by 16 fold, from 256 μg/ml to 16 μg/ml, indicating a shift from frank resistance to intermediate susceptibility for these 3 isolates (Table 4.1). In the case of LES431, MPPA caused the ceftazidime MIC to fall by 4 fold from 256 to 64 μg/ml, although this was still insufficient to enable this isolate to be considered 'clinically susceptible' (Table 4.1). For sensitive isolates MPPA caused a 2 to 8 fold reduction in MIC value as compared to a 4 to 32 fold MIC reduction for resistant isolates, indicating a general tendency towards greater MIC decreases where the MIC was high in the absence of MPPA. The entire gamut of β-lactam resistance mechanisms for all of these isolates is not completely known, nevertheless, some have been identified in earlier work with some of these isolates and deserve mention. Strains of *P. aeruginosa* possess an inducible chromosomal AmpC β-lactamase that can be constitutively derepressed via mutations in the *ampR* regulator and then selected by AmpC labile antibiotics such as ceftazidime. Previous work in this laboratory has revealed that this is the case with C1433 which possesses a SNP in the *ampR* regulatory gene and exhibits relatively high levels of β-lactamase production even in the absence of inducers such as clavulanate (Lewis Stewart, personal correspondence). The LES400 and LES431 isolates have been shown to exhibit differential regulation, relative to PAO1 and each other, of several genes coordinating β-lactam resistance including those encoding Mex systems, porins and chromosomal β-lactamase (Salunkhe *et al*, 2005). Of particular interest were the findings that LES431 was a strong producer of β-lactamase whereas LES400 was not and that LES400, in LB medium, exhibited significant upregulation of drug efflux related genes relative to PAO1, specifically *mexR*, *mexA*, *mexB*, *oprM*, *oprN*, *mexX*, *mexY*, *mexZ*, *mexE* and *mexC* whereas LES431 displayed upregulation of a smaller list of efflux related genes than LES400 in LB relative to PAO1 that included *mexR*, *mexX*, *mexE*, *mexG*, *mexH*, *mexI* and *opmD* (Salunkhe *et al*, 2005). The *oprD* porin gene, encoding a conduit for basic amino-acids and carbapenems (Ochs *et al*, 1999), was downregulated in both LES strains relative to PAO1 in LB (Salunkhe *et al*, 2005). Amongst the efflux associated genes *mexR*, upregulated in both LES strains

(Salunkhe *et al*, 2005), encodes an efflux repressor for the MexAB-OprM system (Evans *et al*, 2001), *mexX*, also upregulated in both LES strains, encodes part of the inner membrane complex of another tripartite efflux system whose other structural components are the inner membrane protein encoded by *mexY* and the porin encoded by *oprM* (Morita *et al*, 2006), both upregulated in LES400 but not in LES431, this efflux system is subject to repression by the product of *mexZ* (Morita *et al*, 2006), upregulated in LES400 but not in LES431 (Salunkhe *et al*, 2005). The *mexE* gene was upregulated in both LES strains whereas its cognate porin, encoded by *oprN*, was upregulated only in LES400, the final component of this tripartite efflux complex, MexF, was not significantly upregulated in either strain (Lomovskaya *et al*, 2001 ; Salunkhe *et al*, 2005). Likewise, the *mexC* gene was upregulated only in LES400 but neither its cognate porin OprJ nor its other inner membrane component, MexD, were significantly dysregulated in either LES strain (Lomovskaya *et al*, 2001 ; Salunkhe *et al*, 2005). Finally, each of the components of the MexGHI-OpmD efflux system were upregulated strain LES431but not in LES400 (Aendekerk *et al*, 2002 ; Salunkhe *et al*, 2005). Assuming expression of these genes in MHB is broadly similar to that in LB, it would be expected that at least 2 multidrug efflux systems functioning in their entirety, MexAB-OprM and MexXY-OprM, would be significantly upregulated in LES400 relative to LES431 with the reverse being true for one other system, MexGHI-OpmD. Of these 3 efflux systems, the anionic cephalosporin ceftazidime is a substrate only of MexAB-OprM, even though MexXY-OprM has been shown to extrude other β-lactam compounds including most of the anionic penicillins in addition to the zwitterionic cephalosporins cefepime and cefpirome (Masuda *et al*, 2000 ; Guénard *et al*, 2014 ; Aendekerk *et al*, 2005). This may indicate that the primary mechanism of ceftazidime resistance in LES400 was increased efflux, possibly bolstered by impermeability, as opposed to enzymatic drug hydrolysis which may have played a greater role in LES431. This would be consistent with the increased degree of synergistic interaction that was observed between ceftazidime and MPPA (predicted to act as a cation chelating permeabiliser) against strain LES400 relative to strain LES 431 (8 vs 4 fold MIC drop). Salunkhe and colleagues have noted synergy between imipenem and the metallo β-lactamase inhibitor EDTA against LES431 as evidenced by a 4 fold decrease in imipenem MIC

and they suggested that this indicated a predominant role of β-lactamase in resistance (Salunkhe *et al,* 2005). Whilst this conclusion is likely true, the logic leading to it seems erroneous given that the chromosomal β-lactamase of *P. aeruginosa* is a class C serine enzyme which inefficiently hydrolyses carbapenems and is catalytically inhibited by the suicide substrate cloxacillin but not by chelators such as EDTA (Jacoby, 2009). This is of importance to the present work as it suggests that the synergy these investigators observed between imipenem and EDTA was likely the result of a membrane permeabilising effect exerted by EDTA, not unlike that of MPPA, rather than being due to inhibition of a metalloenzyme. Such effects may enhance drug uptake and theoretically, may also cause some loss of β-lactamase from the periplasmic space. Mechanisms of β-lactam resistance in other ceftazidime resistant isolates used in the present work, 4 of which have previously been identified as belonging to the LES (A38, S1, S2 and S38) have not been studied but could well overlap with those of LES431 and LES400. Although all other isolates would be considered susceptible to ceftazidime even in the absence of MPPA, it has previously been reported that various mutations may play as yet unknown roles in 'subclinical' resistance to β-lactam antibiotics of *P. aeruginosa* that is overlooked in standard sensitivity tests but may enable a more rapid evolution to clinically relevant resistance in strains harbouring them (Alvarez-Ortega *et al,* 2010). Similar 'subclinical' resistance has also been reported for quinolones (Sanders, 2001) and probably also occurs with other antibiotic classes.

SECTION 4.8.2.2

CIPROFLOXACIN

MPPA demonstrated synergy with the fluoroquinolone ciprofloxacin, also used clinically in the management of pseudomonal infection, against 9 of 14 isolates tested (A36, C1426, J1385, MUC28, LES431, PAO1, PA14, S1 and S38, indifferent interactions with 3 of 14 isolates (C1433, LES400 and S2) and antagonism with a single isolate - J1532 (Table 4.1). Based on the MIC sensitivity interpretative breakpoint of 1 μg/ml as published by the clinical and laboratory standards institute

in 2014 for *P. aeruginosa* and *Acinetobacter spp.* (CLSI, 2014), 2 isolates, S1 and S38, would have been considered clinically resistant to ciprofloxacin, both having an MIC of 4 μg/ml for this drug which MPPA was able to reduce by 8 fold in the case of S1 and by 4 fold in the case of S38, bringing both isolates into the 'clinically susceptible' range (Table 4.1). A further 2 isolates, A38 and S2, had ciprofloxacin MIC values of 2 μg/ml in the absence of MPPA and would have been considered intermediately resistant. MPPA did not alter the ciprofloxacin MIC of isolate S2 and as previously stated, MIC values for the remaining isolate, A38, could not be compared as MPPA alone was completely inhibitory to growth at the concentration employed (250 μg/ml). All remaining isolates were considered susceptible to ciprofloxacin. For isolates A36, C1426, J1385, PAO1 and PA14, the ciprofloxacin MIC was 0.06 μg/ml in the absence of MPPA and in each case, MPPA reduced this 2 fold to 0.03 μg/ml (Table 4.1). For isolate LES431 and MUC28, the ciprofloxacin MIC values were reduced 4 fold in the presence of MPPA from 1 to 0.25 μg/ml and from 0.5 to 0.125 μg/ml, respectively (Table 4.1). For isolate LES400, MPPA caused no change in MIC, which remained at 0.125 μg/ml regardless of whether or not MPPA was present (Table 4.1). Finally, antagonism was evident with J1532, for which MPPA doubled the ciprofloxacin MIC value from 0.06 to 0.125 μg/ml (Table 4.1). As with ceftazidime, strains with a greater degree of synergistic interaction between MPPA and ciprofloxacin were generally those that had higher ciprofloxacin MIC values in the absence of MPPA. However, synergy of ciprofloxacin with MPPA was not universal as was the case with ceftazidime (Table 4.1). No synergy was detected with C1433, LES400 or S2 and antagonism was actually evident in the case of J1532. It is not clear why this should be the case. C1433 appeared highly susceptible to ciprofloxacin at an MIC of 0.03 μg/ml and it may therefore be possible that no synergy could have been observed with this isolate if ciprofloxacin was already performing maximally even in the absence of MPPA. The fact that LES400 was 8 times more susceptible than LES431 to ciprofloxacin is surprising given that 2 multidrug efflux systems capable of extruding fluoroquinolones were previously shown to be upregulated in the former strain relative to the latter (Masuda *et al*, 2000 ; Salunkhe *et al*, 2005). Whilst the MexGHI-OpmD system has been shown to extrude quinolone compounds such as the PQS cell communication molecule and

was found to be upregulated in LES431 but not LES400 relative to PAO1, this efflux system has surprisingly been shown not to increase quinolone resistance in cells expressing it (Aendekerk *et al*, 2005 : Salunkhe *et al*, 2005). This likely suggests that other mechanisms of quinolone resistance are active in LES431, possibly mutations in the drug binding sites on the target topoisomerases (Hooper, 2001).

SECTION 4.8.2.3

COLISTIN

This polymyxin antibiotic was the only test drug used clinically in pseudomonal infections to which all studied isolates were sensitive, based on the MIC sensitivity interpretative breakpoint of 2 μg/ml as published by the clinical and laboratory standards institute in 2014 for *P. aeruginosa* and *Acinetobacter spp.* (CLSI, 2014). MIC values for this agent never varied by more than one doubling dilution between the isolates, all being susceptible at MIC values of 0.25 to 0.5 μg/ml (Table 4.1). Profoundly antagonistic interactions were evident between 250 μg/ml MPPA and colistin for all test isolates and were essentially uniform, bringing about a 64 to 128 fold rise in MIC values to 32 μg/ml – well above the clinical resistance breakpoint of 8 μg/ml. Three explanations could be put forward for this phenomenon and they are not mutually exclusive. The first and most obvious is that MPPA may somehow be interacting physically with colistin in such a way as to render it inactive. In hindsight, this hypothesis seems feasible given that polymyxins have been demonstrated to bind with bilayers of phosphatidic acid, which differs from MPPA (lysophosphatidic acid) only by an extra acyl chain (Beurer *et al*, 1988). The second explanation is that sequestration of Mg^{2+} by MPPA may have lead to the transcriptional activation of the interlinked *phoPQ-oprH*, *pmrAB* and *arn* operons, which coordinate adaptive resistance to polycationic antibiotics such as polymyxins, aminoglycosides and mammalian defence peptides in Gram-negative bacteria (Mulcahy *et al*, 2008). Finally, outer membrane binding sites for MPPA and colistin may overlap, leading to competitive exclusion of colistin by MPPA. Interestingly, in a trial experiment with strain PA14 using a far lower MPPA concentration (~ 8.7

μg/ml or 20 μM) akin to the maximum recorded to occur naturally in the airway (Ediger & Toews, 2001), it was founded that MPPA actually reduced the colistin MIC of PA14 by 2 fold (Table 4.1). This may indicate that there is a window in which MPPA concentrations are sufficiently high to permeabilise membranes or exert other antibiotic potentiating effects but are not high enough to either inactivate colistin or sufficiently deplete Mg^{2+} ions and trigger expression of adaptive resistance genes. This could be of special importance as it may imply that MPPA could be an effective antibiotic synergist at very low, non-toxic, concentrations, not much greater than those that occur naturally. Moreover, since human defence peptides found in the airway, such as LL-37, both promote apoptosis like MPPA and have a mechanism of direct antibacterial action very similar to that of colistin, it seems likely that MPPA must play a role in the immunological response of the airway to infection by pathogens such as *P. aeruginosa* (Barlow *et al*, 2010 ; Ediger & Toews, 2001).

SECTION 4.8.2.4

TOBRAMYCIN

As was the case with colistin, marked antagonism between 250 μg/ml MPPA and tobramycin, an aminoglycoside antibiotic used in the management of pseudomonal infections, was evident for all tested isolates. This was highly unexpected as conditions of divalent cation limitation, as would be induced by MPPA, have long been known to enhance the performance of aminoglycosides against *P. aeruginosa* (Washington *et al*, 1978). Further to this point, it should be taken into account that tobramycin MIC values reported here may be artificially low as it was decided not to supplement the MHB batch used with additional cations out of concern that this may have obscured weak effects of MPPA. Based on the MIC sensitivity interpretative breakpoint of 4 μg/ml as published by the clinical and laboratory standards institute in 2014 for *P. aeruginosa* and *Acinetobacter spp.* (CLSI, 2014), 4 isolates, A38, S1, S2 and S38 would have been considered clinically resistant to tobramycin, all had an MIC of 64 μg/ml for this drug which MPPA increased 8 fold to 512 μg/ml (Table 4.1). All other isolates fell within the clinically susceptible MIC range for

tobramycin with MIC values varying from 0.06 to 4 μg/ml depending on the specific isolate. MPPA increased the MIC values for each of these 10 isolates by 4 to 32 fold, depending on the specific isolate (Table 4.1). For all but 3 of the isolates (A36, PAO1 and PA14), this MIC rise was sufficient to infer a change from sensitivity to intermediate resistance at a breakpoint of 8 μg/ml (C1433, J1385, J1532, LES431, MUC28) or full resistance at a breakpoint of ≥ 16 μg/ml (C1426, LES400). Potential reasons for this antagonism between MPPA and tobramycin are essentially the same as those already proposed for colistin. Aminoglycosides have been shown to bind to phosphatidic acid and other negatively charged phospholipids (Alexander *et al*, 1979). However, in the case of tobramycin, unlike that of colistin, considerable variations in the magnitude of MIC increase were apparent even between strains with comparable MIC values in the absence of MPPA with some remaining within susceptible range and others becoming at least intermediately resistant (Table 4.1). As the presence of different bacterial isolates would not be anticipated to interfere with the stoichiometry of any physicochemical interactions between MPPA and tobramycin, it seems highly improbable that this is the sole factor underpinning the antagonistic interactions observed in these MIC determinations. Again, the observed MIC increase may be due in part to competitive target binding or to activation of the interlinked *phoPQ-oprH*, *pmrAB* and *arn* operons, which govern adaptive resistance to polycationic antibiotics and are inducible through Mg^{2+} depletion by chelators (Mulcahy *et al*, 2008).

SECTION 4.8.2.5

CHLORAMPHENICOL

P. aeruginosa is not generally susceptible to concentrations of chloramphenicol that can be safely achieved in serum and tissues, as an intrinsic species property, thus, it has never been in clinical use for the treatment of pseudomonal infections. Nevertheless, it is of interest to establish whether MPPA may enhance the effects of this drug against *P. aeruginosa*. Synergy was evident with 12 of 14 tested isolates as a 2 to 16 fold decrease in MIC value in the presence of 250 μg/ml MPPA depending

on the specific test isolate (Table 4.1). No change in MIC occurred with isolate MUC28, however, at an MIC of 0.5 µg/ml in the absence of MPPA, this was the most sensitive isolate tested, this may suggest that the drug was already performing maximally against this isolate and that no additive effects could therefore be realised. MIC changes could not be determined for isolate A38 as the concentration of MPPA used in these experiments was enough to completely inhibit its growth in the absence of additional antibiotics. Mechanisms of chloramphenicol resistance for the isolates used in this experiment have not been exhaustively determined. The unusually high sensitivity of LES strains to chloramphenicol observed here was unexpected but in the case of LES400 may have resulted from a lack of the *cat* gene, encoding chloramphenicol acetyltransferase, an enzyme which detoxifies this antibiotic (Jeukens *et al*, 2014 : Winsor *et al*, 2016). Greater resistance of LES400 relative to LES431 probably results from upregulation of both the MexAB-OprM and MexXY-OprM efflux pumps, each of which have been demonstrated to eject chloramphenicol amongst other agents (Salunkhe *et al*, 2005 ; Masuda *et al*, 2001). It is possible that the greater sensitivity of J1532 to chloramphenicol relative to its isogenic counterpart J1385 resulted from the presence of an indel polymorphism in the *mexB* gene which may have abolished MexAB-OprM expression or to a SNP in the *lpxO2* gene which may have altered LPS structure (Stewart *et al*, 2014).

SECTION 4.8.2.6

ERYTHROMYCIN

P. aeruginosa and most other non-fastidious Gram-negative bacteria have not generally been regarded as susceptible to the macrolides. Nevertheless, treatment with some of these agents has produced substantial benefits in CF lung disease. Recent work suggests that under cation limited conditions or in the presence of outer membrane disrupting peptides naturally present within serum and inflammatory secretions, *P. aeruginosa* may in fact be rendered fairly sensitive to these large hydrophobic molecules *in vivo* (Buyck *et al*, 2012 ; Lin *et al*, 2015). As MPPA is thought to act as a membrane disrupting chelator, it was deemed to be of great

interest to test its potential for synergistic interactions with an antibiotic of this group. Disappointingly, synergistic interactions between MPPA and erythromycin were largely modest and only observed in 7 of 14 tested isolates : A36, C1433, LES431, PAO1, PA14, S1 and S38. For A36 and PA14, MPPA reduced the erythromycin MIC 2 fold from 128 to 64 μg/ml and from 64 to 32 μg/ml, respectively (Table 4.1). In the case of C1433, LES431 and PAO1, MPPA appeared to cause 4 fold reductions in MIC, from 128 to 32 μg/ml for the former 2 strains and from 64 to 16 μg/ml for PAO1 (Table 4.1). For 2 isolates, S1 and S38, erythromycin MIC values were reduced 8 fold in the presence of MPPA, from 1024 to 128 μg/ml and from 512 to 64 μg/ml, respectively (Table 4.1). For 5 isolates, erythromycin MICs were unaltered by MPPA remaining at 128, 64, 16, 16 and 1024 μg/ml, respectively, for C1426, J1385, J1532, MUC28 and S2, respectively (Table 4.1). Antagonistic interactions were observed for a single isolate, LES400, as evidenced by a 2 fold increase in MIC from 32 to 64 μg/ml in the presence of MPPA. MIC values could not be compared for a single isolate A38, as MPPA alone completely inhibited its growth (Table 4.1). It is not clear why MPPA variably enhanced erythromycin activity against some isolates but not others. Conditions of cation limitation, as would have been attained in the low cation (78 μM Ca^{2+} and 225 μM Mg^{2+}) MHB used in these experiments upon addition of MPPA at a concentration of 250 μg/ml, have been shown to enhance macrolide activity against *P. aeruginosa* by both increasing outer membrane permeability and decreasing expression of the OprM porin, an essential structural component required in the functioning of both the MexAB-OprM and MexXY-OprM efflux pumps, both of which extrude erythromycin from the cell (Buyck *et al*, 2012 ; Masuda *et al*, 2000). Paradoxically, these investigators reported that downregulation of the OprM porin actually required not only conditions of cation limitation but also the presence of the test macrolide (azithromycin) which would otherwise have had the opposite effect of upregulating OprM in conditions were cation concentration was ample (Buyck *et al*, 2012). The antagonism observed in the present work between MPPA and erythromycin for strain LES400 was therefore very surprising as both of these efflux systems have been demonstrably upregulated in this strain, at least relative to PAO1 during growth in LB (Salunkhe *et al*, 2005). Discrepancies in synergy between MPPA and

erythromycin in the panel of test isolates may therefore be due to the presence of resistance mechanisms other than increased efflux and outer membrane impermeability. Macrolide resistance mechanisms have not been formally assessed in these isolates. Known macrolide specific resistance mechanisms that have been found in pseudomonads include specialised efflux pumps encoded by *mef* and *msr* genes, erythromycin esterases encoded by *ere* genes, macrolide phosphorylases encoded by *mph* genes and 23S rRNA methylases encoded by *erm* genes (Ojo *et al*, 2004). No genes encoding these mechanisms could be found in the subset of tested isolates for which genome sequences were available : C1426, C1433, J1385, J1532, LES400, LES431, PAO1 and PA14 (Stewart *et al*, 2014 ; Winsor *et al*, 2016). The fact that erythromycin has a larger and more hydrophobic structure than other tested antibiotics may account to some extent for the wider variation that has been observed in its interactions with MPPA. It is possible that the greater sensitivity of J1532 to erythromycin relative to its isogenic counterpart J1385 resulted from the presence of an indel polymorphism in the *mexB* gene which may have abolished MexAB-OprM expression or to a SNP in the *lpxO2* gene which may have altered LPS structure (Stewart *et al*, 2014).

SECTION 4.8.2.7

NALIDIXIC ACID

P. aeruginosa is not generally susceptible to concentrations of nalidixic acid that can be safely achieved in serum and tissues, as an intrinsic species property, thus, it has never been in clinical use for the treatment of pseudomonal infections. Nevertheless, it is of interest to establish whether MPPA may enhance the effects of this drug against *P. aeruginosa*. Synergy was evident with 7 of 14 tested isolates as a 2 to 4 fold decrease in MIC value in the presence of 250 µg/ml MPPA depending on the specific test isolate (Table 4.1). No change in MIC occurred with 6 isolates, namely, J1385, J1532, LES400, PAO1, PA14 and S2. 2 fold reductions in MIC in the presence of MPPA were evident for 5 isolates, the MIC being reduced from 64 to 32 µg/ml for A36 and C1433 or from 256 to 128 µg/ml for C1426, MUC28 and S1. 2

isolates displayed a 4 fold reduction in MIC in the presence of MPPA, from 1024 to 256 µg/ml for LES431 and from 512 to 128 µg/ml for S38. As previously stated in regards to ciprofloxacin, the greater resistance of LES431 relative to LES400 may imply that as yet unidentified quinolone resistance mechanisms such as topoisomerase target site mutations are present in the former (Hooper, 2001). It is not clear why MPPA has enhanced the activity of nalidixic acid against some isolates but not others.

SECTION 4.8.2.8

TETRACYCLINE

P. aeruginosa is not generally susceptible to concentrations of tetracycline that can be achieved in serum and tissues, as an intrinsic species property, hence, tetracycline has never been in clinical use for the treatment of pseudomonal infections. Nevertheless, it is of interest to establish whether MPPA may enhance the effects of this drug against *P. aeruginosa*. Synergy was evident with 11 of 14 tested isolates as a 4 to 16 fold decrease in MIC value in the presence of 250 µg/ml MPPA depending on the specific test isolate (Table 4.1). No change in MIC occurred with 2 mucoid isolates, MUC28 and J1532, an MIC of 0.5 µg/ml being recorded in both the absence and presence of MPPA for the former and an MIC of 8 µg/ml being recorded for the latter. MIC changes could not be determined for isolate A38 as the concentration of MPPA used in these experiments was enough to completely inhibit its growth in the absence of additional antibiotics (Table 4.1). For a single isolate, A36, MPPA reduced the tetracycline MIC by 16 fold from 4 to 0.25 µg/ml. In the case of 3 other isolates, tetracycline MIC values were reduced 8 fold, from 32 to 4 µg/ml for C1426 and from 4 to 0.5 µg/ml for both S1 and S38. For the remaining isolates, tetracycline MIC values were reduced 4 fold in the presence of MPPA, from 32 to 8 µg/ml in C1433, from 16 to 4 µg/ml in the cases of both J1385, PA14 and S2, from 2 to 0.5 µg/ml for LES400, from 1 to 0.25 µg/ml for LES431 and from 8 to 2 µg/ml for PAO1 (Table 4.1). It may be of significance that for both strains in which synergy was not observed, MUC28 and J1532, a mucoid phenotype was present. Likewise,

although the isogenic non-mucoid/mucoid strain pair of C1426 and C1433 shared an identical tetracycline MIC of 32 µg/ml in the absence of MPPA, MPPA was able to reduce this by a greater extent in the non-mucoid strain than in the mucoid strain (Table 4.1). This may suggest that mucoid phenotypes reduce the tetracycline enhancing activity of MPPA, although the number of mucoid strains tested is too small to draw any definitive correlations. In interpreting these results it should be borne in mind that tetracyclines themselves are avid chelators of divalent cations and are thought to enter Gram-negative organisms in the first instance, via porins, in the form of cation coordination complexes which then dissociate in the periplasm to yield the free electroneutral molecule which then negotiates the inner membrane by virtue of its lipophilicity (Chopra & Roberts, 2001). This may imply that porins are not required for tetracycline uptake in the presence of MPPA, as competition between MPPA and tetracycline for cation binding would, depending on the chelating affinities of each molecule, result in a level of cationic tetracycline coordination complexes available for porin entry that would be less than that attained in the absence of the competing chelator (MPPA) resulting in antagonism rather than synergy. It therefore seems probable that MPPA, in chelating cations, is simply destabilising the outer membrane, allowing electroneutral tetracycline to bypass it without the need for porin facilitated entry in the form of a cationic complex. Mechanisms of tetracycline resistance have not been defined in this panel of isolates but are likely due, at least in part to the upregulation of Mex pumps. The greater resistance of LES400 relative to LES431 may be due to upregulation of the MexAB-OprM and MexXY-OprM pumps that has been observed in the former relative to the latter (Salunkhe *et al*, 2005). It is possible that the greater sensitivity of J1532 to tetracycline relative to its isogenic counterpart J1385 resulted from the presence of an indel polymorphism in the *mexB* gene which may have abolished MexAB-OprM expression or to a SNP in the *lpxO2* gene which may have altered LPS structure (Stewart *et al*, 2014).

SECTION 4.8.2.9

TRIMETHOPRIM

P. aeruginosa is not generally susceptible to concentrations of trimethoprim that can be achieved in serum and tissues, as an intrinsic species property, hence, trimethoprim has never been in clinical use for the treatment of pseudomonal infections. Nevertheless, it is of interest to establish whether MPPA may enhance the effects of this drug against *P. aeruginosa*. Some *P. aeruginosa* isolates that have evolved in the unique environment of the CF airway have the unusual property of having acquired an increased sensitivity to folate antagonists as a result of syntrophic adaptations (Qin *et al*, 2012). Synergy with trimethoprim was evident with 11 of 14 tested isolates as a 2 to 8 fold decrease in MIC value in the presence of 250 µg/ml MPPA depending on the specific test isolate (Table 4.1). No change in MIC occurred with 2 mucoid isolates, J1532 and MUC28, an MIC of 16 µg/ml being recorded in both the absence and presence of MPPA for the former and an MIC of 64 µg/ml being recorded for the latter. This may be of significance given that no synergistic interactions with MPPA were detected with some other drugs tested against these isolates, namely, tetracycline, erythromycin and nalidixic acid. MIC changes could not be determined for isolate A38 as the concentration of MPPA used in these experiments was enough to completely inhibit its growth in the absence of additional antibiotics (Table 4.1). For 4 isolates, MPPA reduced the trimethoprim MIC 2 fold, from 32 to 16 µg/ml in the cases of C1433 and J1385, from 16 to 8 µg/ml in the case of PAO1 and from 64 to 32 µg/ml in the case of S38. In the case of 6 other isolates, trimethoprim MIC values were reduced 4 fold, from 64 to 16 µg/ml in the cases of A36, LES400 and S1, from 4 to 1 µg/ml in the case of LES431 and from 32 to 8 µg/ml in the cases of PA14 and S2. For a single isolate, C1426, MPPA reduced trimethoprim MIC by 8 fold from 32 to 4 µg/ml (Table 4.1).It is possible that the greater sensitivity of J1532 to trimethoprim relative to its isogenic counterpart J1385 resulted from the presence of an indel polymorphism in the *mexB* gene which may have abolished MexAB-OprM expression or to a SNP in the *lpxO2* gene which may have altered LPS structure (Stewart *et al*, 2014).

SECTION 4.8.2.10

EFFECTS OF MPPA ON ANTIBIOTIC SENSITIVITY ; GENERAL POINTS

Although interactions of MPPA with ceftazidime, colistin and tobramycin were qualitatively consistent across strains, interactions with chloramphenicol, erythromycin, quinolones, tetracycline and trimethoprim varied according to the specific isolate, suggesting that synergistic interactions of MPPA with these agents might be affected by other factors in addition to the membrane permeabilising effects of MPPA. It is of particular interest that whilst mucoid strain J1532 was considerably more susceptible than its non-mucoid isogenic counterpart J1385 to chloramphenicol, erythromycin, tetracycline and trimethoprim, synergy was absent with tetracycline and trimethoprim for J1532, a *mexB* and *lpxO2* mutant (Stewart *et al*, 2014). Although many genes encoding Mex system components were found to be downregulated in response to MPPA, none passed the significance cutoff when filtered by *padj* value. Some of these missed the cutoff by only a narrow margin including *PA14_18780* (*mexQ*) and *PA14_56890* (*mexW*) which are components of the respective MexPQ-OpmE and MexVW-OprM efflux systems, both able to extrude many drugs which are potentiated by MPPA and the former of which is not constitutively expressed (Mima *et al*, 2005 ; Li *et al*, 2003). It may therefore be possible that downregulation of efflux plays a role in MPPA mediated antibiotic potentiation in addition to membrane permeabilisation. The possibility that MPPA directly affects the configuration or energy supply of efflux systems cannot be excluded either and amphipathic molecules of varying charge (including lysolipids) have previously been shown to affect the configuration of membrane channels and some amphipaths already used as drugs for non-infective conditions, such as calcium channel antagonists and the phenothiazines are known efflux pump inhibitors (Martinac *et al*, 1990 ; Ughachukwu & Unekwe, 2012). It is possible that the antibiotic potentiating properties of MPPA observed here will be of use in CF airway and other infections by *P. aeruginosa*.

SECTION 4.9

EFFECTS OF MPPA ON PRODUCTION OF VIRULENCE ASSOCIATED EXOPRODUCTS BY *P. AERUGINOSA* ISOLATES IN AGITATED MHB CULTURES OVER 24 HOURS

SECTION 4.9.1

PYOCYANIN ACCUMULATION IN AGITATED CULTURES

In shaken cultures, MPPA reduced pyocyanin accumulation by 9 of 13 test isolates : C1426, J1385, J1532, LES400, LES431, MUC28, PAO1, PA14 and S2 although this decreased pyocyanin accumulation was statistically significant only for a subset of 5 of these 9 isolates : J1385, LES400, LES431, MUC28 and PA14 (Figure 4.14). Although effects of MPPA on other virulence factors such as pyoverdine, alginate and elastase have been described previously (Laux *et al*, 2002), this appears to be the first time MPPA has been observed to affect pyocyanin production. The observed reductions in pyocyanin production all occurred in strains whose growth was enhanced by MPPA (Figure 4.14). Conversely, MPPA increased pyocyanin accumulation by 4 of 13 test isolates : A36, C1433, S1 and S38 although this rise in pyocyanin accumulation was statistically significant for a subset of only 3 of these 4 isolates : A36, S1 and S38 (Figure 4.14). MPPA has been shown to slow the growth of S1 by extending its lag phase by approximately 4 hours and increasing its generation time from 1.75 to 2.25 hours and to extend the lag phase of S38 by approximately 5 hours although halving its generation time thereafter from 1.5 to 0.75 hours, in the presence of MPPA (Figure 4.14). MPPA halved the doubling time of A36, however, A36 nevertheless appeared to enter stationary phase at approximately the same time regardless of whether or not MPPA was present (Figure 4.14). Therefore, there appears to be an inverse relationship between growth rate and pyocyanin production with MPPA increasing pyocyanin production in isolates whose growth it simultaneously decreases and vice versa. A36 appears to be an exception to this trend, however, it should be noted that this strain appeared to enter stationary growth phase at approximately the same time in MPPA treated and control cultures even though MPPA effectively halved its doubling time during exponential phase

(Figure 4.14). The reasons underlying these differential effects of MPPA on pyocyanin production are not immediately apparent. Nonetheless, it has long been known that pyocyanin production does generally have an inverse correlation with growth rates and levels of oxidative respiration in the producing strain (Whooley & McLoughlin, 1982). Pyocyanin production is also known to be elevated in the presence of high calcium concentrations (Sarkisova *et al*, 2005), implying that the observed reductions in pyocyanin production by this subset of isolates may have been partly due to the Ca^{2+} chelating effects of MPPA. The results of phenotypic microarray studies conducted on PA14 in the absence and presence of MPPA using BioLog PM-1 and PM-2 plates strongly suggest that *P. aeruginosa* is capable of metabolising MPPA as a carbon source (Figures 4.26 & 4.27). MPPA breakdown would be expected to yield fatty acids and glycerol, the former of which can be readily oxidised to succinate, a preferred carbon source in pseudomonads (Rojo, 2010 ; Chung *et al*, 2013). Production of pyocyanin is known to be increased during growth on certain amino-acids whilst being subject to carbon catabolite repression by succinate (Palmer *et al*, 2007 ; Huang *et al*, 2012). This may also provide a partial explanation for the observed reduction in pyocyanin accumulation that occurred in these 9 isolates in the presence of MPPA.

SECTION 4.9.2

SIDEROPHORE ACCUMULATION IN AGITATED CULTURES

In shaken cultures, MPPA reduced pyochelin accumulation by 7 of 13 test isolates : C1433, LES400, LES431, MUC28, PAO1, S2 and S38 although this decreased pyocyanin accumulation was statistically significant only for a subset of 6 of these 7 isolates (Figure 4.15). Conversely, MPPA increased pyochelin accumulation by 6 of 13 test isolates : A36, C1426, J1385, J1532, PA14 and S1 although this rise in pyochelin accumulation was statistically significant for a subset of only 4 of these 6 isolates : A36, C1426, J1532 and PA14 (Figure 4.15). Although effects of MPPA on other virulence factors such as pyoverdine, alginate and elastase have been described previously (Laux *et al*, 2002), this appears to be the first time MPPA has been

observed to affect pyochelin production. It seems intuitive that MPPA, a chelator of ferrous iron (Laux *et al*, 2002) would have an iron depleting effect and thereby induce expression of siderophores. It is therefore unclear why this has been the case only in a minority of the assayed isolates, with the remainder actually appearing to reduce pyochelin production in response to the presence of MPPA. Nevertheless, Laux and colleagues previously discovered that MPPA reduced production of another siderophore, pyoverdine, in strain PAO1, which is in concordance with the present work (Figure 4.15), the opposite effect was observed with another chelator, EDTA, suggesting that this effect was specific to MPPA and not due solely to iron limitation (Laux *et al*, 2002). Pyochelin is a lower affinity siderophore than is pyoverdine and is typically produced earlier on in the growth curve of *P. aeruginosa* which may later switch to producing the higher affinity but metabolically costlier pyoverdine when iron is more severely limited (Dumas *et al*, 2013). *P. aeruginosa* may also be able to acquire ferrous iron from host haemoproteins using redox active phenazines such as phenazine-1-carboxylic acid, which are intermediates in the biosynthesis of pyocyanin (Wang *et al*, 2011). No correlations between pyocyanin and pyochelin production were evident, however, all 5 assayed isolates exhibiting significantly reduced pyoverdine production in response to MPPA (LES400, MUC28, PAO1, S2 and S38) also produced significantly less pyochelin in response to MPPA. Regardless of the statistical significance of the results, 7 of 13 test isolates (53.85 %) increased production of both pyochelin and pyoverdine, 4 of 13 test isolates (30.77 %) decreased production of both siderophores whilst 2 of 13 isolates (15.38 %) increased production of pyoverdine but reduced production of pyochelin (Figure 4.15 & 4.16). Therefore effects on pyochelin and pyoverdine production were correlated in 11 of 13 (84.62 %) of isolates. The answer as to why MPPA increases siderophore production in some isolates whilst having the opposite effect in others remains elusive.

SECTION 4.10

EFFECTS OF MPPA ON PRODUCTION OF OF VIRULENCE ASSOCIATED EXOPRODUCTS BY *P. AERUGINOSA* ISOLATES IN STATIC BIOFILM MHB CULTURES OVER 48 HOURS

SECTION 4.10.1

PYOCYANIN

As pyocyanin production is known to be increased in biofilm cultures and in late exponential to stationary growth phase, it was deemed appropriate to test the effect of MPPA on pyocyanin production by a subset of strains under these conditions (Figure 4.17). MPPA significantly reduced accumulated mean pyocyanin concentrations by 27 % from 0.338 to 0.265 µg/ml in C1426 biofilm cultures. In C1433 biofilm cultures, MPPA significantly reduced accumulated mean pyocyanin concentrations by 39 % from 0.532 µg/ml to 0.325 µg/ml. Biofilm cultures of strain J1532 accumulated 28 % less pyocyanin in the presence of MPPA, mean pyocyanin concentration having been significantly reduced from 1.166 to 0.841 µg/ml. In PAO1 biofilm cultures, MPPA significantly reduced accumulated mean pyocyanin concentrations by 32 % from 0.320 to 0.218 µg/ml. Biofilm cultures of strain PA14 accumulated 36 % less pyocyanin in the presence of MPPA, mean pyocyanin concentration having been significantly reduced from 0.351 to 0.223 µg/ml. For strain J1385, no difference whatsoever was observed in mean pyocyanin concentration. Amongst these strains, C1426, J1532, PAO1 and PA14 also displayed reduced pyocyanin production in response to MPPA when grown in shaken planktonic cultures although in this case, the difference reached statistical significance only for PA14. Unexpectedly, J1385, which produced significantly less pyocyanin in shaken planktonic cultures when treated with MPPA, did not show any difference in pyocyanin production in static biofilm cultures treated with MPPA. In shaken planktonic cultures, MPPA appeared to increase pyocyanin production by C1433, though not significantly so, whereas it significantly reduced pyocyanin production in biofilm cultures of this strain. Potential explanations for reduced pyocyanin production in biofilm cultures are broadly the same as those previously

discussed for planktonic cultures and include increased metabolic rate and carbon catabolite repression.

SECTION 4.10.2

TOTAL EXOPROTEASE

As exoprotease production is known to be increased in biofilm cultures and in late exponential to stationary growth phase, it was deemed appropriate to test the effect of MPPA on exoprotease production by a subset of strains under these conditions (Figure 4.18). MPPA significantly reduced accumulated mean exoprotease concentrations by 38 % in C1426 biofilm cultures. In C1433 biofilm cultures, MPPA significantly reduced accumulated mean exoprotease concentrations by 41 %. Biofilm cultures of strain J1385 accumulated 33 % less exoprotease in the presence of MPPA. In J1532 biofilm cultures, MPPA significantly reduced accumulated mean exoprotease concentrations by 58 %. Biofilm cultures of strain PAO1 accumulated 47 % less exoprotease in the presence of MPPA. For biofilm cultures of strain PA14, mean exoprotease production was significantly reduced by 32 %. Laux and colleagues found that MPPA treated static PAO1 cultures produced a greatly reduced level of exoprotease on skim milk agar plates, the exoprotease found to be produced were not exhaustively identified but were found to include elastase and the staphylolytic protease LasA (Laux *et al*, 2002).In RNA-seq analyses, downregulation of *lasB* was detected in the presence of MPPA but this was not significant (*p-adj* > 0.1) whilst *lasA* transcription was unaltered .This may have been due to the early time-point at which RNA was extracted from cells and it cannot be excluded that significant differences in transcription would have ensued given a longer incubation time. The transcriptomic analysis did however reveal significant downregulation of the *dsbA2* gene in response to MPPA. *dsb* genes have been shown to encode disulphide bond isomerases which are essential for normal folding and catalytic activity of exoproteases including elastase (Malhotra *et al*, 2000 : Braun *et al*, 2001). It is therefore possible that some exoproteases, such as elastase, whilst being transcribed normally, appear deficient in MPPA treated cultures to inactivity

imposed by defective folding or other post-translational modifications. A locus encoding an inhibitor of alkaline protease (*aprI*) was found to be upregulated whilst the outer membrane protein, AprF , which extrudes alkaline protease was downregulated although neither of these dysregulations were significant with respective *p-adj* values > 0.1. Although most of these events, excluding dsbA2 downregulation, do not appear to be significant individually, the total exoprotease assay employed here relies on cleavage of azocasein, a promiscuous protease substrate, and the contributions of individual proteases may be cumulative despite lacking individual significance.

SECTION 4.10.3

ELASTASE

As elastase production is known to be increased in biofilm cultures and in late exponential to stationary growth phase, it was deemed appropriate to test the effect of MPPA on elastase production by a subset of strains under these conditions (Figure 4.19). MPPA significantly reduced accumulated mean elastase concentrations by 52 % in C1426 biofilm cultures. In C1433 biofilm cultures, MPPA significantly reduced accumulated mean elastase concentrations by 52 %. Biofilm cultures of strain J1385 accumulated 51 % less elastase in the presence of MPPA. In J1532 biofilm cultures, MPPA significantly reduced accumulated mean elastase concentrations by 52 %. Biofilm cultures of strain PAO1 accumulated 50 % less elastase in the presence of MPPA. For biofilm cultures of strain PA14, mean elastase production was significantly reduced by 51 %. Laux and colleagues found that MPPA treated static PAO1 cultures produced a greatly reduced level of catalytically inert elastase that could be activated by the addition of exogenous elastase, they found that this effect correlated with *lasB* transcription levels but was not affected by acyl homoserine lactones and suggested that this reduced elastase activity may be due at least partially, to the cation chelating effect of MPPA (Laux *et al*, 2002). In RNA-seq analyses, downregulation of *lasB* was detected in the presence of MPPA but this was not significant (*p-adj*> 0.1) whilst *lasA* transcription was unaltered. This may have

been due to the early time-point at which RNA was extracted from cells. The transcriptomic analysis did however reveal significant downregulation of the *dsbA2* gene in response to MPPA. *dsb* genes have been shown to encode disulphide bond isomerases which are essential for normal folding and catalytic activity of exoproteases including elastase (Malhotra *et al*, 2000 : Braun *et al*, 2001). It is therefore suspected that reduced expression of this gene is at least partly responsible for the reduced elastase activity observed in MPPA treated cultures. As stated previously in regards to the total exoprotease assay, no downregulation of genes involved in type 2 secretion pathways responsible for protease export was evident, making this an improbable cause of reduced elastase activity.

SECTION 4.11

EFFECTS OF MPPA ON PA14 MOTILITY

In the presence of MPPA, strain PA14 mean swim distance was significantly increased by 66 % whilst mean twitch distance was increased by 10 % but this was not found to be statistically significant (Figures 4.20.1 and 4.20.2). This is in fitting with transcriptomic data which reveal significant upregulation of many genes that are involved in flagellum and type IV pilus biogenesis, motility and chemotaxis (Figure 5.1.21). Other phospholipids have previously been shown to act as chemoattractants which stimulate both swimming and twitching motility (Bonner & Shimkets, 2006 : Miller *et al*, 2008). Moreover, both swimming and twitching motility have previously been shown to play roles in biofilm formation. Type IV pili and flagellae may both act as surface adhesins and structural biofilm components whilst twitching motility aids horizontal biofilm expansion and swimming motility enables vertical biofilm expansion (Murray *et al*, 2010, O'Toole & Kolter, 1998 ; Klausen *et al*, 2003). Many genes involved in motility and chemotaxis and the biosynthesis of type IV pili and flagellae are highly upregulated by MPPA in transcriptomic analyses (Figure 5.1.21).

SECTION 4.12

EFFECTS OF MPPA ON BIOFILM FORMATION

The finding that MPPA enhanced biofilm formation by 6 of 7 tested isolates, including reference strain PAO1, was highly unexpected and difficult to explain given that chelators have previously been shown to reduce biofilm formation (Banin *et al*, 2006) and that MPPA appears to enhance chemotactically induce motility (Figure 4.20.1). Laux and colleagues had previously reported that 80 µM MPPA markedly reduced biofilm formation on the surfaces of a 24 well plate inoculated with PAO1 in LB cultures as determined via crystal violet staining and appeared to also reduce formation of readily visible pellicle biofilm under these conditions (Laux *et al*, 2002). They attributed these findings to a marked reduction in alginate production by the organism as confirmed by a carbazole based colorimetric assay for uronic acids (Laux *et al*, 2002). This study was published at a time when the prevailing view was that alginate was the primary exoplolymer involved in biofilm formation and it has subsequently become accepted that other exopolysaccharides such as Pel and Psl, as well as other factors such as eDNA, are more important for biofilm formation by non-mucoid strains, and at least for PAO1, the contribution of alginate is probably negligible (Whitchurch *et al*, 2002 ; Wozniak *et al*, 2003). In principle, carbazole assays detect the uronic acid monomers liberated by acid hydrolysis but are highly sensitive to interference from neutral sugars and it has been suggested that the positive results obtained when these assays are applied to the exopolymeric substance of PAO1 biofilms, and possibly also biofilms of other non-mucoid strains, are due to the presence of eDNA and not alginate, as was once widely believed to be the case (Whitchurch *et al*, 2002 ; Wozniak *et al*, 2003). The relative contributions of Pel and Psl polysaccharides to the biofilm matrix is strain dependent, some strains produce both in a redundant fashion, such that loss of either single polysaccharide does not substantially alter biofilm formation, other strains, including PAO1, utilise primarily Psl but can and do produce smaller quantities of Pel under certain circumstances whereas a minority of tested strains, as typified by PA14, are wholly reliant on Pel for biofilm formation and lack the *psl* gene cluster (Colvin *et al*, 2011b ; Mann & Wozniak, 2012). Curiously, PA14 was the only tested

isolate which did not exhibit enhanced biofilm formation in the presence of MPPA in the present work (Figure 4.21). This may be of significance given that all other test isolates are genetically capable of Psl production (Stewart *et al*, 2014 ; Colvin *et al*, 2011b). Many of the sugar nucleotide monomers used to produce Psl polysaccharide are also involved in the biosynthesis of A-band LPS and functional redundancy has been noted between genes involved in LPS production (*wbpW*) and Psl (*pslB*) polysaccharide (Byrd *et al*, 2009). In RNA-seq analyses with strain PA14, *wbpW* (*PA14_71970*) was found to be upregulated although this was not deemed significant after *p* value adjustment - *padj* > 0.1. Significant upregulation of several other genes involved in the synthesis and export of LPS components such as *orfK*, *orfN*, *wzz*, *wzt* and *eno* however, implies, that LPS is indeed overexpressed in response to MPPA (Figures 5.1.1 & 5.1.19). Upregulation of LPS linked genes in response to MPPA makes sense given that MPPA has been shown to chelate cations required in LPS bridging, presumably inducing its loss from the outer membrane (Krogfelt *et al*, 2000). It is possible that similar upregulation of LPS genes in strains other than PA14 occurs, increasing the cellular pool of sugar nucleotides available for LPS biosynthesis, and potentially also increasing biosynthesis of Psl polysaccharide, although this is purely speculative. Equally, effects of MPPA on the structure of LPS itself may affect biofilm formation ; absence of the O-polysaccharide, specifically the B-band, has previously been demonstrated to facilitate adhesion of *P. aeruginosa* to polystyrene and biofilm formation ranked by A and B-band O-polysaccharide expression was found to be as follows A^+B^->A^-B^->A^+B^+>A^-B for PAO1 and its mutants (Beveridge *et al*, 1997). Abbreviation of core polysaccharide has also been found to enhance both adhesion and cohesion of cells (Lau *et al*, 2009). Therefore it seems feasible that removal of LPS components (B-band LPS) by MPPA might increase adhesion or alternatively, that enhanced expression of A-band LPS in response to this lipid may increase adhesion. In this regard, it is of interest to note that strain PA14, the only tested strain found not to increase biofilm formation in response to MPPA, asides from lacking Psl, is also unusual in that it lacks A-band LPS due to the presence of SNPs in the *wbpX* and *migA* genes (Hao *et al*, 2015). Several other possible explanations also exist for the increased biofilm formation that was observed to occur in the presence of MPPA. Whilst biofilm formation and some

forms of active motility are often considered to be opposing and mutually exclusive behaviours, flagellae and type IV pili have been implicated in certain stages of biofilm formation, in which they dually function as adhesins and structural components, they are also important for horizontal expansion of the biofilm upon a substratum as well as in vertical differentiation and dispersal, respectively (Murray *et al*, 2010, O'Toole & Kolter, 1998 ; Klausen *et al*, 2003). Therefore, upregulation of genes encoding type IV pilus and/or flagellum biogenesis in response to MPPA (Figures 5.1.21) may contribute to enhanced biofilm formation. The diguanylate cyclase GcbA (*PA14_64050*) is greatly downregulated in response to MPPA (Figure 5.1.33). GcbA has been shown to be essential for the post-transcriptional activation of BdlA which is in turn essential for biofilm dispersal (Petrova *et al*, 2014). Downregulation of this gene in response to MPPA may thus have increased biofilm biomass determinations in these experiments. The *PA14_33530* locus is highly upregulated in the presence of MPPA and encodes an adhesion protein that may be involved in biofilm formation (Ulrey *et al*, 2014). It should be noted that cells growing in the biofilm mode exhibit differential gene regulation that may differ temporospatially not only from that of cells grown in planktonic cultures but also from that of cells growing in different regions of the same biofilm. Therefore, the fact that transcriptomic analyses in the present work were carried out using RNA extracted from planktonic cultures at a set time-point may place considerable limitations on their predictive powers in regards to biofilm phenotypes.

SECTION 4.13

VIRULENCE MODULATION ; GENERAL POINTS & FUTURE DIRECTIONS

Overall, it is not clear whether or not the effects of MPPA observed here on virulence factor elaboration by *P. aeruginosa* would support its use as a therapeutic agent in CF airway infections. Although consistently reduced expression of elastase and other exoproteases would seem to offer therapeutic promise, the effects of MPPA upon the production of other virulence factors such as pyocyanin and siderophores, at least in planktonic cultures, has been erratic. Likewise, the apparent

ability of MPPA to increase biofilm formation observed here, although at odds with previous findings (Laux *et al*, 2002) may be cause for concern. It may be of interest to test the effects of MPPA in an *in vivo* infection model of *P. aeruginosa* airway model such as that reported by Carter and colleagues, alone and in combination with standard antibiotics (Carter *et al*, 2010).

SECTION 4.14

PHENOTYPE MICROARRAY DATA

SECTION 4.14.1

CARBON SOURCE UTILISATION

The fact that strain PA14 grew in the negative control wells of carbon utilisation assay plates PM-1 and PM-2 only when 250 µg/ml MPPA was present can be taken as evidence that MPPA can be catabolised by this organism as a sole carbon source (Figures 4.26 & 4.27). This may also provide at least a partial explanation as to why MPPA appeared to enhance the growth rate of most *P. aeruginosa* isolates tested (Figures 4.1 – 4.13). Many carbon sources in these assay plates failed to support the growth of PA14 in the absence of MPPA, lending further credence to this idea. It appears feasible that MPPA could be broken down to yield free fatty acids which could be oxidised to provide succinate as a carbon source along with glycerol. The fact that PA14 failed to use many carbon sources present in these plates, growing only in wells containing these when supplemented with MPPA, lends further credence to this notion. In other cases, PA14 was able to grow on certain carbon sources but this growth was increased in the presence of MPPA, again, it can be inferred that MPPA, or its breakdown products, act as a carbon source. In the case of some carbon sources on which PA14 grew especially well, mostly TCA cycle intermediates and a select few amino acids, the addition of MPPA appeared to slightly reduce final growth yields but this did not reach significance in any case (Figures 4.26 & 4.27). The phenotypes observed here are in fitting with

transcriptomic findings reported in chapter 5, which imply that MPPA is utilised as a preferred carbon source.

SECTION 4.14.2

CHEMICAL SENSITIVITIES

MPPA appeared to reduce the sensitivity of strain PA14 to various challenge agents in the PM-15 and PM-17 phenotype microarray plates. MPPA significantly increased growth in wells (A3-A4, PM-15) containing procaine at the 2 highest test concentrations (Figure 4.28 & 4.29). Procaine is one of a group of cationic amphiphilic agents used as a local anaesthetics that have been found to possess antibacterial effects (Johnson *et al*, 2008). There is evidence that the antibacterial effects of these anaesthetic compounds are due to inner membrane disruption (Leung & Rawal, 1977). Moreover, oppositely charged amphipathic molecules have been shown to differentially activate mechanosensitive ion channels with the result being that cationic agents such as procaine induce membrane cupping, in contrast neutral or anionic agents, including lysolipids, induce membrane crenation (Martinac *et al*, 1990). It is not clear from the phenotype microarray data whether the increased growth in the presence of MPPA is genuinely due to antagonism of the antibacterial effects of procaine or simply to increased growth rate, however, assuming the former to be the case, possible explanations may include reduced membrane electronegativity and procaine binding due to induction by MPPA of the phoPQ-oprH operon (Figure 5.1.19) or opposing effects on membrane configuration by the oppositely charged amphipaths although mechanisms of procaine resistance have not been defined. MPPA also enhanced growth in well D8, containing the cationic quaternary ammonium detergent domiphen bromide at the highest test concentration (Figure 4.28). Again it is uncertain whether this is an incidence of true antagonism or explicable as a result of enhanced baseline growth rate. Quaternary ammonium salts are cationic membrane active agents and it is surprising that phoPQ-oprH has not thus far been implicated in resistance towards these agents (Loughlin *et al*, 2002). There is some evidence for cross resistance occurring between quaternary

ammonium salts and EDTA, a compound which chelates LPS bridging cations in a manner similar to MPPA (Loughlin *et al*, 2002 ; Laux *et al*, 2002). MPPA also appeared to increase growth in the presence of 3,4-dimethoxybenzyl alcohol, sodium azide, menadione, 2-nitroimidazole and hydroxyurea, $_{D}$-serine, ethionamide, 4-aminopyridine, 3-amino-1,2,4-triazole, niaproof, lithium chloride, DL-methionine hydroxamate and cefsulodin (Figure 4.29). For most of these compounds, reasons for antagonism with MPPA are not at all clear, however, the fact that MPPA increased PA14 growth in the presence of cefsulodin was unexpected given that MPPA has been shown to exert marked synergy with other β-lactam agents such as ceftazidime, piperacillin and ampicillin in the present work and elsewhere (Krogfelt *et al*, 2000). It may be noteworthy that cefsulodin is highly unusual amongst β-lactams in that it preferentially binds PBPs 1A and 1B and has a very narrow spectrum of antibacterial action that is almost exclusively limited to *P. aeruginosa* (Yousif *et al*, 1985). It might be possible that antagonism of MPPA with azide results from downregulation of cytochrome oxidases by MPPA (Figures 5.1.23 & 5.1.38) and that uptake of amino-compounds, lithium chloride, hydroxamate may be influenced negatively by downregulation of amino-acid transporters (Figure 5.1.3), inorganic ion transporters and the PiuA siderophore receptor (Figure 5.1.25). Some resistance to membrane stress may also be conferred by downregulation of the *PA14_71070* which breaks down the osmoprotectant glycine betaine (Figure 5.1.15).

CHAPTER 5

THE TRANSCRIPTOMIC RESPONSE TO MPPA

SECTION 5.1

INTRODUCTION

In chapter 4, it was established that MPPA has a marked effect on several virulence related *P.aeruginosa* phenotypes, including production of pyocyanin, the fluorescent siderophores pyochelin and pyoverdine, elastase and other exoproteases as well as biofilm formation and antibiotic sensitivities. The mechanisms underlying these phenotypic effects remain elusive. In this chapter, RNA extracted from PA14 cultures treated with MPPA is sequenced using the Ion Torrent PGM platform in order to deduce the transcriptomic response to MPPA in hopes that it may yield clues as to the mechanism by which MPPA exerts its phenotypic effects.

SECTION 5.2

MATERIALS AND METHODS

SECTION 5.2.1

CULTURE CONDITIONS

Growth conditions were optimised during a trial run prior to RNA extraction, using pyocyanin production as a surrogate marker of MPPA activity. 15 mg of MPPA was weighed and added to a small round bottomed flask that had been pre-cleaned with 70 % ethanol and dried in an oven. 5 ml of chloroform was then added to the flask and swirled to fully suspend the MPPA. The chloroform was then evaporated to dryness using a Buchi rotary evaporator with rotor set to 200 rpm and water bath at 55^{o}C, residual chloroform was then removed under a nitrogen stream. The flask was then sealed with parafilm to prevent entry of contaminants before being placed in a UV crosslinker oven set to full power for 30 minutes in order to kill any remaining microbes. 10 ml volumes of sterile PBS were decanted into each of two 50 ml Falcon tubes which were then capped and heated in a water bath set to 70^{o}C in order to raise the temperature of the PBS to levels exceeding the transition temperature of MPPA (~50^{o}C). The round bottomed flask containing dried MPPA lipid film was then opened in a laminar flow cabinet and 10 ml of the pre-heated PBS was aseptically added and swirled immediately after retrieval from the water bath. This enabled the lipid film to be solubilised to clarity. The resulting 10 ml aliquot of MPPA solution was then pipetted into a Falcon tube containing 40 ml of MHB to obtain a final concentration of 300 mg/l MPPA, whilst 10 ml of warmed PBS was added to another Falcon tube containing 40 ml of MHB as a control. 15 ml volumes of MHB containing 300 mg/l were then transferred to each of three sterile 250 ml conical flasks, matched controls were prepared using the MPPA free MHB. Triplicate PA14 cultures each grown in 5 ml MHB for 16 hours at 250 rpm/37^{o}C from separate isolated colonies on a fresh LB streak plate, were then used to inoculate the conical flasks in 3 MPPA/control pairs to obtain 3 biological replicates for each condition – 100 µl of overnight culture was added to each flask. The flasks were then sealed with

sterile aluminium foil attached with autoclave tape and incubated at 37ºC with 250 rpm agitation. A_{695} values of 100 µl aliquots withdrawn from each conical flask at 20 minute intervals were then recorded for 8 hours to obtain approximate measures of pyocyanin concentration over time and triplicate cfu/ml spread plate counts were also recorded at these intervals to normalise for growth (data not shown). The time point at which there was a maximal mean difference in A_{695} values between MPPA treated and matched control cultures after correction for differences in growth was taken as the time point at which MPPA was exhibiting maximal effect on the log phase cultures – this corresponded to 4 hours and 40 minutes. Cultures for RNA extraction were thus prepared exactly as described for the trial run and then harvested after 4 hours and 40 minutes of growth by adding 1 ml of culture (~ 1 x 10^9 cfu) to a 50 ml Falcon tube containing 2 ml of Qiagen RNA protect bacteria reagent. The tubes were then capped and shaken before being left at room temperature for 5 minutes and centrifuged at 4,200 rpm for 10 minutes. Supernatants were then siphoned off and the resulting cell pellets, 3 MPPA treated and 3 matched controls, were immediately stored at -80ºC.

SECTION 5.2.2

RNA PREPARATION, SEQUENCING & DESeq2 ANALYSIS

These steps were kindly carried out by Dr. Alison MacFadyen. RNA was extracted from cell pellets using an Ambion RiboPure Bacteria Kit and depleted using the Ambion MicrobExpress Kit (Life Technologies, Paisley, UK). rRNA depleted samples were reverse transcribed to cDNA libraries and amplified using the Ion Total RNA-Seq Kit (version 2) prior to being loaded on an Ion 316 chip for sequencing using the Ion Torrent Personal Genome Machine (Life Technologies, Paisley, UK). The sequenced genes were mapped to the UCBPP-PA14 reference genome prior to filtering and primary statistical analysis being undertaken with the DESeq2 package (Bioconductor.org).

SECTION 5.2.3

FUNCTIONAL ANALYSES

To enable systematic gene classification and detection of functional enrichment, significantly dysregulated genes were mapped to the PAO1 reference genome and assigned to 1 of 18 functional categories based on COG (cluster of orthologous groups of proteins) annotations from the *Pseudomonas* genome project (Winsor *et al*, 2016; http://pseudomonas.com/). Only 23 genes not mapping to any of the 18 categories were identified, these genes were assigned to 1 of 2 arbitrarily created categories ; W for genes not otherwise classified despite having PAO1 orthologs or X for genes not otherwise assigned due to the lack of a PAO1 ortholog. Enrichment for upregulated and downregulated genes in each functional category was determined using Fisher's Exact Test. Enrichment, or derichment, respectively, were considered to have occurred where dysregulated genes in a given functional category were proportionately over or under represented relative to their abundance in the PAO1 genome as a whole, with statistical significance at the $\alpha = 0.05$ level. Functions and pathway involvement of genes were also determined using annotations from the *Pseudomonas* genome project database (Winsor *et al*, 2016 ; http://pseudomonas.com/), KEGG database (Kanehisa *et al*, 2000 ; Kanehisa *et al*, 2016) and searches of the open literature. Dysregulated genes were also mapped to the extracytoplasmic function sigmulome of strain PA14 as determined previously using RNA and ChIP sequencing results together with global motif searches (Schulz *et al*, 2015). Enrichment for upregulated and downregulated genes in each primary sigmulon was determined using Fisher's Exact Test. Enrichment, or derichment, respectively, were considered to have occurred where dysregulated genes in a given primary sigmulon were proportionately over or under represented relative to their abundance in the PA14 genome as a whole, with statistical significance at the $\alpha = 0.05$ level.

SECTION 5.3

Transcriptomic data can yield vital clues as to the effects and possible mechanism of action of bioactive agents ; clustering of many genes that are significantly dysregulated (upon exposure to that agent) into one functionally linked category may indicate that a particular suite of cell processes or stress responses is being triggered, for example, amino-acid metabolism or membrane biogenesis. The effect of MPPA upon the transcriptome of *P. aeruginosa* PA14 was therefore evaluated by dividing all genes significantly dysregulated in response to MPPA into COGs after RNA sequencing using Ion Torrent, a next generation sequencing platform which operates by detecting the H^+ ion release coupled to the incorporation of dNTPs to extending DNA strands . For each COG grouping, statistically significant enrichments and derichments were analysed. Significant associations detected in this experiment were upregulation of genes involved in secretion (category U), motility (category N) and translation/ribosome biogenesis (category J) alongside downregulation of genes involved in lipid trafficking/metabolism (category I), amino-acid transport/metabolism (category E) and energy production/conversion (category C). These findings potentially offer an explanation for the observed phenotypic changes of increase in growth rate, motility and reduced pyocyanin production by *P. aeruginosa* as triggered by MPPA and are in fitting with the the results of carbon catabolite microarrays which indicate that PA14 can utilise MPPA as a sole carbon source. It appears possible that these phenotypes may result from carbon catabolite repression induced by MPPA breakdown products. Although unproven, it remains possible that downregulation of *dsb* genes could account for the observed phenotype of abberent protease production by MPPA exposed *P. aeruginosa* cultures observed here and in previous work which has not yet been mechanistically explained (Laux *et al*, 2002).

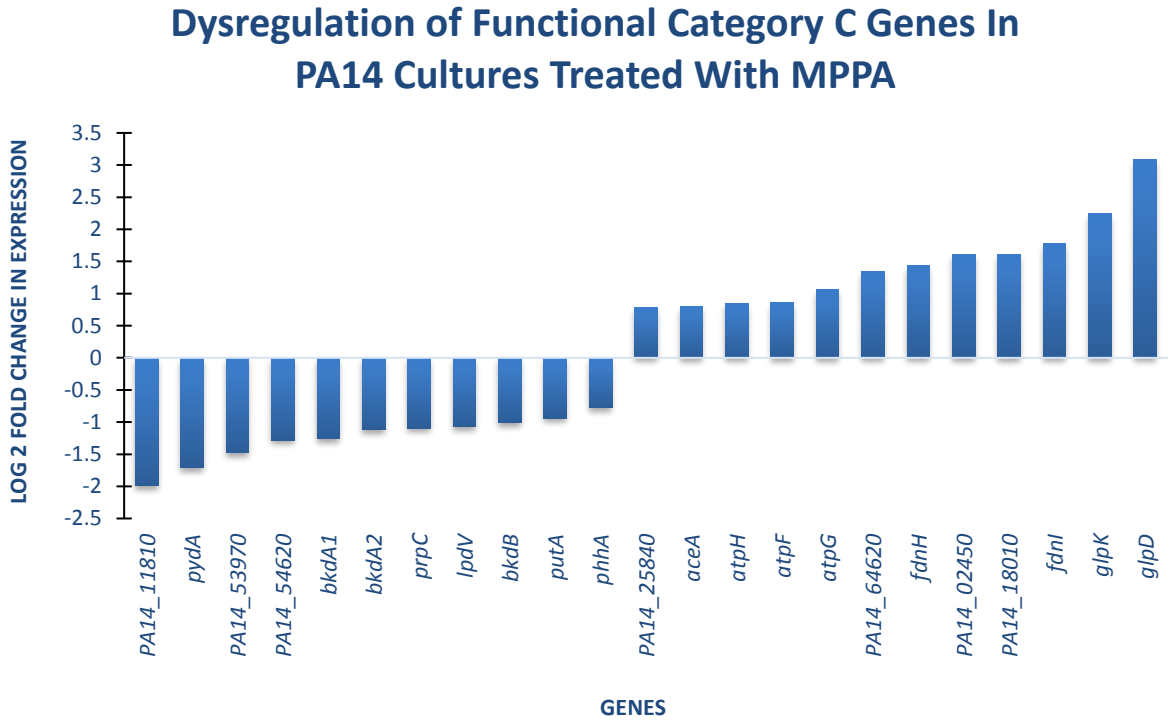


Figure 5.1.1
Quantitative Expression of PA14 Genes Involved In Energy Production and Conversion (Functional Category C) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category C Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

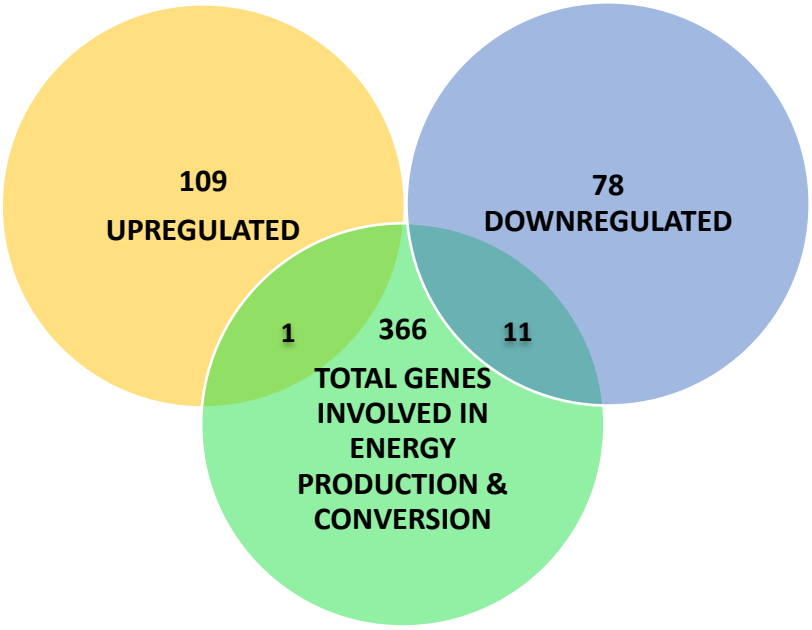


Figure 5.1.2
Number of PA14 Genes Involved In Energy Production and Conversion (Functional Category C) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category C Orthologs In Strain PAO1

366 genes, equivalent to 6.22 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 12 of 109 (11.01 %) significantly upregulated genes and 11 of 78 (14.1 %) significantly downregulated genes. This indicates enrichment of downregulated genes in functional category C ($p > 0.05$). Conversely, there is no enrichment/derichment of upregulated genes ($p < 0.05$).

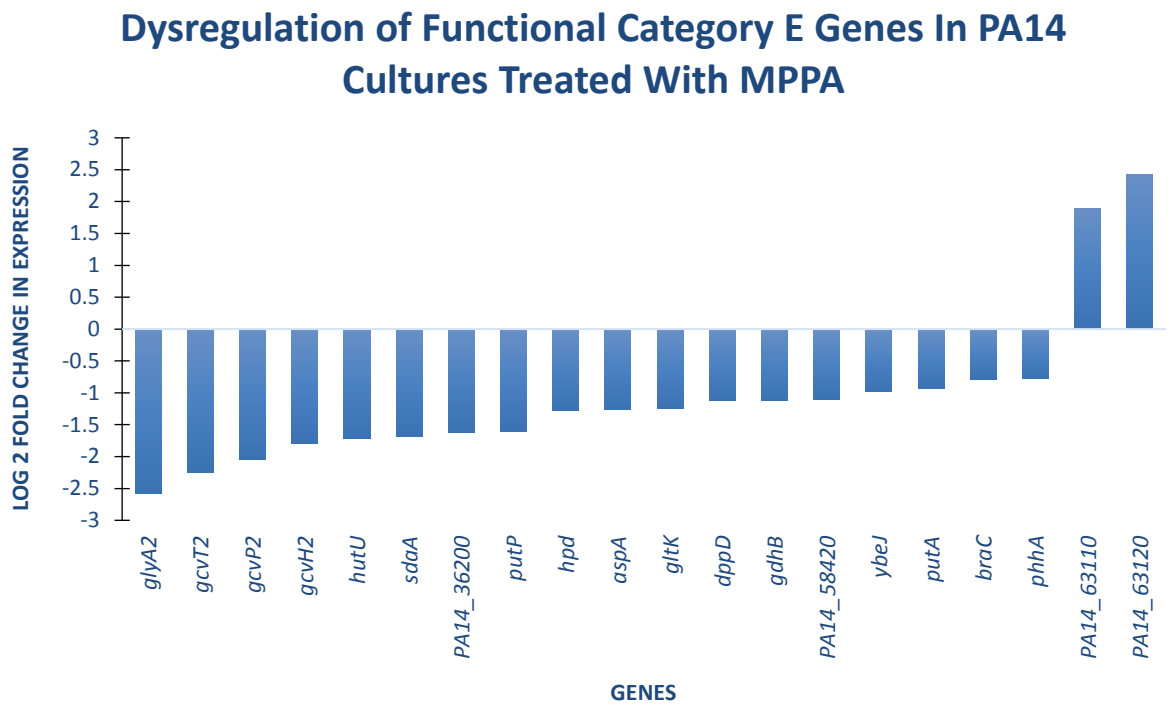


Figure 5.1.3
Quantitative Expression of PA14 Genes Involved In Amino Acid Metabolism and Transport (Functional Category E) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category E Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

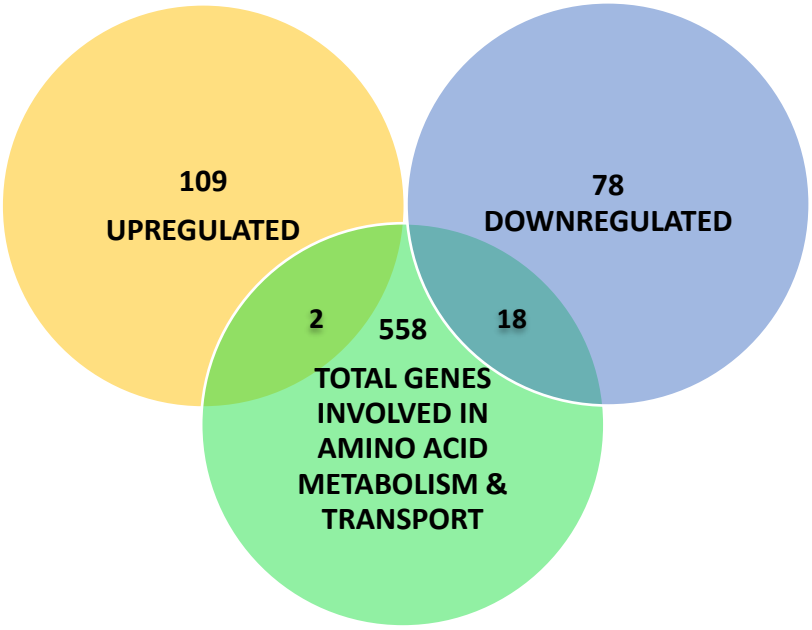


Figure 5.1.4
Number of PA14 Genes Involved In Amino Acid Metabolism and Transport (Functional Category E) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category E Orthologs In Strain PAO1

558 genes, equivalent to 9.48 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 2 of 109 (1.83 %) significantly upregulated genes and 18 of 78 (23.08 %) significantly downregulated genes. This indicates derichment of upregulated genes in functional category E ($p < 0.05$) and also enrichment of downregulated genes ($p < 0.05$).

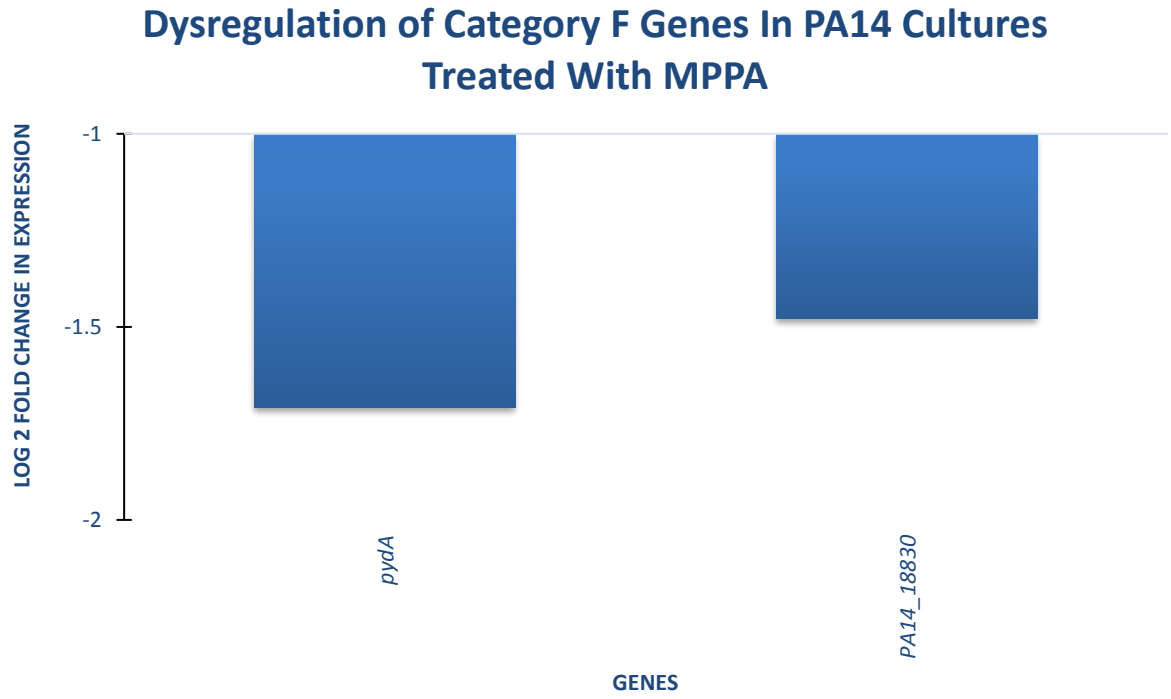


Figure 5.1.5
Quantitative Expression of PA14 Genes Involved In Nucleotide Metabolism and Transport (Functional Category F) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category F Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

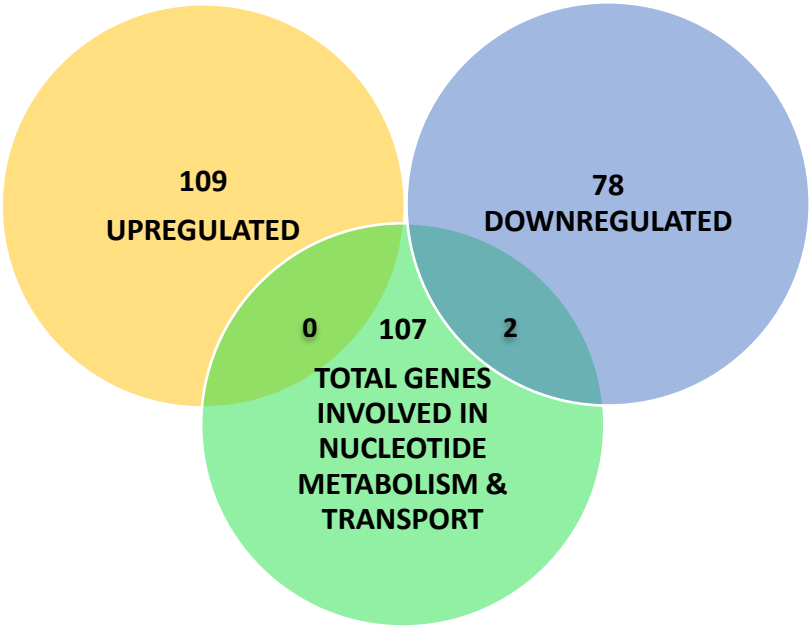


Figure 5.1.6
Number of PA14 Genes Involved In Nucleotide Metabolism and Transport (Functional Category F) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category F Orthologs In Strain PAO1

107 genes, equivalent to 1.82 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 0 of 109 (0%) significantly upregulated genes and 2 of 78 (2.56 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category F ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

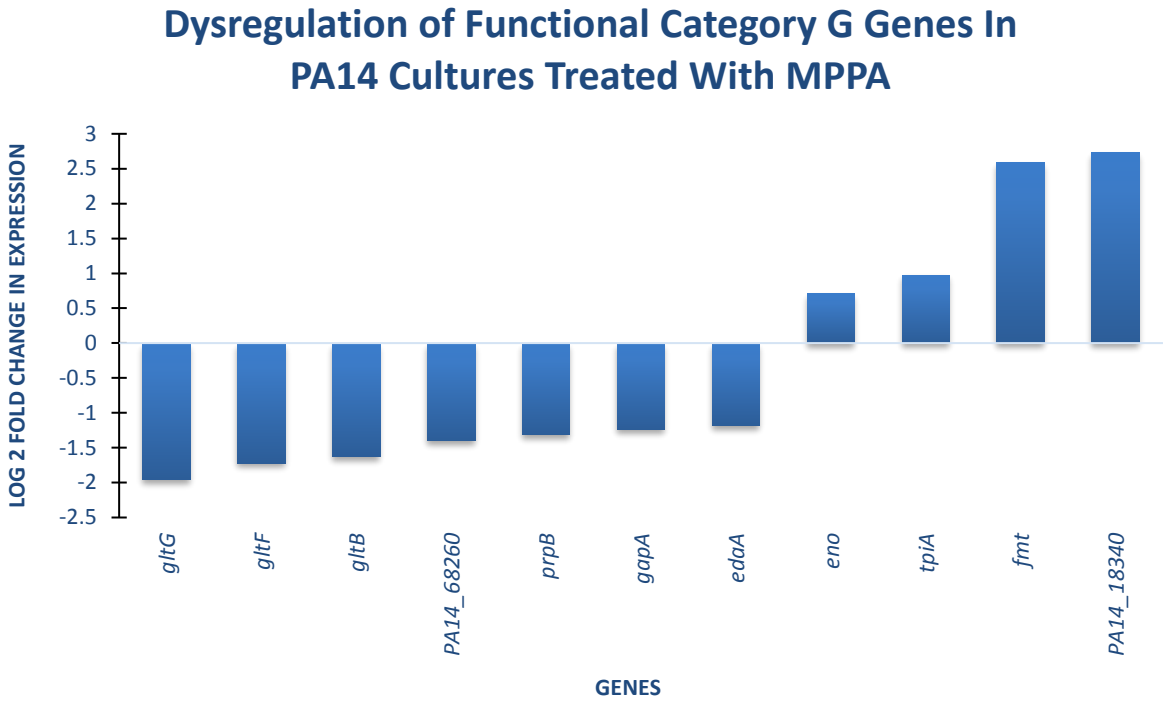


Figure 5.1.7
Quantitative Expression of PA14 Genes Involved In Carbohydrate Metabolism and Transport (Functional Category G) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category G Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

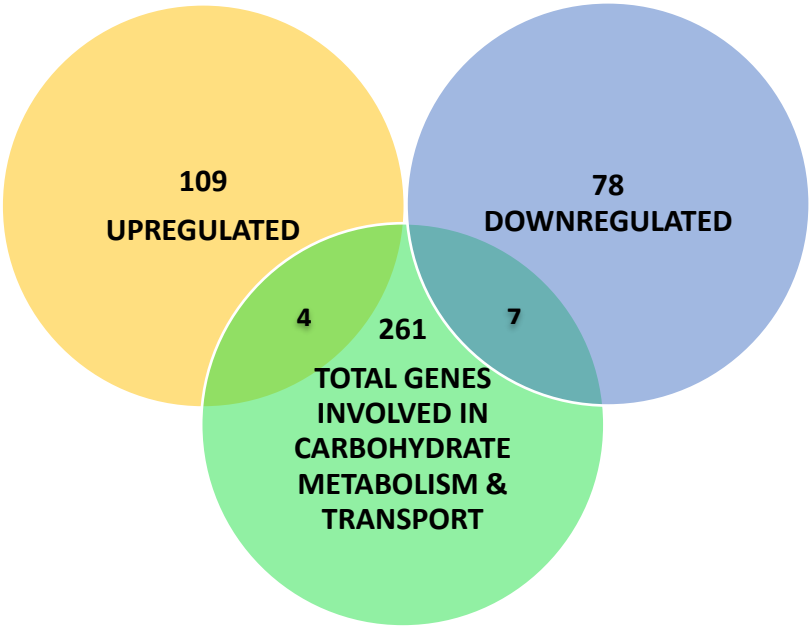


Figure 5.1.8
Number of PA14 Genes Involved In Carbohydrate Metabolism and Transport (Functional Category G) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category G Orthologs In Strain PAO1

261 genes, equivalent to 4.43 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 4 of 109 (3.67 %) significantly upregulated genes and 7 of 78 (8.97 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category G ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

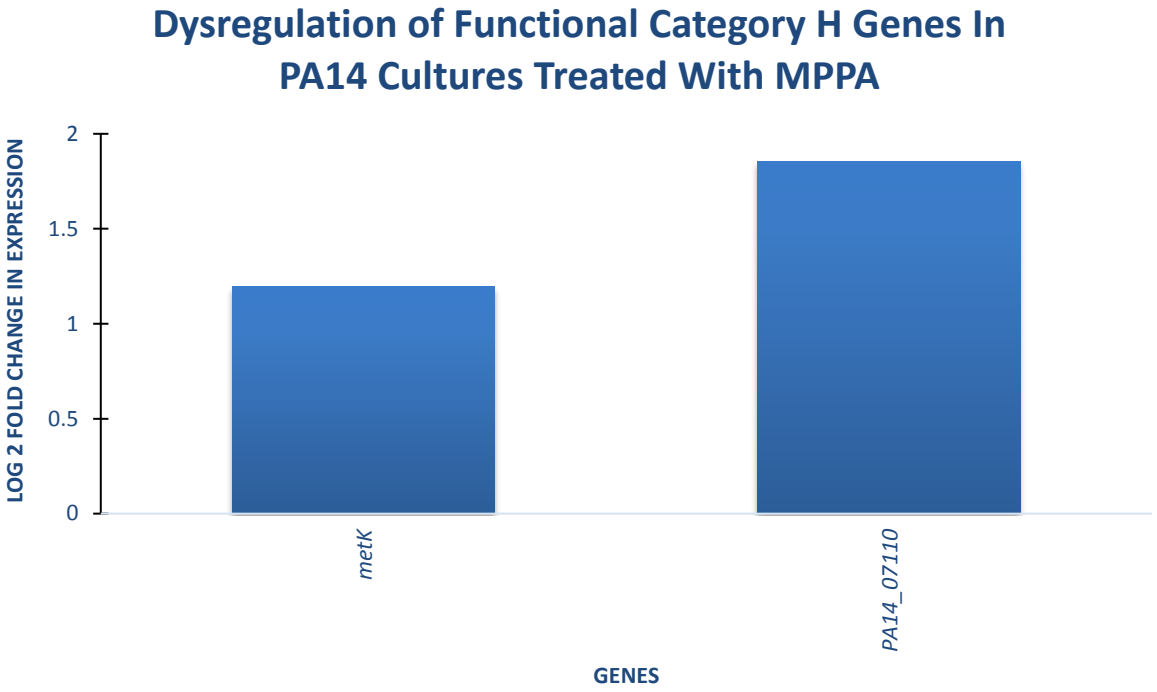


Figure 5.1.9

Quantitative Expression of PA14 Genes Involved In Carbohydrate Metabolism and Transport (Functional Category H) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category H Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

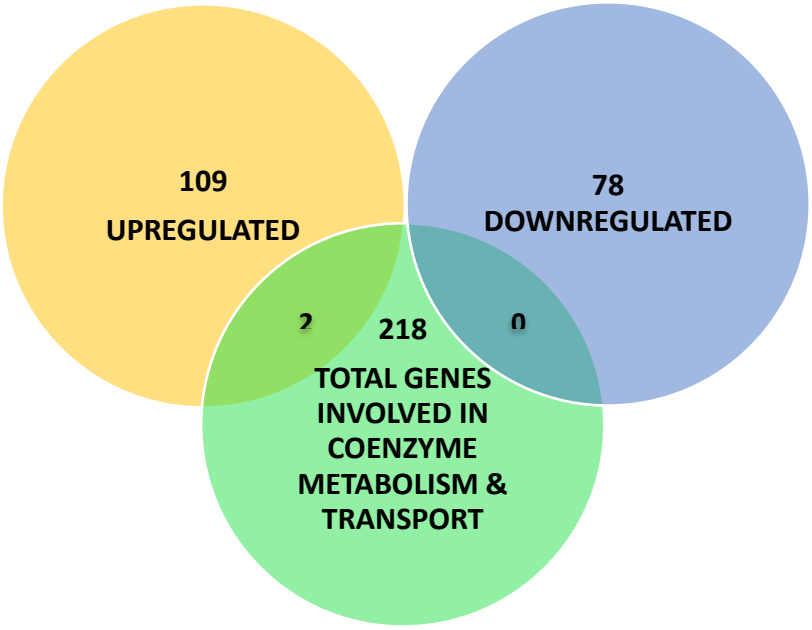


Figure 5.1.10
Number of PA14 Genes Involved In Coenzyme Metabolism and Transport (Functional Category H) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category H Orthologs In Strain PAO1

218 genes, equivalent to 3.7 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 2 of 109 (1.83 %) significantly upregulated genes and 0 of 78 (0 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category H ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

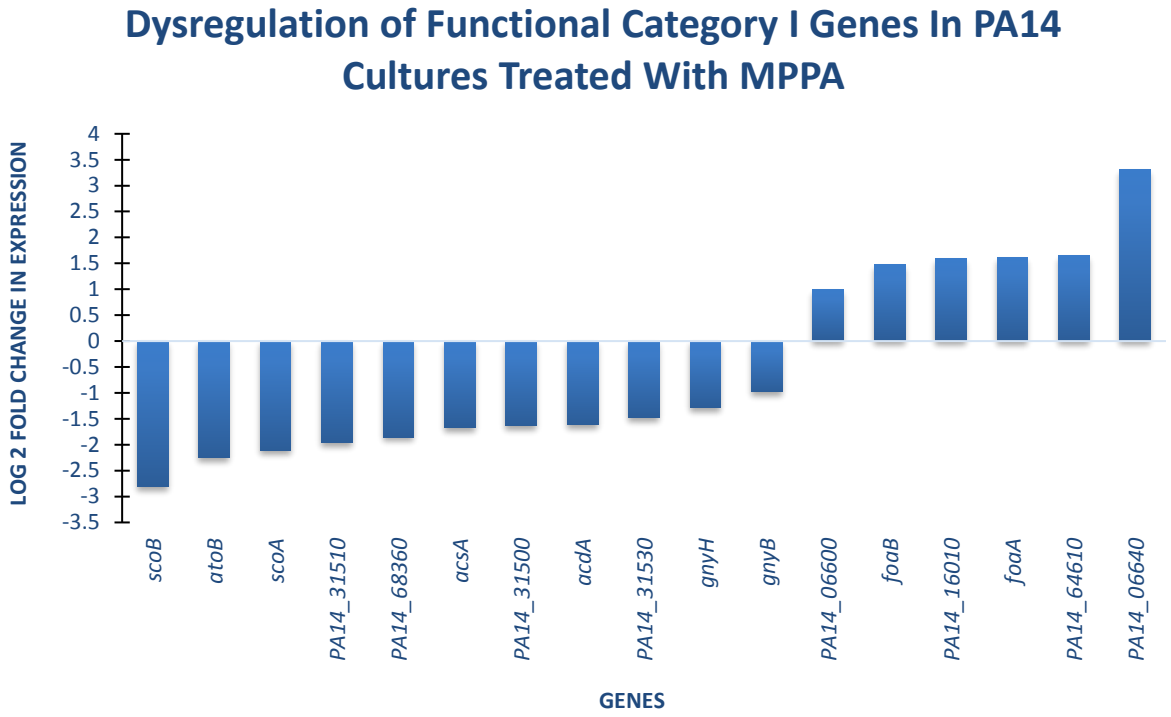


Figure 5.1.11

Quantitative Expression of PA14 Genes Involved In Lipid Metabolism and Transport (Functional Category I) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category I Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

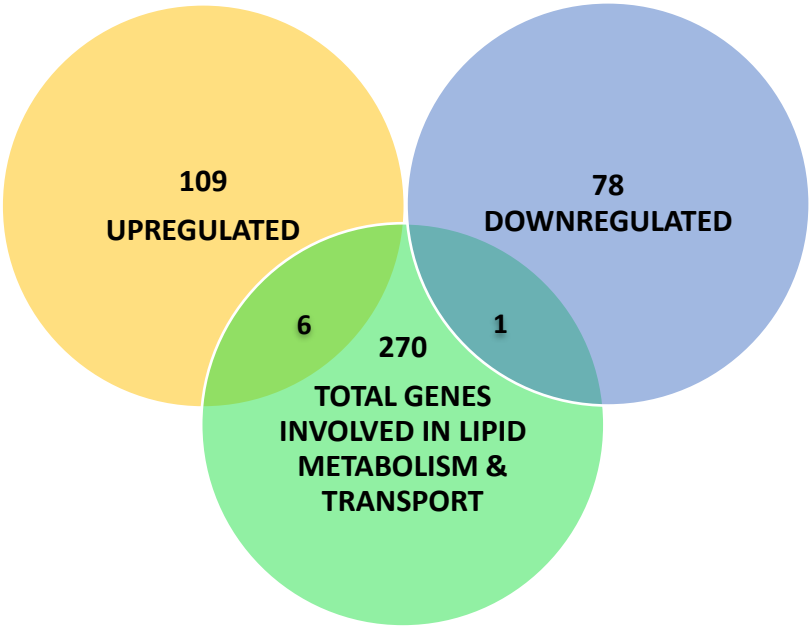


Figure 5.1.12
Number of PA14 Genes Involved In Lipid Metabolism and Transport (Functional Category I) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category I Orthologs In Strain PAO1

270 genes, equivalent to 4.59 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 6 of 109 (5.5 %) significantly upregulated genes and 11 of 78 (14.1 %) significantly downregulated genes. This does indicate enrichment of downregulated genes in functional category I ($p < 0.05$) but neither enrichment nor derichment of upregulated genes ($p > 0.05$).

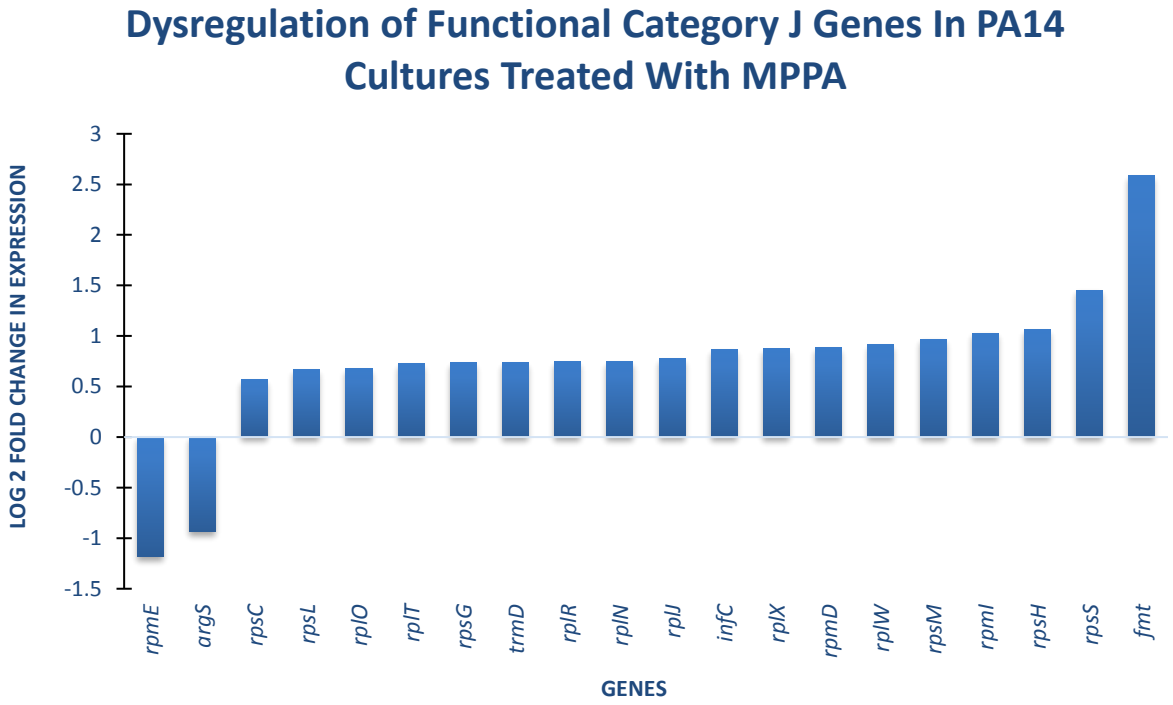


Figure 5.1.13

Quantitative Expression of PA14 Genes Involved In Translation and Ribosome Biogenesis (Functional Category J) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category J Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

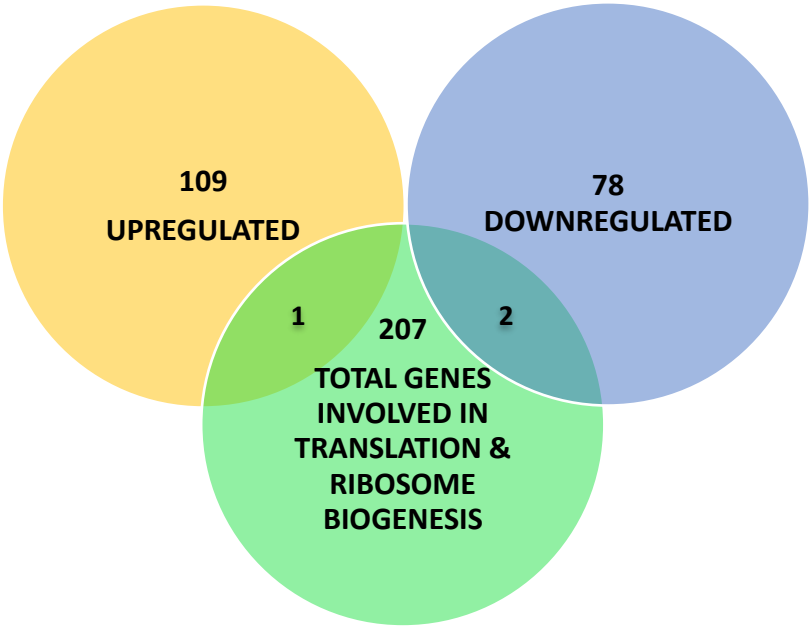


Figure 5.1.14
Number of PA14 Genes Involved In Translation and Ribosome Biogenesis (Functional Category J) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category J Orthologs In Strain PAO1

207 genes, equivalent to 3.52 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 18 of 109 (16.51 %) significantly upregulated genes and 2 of 78 (2.56 %) significantly downregulated genes. This constitutes neither enrichment nor derichment of downregulated genes in functional category J ($p > 0.05$) but does indicate enrichment of upregulated genes ($p < 0.05$).

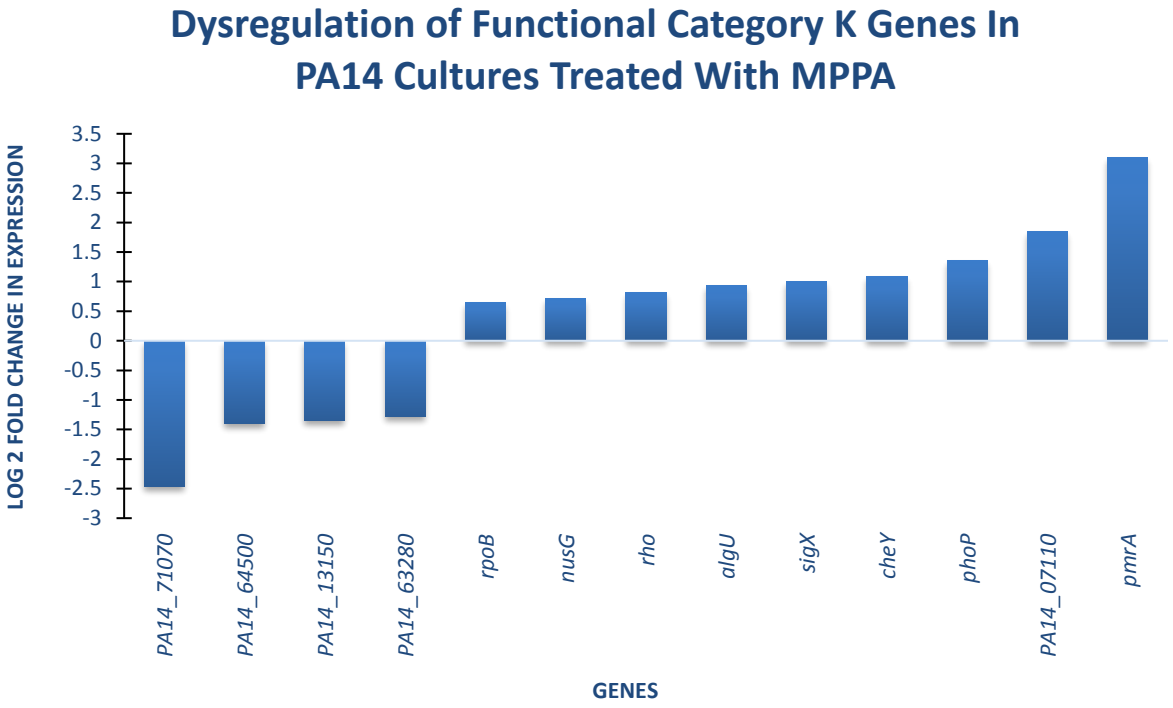


Figure 5.1.15

Quantitative Expression of PA14 Genes Involved In Transcription (Functional Category K) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category K Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

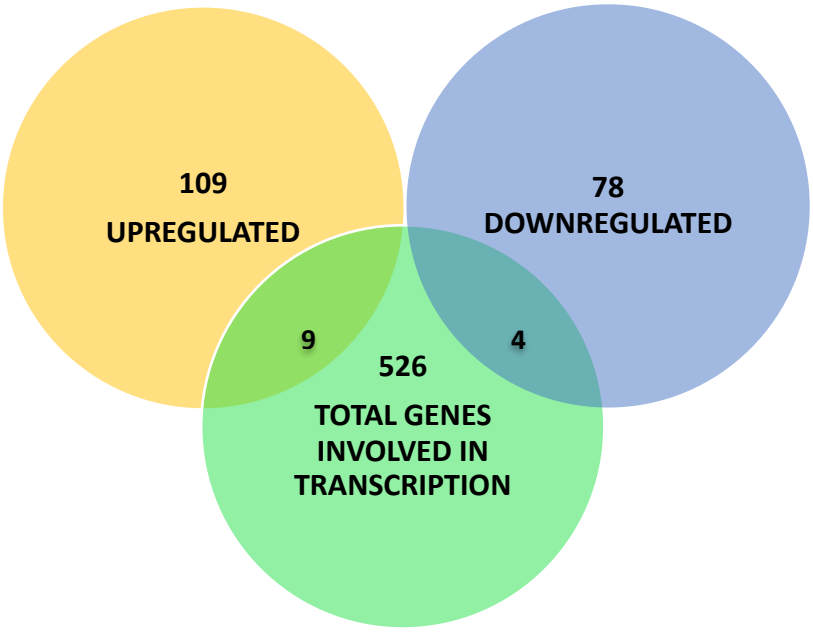


Figure 5.1.16
Number of PA14 Genes Involved In Transcription (Functional Category K) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category K Orthologs In Strain PAO1

526 genes, equivalent to 8.94 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 9 of 109 (8.26 %) significantly upregulated genes and 4 of 78 (5.13 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category K ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

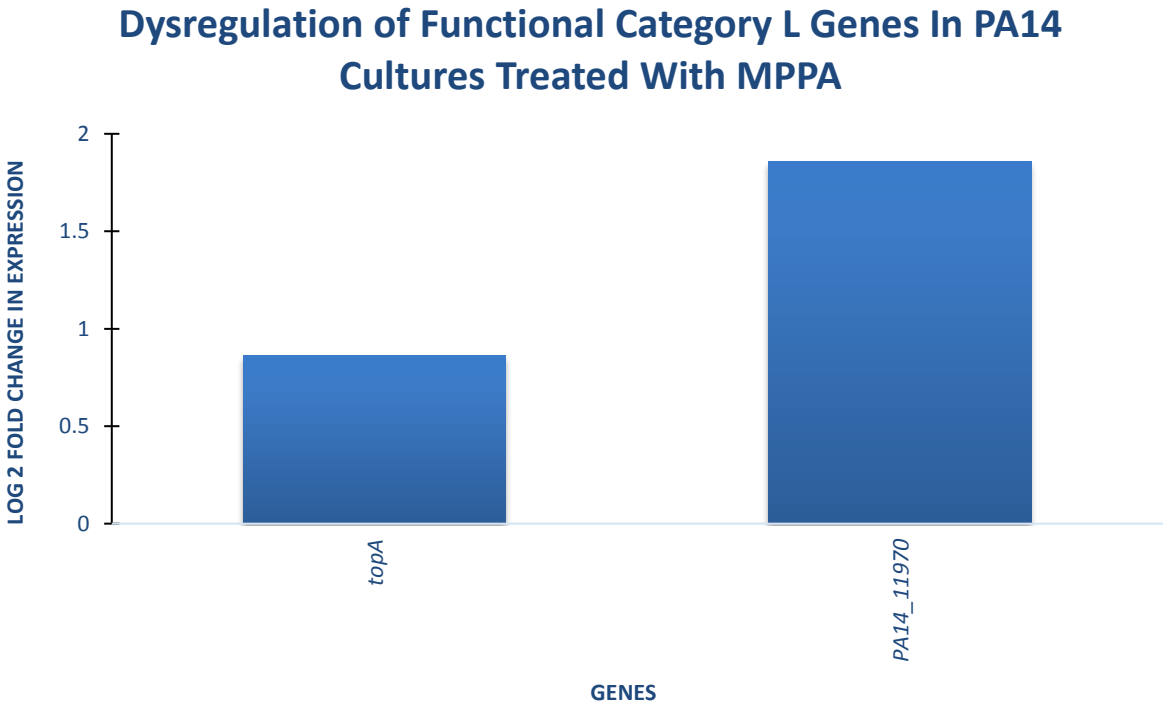


Figure 5.1.17
Quantitative Expression of PA14 Genes Involved In DNA Replication, Recombination & Repair (Functional Category L) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category L Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

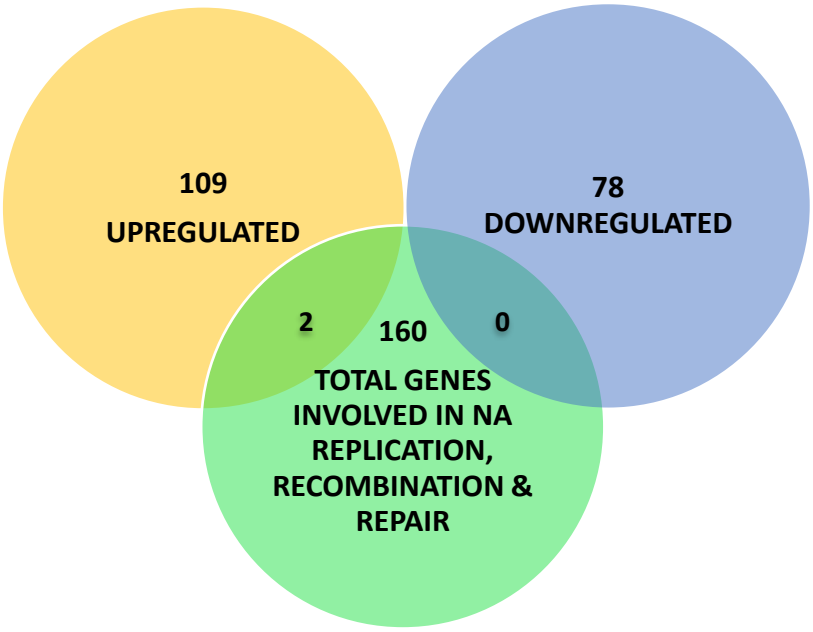


Figure 5.1.18
Number of PA14 Genes Involved In Nucleic Acid Replication, Recombination and Repair (Functional Category L) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category L Orthologs In Strain PAO1

160 genes, equivalent to 2.72 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 2 of 109 (1.83 %) significantly upregulated genes and 0 of 78 (0 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category L ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

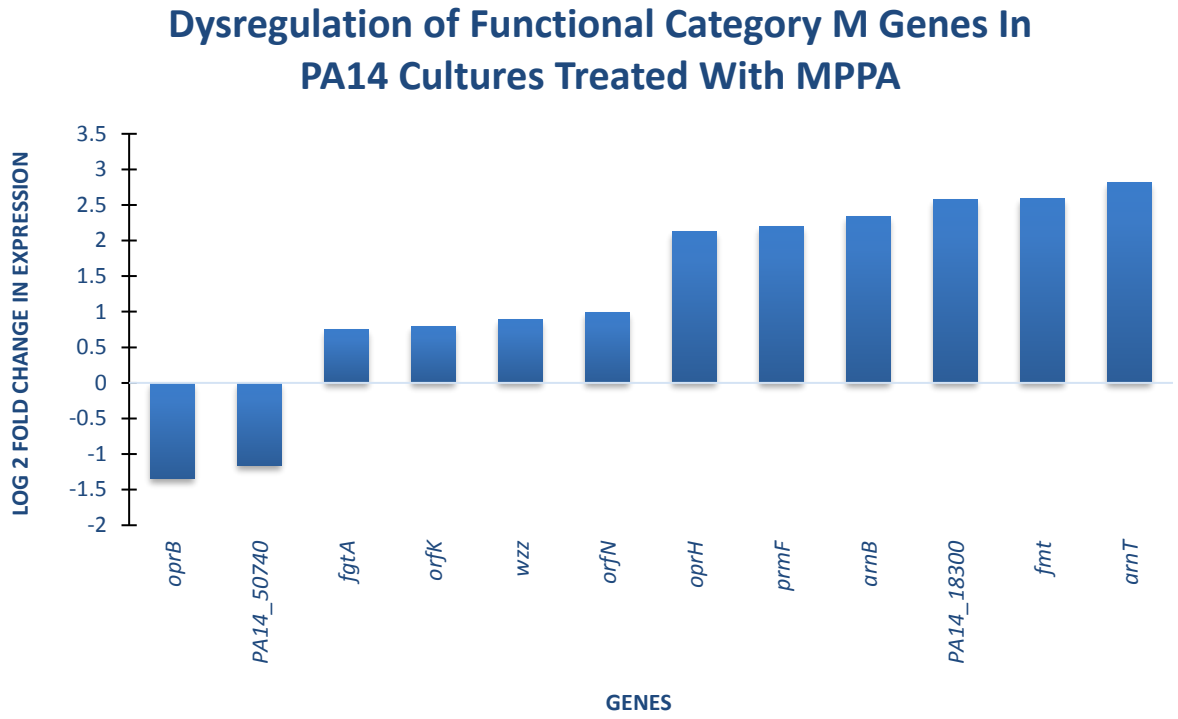


Figure 5.1.19
Quantitative Expression of PA14 Genes Involved In Cell Envelope Biogenesis and Repair (Functional Category M) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category M Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

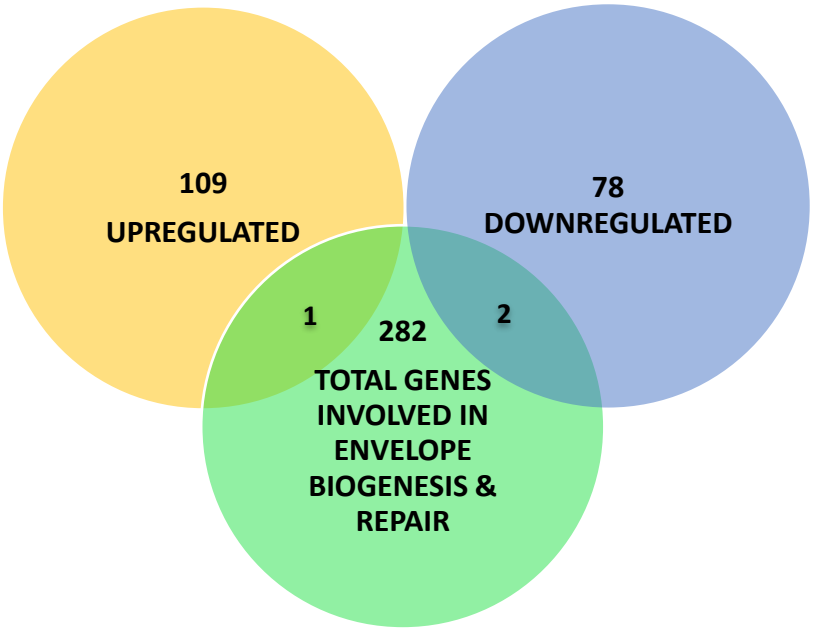


Figure 5.1.20
Number of PA14 Genes Involved In Cell Envelope Biogenesis and Repair (Functional Category M) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category M Orthologs In Strain PAO1

282 genes, equivalent to 4.79 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 10 of 109 (9.17 %) significantly upregulated genes and 2 of 78 (2.56 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category M ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

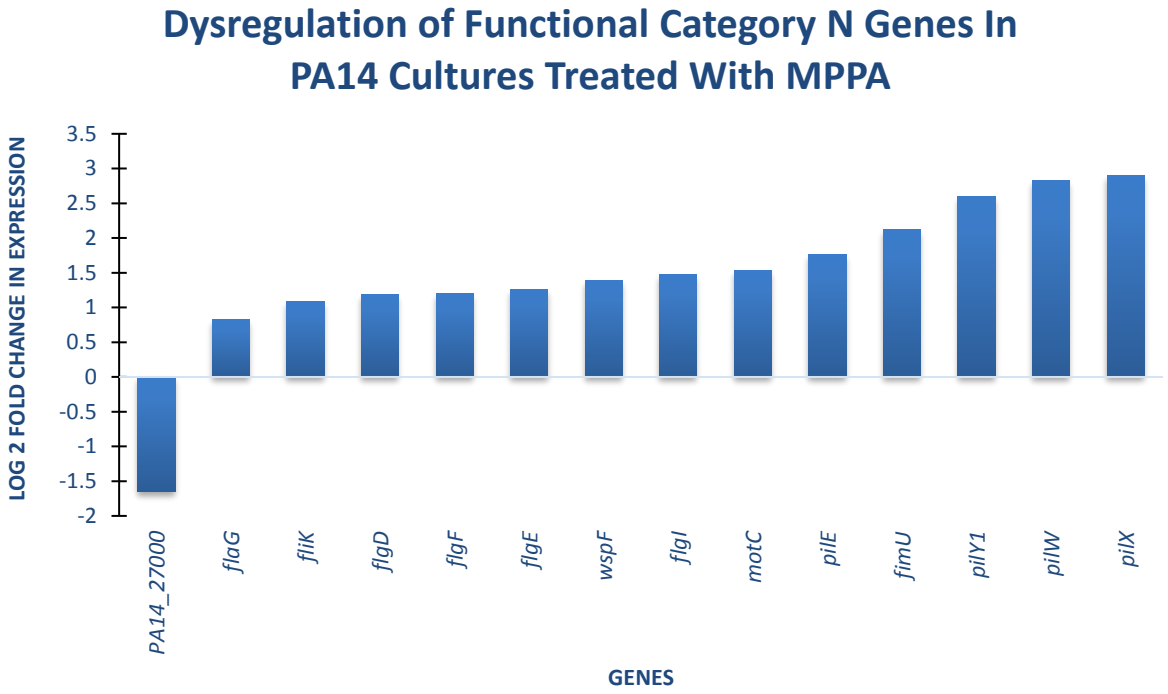


Figure 5.1.21
Quantitative Expression of PA14 Genes Involved In Cell Motility (Functional Category N) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category N Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

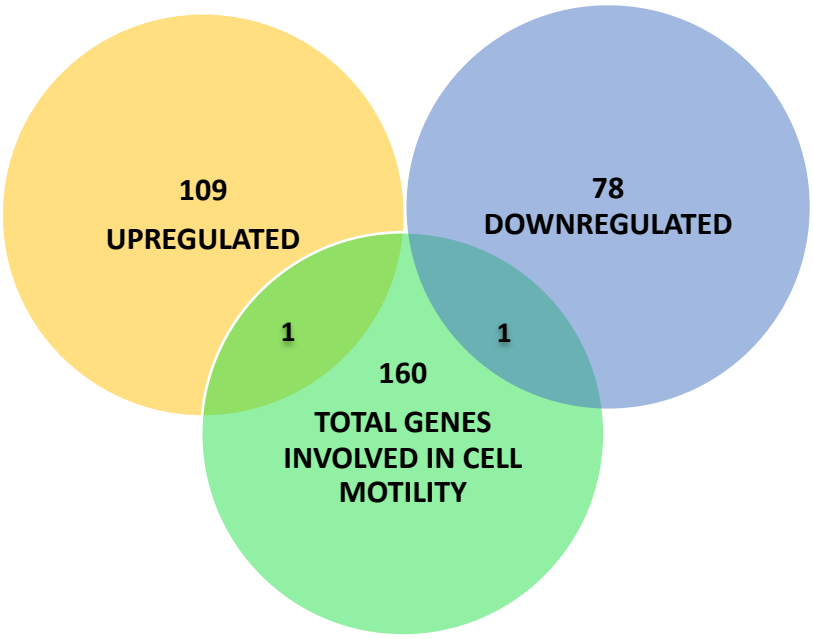


Figure 5.1.22
Number of PA14 Genes Involved In Cell Motility (Functional Category N) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category N Orthologs In Strain PAO1

160 genes, equivalent to 2.72 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 13 of 109 (11.93 %) significantly upregulated genes and 1 of 78 (1.28 %) significantly downregulated genes. This constitutes neither enrichment nor derichment of downregulated genes in functional category N ($p > 0.05$) but does indicate enrichment of upregulated genes ($p < 0.05$).

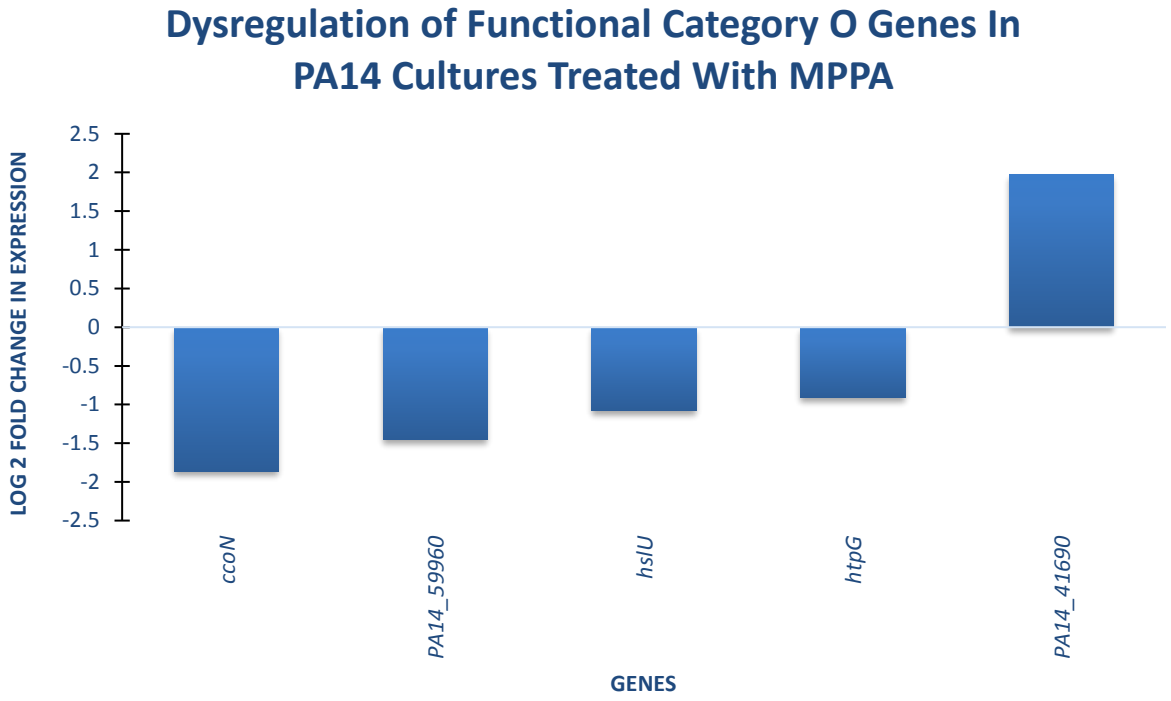


Figure 5.1.23
Quantitative Expression of PA14 Genes Involved In Post-Translational Modification, Protein Turnover and Chaperoning (Functional Category O) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category O Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

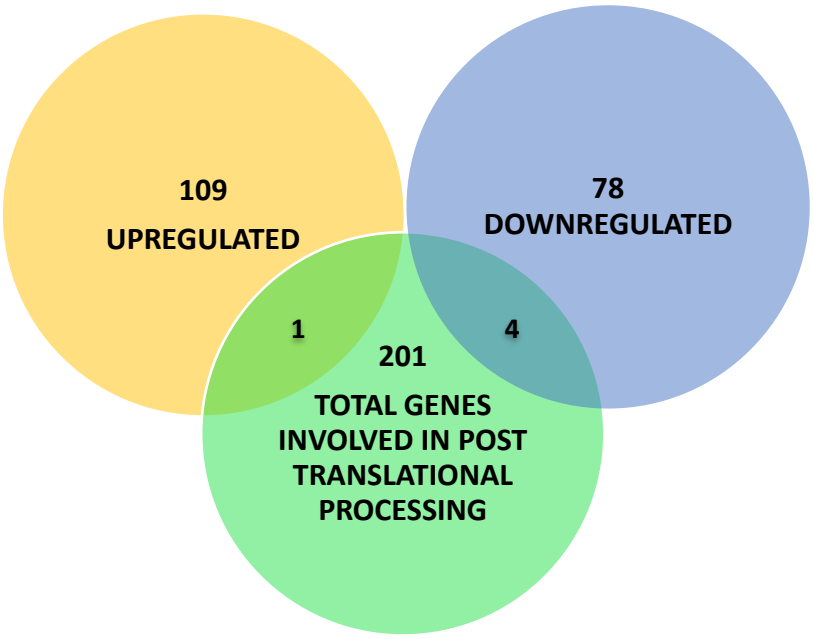


Figure 5.1.24
Number of PA14 Genes Involved In Post-Translational Modification, Protein Turnover and Chaperoning (Functional Category O) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category O Orthologs In Strain PAO1

201 genes, equivalent to 3.41 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 1 of 109 (0.92 %) significantly upregulated genes and 4 of 78 (5.13 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category O ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

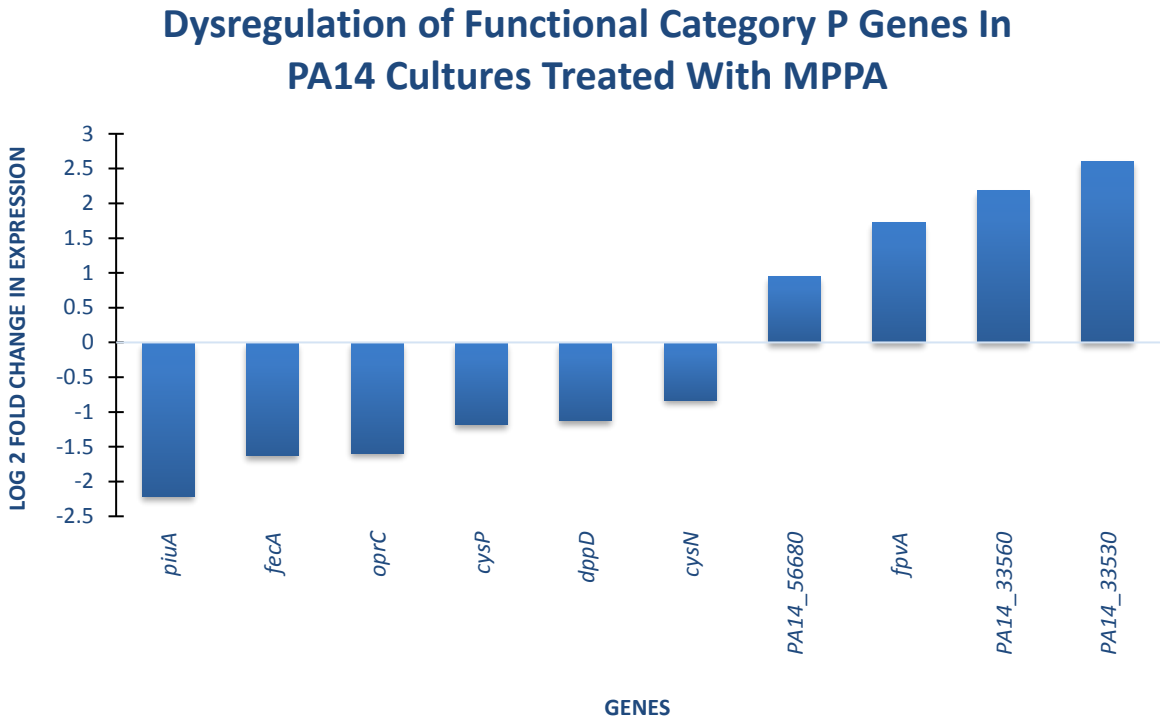


Figure 5.1.25
Quantitative Expression of PA14 Genes Involved In Inorganic Ion Metabolism and Transport (Functional Category P) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category P Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

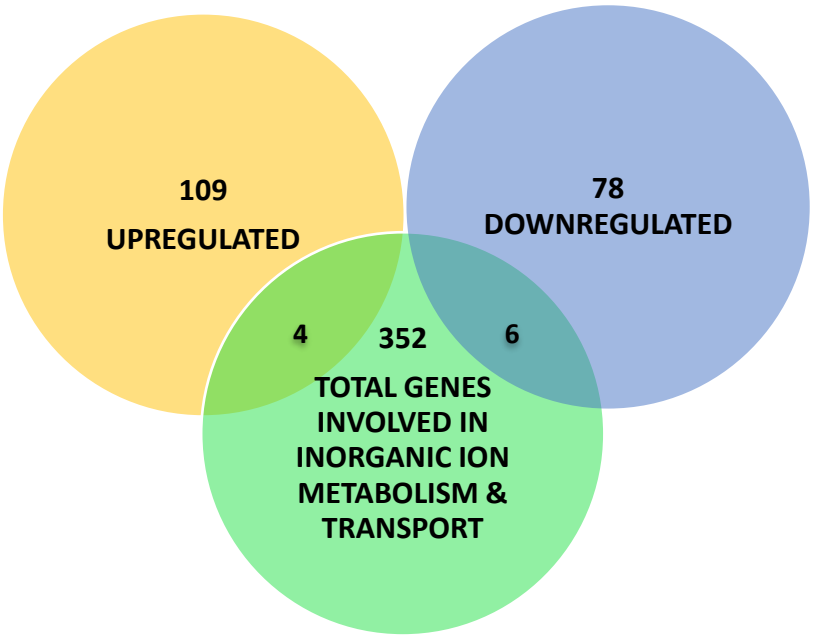


Figure 5.1.26
Number of PA14 Genes Involved In Inorganic Ion Metabolism and Transport (Functional Category P) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category P Orthologs In Strain PAO1

352 genes, equivalent to 5.98 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 4 of 109 (3.67 %) significantly upregulated genes and 6 of 78 (7.69 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category P ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

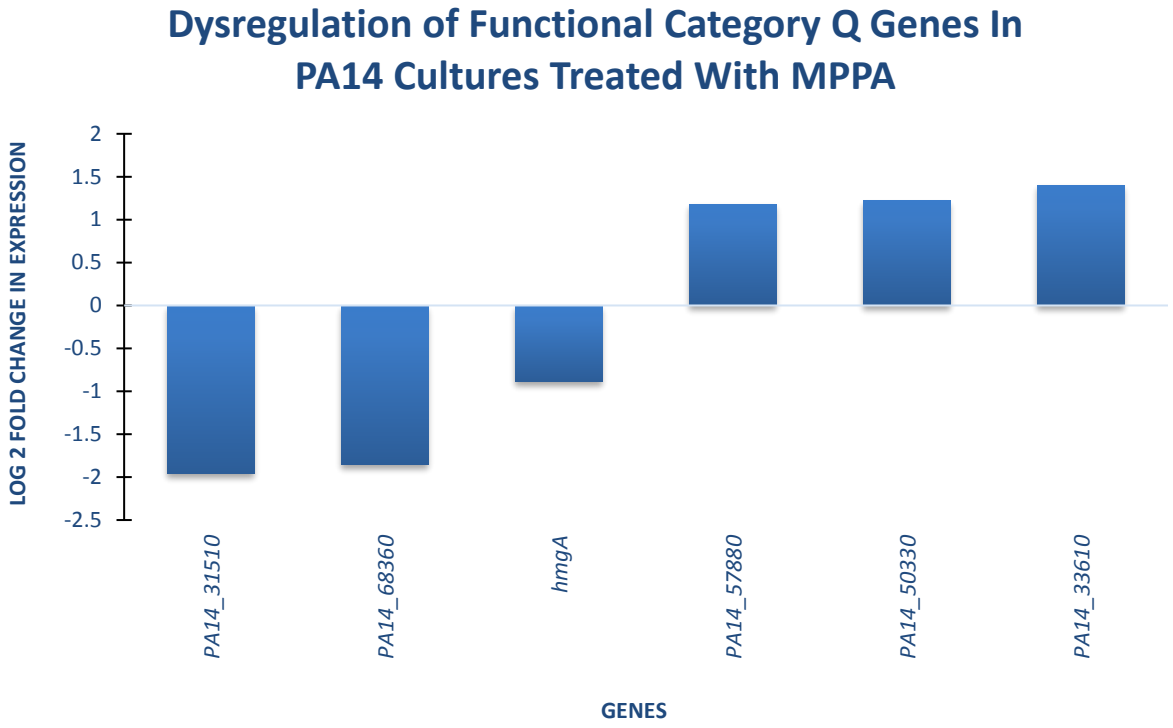


Figure 5.1.27
Quantitative Expression of PA14 Genes Involved In Secondary Metabolite Synthesis and Transport (Functional Category Q) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category Q Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

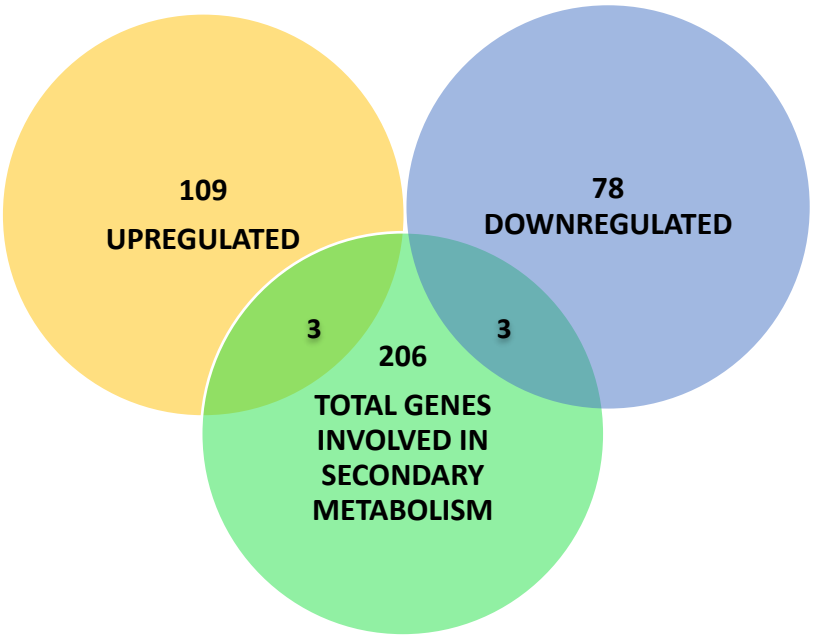


Figure 5.1.28
Number of PA14 Genes Involved In Secondary Metabolite Synthesis and Transport (Functional Category Q) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category Q Orthologs In Strain PAO1

206 genes, equivalent to 3.5 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 3 of 109 (2.75 %) significantly upregulated genes and 3 of 78 (3.85 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category Q ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

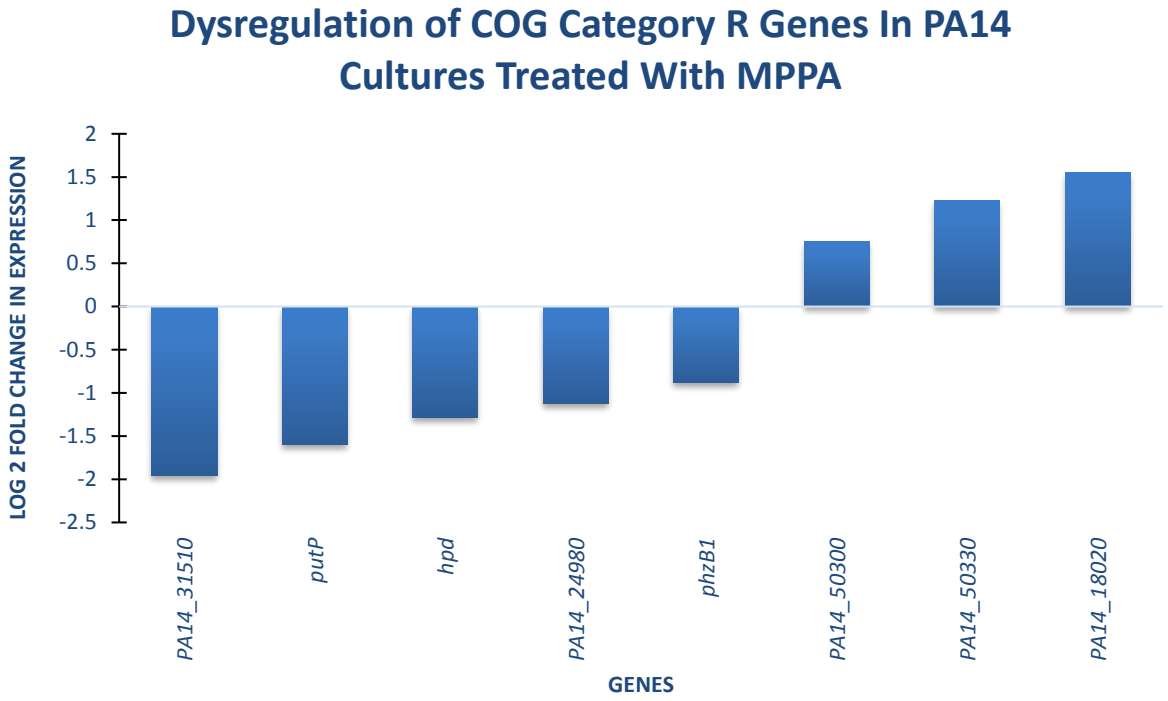


Figure 5.1.29

Quantitative Expression of PA14 Genes With General Functional Predictions Only (Functional Category R) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category R Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

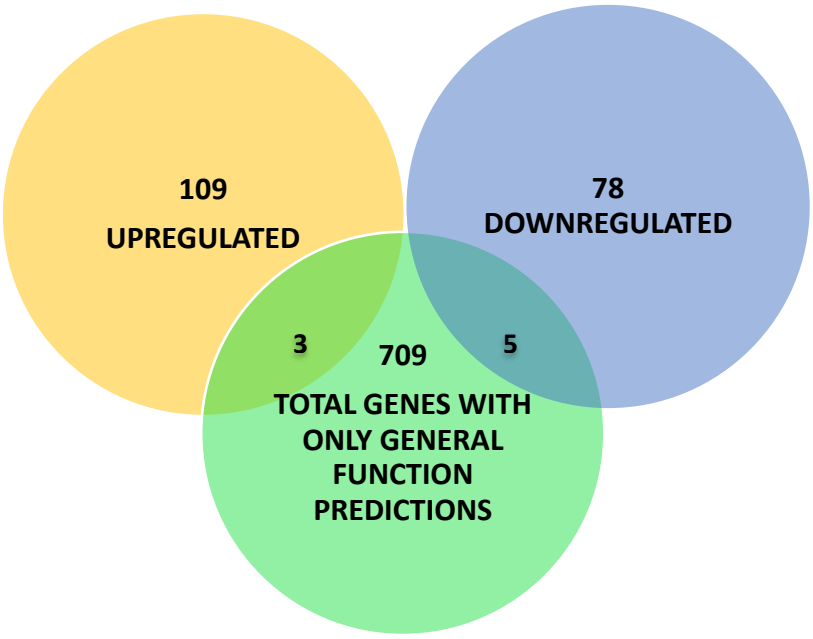


Figure 5.1.30
Number of PA14 Genes With Only General Functional Predictions (Functional Category R) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category R Orthologs In Strain PAO1

709 genes, equivalent to 12.05 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 3 of 109 (2.75 %) significantly upregulated genes and 5 of 78 (6.41 %) significantly downregulated genes. This constitutes neither enrichment nor derichment of downregulated genes in functional category R ($p > 0.05$) but does indicate derichment of upregulated genes ($p < 0.05$).

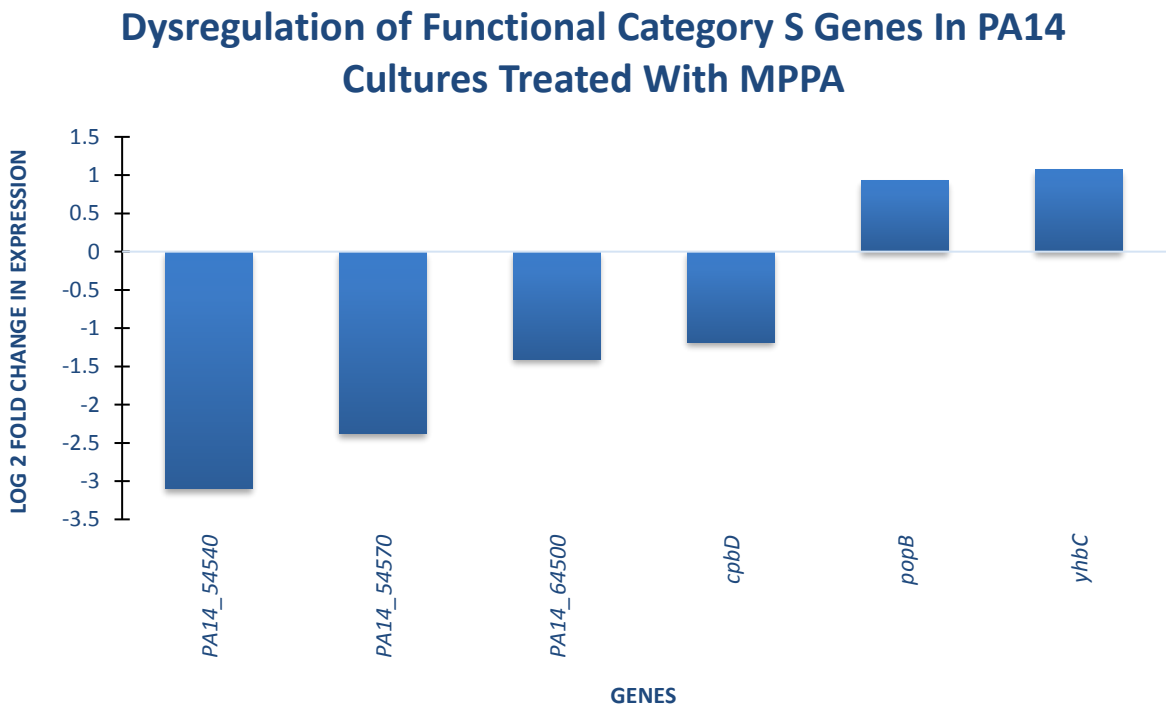


Figure 5.1.31

Quantitative Expression of PA14 Genes With Unknown Function (Functional Category S) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category S Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

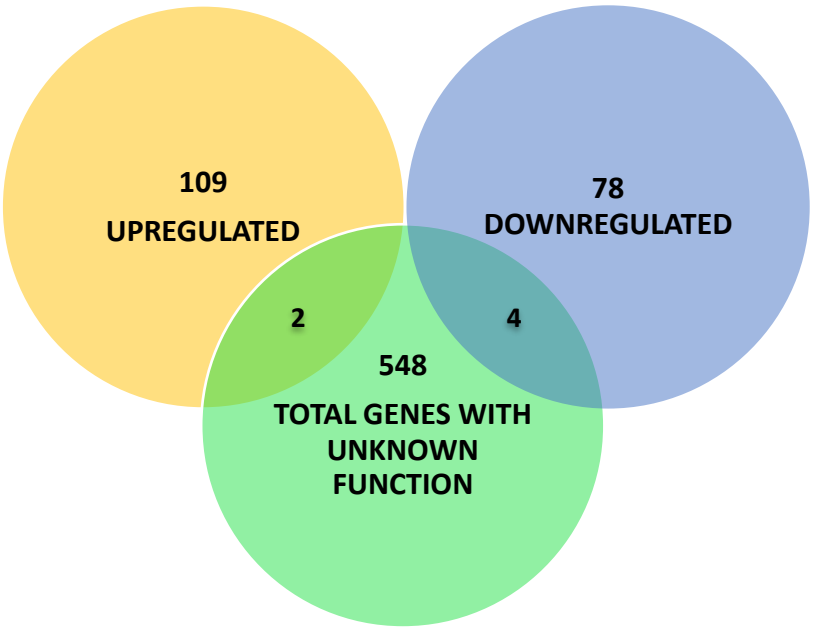


Figure 5.1.32

Number of PA14 Genes With Unknown Function (Functional Category S) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category S Orthologs In Strain PAO1

548 genes, equivalent to 9.31 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 2 of 109 (1.83 %) significantly upregulated genes and 5 of 78 (5.13 %) significantly downregulated genes. This constitutes neither enrichment nor derichment of downregulated genes in functional category S ($p > 0.05$) but does indicate derichment of upregulated genes ($p < 0.05$).

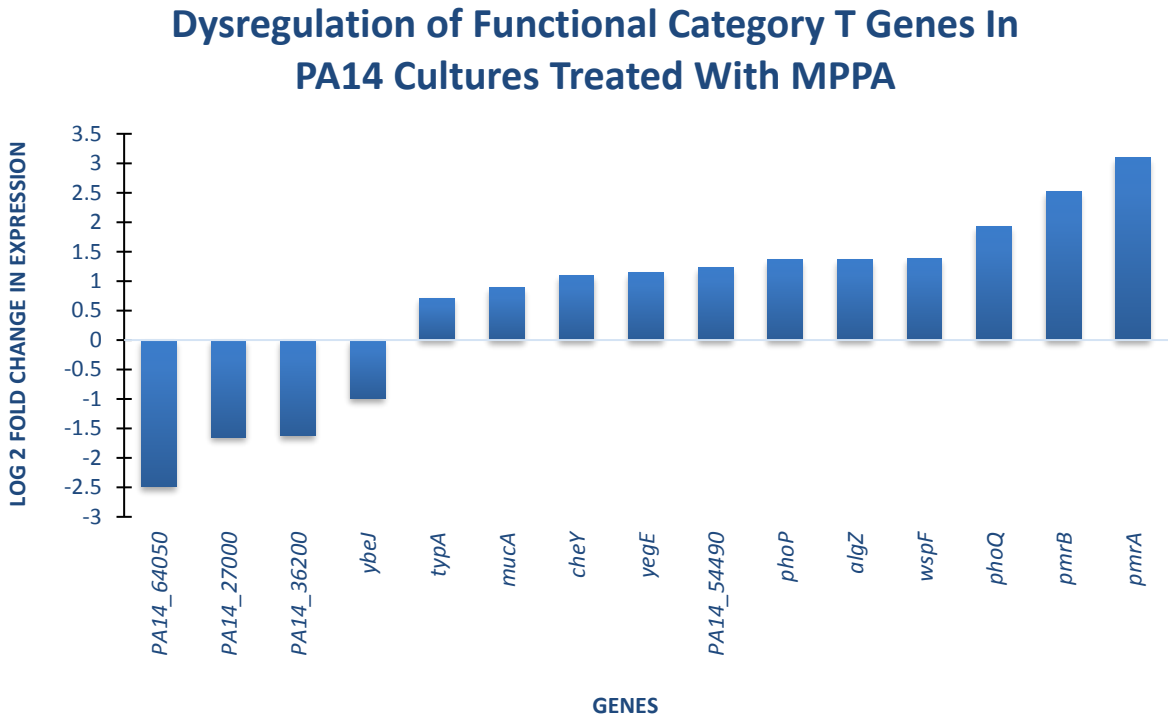


Figure 5.1.33
Quantitative Expression of PA14 Genes Involved In Signal Transduction (Functional Category T) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category T Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

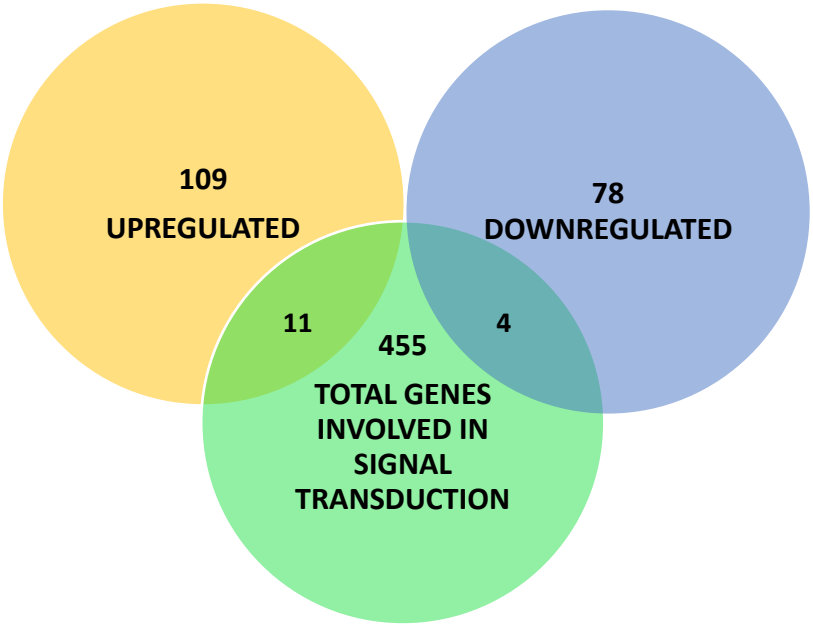


Figure 5.1.34
Number of PA14 Genes That Are Involved In Signal Transduction (Functional Category T) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category T Orthologs In Strain PAO1

455 genes, equivalent to 7.73 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 11 of 109 (10.1 %) significantly upregulated genes and 4 of 78 (5.13 %) significantly downregulated genes. This neither constitutes enrichment/derichment of downregulated genes in functional category T ($p > 0.05$) nor of upregulated genes ($p > 0.05$).

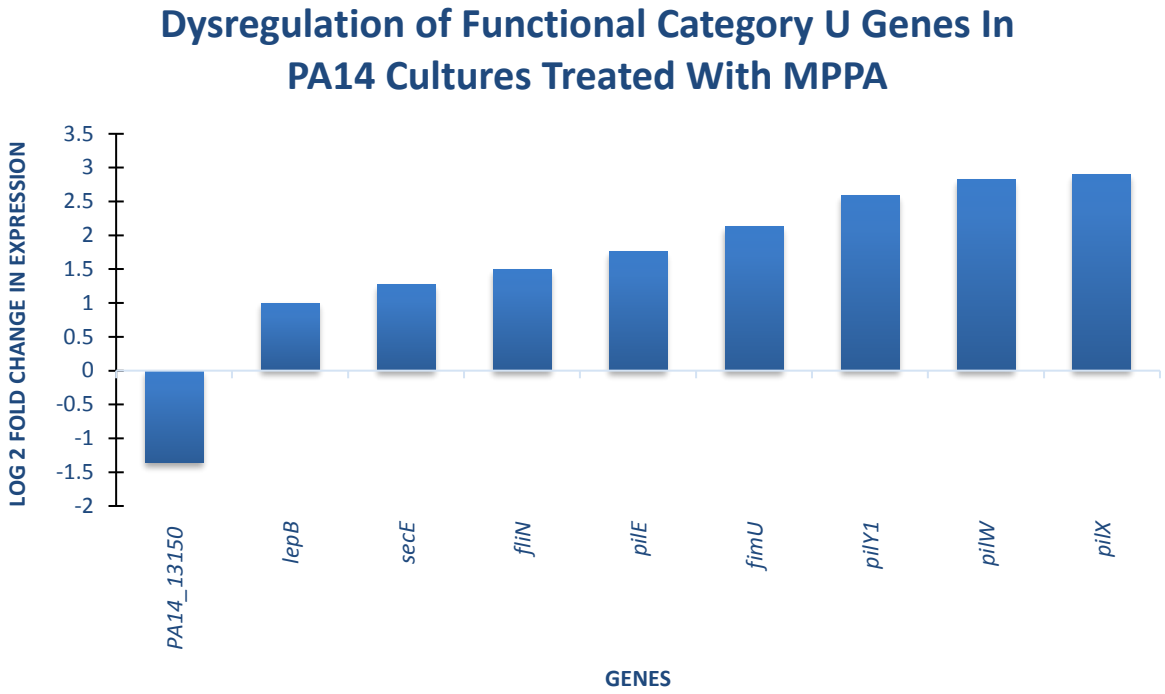


Figure 5.1.35

Quantitative Expression of PA14 Genes Involved With Secretion (Functional Category U) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category U Orthologs In Strain PAO1

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

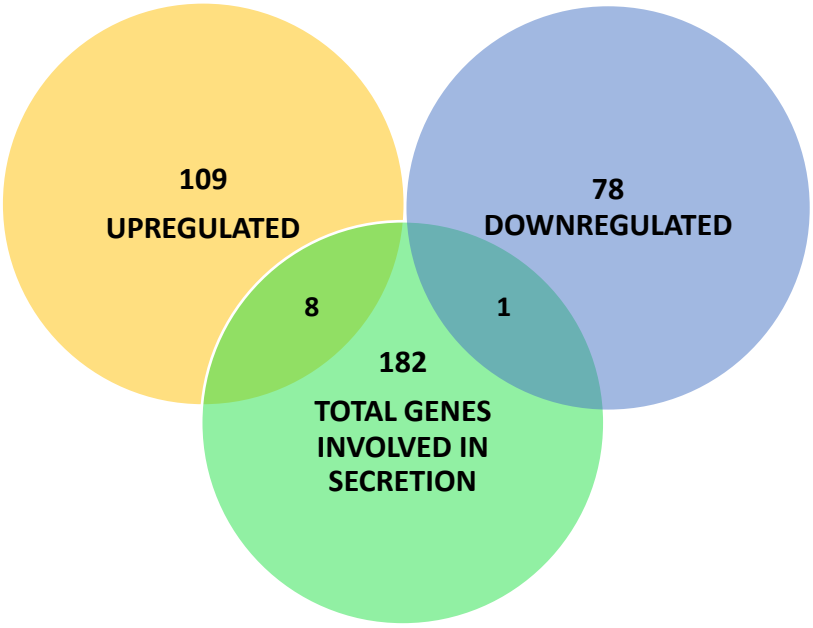


Figure 5.1.36

Number of PA14 Genes Involved With Secretion (Functional Category U) That Are Significantly Dysregulated on Exposure to MPPA, Represented As A Proportion of The Total Number of Category U Orthologs In Strain PAO1

182 genes, equivalent to 3.09 % of the whole PAO1 genome of 5886 open reading frames, belong to this functional category relative to 8 of 109 (7.34 %) significantly upregulated genes and 1 of 78 (1.28 %) significantly downregulated genes. This constitutes neither enrichment nor derichment of downregulated genes in functional category U ($p > 0.05$) but does indicate enrichment of upregulated genes ($p < 0.05$).

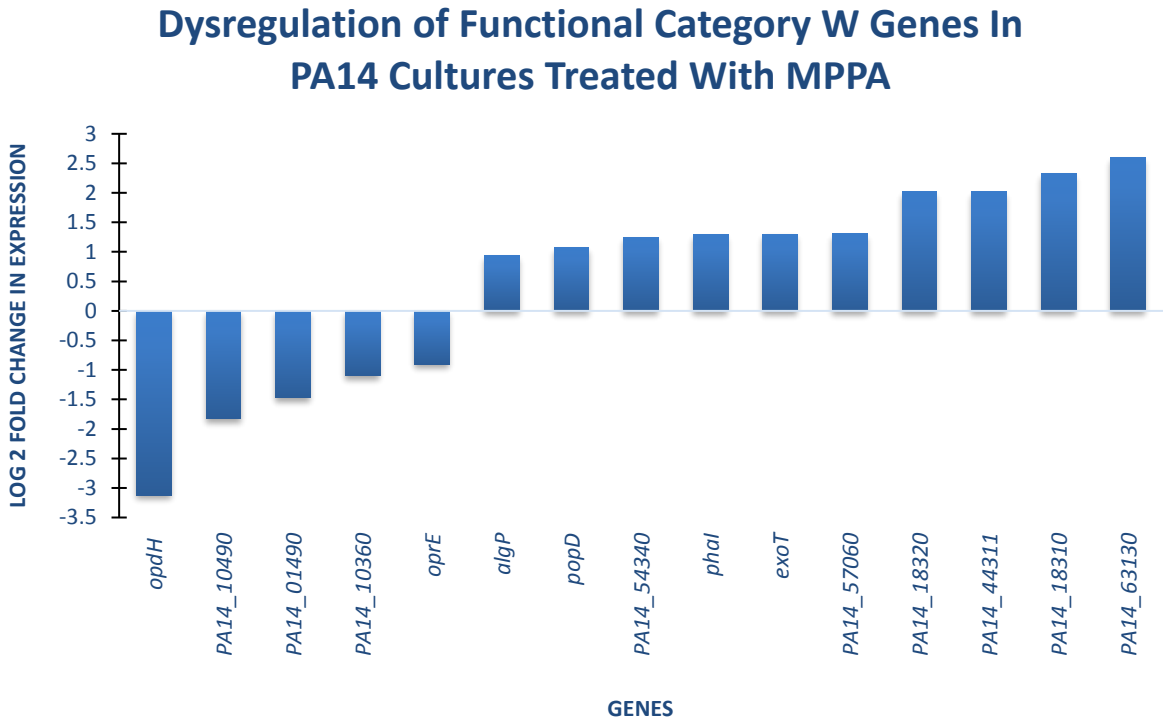


Figure 5.1.37
Quantitative Expression of PA14 Genes Not Otherwise Classified (Functional Category W) That Are Significantly Dysregulated on Exposure to MPPA

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

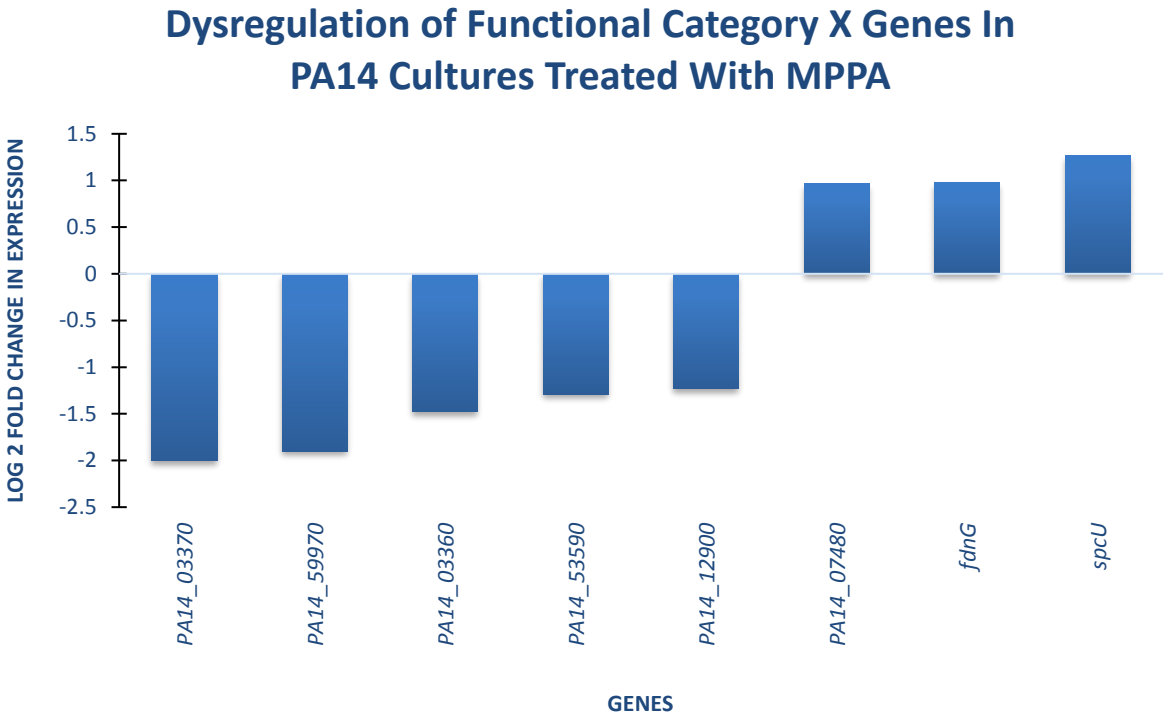


Figure 5.1.38

Quantitative Expression of PA14 Genes Without PAO1 Orthologs (Functional Category X) That Are Significantly Dysregulated on Exposure to MPPA

All genes presented had an adjusted *p* value of less than 0.1 when analysed with the DESeq2 package.

Significantly dysregulated genes were grouped into functional categories based on clusters of orthologous groups (COGS) and functional enrichments (or derichments) were determined for each COG, normalised to their relative abundances in the PAO1 genome as a whole (Figures 5.1.1 – 5.1.38). COG annotations were taken from the *Pseudomonas* genome project database (Winsor et al, 2016 ; http://pseudomonas.com) . Functions of individual significantly dysregulated genes are outlined in table 5.1 and these were also taken from the *Pseudomonas* genome project and KEGG databases (Kanehisa et al, 2000 ; Kanehisa et al, 2016).

RESULTS – SIGMULON GROUPINGS

Enrichment of multiple genes governed by a single sigma factor in response to a bioactive agent may indicate its involvement in response to that agent. As MPPA is suspected of affecting envelope structure, MPPA dysregulated genes were mapped according to extracytoplasmic sigma factors known to govern them in attempt to screen for the possible involvement of these sigma factors in response to MPPA. The genes listed in table 5.1 have been grouped according to extracytoplasmic function sigmulons as determined by RNA and chromatin immunoprecipation sequencing experiments previously published by Schulz and colleagues (Schulz *et al*, 2015). Stastically significant associations were found for genes within the *rpoD*, *rpoN* and *sigX* sigmulons indicating possible regulatory functions for these genes occurring in response to MPPA, suggesting a role for these genes in the MPPA master regulome.

Upregulated In Response To MPPA		KEY				DOWNREGULATED			UPREGULATED			
LOCUS	GENE	AlgU‡	FliA	PvdS	RpoH	RpoN	RpoS	SigX‡	RpoD	FpvI	FecI	Total
PA14_08910	rpsC 50S ribosomal protein S3					X		X				
PA14_08760	rpoB RNA polymerase β chain											
PA14_08790	rpsL 30S ribosomal protein S12					X		X				
PA14_09040	rplO 50S ribosomal protein L15					X						
PA14_17320	eno enolase											
PA14_67560	typA GTP binding protein					X						
PA14_08710	nusG transcription antitermination factor					X						
PA14_28680	rplT 50S ribosomal protein L20								X			
PA14_08810	rpsG 30S ribosomal protein S7											
PA14_15990	trmD tRNA (guanine-N1) methyltransferase					X						
PA14_50300	hypothetical protein					X						
PA14_09010	rplR 50S ribosomal protein L18					X						
PA14_08950	rplN 50S ribosomal protein L14					X		X				
PA14_08740	rplJ 50S ribosomal protein L10					X						
PA14_25840	putative electron transfer flavoprotein – ubiquinone oxidoreductase								X			
PA14_23370	orfK UDp-N-acetylglucosamine 2-epimerase								X			
PA14_30050	aceA isocitrate lyase											
PA14_69190	rho transcription termination factor							X				
PA14_50280	flaG hypothetical protein		X			X		X				
PA14_73280	atpH ATP synthase δ chain							X				
PA14_25110	topA DNA topoisomerase I											
PA14_73290	atpF ATP synthase B chain							X				
PA14_28660	infC translation initiation factor IF-3								X			
PA14_08960	rplX 50S ribosomal protein L24					X		X				
PA14_09030	rpmD 50S ribosomal protein L30					X						
PA14_54420	mucA anti-sigma factor	X										

PA14_23360	wzz O-Ag chain length regulator											
PA14_08870	rplW 50S ribosomal protein L23					X		X				
PA14_54430	algU sigma factor	X	X									
PA14_42450	popB translocator					X		X	X			
PA14_69370	algP alginate regulator								X			
PA14_56680	putative ferrous iron transport protein B											
PA14_09080	rpsM 30S ribosomal protein S13											
PA14_07480	putative reverse transcriptase				X	X						
PA14_62830	tpiA triosephosphate isomerase				X							
PA14_63605	fdnG formate dehydrogenase-O major subunit											
PA14_23460	orfN group IV glycosyltransferase											
PA14_54350	lepB signal peptidase I								X			
PA14_41575	sigX ECF sigma factor							X	X			
PA14_06600	putative acyl CoA dehydrogenase						X					
PA14_28670	rpmI 50S ribosomal protein L35					X			X			
PA14_73250	atpG ATP synthase γ chain							X				
PA14_08990	rpsH 30S ribosomal protein S8											
PA14_42440	popD outer membrane translocator								X			
PA14_62780	yhbC conserved hypothetical protein											
PA14_45830	fliK flagellar hook length control protein					X						
PA14_45620	cheY two component regulator		X									
PA14_49160	yegE conserved hypothetical protein		X					X				
PA14_50460	flgD flagellar basal rod modification protein					X						
PA14_57880	putative toluene ABC efflux pump							X				
PA14_07090	metK methionine adenosyltransferase											
PA14_50440	flgF flagellar basal body rod protein					X						
PA14_50330	hypothetical protein		X									
PA14_54490	hypothetical protein											
PA14_54340	hypothetical protein						X					

PA14_50450	flgE flagellar hook protein					**X**						
PA14_51520	spcU exotoxin chaperone								**X**			
PA14_08695	secE secretion protein					**X**		**X**				
PA14_66880	phaI putative polyhydroxyalkanoate granule protein											
PA14_00560	exoT exoenzyme T			**X**								
PA14_57060	conserved hypothetical protein	**X**		**X**								
PA14_64620	putative oxidoreductase											
PA14_49180	phoP two component regulator											
PA14_69480	algZ alginate synthesis protein (fimS)											
PA14_16480	wspF methylesterase (cheB like)											
PA14_33610	probable non-ribosomal peptide synthetase		**X**	**X**				**X**		**X**		
PA14_63580	fdnH nitrate inducible formate dehydrogenase β subunit											
PA14_08890	rpsS 30S ribosomal protein S19					**X**		**X**				
PA14_50410	flgI flagellar P ring protein precursor					**X**						
PA14_25090	foaB fatty acid oxidation complex β subunit								**X**			
PA14_45790	fliN flagellar motor switch protein					**X**						
PA14_45560	motC chemotaxis protein		**X**									
PA14_18020	putative phenazine biosynthesis protein phzF family											
PA14_16010	putative long chain acyl CoA thioester hydrolase											
PA14_02450	putative NAD(P) transhydrogenase subunit $\alpha 1$											
PA14_25080	foaA fatty acid oxidation complex α subunit								**X**			
PA14_18010	glpK glycerol kinase											
PA14_64610	putative fatty acid desaturase											
PA14_33680	fpvA ferripyoverdine receptor							**X**		**X**		
PA14_60320	pilE type IV fimbrial biogenesis protein											
PA14_63570	fdnI nitrate inducible formate dehydrogenase γ subunit											
PA14_07110	putative arsR family transcriptional regulator											

PA14_11970	methylpurine DNA glycosylase family protein								X			
PA14_63110	putative S-adenosylmethionine decarboxylase proenzyme											
PA14_49170	phoQ two component sensor											
PA14_41690	conserved hypothetical protein											
PA14_18320	arnE putative inner membrane protein								X			
PA14_44311	hypothetical protein											
PA14_60280	fimU type IV fimbrial biogenesis protein											
PA14_49200	oprH low magnesium outer membrane protein						X					
PA14_33560	putative adhesion protein		X									
PA14_18360	prmF glycosyltransferase (arnC)								X			
PA14_17960	glpK glycerol kinase						X					
PA14_18310	arnF inner membrane protein								X			
PA14_18370	arnB UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase								X			
PA14_63120	putative spermidine synthase											
PA14_63160	pmrB two component sensor											
PA14_18300	putative nucleotide sugar dehydrogenase								X			
PA14_18350	fmt transformylase (arnA)						X					
PA14_60310	pilY1 type IV fimbrial biogenesis protein											
PA14_63130	conserved hypothetical protein											
PA14_33530	periplasmic ABC transporter domain		X					X				
PA14_18340	arnD 4-amino-4-deoxy-α-L-arabinose undecaprenyl phosphate biosynthesis								X			
PA14_18330	arnT 4-amino-4-deoxy-L-arabinose transferase								X			
PA14_60290	pilW type IV fimbrial biogenesis protein											
PA14_60300	pilX type IV fimbrial biogenesis protein											
PA14_17930	glpD glycerol-3-phosphate dehydrogenase					X						
PA14_63150	pmrA two component regulator											

PA14_06640	putative acyl CoA dehydrogenase					X						
	TOTAL No. UPREGULATED GENES	**3**	**9**	**3**	**2**	**27**	**5**	**19**	**21**	**2**	**0**	**91**
	% UPREGULATED GENES	**2.75**	**8.3**	**2.75**	**1.835**	**24.77**	**4.59**	**17.4**	**19.266**	**1.83**	**0**	**83.5**
	TOTAL No. GENES IN PA14 GENOME	**341**	**316**	**84**	**228**	**680**	**272**	**347**	**867**	**18**	**26**	**3179**
	% GENES IN PA14 GENOME	**5.77**	**5.4**	**1.42**	**3.861**	**11.52**	**4.61**	**5.88**	**14.682**	**0.3**	**0.4**	**53.8**
Downregulated In Response To MPPA		**KEY**			**DOWNREGULATED**				**UPREGULATED**			
LOCUS	**GENE/FUNCTION**	**AlgU‡**	**FliA**	**PvdS**	**RpoH**	**RpoN**	**RpoS**	**SigX‡**	**RpoD**	**FpvI**	**FecI**	**Total**
PA14_54520	opdH porin											
PA14_54540	conserved hypothetical protein											
PA14_38640	scoB putative CoA transferase subunit B								X			
PA14_33010	glyA2 serine hydroxymethyltransferase					X						
PA14_64050	gcbA diguanylate cyclase	X	X			X						
PA14_71070	putative AraC transcriptional regulator					X						
PA14_54570	conserved hypothetical protein											
PA14_33040	gcvT2 glycine cleavage protein T2	X										
PA14_38630	atoB acetyl CoA acetyltransferase											
PA14_58570	piuA OM ferrisiderophore receptor											
PA14_38660	scoA putative CoA transferase subunit A								X			
PA14_33000	gcvP2 glycine cleavage protein P2					X						
PA14_03370	conserved hypothetical protein		X					X				
PA14_11810	putative aldehyde dehydrogenase					X						
PA14_31510	putative short chain dehydrogenase											
PA14_23000	gltG permease of ABC sugar transporter					X			X			
PA14_59970	conserved hypothetical protein							X				
PA14_10500	ccoN cytochrome oxidase cbb3 subunit											
PA14_68360	putative β-ketoacyl synthase							X				
PA14_10490	Hypothetical protein											
PA14_32985	gcvH2 glycine cleavage protein H2					X						
PA14_22990	gltF permease of ABC sugar transporter					X			X			

PA14_67350	hutU urocanase					X						
PA14_05740	pydA dihydroorotate dehydrogenase				X	X	X		X			
PA14_33030	sdaA L-serine dehyddratase	X				X						
PA14_52800	acsA acetyl CoA synthetase								X			
PA14_27000	putative chemotaxis protein		X									
PA14_31500	acyl CoA synthase / fatty acid ligase											
PA14_36200	binding protein of ABC transporter				X							
PA14_22980	gltB ABC sugar transporter domain								X			
PA14_13430	fecA Fe^{3+} dicitrate transporter											
PA14_31540	acdA putative acyl CoA dehydrogenase											
PA14_54150	putP sodium/proline symporter											
PA14_15070	oprC copper uptake porin											
PA14_31530	putative acyl CoA thiolase											
PA14_03360	conserved hypothetical protein											
PA14_18830	putative lyase							X	X			
PA14_53970	probable aconitate hydratase								X			
PA14_01490	putative haemolysin											
PA14_59960	putative disulphide isomerase		X									
PA14_64500	putative transcriptional regulator											
PA14_68260	c4 dicarboxylate binding protein					X						
PA14_13150	putative transcriptional regulator						X					
PA14_32790	hypothetical protein							X	X			
PA14_23030	oprB glucose uptake porin								X			
PA14_53940	prpB carboxyphosphoenolpyruvate phosphonomutase								X			
PA14_53590	hypothetical protein							X				
PA14_63280	putative transcriptional regulator											
PA14_53070	hpd 4-hydroxyphenylpyruvate dioxygenase											
PA14_38470	gnyH putative enoyl CoA hydratase								X			

PA14_54620	Probable aldehyde dehydrogenase											
PA14_71650	aspA aspartate ammonia lyase								X			
PA14_35530	bkdA1 2-oxoisovalerate dehydrogenase α subunit						X		X			
PA14_23010	gltK putative ATP binding unit of ABC transporter					X			X			
PA14_22890	gapA glyceraldehyde phosphate dehydrogenase											
PA14_12900	histone like protein HU form N	X										
PA14_45110	sulphate binding protein of ABC transporter											
PA14_66710	rpmE 50S ribosomal protein L31											
PA14_23090	edaA 2-keto-3-deoxy-6-phosphogluconate aldolase											
PA14_53250	cpbD chitin binding protein precursor											
PA14_50740	conserved hypothetical protein							X				
PA14_24980	conserved hypothetical protein											
PA14_58470	dppD putative dipeptide ABC transporter					X						
PA14_35520	bkdA2 2-oxoisovalerate dehydrogenase β subunit						X		X			
PA14_24445	gdhB NAD dependent glutamate dehydrogenase											
PA14_58420	putative binding protein unit of ABC dipeptide					X						
PA14_53950	prpC citrate synthase 2								X			
PA14_10360	conserved hypothetical protein			X								
PA14_66790	hslU heat shock protein				X	X						
PA14_35490	lpdV lipoamide dehydrogenase val											
PA14_35500	bkdB lipoamide acyltransferase						X		X			
PA14_46910	ybeJ putative binding protein of ABC transporter					X					X	
PA14_38460	gnyB acyl CoA carboxyltransferase β chain								X			
PA14_54170	putA proline dehydrogenase											
PA14_66750	argS arginyl tRNA synthetase											
PA14_43850	htpG heat shock protein				X							
PA14_03800	oprE anaerobically induced porin					X						
PA14_38510	hmgA homogentisate 1-2-dioxygenase											
PA14_09470	phzB1 phenazine biosynthesis protein							X				

PA14_57710	cysN ATP sulphurylase GTP binding unit/APS kinase				**X**							
PA14_50520	braC branched chain amino-acid transporter											
PA14_52990	phhA phenylalanine-4-hydroxylase						**X**		**X**			
	TOTAL No. DOWNREGULATED GENES	**4**	**4**	**1**	**5**	**18**	**6**	**8**	**21**	**0**	**1**	**68**
	% DOWNREGULATED GENES	**5.13**	**5.13**	**1.28**	**6.4**	**23.08**	**7.69**	**10.27**	**26.92**	**0**	**1.28**	**87.18**
	TOTAL No. GENES IN PA14 GENOME	**341**	**316**	**84**	**228**	**680**	**272**	**347**	**867**	**18**	**26**	**3179**
	% GENES IN PA14 GENOME	**5.77**	**5.4**	**1.42**	**3.861**	**11.52**	**4.61**	**5.88**	**14.682**	**0.3**	**0.4**	**53.8**

‡ = statistically significant upregulation of ECF σ factor (*padj* < 0.1 in DESeq2 analysis)

Table 5.1.

Grouping of Genes That Are Differentially Expressed In Response To MPPA Into Extracytoplasmic Function Primary Sigmulons (adapted from Schulz *et al*, 2015)

Sigmulons were analysed for enrichment or derichment of MPPA regulated genes relative to the abundance of genes in each sigmulon as a percentage of the PA14 genome as a whole (5,905 open reading frames). Enrichment was evident for both upregulated ($p < 0.05$) and downregulated ($p < 0.05$) genes in the *rpoN* sigmulon, for upregulated genes in the *sigX* sigmulon ($p < 0.05$) and for downregulated genes in the *rpoD* sigmulon ($p < 0.05$). No other statistically significant associations were found.

SECTION 5.4

DISCUSSION

SECTION 5.4.1

FUNCTIONAL CATEGORY C – ENERGY METABOLISM

It is apparent that many of the functional category C genes that are downregulated in response to MPPA treatment are involved in amino acid metabolism, glycolysis and/or the TCA cycle, conversely, no upregulated genes in this category have roles in either glycolysis or amino acid metabolism (Figure 5.1.1). Downregulated genes within this category demonstrate significant enrichment (Figure 5.1.2). Although a subset of functional category C genes that are upregulated in response to MPPA exposure, comprising *aceA* and *fdnH*, encode products that function in the TCA cycle, this involvement is invariably at the level of glyoxylate/dicarboxylate metabolism. This might imply that carbon is diverted into the glyoxylate shunt after a block in the TCA cycle that is induced in response to MPPA. As many genes involved in type 3 secretion are also upregulated in response to MPPA (Figure 5.1.35) it may be of interest that *aceA* encodes an isocitrate lyase which has been shown to upregulate the T3SS in *P. aeruginosa* without input from *exsA*, the master regulator of type 3 secretion in this species (Chung *et al*, 2013). In this regard it should also be noticed that deletion of *prpC*, a methylcitrate synthetase downregulated in this dataset (Figure 5.1.1) has been shown to induce type 3 secretion (Rietsch & Mekalanos, 2006). MPPA is a glycerophospholipid and some of the functional category C genes upregulated in response to it, namely *glpD*, *glpK* and *PA14_18010* appear to be involved in the metabolism of glycerol and glycerolipids (Figure 5.1.1). This, could suggest that under the experimental conditions employed, *P. aeruginosa* is utilising MPPA breakdown products as a preferred carbon source. The fact that many genes involved in the metabolism of fatty acids (Figure 5.1.6), the other key product of glycerolipid metabolism, are also upregulated, may lend further credence to this hypothesis. Moreover, catabolite repression resulting from use of glycerophospholipid derived carbon sources may explain the observed

downregulation of key TCA cycle genes (Figure 5.1.1). The medium employed in these studies, MHB, whilst comparatively rich, is based on starch, beef extracts and casein hydrolysate which would supply sugars and amino acids as carbon sources. Although noted for their nutritional versatility, pseudomonads are unusual in that they prefer certain organic acids as carbon sources as opposed to glucose which is favoured by both *Enterobacteriaceae* and *Firmicutes* (Rojo, 2010). Metabolism of fatty acid breakdown products would yield 2 carbon organic acids that *P. aeruginosa* could, together with glycerol, assimilate in preference to the sugars and amino acids that are provided by MHB (Zarzycki Siek *et al*, 2013). Growth on fatty acids or 2 carbon units has been shown to trigger an *aceA* dependent TCA block accompanied by flux of carbon into the glyoxylate shunt ; this would be consistent with this dataset (Chung *et al*, 2013). It has previously been observed that *P. aeruginosa* strains adapted to cystic fibrosis lungs have an enhanced ability to exploit glycerol as a carbon source (Daniels *et al*, 2014). This may explain the observation that MPPA especially enhanced the growth of certain hypervirulent LES isolates (Figure 4.6 – 4.7). Several of the functional category C genes that are upregulated in response to MPPA exposure are involved in oxidative respiration (Figure 5.1.1) and this is in fitting with phenotypic findings that would imply a faster growth rate, amongst some non-mucoid strains at least and especially those belonging to the LES.

SECTION 5.4.2

FUNCTIONAL CATEGORY E – AMINO ACID METABOLISM & TRANSPORT

All functional category E genes appear to be downregulated in response to MPPA apart from only 2, indicating derichment of upregulated genes within this category and enrichment of downregulated genes (Figure 5.1.4), *PA14_63110* and *PA14_63120*, which together form an operon and are highly upregulated (Figure 5.1.3). This operon is involved in biosynthesis of spermidine, a cationic polyamine that has been shown to act as a substitute for Ca^{2+} and Mg^{2+} ions in stabilising LPS molecules in conditions of membrane stress in which these divalent cations have been depleted (Johnson *et al*, 2012). Given the chelating properties of MPPA, the

upregulation of this spermidine biosynthesis operon is hardly surprising (Krogfelt *et al*, 2000). It is not clear why all of the other genes in functional category E are downregulated in response to MPPA, nonetheless, the fact that they do all function in the assimilation and metabolism of amino acids and/or peptides makes it tempting to speculate that these genes are being repressed because a preferable carbon source is available in the form of MPPA breakdown products. These MPPA breakdown products, however, cannot provide nitrogen, essential for the growth of *P. aeruginosa*, meaning that the test medium, MHB, would be the only possible nitrogen source in these experiments. It is possible that in MPPA treated cultures, certain amino acids are required only as a source of nitrogen whereas in matched MPPA free controls they may be used as a source of both carbon and nitrogen, thus explaining a lesser reliance on at least some amino acids in the presence of MPPA. Although they are not the preferred carbon source for pseudomonads, certain amino acids are nevertheless more easily assimilated than sugars, the only other carbon source that MHB could provide (Rojo, 2010). Downregulation of genes involved in amino acid metabolism may have ramifications for other *P. aeruginosa* behaviours including expression of virulence factors. The 4 functional category E genes that exhibit the greatest down regulation in response to MPPA (*glyA2*, *gcvT2*, *gcvP2* and *gcvH2*) are part of a glycine cleavage system whose expression along with that of *sdaA*, another functional category E gene that is downregulated in response to MPPA and encodes a serine dehydratase, is positively correlated with pyocyanin production ; all of these metabolic feats are dependent upon the expression of *PA14_32940*, a locus encoding a putative TyrR family enhancer binding protein that interacts with *rpoN* (Lundgren *et al*, 2013). *PA14_32940* is downregulated upon exposure to MPPA but not to a level reaching statistical significance - $padj > 0.1$. *hpd* and *phhA*, two other functional category E genes that are downregulated in response to MPPA, are involved in the catabolism of aromatic amino acids which can be precursors in phenazine biosynthesis and these are both upregulated by *phhR*, a homolog of the *PA14_32940* locus -sharing 44 % identity (Palmer *et al*, 2010 ; Palmer *et al*, 2007). Again *phhR* is downregulated in response to MPPA, but not significantly so – $padj > 0.1$. It may be inferred from these collective findings that use of certain amino acids, including glycine, serine, tyrosine and phenylalanine, as carbon sources in *P.*

aeruginosa supports pyocyanin production and by extension that MPPA provides an alternative carbon source thus repressing pyocyanin biosynthesis. This is in fitting with the observation that MPPA substantially reduces pyocyanin production in various *P. aeruginosa* strains (Figures 4.14 & 4.17).

SECTION 5.4.3

FUNCTIONAL CATEGORY F – NUCLEOTIDE METABOLISM & TRANSPORT

Only 2 genes in functional category F are significantly dysregulated in response to MPPA treatment – both negatively (Figure 5.1.5). This does not represent either enrichment or derichment in this category (Figure 5.1.6). The first of these is *pydA*, encoding a dihydropyrimidine dehydrogenase that functions in the biosynthesis of β alanine, pantothenate and coenzyme A. It is unclear why this gene is downregulated, nonetheless, as already stated in previous sections with reference to functional categories C and E, it is possible that the provision of an alternative carbon source, resulting from MPPA breakdown, could enable *P. aeruginosa* to be less dependent on the availability of amino acids (including β alanine) for growth whilst also providing alternative precursors for metabolism of other molecules, which could plausibly include coenzyme A and panothenate. *PA14_18830*, the only other downregulated gene in functional category F, encodes an adenylosuccinate lyase with predicted involvement in the biosynthesis of purines, alanine, aspartate and glutamate. Again it is difficult to explain why this gene is downregulated in response to MPPA although this may also be partly due to a reduced requirement for amino acids.

SECTION 5.4.4

FUNCTIONAL CATEGORY G – CARBOHYDRATE METABOLISM & TRANSPORT

Within functional category G, several genes are significantly dysregulated in response to MPPA ; 7 in negatively and 4 positively (Figure 5.1.7). No functional enrichment or derichment was detectable for either upregulated or downregulated genes in this category (Figure 5.1.8). The *gltGF* genes are most highly downregulated and together encode the permease domain of an ABC glucose transporter whilst *gltB* encodes the periplasmic substrate binding domain of the same transporter. Downregulation of these genes would make sense given the probable availability of alternative carbon sources derived from MPPA. *PA14_68260* encodes a periplasmic c4-dicarboxylate binding protein and might be downregulated due to the availability of smaller carboxylate units, although this is not certain. The *prpB* locus, encoding a 2-methylisocitrate lyase predicted to function in propanoate metabolism is the next most highly downregulated gene in this category ; this gene belongs to the same operon as *prpC* which is also downregulated (Figure 5.1.1). The *prp* genes as well as some of the *glt* genes have also been implicated in the metabolic repression of type 3 secretion in *P. aeruginosa* (Rietsch & Mekalanos, 2006). This could bear relevance to the fact that many T3SS genes are upregulated in response to MPPA. The next most downregulated functional category G gene, *gapA*, encodes a glyceraldehyde-3-phosphate dehydrogenase with predicted involvement in glycolysis as well as in the biosynthesis of amino acids and secondary metabolites, therefore it is not unexpected that this gene would be downregulated in response to MPPA, which provides carbon sources which are preferentially utilised by *P. aeruginosa*, instead of sugars and amino acids (Rojo, 2010). Following on from this, *edaA* is the final downregulated gene in this category. The product of this gene is a bifunctional keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase which catalyses the conversion of glucose to pyruvate in the Entner Doudoroff pathway (Fang-Ma *et al*, 1998). Since glucose is not likely being immediately utilised in MPPA treated cultures, it seems plausible that this enzyme would be redundant under such conditions. The first upregulated gene in functional category G is *eno*, which encodes a phosphoenolpyruvate hydratase. Upregulation of this gene in response to

MPPA seems paradoxical ; its product is a phosphoenolpyruvate hydratase involved in glycolysis, a pathway in which all other functional category G genes, with the notable exception *tpiA*, are downregulated in MPPA exposed cultures. Upregulation of *eno* might be explained by the fact that it has additional functions in lipopolysaccharide biosynthesis and belongs to an operon (*PA14_17290 – PA14_17340*) involved in cell division and envelope biogenesis in which all other members are positively expressed in response to MPPA, or at least not significantly downregulated (Walsh *et al*, 1999). The observation that *tpiA*, a glycolysis associated gene encoding a triosephosphate isomerase, is upregulated in response to MPPA is similarly paradoxical but may be explained in two ways. Firstly, it too, has additional functions in membrane maintenance (Fernandez *et al*, 2013). Secondly, it plays an essential role in glycerol catabolism by catalysing the interconversion of dihydroxyacetone phosphate, which might otherwise be regarded as a dead end metabolite, to glyceraldehyde 3 phosphate (Merlo *et al*, 2007). As previously mentioned in regards to functional category C, many other genes functioning in glycerol catabolism are upregulated in response to MPPA, implying that glycerol derived from this lipid is being utilised as a carbon source. The only other 2 functional category G genes to be upregulated are *fmt* (*arnA*) and *PA14_18340* (*arnD*), both of which are involved in a response to membrane stress induced by chelating agents and cationic antibiotics such as aminoglycosides, polymyxins and even host defence peptides, all of which enter Gram- negative cells via membrane binding followed by self-promoted uptake (Mulcahy *et al*, 2008 ; Lewenza, 2013 ; Hancock & Brinkman, 2002). All genes in this 8 member operon are highly upregulated in response to MPPA but are spread across different functional categories. This could partially explain the strongly antagonistic interactions MPPA exerts with both tobramycin and colistin, in spite of its additive or synergistic effects with anionic or neutrally charged antibiotics which enter Gram-negative organisms via porins rather than via self-promoted uptake (Table 4.1).

SECTION 5.4.5

FUNCTIONAL CATEGORY H – COENZYME METABOLISM & TRANSPORT

Only 2 genes are significantly dysregulated in this category, both upwardly, they belong to the same operon (Figure 5.1.9). This is not indicative of functional enrichment/derichment (Figure 5.1.10). The first of these functional category H genes, *metK*, encodes a methionine adenosyltransferase involved in the biosynthesis of the sulphur containing amino acids, cysteine and methionine. It is not clear why this locus is upregulated; most other amino acid metabolism genes are downregulated in response to MPPA, only 2 genes (*PA14_63110* and *PA14_63120*) in functional category E, which is specifically dedicated to amino acid metabolism, are upregulated (Figure 5.1.3). Together, these 2 genes constitute an operon which functions in the biosynthesis of membrane stabilising spermidine – methionine is a necessary precursor in spermidine biosynthesis and this may provide an explanation as to why *metK* is upregulated (Kreamer *et al*, 2015). Furthermore, several genes – distributed across various functional categories, that are dysregulated in response to MPPA, are involved in the metabolism of coenzyme A, a process in which methionine is also involved.

SECTION 5.4.6

FUNCTIONAL CATEGORY I – LIPID METABOLISM & TRANSPORT

Of the significantly dysregulated genes in functional category I, 11 are downregulated and 6 are upregulated (Figure 5.1.11). This indicates enrichment of downregulated genes within this category but neither enrichment nor derichment of upregulated genes (Figure 5.1.12). Amongst the downregulated genes *PA14_31510* encodes a putative short chain dehydrogenase and oxidoreductase with predicted involvement in the breakdown of glucose and ribitol. It cannot be said for certain why this gene is downregulated in response to MPPA exposure although its role in sugar metabolism may be a factor as there appears to be catabolic repression of many

other genes involved in glucose breakdown pathways. *PA14_31500,* belongs to the same operon and encodes an AMP binding enzyme with predicted involvement in glycolysis, secondary metabolite biosynthesis and propanoate metabolism. It is possible that its downregulation is also the result of carbon catabolite repression from MPPA breakdown products. *PA14_31530* also belongs to this operon and encodes an acyl-CoA thiolase with predicted involvement in the catabolism of pyruvate, glyoxylate/dicarboxylates, fatty acids, benzoate, various amino acids and also in the biosynthesis of antibiotics and other secondary metabolites. The final member of this operon, *acdA*, encodes an acyl-CoA dehydrogenase involved in geraniol catabolism and secondary metabolite biosynthesis. It is possible that this entire operon is downregulated as a result of catabolite repression imposed by MPPA breakdown products and although fatty acid degradation is a predicted function of *PA14_31530*, other, more specific enzymes which are upregulated, such as FoaA and FoaB, may fulfil this role instead. The *PA14_68360* locus encodes a β-ketoacyl synthase that is involved in fatty acid biosynthesis, it would seem likely that this enzyme is downregulated because MPPA breakdown products provide a readily available exogenous source of fatty acids. *acsA* encodes an acetyl CoA synthetase predicted to be involved in propanoate metabolism, glycolysis (pyruvate metabolism) and biosynthesis of secondary metabolites, transcription of this gene has previously been shown to be repressed where certain 2 carbon compounds (succinate) are available as a carbon source (Kretzschmar *et al*, 2010). Therefore, downregulation of this gene may also be the result of carbon catabolite repression. The next 2 downregulated genes share an operon ; the *gnyH* gene encodes γ-carboxygeranoyl-CoA hydratase, an enzyme with predicted functions in the catabolism of branched chain amino acids and acyclic isoprenoids, *gnyB* is located immediately upstream in the same operon and encodes the β-subunit of an acyl-CoA carboxyltransferase, an enzyme with predicted functions in the biosynthesis of fatty acids, aflatoxin, tetracyclines and malonyl-CoA and in the metabolism of acyclic isoprenoids, pyruvate, propanoate and branched chain amino acids. It is probable that the enzymes encoded by these genes are surplus to requirement in the presence of MPPA breakdown products. The first upregulated locus in functional category I, *PA14_06600*, encodes a probable acyl-CoA dehydrogenase. This enzyme could have roles in the catabolism of MPPA

breakdown products. *PA14_06640*, the most highly upregulated gene in this functional category, also encodes an acyl-CoA dehydrogenase and is likely upregulated for reasons similar to those previously mentioned for *PA14_06600* which is located 2 genes upstream. *foaB*, alternatively named *fadA*, encodes the β-subunit of a fatty acid oxidation complex with predicted functions in the catabolism of fatty acids, branched chain amino acids, geraniol, benzoate and ethylbenzene. *foaA*, alternatively named *fadB*, shares an operon with *foaB* and encodes the α-subunit of the same fatty acid oxidation complex, it has predicted functions in the metabolism of branched chain amino acids, lysine, tryptophan, β alanine, butanoate, propanoate, butanoate, geraniol, caprolactams, toluene, limonene and pinene, it also has predicted functions in the biosynthesis of secondary metabolites. The *foaAB* genes are likely upregulated to enable β-oxidation of long chain fatty acids derived from MPPA lysis to succinyl-CoA for routing to central metabolism (Kazakov *et al*, 2009).

SECTION 5.4.7

FUNCTIONAL CATEGORY J – TRANSLATION & RIBOSOME BIOGENESIS

20 genes in this functional category are significantly dysregulated, 18 positively and 2 negatively (Figure 5.1.13). This indicates enrichment of upregulated genes in this category (Figure 5.1.14). It is very difficult to assess how and why genes belonging to this category may be dysregulated as their functions are so broad and generalised. Nevertheless, enrichment of upregulated genes in this category may suggest that there is an overall increase in translation occurring in response to MPPA and this would be in fitting with increased metabolism and growth rates (Figures 4.1 – 4.13).

SECTION 5.4.8

FUNCTIONAL CATEGORY K – TRANSCRIPTION

13 genes within functional category K are significantly dysregulated, 9 positively and 4 negatively (Figure 5.1.15). No enrichment or derichment of gene abundance relative to the PAO1 genome as a whole was detectable for this category (Figure 5.1.16). The most highly downregulated locus belonging to functional category K is *PA14_71070* which encodes an AraC family transcriptional activator of the breakdown of glycine betaine, a choline oxidation product which can be used as a source of both carbon and nitrogen or as an osmoprotectant by *P. aeruginosa* (Wargo *et al*, 2008). Since MPPA likely induces membrane stress and provides a source of readily available carbon, downregulation of this gene would seem logical as it would allow glycine betaine to be sequestered in the cytoplasm as an osmoprotectant rather than be catabolically squandered under conditions of membrane stress where alternative nutrients are ample (Wargo, 2013). The next most downregulated gene in this functional category is *PA14_64500*, a MerR family transcriptional regulator responsive to cyclic-di-GMP ; the product of this gene has been shown to increase resistance to the antibiotics tobramycin, trimethoprim and chloramphenicol in *P. aeruginosa* grown either planktonically or in biofilms via a mechanism that depends only partially on overexpression of the mexAB-oprM and mexEF-oprN efflux systems (Liao *et al*, 2013). Downregulation of the *PA14_64500* locus in MPPA treated cultures would therefore make sense with regard to the finding that MPPA exerts marked synergy with both trimethoprim and chloramphenicol as well as other drugs that are known substrates of these pumps (Table 4.1). However, none of the genes encoding components of these tripartite mex systems were significantly dysregulated in MPPA treated cultures. It can thus be inferred that if lack of *PA14_64500* expression is influencing the MICs of these antibiotics in the presence of MPPA, it is by an unknown mechanism probably not directly involving these mex systems. *PA14_64500* has also been shown to increase *P. aeruginosa* susceptibility to colistin in both planktonic and biofilm cultures via a mechanism involving repression of the *phoPQ-oprH* operon (Chambers & Sauer, 2013). This would be consistent with the observations that MPPA treated cultures show exhibit heightened

expression of all genes in this operon as well as other operons subject to its control (*pmr* and *arn*) and that colistin is one of few antibiotics whose action against *P. aeruginosa* is antagonised rather than enhanced by MPPA (Figures 5.1.20, 5.1.33 & Table 4.1). Following on from this, the next 2 most downregulated gene in functional category K are *PA14_13150* and *PA14_63280* probable members of the LuxR and AraC families, respectively, with as yet unknown function. The first and least upregulated functional category K gene upregulated in MPPA treated cultures is *rpoB*, encoding the β subunit of DNA directed RNA polymerase, due to the vast array of processes in which this enzyme is involved, it is very difficult to speculate as to why it is upregulated in the presence of MPPA but it might nevertheless be surmised that MPPA has induced a net increase in transcription, although the significance of this cannot be known. *nusG* and *rho* are the next 2 functional category K genes upregulated in response to MPPA, these are antitermination and termination factors of transcription, respectively which may further imply a net increase in transcriptional activity in MPPA exposed organisms. It may be noteworthy that *secE*, another upregulated gene, is located immediately adjacent to *nusG* in the same operon. The next most upregulated gene in functional category K is *algU*, encoding a σ22 family ECF factor that acts as an activator of alginate biosynthesis (Martin *et al*, 1993). Upregulation of this gene may be consistent with the MPPA induced phenotype of increased biofilm formation by mucoid strains (Figures 4.21 & 4.23.2). Upregulation of *algU* has previously been noted to occur in response to other membrane and cell wall active compounds and is perhaps not surprising under the present conditions (Wood & Ohman, 2009). Genes under the control of *algU* can be discerned from table 5.1. *sigX* is yet another ECF σ factor that is upregulated upon MPPA exposure and dysregulated genes within its primary regulon are also documented in table 5.1. Interestingly, expression of oprF, the most abundant porin and structural outer membrane protein in *P. aeruginosa*, is controlled by *sigX* (Brinkman *et al*, 1999). Unexpectedly, however, the *oprF* gene itself is not differentially regulated in response to MPPA at a statistically significant level. The next most upregulated gene in functional category K is *cheY*, a two component response regulator governing chemotaxis and essential for swimming motility (Güvener *et al*, 2006). Phospholipids have previously been shown to act as

chemoattractants which stimulate swimming motility in various bacterial species in a *che* dependent manner which may go some way towards explaining why this gene is upregulated (Bonner & Shimkets, 2006). Several other motility related genes are also upregulated in response to MPPA, lending further credence to this hypothesis (Figure 5.1.22). *phoP* is the next most highly upregulated gene in MPPA treated cultures; all genes in this operon are highly upregulated as are those within the *pmrAB* and *arnBCADTEF* operons which are under its regulatory control – collectively these genes would be expected to bolster cell envelope integrity under conditions of cation limitation and membrane stress, such as those induced by MPPA (Fernandez *et al*, 2010). Note that *pmrA* has also been assigned to functional category K on account of its regulatory functions. Finally, *PA14_07110* is an ArsR family transcriptional regulator of unknown function, it shares an operon with *metK*, also upregulated but assigned to functional category H.

SECTION 5.4.9

FUNCTIONAL CATEGORY L – DNA REPLICATION, RECOMBINATION & REPAIR

Two genes in functional category L are upregulated in response to MPPA (Figure 5.1.17). This could not be taken to indicate either enrichment or derichment of genes in this functional group (Figure 5.1.18). The first of these, *topA*, encodes DNA topoisomerase I, an enzyme which functions in both the breakage and the re-annealing of the phosphate backbone of DNA molecules (Khodursky *et al*, 2000). It is not known why this gene is upregulated in response to MPPA but it could be possible that the observed increase in expression is simply the result of a faster growth rate – and by extension greater DNA replication, in MPPA treated PA14. Other topoisomerases (II and IV) constitute the targets of quinolone and coumarin antibiotics, the relevance of this is not clear, but an additive effect of MPPA on the activity of the quinolones ciprofloxacin and nalidixic acid has been observed for some strains (Table 4.1). The only other gene in this functional category, *PA14_11970*, encodes a 3-methyladenine DNA glycosylase predicted to function in base excision repair. Again, it cannot be said with any certainty why this gene is

upregulated but this could also be the result of a general increase in replication and metabolic rate.

SECTION 5.4.10

FUNCTIONAL CATEGORY M – CELL ENVELOPE BIOGENESIS & REPAIR

10 genes are upregulated within this functional category whilst the other 2 are downregulated (Figure 5.1.19). This does not indicate a statistically significant difference in abundance of either up or downregulated genes in this category relative to the PAO1 genome as a whole (Figure 5.1.20). The first of the downregulated genes *oprB* encodes a porin involved in glucose uptake; this porin is likely functionally redundant in the presence of MPPA breakdown products (fatty acids) that provide preferable carbon sources (Wylie & Worobec, 1995 ; Rojo, 2010). The only other downregulated locus, *PA14_50740*, encodes a conserved hypothetical outer membrane protein of unknown function. The first gene that is upregulated in functional category M, *fgtA*, encodes a flagellar glycosyltransferase (Verma *et al*, 2006). Upregulation of this gene would be consistent with the observation that several genes involved in motility and flagellar biogenesis are also upregulated and the fact that MPPA increases swimming motility (Figures 5.1.21). *orfK* is the next most upregulated gene in functional category M and its product is a UDP-N-acetylglucosamine-2-epimerase involved in O-antigen biosynthesis (Hare *et al*, 2012). It is conceivable that this enzyme may be upregulated in order to increase outer membrane integrity in the face of membrane stressing agents – phenotypic results obtained by other investigators (Krogfelt *et al*, 2000), strongly suggest that certain mucoid *P. aeruginosa* strains isolated from CF airways are sensitive to MPPA as a result of stunted O-antigen profiles that are likely acquired during chronic infection in order to evade immune surveillance (Hancock *et al*, 1983). *wzz* is the next most upregulated gene in this functional category and encodes the O-antigen chain length regulator, it is therefore most likely upregulated for the same reasons as the aforementioned *orfK* (Burrows *et al*, 1997). Homologs of *wzz* in *Salmonella enterica* serovar Typhimurium are subject to regulatory control by the *pmrAB*

operon, possibly denoting another regulatory link amongst MPPA upregulated genes (Farizano *et al*, 2012). The next most highly upregulated gene in functional category M, *orfN*, encodes an O-antigen glycosyltransferase involved in LPS biosynthesis, further suggesting a response to membrane stress. *oprH* is a member of the *phoPQ-oprH* operon which responds to membrane stress and Mg^{2+} limitation – oprH is an outer membrane protein whose expression is activated via the phoP regulator when its cognate phoQ sensor detects Mg^{2+} depletion, oprH then binds to LPS where it acts as a functional substitute for divalent cations in maintaining stability (Edrington *et al*, 2011). This operon is important in adaptive resistance to polycationic antibiotics such as polymyxins, aminoglycosides and host defence peptides as well as in cellular responses to membrane permeabilising cation chelators such as EDTA (Nicas & Hancock, 1980 ; Mulcahy *et al*, 2008). Therefore it makes sense that this operon is upregulated in response to MPPA, a cation chelator (Krogfelt *et al*, 2000). This operon also activates the *pmrAB* and *arn* operons enabling, respectively, the synthesis of spermidine, which, like oprH can function in place of cations in LPS stabilisation and the modification of lipid A with aminoarabinose residues which reduce its electronegativity and thereby its affinity for cationic antimicrobials (Mulcahy *et al*, 2008 ; Johnson *et al*, 2012). All genes in these interlinked operons are highly upregulated in response to MPPA but are distributed across various functional categories; this may explain the profound antagonism observed between MPPA and cationic antibiotics with self-promoted uptake mechanisms such as colistin and tobramycin (Table 4.1). All other functional category M genes that are upregulated in response to MPPA belong to the *arn* operon (Figure 5.1.20).

SECTION 5.4.11

FUNCTIONAL CATEGORY N – CELL MOTILITY & CHEMOTAXIS

14 genes in this category were significantly dysregulated, 1 negatively and 13 positively (Figure 5.1.21). This indicates enrichment for the upregulated genes in this functional category (Figure 5.1.22). *PA14_27000* is the only downregulated locus in this functional category and encodes a chemotaxis transducer, it is not known why

this gene is downregulated in response to MPPA. Amongst the upregulated genes in functional category N, *flaG*, *fliK* and the *flgDEFI* genes all encode various structural components of the flagellum. It may be the case that these are upregulated in order to enable chemotaxis towards MPPA or its fatty acid breakdown products; fatty acids are well documented to act as chemoattractants that can induce both swimming and twitching motility in various bacterial species including *P. aeruginosa*, regardless of whether or not they are capable of utilising these molecules as a carbon source (Bonner & Shimkets, 2006 ; Miller *et al*, 2008). *wspF* encodes a chemotaxis specific methylesterase whilst *motC* encodes a flagellar motor protein, adding further weight to this hypothesis. Moreover, *fimU* and the *pil* genes all encode products that are involved in type IV pilus biogenesis, suggesting that twitching, as well as swimming motility, may be employed in MPPA driven chemotaxis, this is not inconsistent with the observed phenotypes (Figures 4.20.1 & 4.20.2). Phenotypic data generated from crystal violet binding assays undertaken as part of the present work have suggested that MPPA may increase biofilm formation on polystyrene surfaces by some strains including PAO1 but not PA14 (Figure 4.21). This is at odds with the earlier findings of Laux and colleagues who found that MPPA markedly reduced biofilm formation, at least by strain PAO1 (Laux *et al*, 2002). Some of this discrepancy may be explained by the fact that a different medium was employed – MHB rather than LB or by the fact that a higher MPPA concentration was applied. Field emission scanning electron micrographs of MPPA treated biofilms formed on glass coverslips by various *P. aeruginosa* strains, including PAO1, PA14 and an isogenic pair of non-mucoid/mucoid CF isolates – J1385 and J1532, generally show no obvious differences in biofilm biomass when compared to matched untreated controls, however, the production of exopolymeric substance appeared to be increased in J1532 biofilms treated with MPPA relative to matched controls (Figures 4.21.1 – 4.25.2). Whilst biofilm formation and some forms of active motility are often considered to be opposing and mutually exclusive behaviours, flagellae and type IV pili have been implicated in certain stages of biofilm formation, in which they dually function as adhesins and structural components, they are also important for horizontal expansion of the biofilm upon a substratum as well as in vertical differentiation and dispersal, respectively (Murray *et al*, 2010, O'Toole & Kolter,

1998 ; Klausen *et al*, 2003). It should also be noted that crystal violet stain is not specific for exopolysaccharide and may therefore have stained pili, flagellae and other proteinaceous appendages within biofilm, many of which are likely to be highly glycosylated in any case. The *pel* polysaccharide gene cluster members (*PA14-24480 – PA14_24560*), with the possible exception of *pelD*, were not significantly dysregulated in response to MPPA although all but *pelC* and *pelE* were downregulated ; this may suggest that any increase in biofilm formation induced by MPPA is not due to greater production of this exopolysaccharide and this hypothesis may be supported by the observation that strain PA14, which lacks the *psl* gene cluster and therefore is not known to be capable of producing polysaccharides other than pel and alginate, was the only strain tested that did not increase biofilm formation in the presence of MPPA (Figure 4.21 ; Colvin *et al*, 2012b). One alternative explanation for the observed increase in biofilm formation is that it may merely have been a result of greater growth in MPPA treated cultures possibly combined with wider horizontal expansion of biofilms due to increased swimming and twitching motility.

SECTION 5.4.12

FUNCTIONAL CATEGORY O – POST-TRANSLATIONAL MODIFICATION, TURNOVER & CHAPERONING

This functional category includes 5 genes that are significantly dysregulated in response to MPPA exposure; 1 upregulated and 4 downregulated (Figure 5.1.23). Neither upregulated nor downregulated genes in this category were enriched or deriched (Figure 5.1.24). The only upregulated gene, *PA14_41690*, encodes a hypothetical protein of unknown cellular localisation that is predicted to be a zinc dependent protease with an aspartic peptidase domain. Although it is not clear why this gene is upregulated, transposon insertions within its PAO1 ortholog can diminish resistance to polymyxins in phoQ mutants (Gutu *et al*, 2013). This might imply that the probable protease encoded by this locus somehow modifies the cell envelope in response to membrane damaging agents, which might include MPPA. Total protease activity in the supernatants of MPPA treated cultures was found to be considerably

lower than in matched controls (Figure 4.18) which is in fitting with the observations of other reports (Laux *et al*, 2002). The first downregulated gene in this functional category, *ccoN*, encodes cbb3 type cytochrome C oxidase subunit I. Downregulation of this gene might be explained by the fact that cbb3 type cytochrome C oxidases are known to be expressed primarily in hypoxic conditions (Pitcher & Watmough, 2004). Although matched control cultures not treated with MPPA had an ample oxygen supply, several genes upregulated in response to MPPA are involved in oxidative respiration, suggesting that this is increased in response to MPPA. Another possible explanation for downregulation of this gene is that cytochrome C oxidases use copper in their catalytic activity (Lee *et al*, 2011). The oprC copper acquisition porin is downregulated in response to MPPA, indicating that copper may be scarce in MPPA treated cultures (Figure 5.1.25). The next gene downregulated in this functional category, *PA14_59960*, encodes a protein disulphide isomerase and oxidoreductase (DsbA2) of unknown function whose structure has only recently been solved, *P. aeruginosa* also possesses a better characterised homolog of this protein (DsbA1) that is known to be required for the correct folding of many virulence related proteins, including elastase (Arts *et al*, 2013). Reasons for the downregulation of this locus in response to MPPA treatment are not apparent and its functions likely are not entirely identical to those of *dsbA1* as Arts and colleagues found that expression of DsbA2 from a plasmid could not complement the phenotype of a *dsbA1* mutant (Arts *et al*, 2013). Nonetheless, if DsbA2 turns out to have functions in the folding of protein virulence factors such as elastase then this might at least partially explain the reduced expression of exoproteases and other virulence factors that was observed to occur in response to MPPA (Figures 4.18 & 4.19). *hslU* and *htpG* both encode cytoplasmic heat shock proteins, it is not clear how or why these genes are downregulated.

SECTION 5.4.13

FUNCTIONAL CATEGORY P – INORGANIC ION METABOLISM & TRANSPORT

10 genes within this functional category are significantly dysregulated in response to MPPA, 6 downwardly and 4 upwardly (Figure 5.1.25). There were no statistically significant differences of gene abundance in this category relative to the whole PAO1 genome sequence (Figure 5.1.26). The first downregulated gene in this category, *piuA*, encodes a TonB dependent Fe^{3+} siderophore (hydroxamate) receptor. Downregulation of this gene in response to MPPA is unexpected given that this lipid is known to chelate Fe^{2+} ions, albeit much less efficiently than Ca^{2+} and Mg^{2+} ions (Laux *et al*, 2002). The next downregulated gene in functional category P, *fecA*, encodes an outer membrane receptor of ferrous dicitrate, this is again surprising given the iron chelating effects of MPPA but might be explained by the fact that citrate synthase, prpC, is downregulated in response to MPPA (Figure 5.1.1). The next downregulated gene in functional category P, *oprC*, encodes the copper uptake porin. It cannot be known how or why this gene is downregulated in response to MPPA. It might be of interest to note that several genes with cytochrome C oxidase (copper dependent) related products are also downregulated including *ccoN*, *PA14_03360* and *PA14_03370* (Figures 5.1.23 & 5.1.38). The next downregulated gene in functional category P, *cysP*, encodes the substrate binding unit of an ABC sulphate transporter; it is not clear how or why this gene is downregulated. The next gene to be downregulated in response to MPPA is *dppD* encoding the ATP unit of an ABC dipeptide transporter that is also involved in glycine/glutamate uptake, it is possible that this downregulation is the result of catabolite repression. Following on from this, *cysN*, the next most downregulated gene in functional category P, encodes a bifunctional enzyme with sulphate adenylyltransferase and kinase activity that is predicted to be involved in the biosynthesis of cysteine, purines and selenocompounds. It is not clear why this gene is downregulated. The first upregulated locus in functional category P, *PA14_56680*, encodes ferrous iron transport protein B, located in the inner membrane. This protein is known to be involved in the uptake of iron which has previously entered the periplasmic space in ferric form complexed with dicitrate via FecA and has then been converted to the

more soluble ferrous oxidation state (Marshall *et al*, 2009). It may therefore be possible that *PA14_56680* is upregulated in order to compensate for the downregulation of *fecA*. *fpvA* encodes an outer membrane protein that acts a TonB dependent receptor for ferripyoverdine and certain pyocins (de Chial *et al*, 2003). *fpvA* itself is also involved in regulatory control of pyoverdine biosynthesis (Shen *et al*, 2002). This is in fitting with the observation that production of pyoverdine is increased by some strains, including PA14, in response to MPPA (Fig 4.16), it could therefore be possible that this receptor is upregulated to enable greater uptake of ferripyoverdine in PA14 and some other strains. *PA14_33560* encodes an inner membrane adhesion protein, this may be of relevance to the observation that MPPA was noted to increase biofilm formation (Figure 4.21). *PA14_33530* belongs to the same operon and encodes a hypothetical protein of unknown function and cellular localisation that has nonetheless been predicted to function in metal ion transport.

SECTION 5.4.14

FUNCTIONAL CATEGORY Q – SECONDARY METABOLITE SYNTHESIS & TRANSPORT

There are 6 significantly dysregulated genes within functional category Q, half downregulated and the other half upregulated (Figure 5.1.27). There were no statistically significant differences of gene abundance in this category relative to the whole PAO1 genome sequence (Figure 5.1.28). Amongst the downregulated genes, *PA14_31510* encodes a putative short chain dehydrogenase and oxidoreductase with predicted involvement in the breakdown of glucose and ribitol. It cannot be said for certain why this gene is downregulated in response to MPPA exposure although its role in sugar metabolism may be a factor as there appears to be catabolic repression of many other genes involved with this. *PA14_68360* encodes a β-ketoacyl synthase involved in fatty acid biosynthesis – this is most likely downregulated due to the ready availability of fatty acids derived from MPPA. *hmgA* encodes homogentisate 1,2-dioxygenase, an enzyme that is involved in the catabolism of aromatic amino-acids. Downregulation of this gene may also be explicable in terms of carbon catabolite repression and could possibly be linked to the observed decrease of

pyocyanin production in MPPA treated cultures, as aromatic amino acids are important precursors in phenazine biosynthesis (Palmer *et al*, 2010). Amongst the upregulated genes in functional category Q, *PA14_57880* encodes the ATP binding subunit of a probable ABC transporter possibly involved in lipid uptake. *PA14_50330* encodes a hypothetical protein with predicted S-adenosyl-L-methionine dependent methyltransferase activity. It is unknown why this protein is upregulated. Finally, *PA14_33610* encodes a non -ribosomal peptide synthase with phosphoantetheine binding domains.

SECTION 5.4.15

FUNCTIONAL CATEGORY R – GENES WITH GENERAL FUNCTION PREDICTIONS ONLY

Of the genes assigned to functional category R, 5 are downregulated whilst 3 are upregulated (Figure 5.1.29). This indicates derichment of upregulated genes in this functional category (Figure 5.1.30). The first downregulated gene, *PA14_31510*, is also assigned to functional category Q and has already been discussed in section. *putP* and *hpd*, downregulated within functional category R, have also been assigned to functional category E and possible reasons for their downregulation have already been discussed in section. *PA14_24980* encodes a hypothetical protein predicted to bind pyridoxal phosphate and molybdenum ions – no reasons are evident for the downregulation of this locus. *phzB1* encodes a gene belonging to the *phz1* operon that is involved in the biosynthesis of phenazine ; its downregulation may partially contribute to the reduced pyocyanin production that has been observed in MPPA treated cultures, however, neither this gene nor its operon are essential for phenazine biosynthesis as the *P. aeruginosa* genome encodes an alternative phenazine biosynthetic cluster, *phz2* (Recinos *et al*, 2012). The first upregulated gene in functional category R, *fgtA*, is also assigned to functional category M and its downregulation has been considered in section. The next most upregulated gene in functional category R, *PA14_50330*, encodes a hypothetical protein of unknown function whose operon is flanked downstream by several flagellar structural genes beginning with the flagellar hook gene *flgL* and upstream by the aforementioned

flagellar glycosyltransferase gene *fgtA*. It is therefore tempting to hypothesise that the product of this gene may have hitherto unknown roles in flagellar assembly or modification. Finally, *PA14_18020* encodes a *phzF* family phenazine biosynthetic protein, it is not known how or why this gene is upregulated by MPPA.

SECTION 5.4.16

FUNCTIONAL CATEGORY S – GENES WITH UNKNOWN FUNCTIONS

4 genes are downregulated in functional category S and 2 are upregulated (Figure 5.1.31). This indicates derichment of upregulated genes in this category (Figure 5.1.32). The first downregulated gene in this category is *PA14_54540* whose product is a hypothetical protein of unknown function and cellular localisation, this locus is immediately upstream of the *opdH* gene within the same operon, which is also downregulated and encodes a tricarboxylate uptake porin (Figure 5.1.37), it is possible that this protein forms the periplasm spanning domain of this tricarboxylate transporter. The next downregulated gene in this category, *PA14_54570*, is a hypothetical inner membrane protein belonging to the same operon and probably also forms part of this transporter. The next downregulated gene is *PA14_64500* which also has an assignation in functional category K and has already been discussed in section. *cbpD* encodes a chitin binding protein, it is not clear why this gene is downregulated but catabolite repression may play a role, given that chitin is a polymer composed of sugars. *popB* encodes a translocator protein that is part of the type III injectisome ; its upregulation is not surprising considering that several other genes within the type III secretion regulon are upregulated and the fact that Ca^{2+} depletion is known to induce expression of these genes (Hauser, 2009). Finally, *yhbC* encodes a ribosome maturation protein. It is not obvious how or why this gene is upregulated in response to MPPA, however, a partial explanation may lie with the finding that significant enrichment was found for MPPA upregulated genes involved in translation (Figure 5.1.13).

SECTION 5.4.17

FUNCTIONAL CATEGORY T – SIGNAL TRANSDUCTION

In functional category T, 4 loci are downregulated in response to MPPA whilst 7 others are upregulated (Figure 5.1.33). There were no statistically significant differences of gene abundance in this category relative to the whole PAO1 genome sequence (Figure 5.1.34). The most downregulated locus is *PA14_64050* which encodes a diguanylate cyclase that has been shown to stimulate swimming motility and initial surface attachment in the earliest steps of biofilm formation, however, this gene is not essential for these behaviours and is not involved in later stages of biofilm maturation (Petrova *et al*, 2014). This makes downregulation of this locus seem consistent with the decreased biofilm formation that was observed to occur in strain PA14 in response to MPPA (Figure 4.21), however, this gene has also been shown to be necessary for normal biofilm dispersal as it is involved in the post-translational activation of BdlA, a chemotactic transducer that is required for the dispersal of *P. aeruginosa* biofilms (Petrova *et al*, 2015). This may provide at least a partial explanation for the increased biofilm formation observed in other strains, where lack of dispersal might be additive to other factors such as increased Psl or alginate expression. The next most downregulated gene in functional category T, *PA14_27000*, encoding a chemotaxis transducer, has also been assigned to functional category N, in which it is the only downregulated gene (Figure 5.1.21), it is not clear why this gene is downregulated. *PA14_36200* is the next downregulated gene in functional category T and encodes a probable periplasmic substrate binding component of an ABC transporter, the fact that it is located immediately upstream of an operon encoding amino acid ABC transporter permeases suggests that its substrates are probably amino acids which would make sense given the likelihood that MPPA induces carbon catabolite repression of amino acids. The final gene to be downregulated in this category, *ybeJ*, is likely repressed by MPPA for the same reasons as it encodes the periplasmic binding domain of a glutamate/aspartate ABC transporter. The first upregulated gene in functional category T is *typA*, whose product is a GTPase known to activate type III secretion and induce biofilm formation in *P. aeruginosa* whilst also increasing resistance to certain antibiotics

including β-lactams, tetracyclines, polymyxins and the cationic host defence peptide LL-37 (Neidig *et al*, 2013). Upregulation of this gene in response to MPPA therefore seems to be in fitting with the finding that many type III secretion genes are also overexpressed in response to MPPA (Figures 5.1.37 & 5.1.38) and that MPPA appeared to enhance biofilm formation (Fig 4.21) and colistin resistance (Table 4.1). Nevertheless, MPPA increased rather than reduced the susceptibility of *P. aeruginosa* to β-lactams and tetracycline (Table 4.1) ; it is probable that any increase in resistance to these agents afforded by overexpression of TypA is annulled by the outer membrane permeabilising effects of MPPA. The second functional category T gene to be upregulated in response to MPPA is *mucA*, the product of this gene binds to and represses the ECF σ factor AlgU, thereby decreasing alginate production (Martin *et al*, 1993). This would also be consistent with the reduced level of alginate production observed to occur in MPPA treated cultures in the previous report of Laux and colleagues, although it is likely that a great deal of the uronic acids detected by these investigators may have came from other sources such as eDNA (Laux *et al*, 2002 ; Whitchurch *et al*, 2002). The next most upregulated gene in this category, *cheY*, is co-assigned to functional category K and its upregulation has already been discussed. *yegE* is located immediately upstream of the *phoPQ-oprH* operon and encodes an inner membrane sensor protein with diguanylate cyclase activity, *yegE* insertion mutants exhibit reduced biofilm formation (Kulesekara *et al*, 2006). This may indicate that upregulation of *yegE* played a role in the increased biofilm formation that was observed to occur in response to MPPA exposure (Fig 4.21). *PA14_54490* encodes a hypothetical cytoplasmic protein of unknown function which possesses a metal dependent hydrolase domain, the implications of its upregulation in response to MPPA are not clear. The *phoPQ* and *pmrAB* operons have already been discussed in regards to functional categories K and M whilst *wspF* has been previously been considered as part of functional category N. *algZ*, sometimes designated as *fimS*, forms a 2 component regulatory system with *algR*, it plays a non-essential role in alginate production but is known to be essential for twitching motility (Whitchurch *et al*, 1996). Twitching motility by PA14 is increased in the presence of MPPA, though not significantly (Figure 4.20.1).

SECTION 5.4.18

FUNCTIONAL CATEGORY U – SECRETION

Within functional category U, only a single gene is downregulated in response to MPPA whereas 8 are upregulated (Figure 5.1.35). This indicates enrichment of upregulated genes in functional category T (Figure 5.1.36). The only downregulated locus, *PA14_13150* encodes a LuxR family transcriptional regulator of undefined function and cellular localisation, it is not clear how or why this gene is downregulated in response to MPPA. The first upregulated gene in functional category U, *lepB*, encodes one of only two type I signal peptidases known to be present in the *P. aeruginosa* genome and no fewer than 801 proteins, equivalent to 14.4 % of the PAO1 genome, include a type I signal peptide (Waite *et al*, 2012 ; Lewenza *et al*, 2005). The next gene in functional category U to be upregulated in response to MPPA is *secE*, a protein translocase involved in the general secretory (sec) pathway which translocates proteins bearing a type I or II signal peptide (Ma *et al*, 2003). *fliN* encodes a flagellar motor switch and its upregulation in response to MPPA is not surprising given that other flagellar genes are also upregulated and swimming motility increased in the presence of MPPA (Figures 4.20.1 & 5.1.21). This further supports the hypothesis that MPPA or its breakdown products may act as chemoattractants that stimulate motility. The *pilE,W,X,Y1* genes are all involved in the biogenesis of type IV pili, which are involved in twitching motility and surface attachment, as is *fimU* which belongs to the same operon (O'Toole & Kolter, 1998).

SECTION 5.4.19

FUNCTIONAL CATEGORY W – GENES NOT OTHERWISE CLASSIFIED

This functional category has been created for genes that are significantly dysregulated in response to MPPA and have PAO1 orthologs but have not been assigned COG annotations. It comprises 5 downregulated and 10 upregulated loci. The first downregulated gene in this category, *opdH*, encodes a porin that is involved in the uptake of tricarboxylates including aconitate, citrate and isocitrate, this porin

also acts as a conduit for ceftazidime, a cephalosporin which is unique in that it has an unusually high molecular weight due to its bulky side chains, opdH does not appear to be involved in the uptake of other β-lactam compounds with lower molecular weight including imipenem, cefotaxime, ceftriaxone and cefepime (Tamber *et al*, 2007). It is not clear why this porin is downregulated in response to MPPA but this may be a result of carbon catabolite repression or a general membrane stress response aimed at maintaining outer membrane integrity. *PA14_10490* encodes a hypothetical protein, neither its function nor cellular localisation are known. *PA14_01490* encodes an aegerolysin, RahU, which has been shown to increase biofilm formation and, at high levels, impair monocyte chemotaxis (Rao *et al*, 2011). Lysophosphocholine, a host derived inflammatory lipid related to MPPA, has been shown to upregulate expression of *PA14_01490* whereas some oxidised lipids downregulate this gene – this could indicate substantial oxidation of MPPA has occurred in the present experiment (Rao *et al*, 2011). *PA14_10360* encodes another hypothetical protein with unknown function and localisation. *oprE* encodes a porin that is inducible in hypoxic conditions, its homology with *oprD* implies that it might be involved in the uptake of amino acids (Yamano *et al*, 1993). Possible reasons for downregulation of oprE are catabolite repression and an increase in oxidative metabolism induced by MPPA although it should be noted that conditions in matched MPPA control cultures were by no means anaerobic. The next gene in this functional category to be upregulated in response to MPPA, *algP*, encodes a histone like protein involved in alginate biosynthesis, although essential for mucoidy, it is not always upregulated in mucoid strains and can even be downregulated (Firoved & Deretic, 2003). *popD* encodes a translocator protein that is part of the type III injectisome ; several other proteins involved in type III secretion are upregulated in response to MPPA and are dispersed across different functional categories (Figures 5.1.31, 5.1.37 & 5.1.38). *PA14_54340* encodes a hypothetical protein of unknown function and cell localisation, it is located immediately upstream of *lepB*, discussed as part of functional category U (Figure 5.1.33). *phaI* encodes a putative polyhydroxyalkanoate granule associated protein, it is not clear why this gene is upregulated by MPPA. *exoT* encodes exotoxin T, an effector protein of the type III secretion system, its upregulation is not surprising

given that several other proteins involved in type III secretion are upregulated in response to MPPA and are dispersed across different functional categories (Figures 5.1.31, 5.1.37 & 5.1.38). *PA14_57060* encodes a hypothetical protein of unknown function and cell localisation. *PA14_18320* and *PA14_18310* both belong to the *arn* operon, all genes in this operon are upregulated and spread across various functional categories, its function has already been discussed in section. *PA14_44311* encodes a hypothetical cytoplasmic protein of unknown function. *PA14_63130* is a hypothetical cytoplasmic protein of unknown function which belongs to the *pmrAB* operon, previously discussed in section.

SECTION 5.4.20

FUNCTIONAL CATEGORY X – GENES WITHOUT PAO1 ORTHOLOGS

This functional category was created for genes that are significantly dysregulated in response to MPPA but have no PAO1 ortholog and hence no COG annotation, it consists of 5 downregulated and 3 upregulated genes. *PA14_03370* encodes a hypothetical inner membrane protein predicted to function as a ubiquinol cytochrome C chaperone. *PA14_59970* encodes a hypothetical protein with unknown function and cellular localisation, it is located immediately downstream of *PA14_59960*, discussed in section. *PA14_03360* is located immediately upstream of *PA14_03370*, it encodes a hypothetical protein with unknown function or cell localisation but has been predicted to function as a ubiquinol cytochrome C chaperone. *PA14_53590* encodes a hypothetical cytoplasmic protein of unknown function. *PA14_12900* encodes a DNA binding protein with integration host factor like motifs. It is not clear how or why any of these genes are downregulated in response to MPPA. The first locus upregulated in functional category X, *PA14_07480*, encodes reverse transcriptase, again, it is not clear why this gene is upregulated. The next upregulated locus, *fdnG*, encodes nitrate inducible formate dehydrogenase subunit O, although involved in glyoxylate metabolism its upregulation is unexpected since it generally functions in anaerobic metabolism (Wu *et al*, 2005). *fdnH*, also upregulated, belongs to the same operon and is assigned to functional category C. *spcU* encodes a

chaperone of exotoxin U, a phospholipase effector that is part of the type III secretion system, upregulation of this gene is not surprising as conditions of calcium limitation and diversion of carbon into the glyoxylate shunt are can both stimulate type III secretion genes (Hauser, 2009 ; Chung *et al*, 2013).

SECTION 5.4.21

ROLE OF EXTRACYTOPLASMIC FUNCTION SIGMA FACTORS

The role of ECF σ factors in the transcriptional response to MPPA is not clear. All apart from *rpoS*, the stationary phase sigma factor and *fecI*, a regulator of iron transport, are positively expressed in MPPA treated cultures. However, of these positively expressed ECF σ factors, only 2, *algU* and *sigX*, are themselves significantly upregulated and the observed downregulation of *rpoS* and *fecI* does not reach statistical significance either. When MPPA regulated genes were grouped into primary ECF sigmulons the only significant findings were enrichment for downregulated genes in the *rpoD* sigmulon, for upregulated genes in the *sigX* sigmulon and for both up and downregulated genes in the *rpoN* sigmulon. This would suggest that transcriptomic responses to MPPA are partially controlled by these 3 σ factors. Involvement of the other σ factors cannot be ruled out, however, due to the possibility of regulatory crosstalk.

CHAPTER 6

DEVELOPMENT OF MPPA NANO-FORMULATIONS WITH POTENTIAL ROLES IN ANTIBIOTIC DELIVERY

SECTION 6.1

INTRODUCTION

Lipid vesicles have a long history of use in the delivery of drugs, including the application of antibacterial agents to the airway in cystic fibrosis (Alhariri *et al*, 2013). There are several potential advantages to the use of such drug delivery systems in cystic fibrosis. Some of the antibiotics used to treat pseudomonal infections of the cystic fibrosis airway are comparatively toxic and have narrow therapeutic indices ; polymyxins such as colistin are potentially neurotoxic whilst aminoglycosides such as tobramycin are ototoxic, both classes of agent exhibit nephrotoxicity (Falagas & Kasiakou, 2006 ; Mingeot-Leclercq & Tulkens, 1999). Encapsulation of drugs within lipid vesicles has the potential to mitigate toxicity and allow high concentrations to be attained locally at the site of infection (Karlowsky & Zhanel, 1992 ; Alipour *et al*, 2009). Drug encapsulation may also have the potential to protect drugs from inactivation by polyanionic components of sputum (Alipour *et al*, 2009). Antibiotics have deleterious effects on the host microflora that may on occasion lead to pathology as the drugs may exert positive selective pressure for pathogens such as *Clostridium difficile, Candida sp.* and multi-drug resistant organisms of the 'ESKAPE' group (Pendleton *et al*, 2013). This is especially likely with some of the broader spectrum agents such as cephalosporins, fluoroquinolones and carbapenems, many of which are employed out of necessity to treat recalcitrant infections of the airways in cystic fibrosis patients (Dancer *et al*, 2013 ; Nilholm *et al*, 2015). Evidence is also mounting to suggest that dysbiosis of the host microbiome, possibly linked to antibiotic use, may have hitherto unknown roles in the pathology of numerous and diverse chronic ailments ranging from asthma to inflammatory bowel disease that have not traditionally been construed as 'infectious conditions' (Huang & Boushey, 2015 ; Joossens *et al*, 2011). Therefore, a potential advantage of using lipid nano-vesicles for antibiotic delivery or, indeed any inhaled drug delivery system, is that the drugs are not distributed systemically and therefore do not interact with the host microflora at distant anatomic sites such as the bowel, nasopharynx and skin. Liposomes have been the most widely used lipid based drug delivery system to date. Several liposomal antibiotic formulations for nebulisation in

cystic fibrosis lung disease have shown therapeutic advantages over free drug and are now in advanced clinical trials such as ARIKACE™ (liposomal amikacin), further highlighting the potential of this approach to drug delivery (Meers *et al*, 2008 ; Clancy *et al*, 2013). Herein, the preliminary development of a lipid nano-vesicle formulation based upon the bioactive lysophopholipid MPPA is described, some physicochemical parameters of the formulation are also defined (size distribution, drug encapsulation and charge) and its *in vitro* bactericidal effects against polystyrene grown *P. aeruginosa* biofilms are depicted in time-kill analyses.

SECTION 6.2

MATERIALS AND METHODS

SECTION 6.2.1

FORMULATION OF NANO-VESICLES ENCAPSULATING ANTIBIOTIC

Lipid nano-vesicles encapsulating ceftazidime were prepared using modifications of the popular thin-film hydration method (Rukholm *et al*, 2006). 20 mg of MPPA was weighed out into a round bottomed flask that had previously been cleaned sequentially with boiling water and 70 % ethanol before being dried in an oven. The lipid was then suspended in 10 ml of chloroform. The chloroform was then removed by rotary evaporation at 250 rpm in a water bath set to 60˚C to obtain a dry, uniform lipid film. Any excess traces of chloroform were then removed briefly by applying a nitrogen stream. The mouth of the round bottomed flask was then sealed with cling film and it was transferred to a UV cross-linker oven set to full power for 45 minutes in order to kill any surviving contaminants and further ensure sterility. A solution of 10 mg/ml ceftazidime hydrate was prepared in PBS and filter sterilised by passage through a syringe driven 0.2 µM pore size filter. 4 ml of 10 mg/ml ceftazidime solution was then added to the flask containing dried sterile lipid film. The film was then completely and uniformly suspended via pipetting vigorously and scraping with a flame sterilised spatula. The 4 ml of resulting suspension were then divided into 4

equal aliquots of 1 ml in 1.5 ml microcentrifuge tubes. These tubes were then placed into a foam float in a sonic water bath set above the phase transition temperature of MPPA at 50 ˚C and left for 30 minutes to enable vesicle formation. After 30 minutes, sonication was stopped and the tubes were incubated in the water bath for a further hour. The tubes were then placed into a fridge at 4˚C for 16 hours before being centrifuged at 14,000 rpm and 4 ˚C for 2 hours to allow the nano-vesicles to pellet. As MPPA is an amphiphilic lipid, not all vesicles were centrifuged out and a constant but undefined quantity remained solubilised in the aqueous supernatants, which were then discarded. Matched pellets of drug free nano-vesicles were prepared in parallel by hydrating an equivalent lipid film with sterile PBS containing no ceftazidime. Pilot experiments were also carried out using lipid films prepared at a 2:1 w/w lipid to cholesterol ratio by adding 10 mg of cholesterol when preparing lipid films and solubilising with a 80:20:2 v/v solution of chloroform:methanol:distilled water, to enable miscibility of the hydrophobic cholesterol and amphiphilic chloroform insoluble MPPA.

SECTION 6.2.2

ASSAYING ENCAPSULATION EFFICIENCY

Encapsulation efficiency was assayed by adapting a UV-visible spectrophotometric method previously reported in the literature (Devkhile & Shaikh, 2011). Pelleted formulation was resuspended by adding 1ml per microfuge tube of 0.2 % Triton X-100 and pipetting to lyse vesicles. Tubes were then transferred to a foam float and placed in a water bath set to 50˚C for 1 hour to further disrupt vesicles. A series of standard ceftazidime hydrate solutions in PBS ranging in concentration from 4 to 16 µg/ml were prepared in triplicate and their absorbances were measured in a quartz cuvette using a UV-visible spectrophotometer set at 256 nm, which a spectral scan on the 16 µg/ml sample had previously determined to equate to the λ-max value of ceftazidime hydrate, these triplicate absorbance values were then used to create a calibration plot. Suspensions of disrupted vesicles were then diluted 50 fold in PBS and transferred to the quartz cuvette for triplicate absorbance readings. A solution of

0.2 % Triton X-100 that had been diluted 50 fold was used as a blank to normalise for any background absorbance resulting from the detergent. Unknown ceftazidime concentrations were then determined using the calibration plot. 2 tubes per batch were assayed to ensure uniform encapsulation efficiency.

SECTION 6.2.3

BIOACTIVITY OF FORMULATION AGAINST BIOFILMS – FORMULATION MIC VALUES

This experiment was carried out using modifications of a resazurin based method previously reported in the literature (Lipuma *et al*, 2009). Biofilms of 6 *P. aeruginosa* strains were cultured on the well surfaces of a 96 well microtitre plate. Single colonies of each strain were picked and grown overnight at 37˚C with agitation at 250 rpm in universals containing 5 ml aliquots of LB. These cultures were then diluted to an A_{600} value of 0.2 ± 0.02 in fresh LB and then further diluted 30 fold to obtain a starter inoculum containing ~ 1 x 10^7 cfu/ml. 125 µl aliquots of this inoculum were then transferred to the inner wells of a 96 well plate. 200 µl of sterile water was added to the outer wells to minimise evaporation and lids were attached before the plates were sealed with parafilm and incubated statically at 37 ˚ C for 48 hours to allow biofilm formation upon the wells. After this time the plates were opened and culture from the inner wells was removed via pipetting. The inner wells were then rinsed twice with 180 µl volumes of sterile PBS to remove residual planktonic growth. Empty pelleted formulations and pelleted formulation incorporating ceftazidime were thoroughly resuspended in 1 ml of cation supplemented MHB and then serial diluted in a fresh 96 well plate to obtain 6 replicates of a descending concentration series ranging from 512 to 4 µg/ml encapsulated drug, in 200 µl volumes. Wells in columns 9-11 of the plate were filled in triplicate with cation supplemented MHB alone as a growth control or with the drug free nano-vesicles at the highest test concentration, equating to the quantity of nano-vesicles that would have been present in the wells containing 512 µg/ml of encapsulated ceftazidime. A multi-channel pipette was then used to transfer these

dilutions to wells of the other microtitre plate containing freshly rinsed biofilms. Lids were attached and the plates were then resealed with parafilm and incubated statically at 37˚C for 24 hours. After this time, plates were removed from incubation and 20 µl of 0.03 % resazurin metabolic indicator solution was added to each inner well. Plates were then resealed with lids and parafilm and incubated statically at 37 ˚ C for a further 16 hours. After this time, plates were removed from incubation and absorbances at 570 nm and 600 nm wavelengths were read and used to calculate the percentage reduction of resazurin as a surrogate measure of growth - due to the natural opacity of the nano-vesicle formulation, using the following equation (O'Brien *et al,* 2000 ; Alamar Blue Technical Datasheet)

$$\frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where: O1 = molar extinction coefficient (80586 L mol^{-1} cm^{-1}) of oxidized resazurin at 570nm

O2 = molar extinction coefficient (117216 L mol^{-1} cm^{-1}) of oxidized resazurin at 600nm

R1 = molar extinction coefficient (155677 L mol^{-1} cm^{-1}) of reduced resazurin at 570nm

R2 = molar extinction coefficient (14652 L mol^{-1} cm^{-1}) of reduced resazurin at 600nm

N1 = absorbance of negative control well at 570nm

N2 = absorbance of negative control well at 600nm

Mean percentages of resazurin reduction were recorded for each strain and experimental condition on bar charts with standard error.

SECTION 6.2.4

BIOACTIVITY OF FORMULATION AGAINST BIOFILMS – TIME-KILL EXPERIMENTS

Biofilms of 6 *P. aeruginosa* strains were cultured on the well surfaces of a 96 well microtitre plate. Single colonies of each strain were picked and grown overnight at 37˚C with agitation at 250 rpm in universals containing 5 ml aliquots of LB. These cultures were then diluted to an A_{600} value of 0.2 ± 0.02 in fresh LB and then further diluted 30 fold to obtain a starter inoculum containing ~ 1 x 10^7 cfu/ml. 125 µl aliquots of this inoculum were then transferred to the inner wells of a 96 well plate. 200 µl of sterile water was added to the outer wells to minimise evaporation and lids were attached before the plates were sealed with parafilm and incubated statically at 37 ˚ C for 48 hours to allow biofilm formation upon the wells. After this time the plates were opened and culture from the inner wells was removed via pipetting. The inner wells were then rinsed twice with 180 µl volumes of sterile PBS to remove

residual planktonic growth. Pelleted formulation incorporating ceftazidime were thoroughly resuspended in 1 ml of cation supplemented MHB and then diluted to the desired test concentration of encapsulated antibiotic. This varied according to strain and was defined as the concentration of encapsulated drug that equated to 4 times the formulation MIC that had been predetermined for biofilms of that strain in endpoint biofilm MIC analyses. A control dilution of free drug in cation supplemented MHB was compared at either the same concentration, or at its own MIC as predetermined for biofilms of that strain in endpoint biofilm MIC analyses, whichever was greater for the strain being tested. Diluted free and encapsulated ceftazidime were each transferred to 21 rinsed biofilm containing wells of the aforementioned 96 well plate in 200 µl volumes per well to provide 3 biological replicates for each strain and challenge condition at 7 time-points of 1 , 2 , 3 , 4 , 5 , 6 and 24 hours. 3 other biofilm containing wells were then filled with 200 µl of 5 mg/ml filter sterilised cellulase solution in 0.05 M citrate buffer (pH 6.0) to aid dispersal of the biofilms. A plate lid was then attached and sealed with parafilm before the plate was incubated statically for 1 hour at 37˚C, The plate was then quickly removed from incubation and opened. Walls of each of the wells containing cellulase in citrate buffer were scraped with 200 µl pipette tips and the cellulase solution was pipetted up and down several times to further disperse adherent biofilms. 20 µl aliquots from each of these wells were then transferred to a separate 96 well plate with wells containing 180 µl of sterile PBS. 10 fold serial dilutions were then prepared across the rows of this plate down to 10^{-7} and 50 µl aliquots from each triplicate well in the 10^{-5} to 10^{-7} range were then spread onto triplicate MH agar plates with a cooled flame sterilised steel plate spreader. Plates were then incubated at 37 ˚ C overnight and colonies were counted after incubation. At the same time, triplicate wells containing free or encapsulated drug had their contents removed with a multi-channel pipette and replaced with 200 µl volumes of 5 mg/ml cellulase in 0.05 M citrate buffer before the lid was re-attached to the 96 well plate and it was again incubated at 37˚C for a further hour. After this time, the plate was removed and the process was repeated every hour for 6 hours and again at 24 hours to obtain triplicate colony counts for each strain and test condition at each of 8 time-points corresponding to the length of exposure to the challenge agent (0 , 1 , 2 , 3 , 4 , 5 , 6 and 24 hours). With longer

incubation times, progressively lower dilutions were plated, to account for the anticipated decimation of the bacteria by the antibiotic. A separate experiment in a fresh 96 well plate was carried out for each of the 6 test strains. Mean viable cfu/ml counts for each strain and test condition were plotted versus exposure time on semi-log graphs to obtain time-kill curves.

SECTION 6.3

RESULTS

In order to determine encapsulation efficiency and particle size distribution for the MPPA/ceftazidime formulation, UV visible spectrophotometry and Nanosight particle tracking technology were employed, respectively. This revealed that drug encapsulation was fairly consistent between independently prepared batches, always being in the range of 4-5 % of total loaded drug. Nanosight tracking revealed that particle size distribution was invariably polydisperse across batches tested and did not appear to be affected by drug encapsulation, as size distributions were similar for drug free control vesicles.

SECTION 6.3.1

ENCAPSULATION EFFICIENCY

Percentage encapsulation efficiencies for ceftazidime hydrate were found to vary between 6 independent batches of pelleted MPPA formulation from 4.34 to 4.95 %, corresponding to drug contents of 434 to 494 µg per pellet (Figure 6.1 ; Table 6.1).

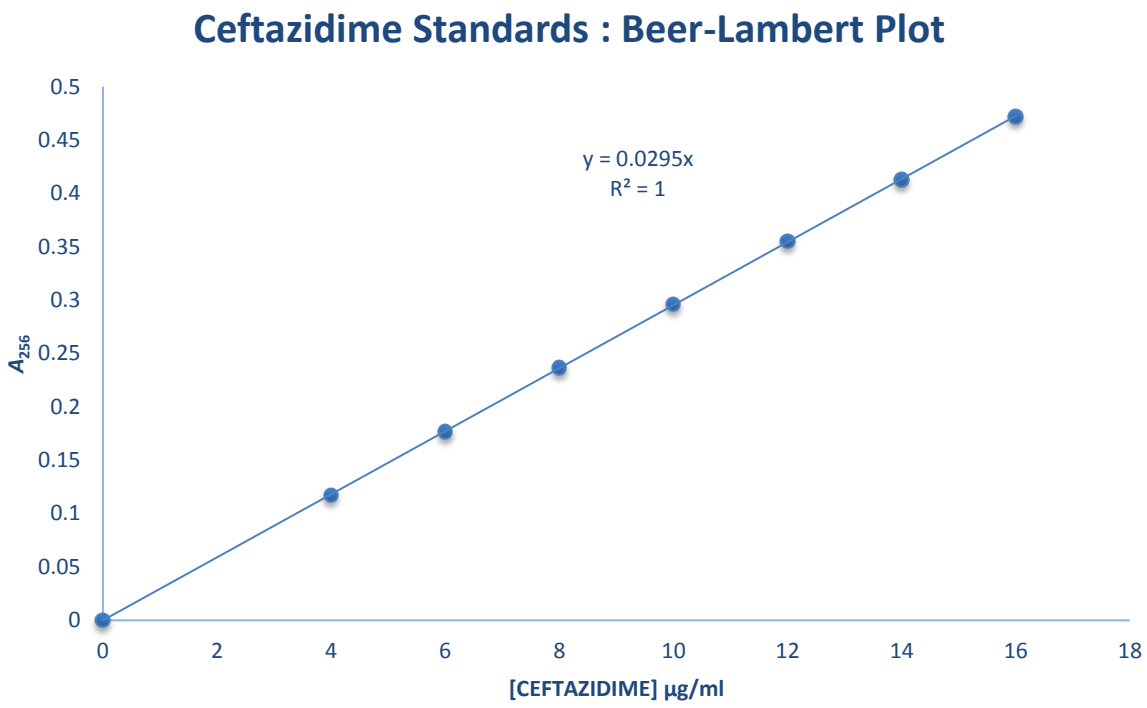


Figure 6.1

Calibration plot of ceftazidime hydrate standards, graphed as mean absorbances observed at the predetermined λ max of 256 nm (n = 3). Error bars omitted for clarity. Mean recorded absorbances (n = 3) of each formulation batch, tested at a 1 in 50 dilution were used to extrapolate the unknown drug encapsulation efficiency.

BATCH No.	A_{256}	ENCAPSULATION EFFICIENCY (%)	µg DRUG/PELLET
1	**0.292**	**4.95**	**495**
2	**0.260**	**4.41**	**441**
3	**0.282**	**4.78**	**478**
4	**0.256**	**4.34**	**434**
5	**0.279**	**4.73**	**473**
6	**0.286**	**4.85**	**485**
MEAN	**0.276**	**4.68**	**468**
S.E.M	**0.005418**	**0.091703**	**9.170346**

Table 6.1
Ceftazidime encapsulation efficiencies of 6 independently prepared batches of MPPA formulation as extrapolated from calibration plot. Drug was loaded at a concentration of 10 mg/ml in sterile PBS.

SECTION 6.3.2

PARTICLE SIZE DISTRIBUTION

Particle size distribution variances were analysed between 3 independently prepared batches of pelleted MPPA empty formulation (Figures 6.2.1 - 6.2.3) and drug loaded formulation (Figures 6.3.1 - 6.3.3) using a Nanosight NTA particle tracking system. Differences in size distribution are summarised in table 6.2. For empty nano-vesicles, mean particle size varied between 147 and 247 nm in diameter and modal particle size was found to vary between 74 and 197 nm in diameter. Cumulative size distribution values for empty nano-vesicles varied from 54 to 125, 101 to 232 and 272 to 397 nm, respectively at the 10 , 50 and 90 % deciles. In batch standard deviations varied from 82 to 110 nm for empty formulations. For drug loaded nano-vesicles, mean particle size varied between 145 and 249 nm in diameter and modal particle size was found to vary between 76 and 96 nm in diameter. Cumulative size distribution values for empty nano-vesicles varied from 65 to 72, 116 to 187 and 260 to 582 nm, respectively at the 10 , 50 and 90 % deciles. In batch standard deviations

varied from 87 to 202 nm for drug loaded formulations.

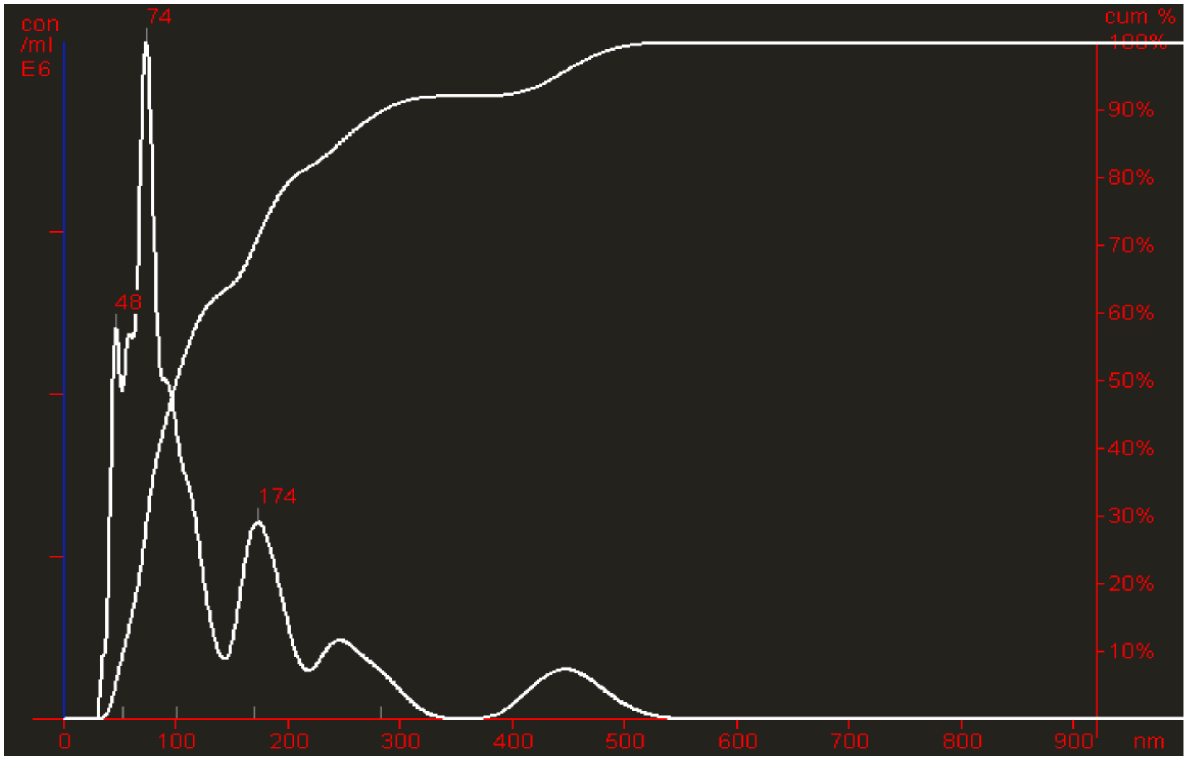


Figure 6.2.1

Particle size distribution of empty MPPA nano-vesicle formulation batch 1. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 3.16 x 10^8 per ml dH_2O.

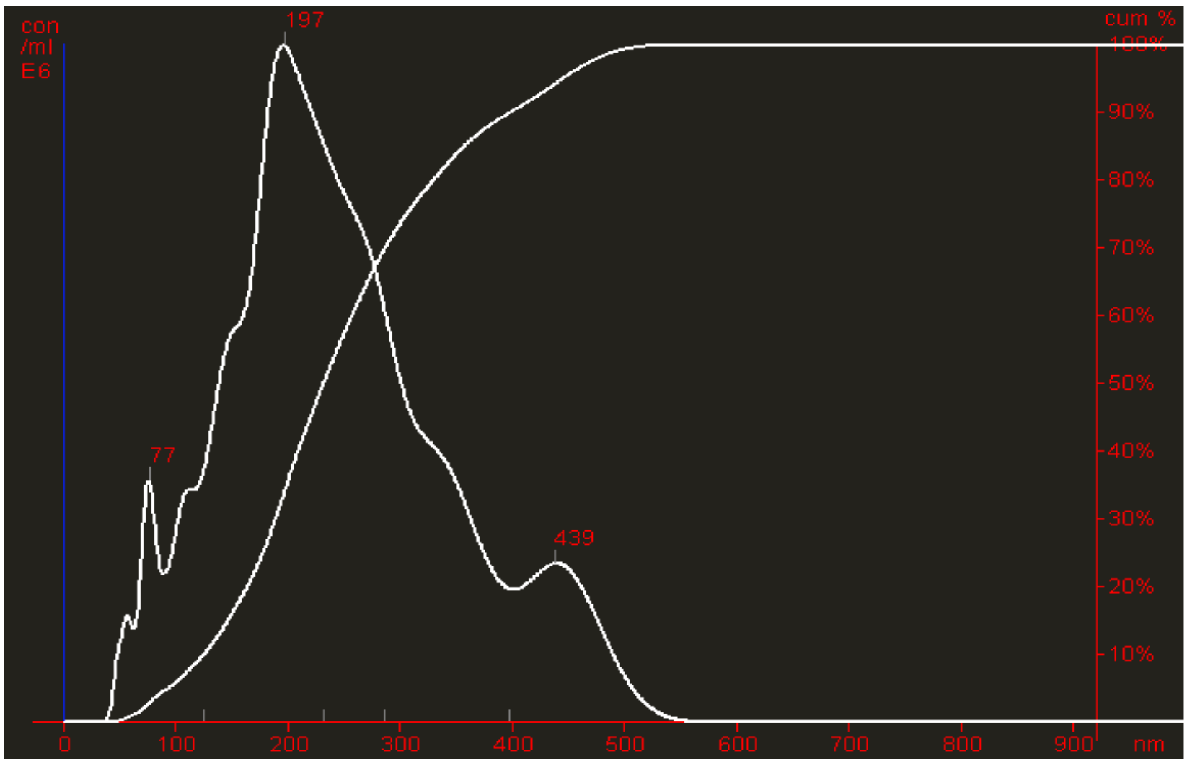


Figure 6.2.2

Particle size distribution of empty nano-vesicle formulation batch 2. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 2.02 x 10^8 per ml dH_2O.

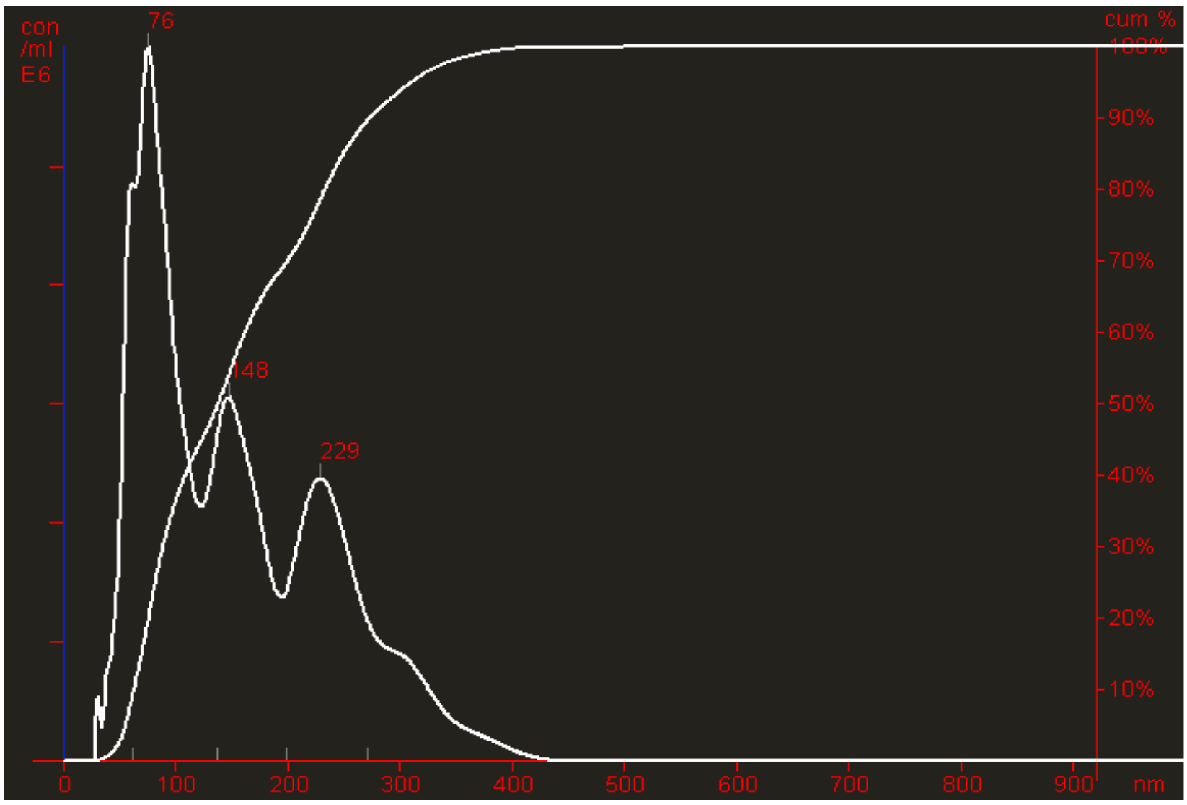


Figure 6.2.3

Particle size distribution of empty MPPA nano-vesicle formulation batch 3. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 6.99 x 10^8 per ml dH_2O.

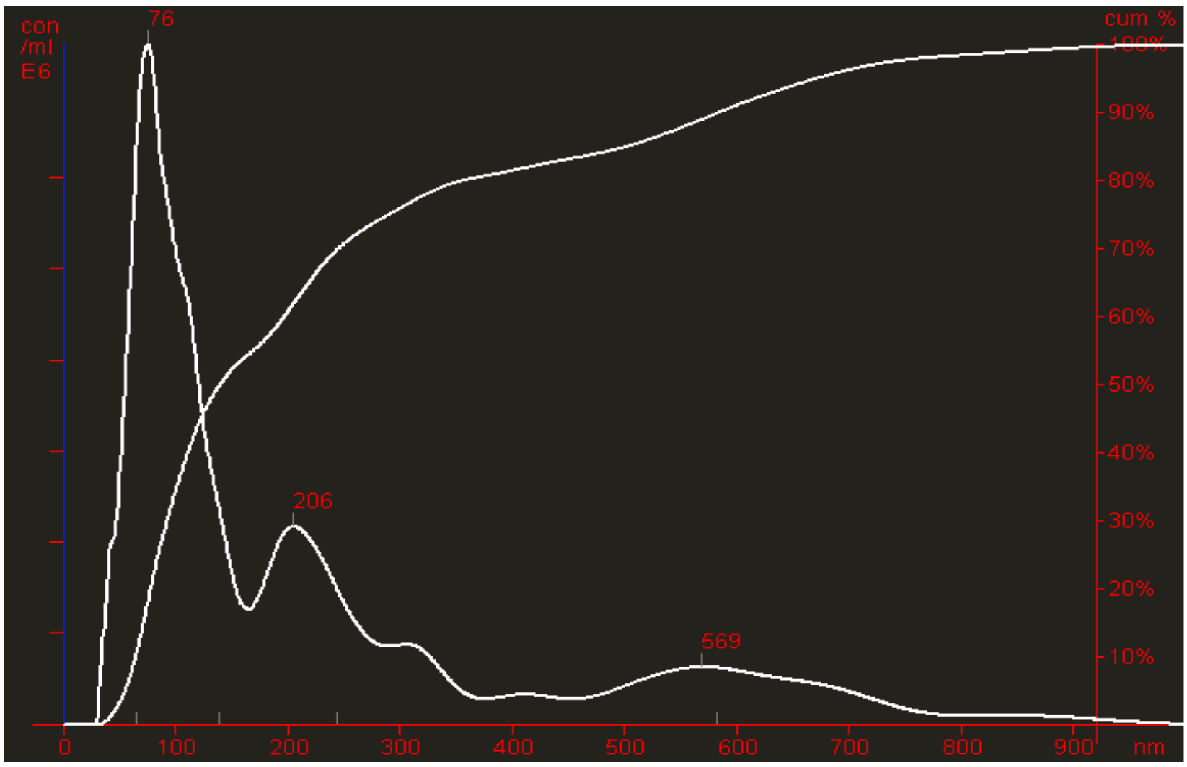


Figure 6.3.1

Particle size distribution of ceftazidime loaded MPPA nano-vesicle formulation batch 1. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 9.5 x 10^8 per ml dH_2O.

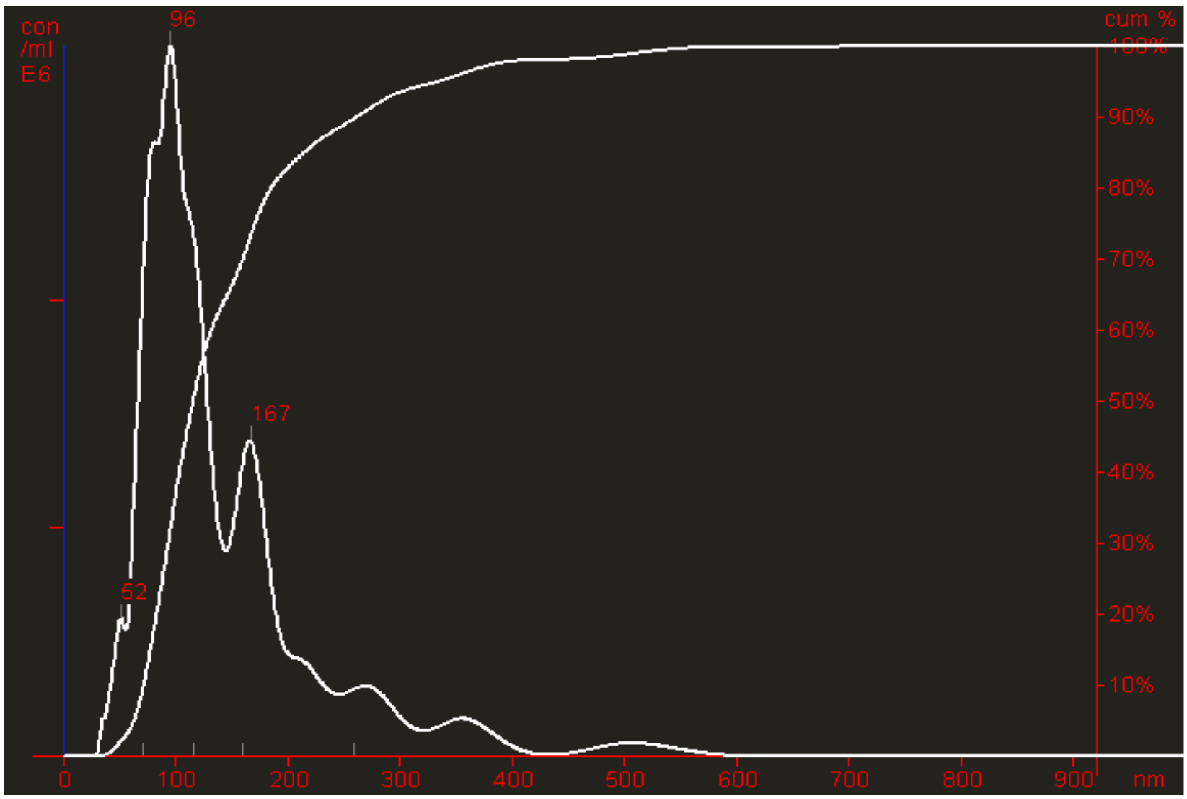


Figure 6.3.2

Particle size distribution of ceftazidime loaded MPPA nano-vesicle formulation batch 2. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 2.96 x 10^8 per ml dH_2O.

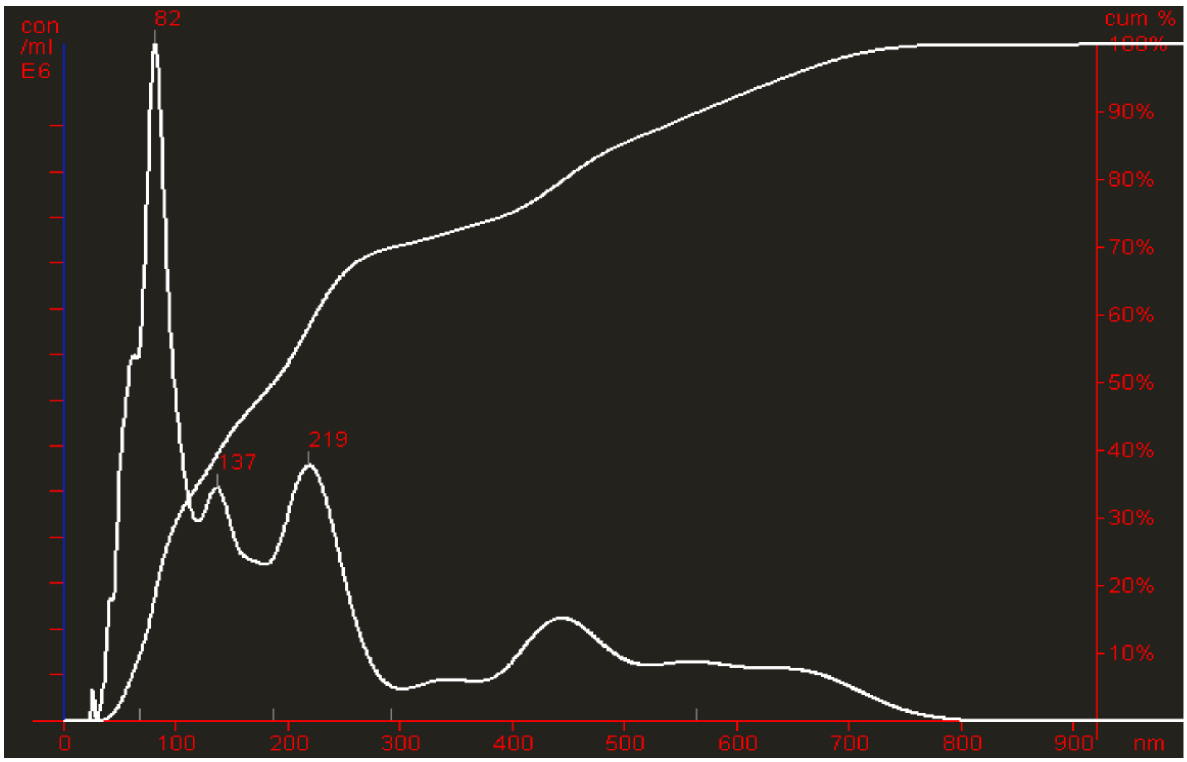


Figure 6.3.3

Particle size distribution of ceftazidime loaded MPPA nano-vesicle formulation batch 3. Particle size (nm) is denoted on the X- axis whilst cumulative decile percentages of particles are plotted up the right Y-axis. Particle concentration is 1.83 x 10^9 per ml dH_2O

BATCH No.	MEAN (nm)	MODE (nm)	D_{10} (nm)	D_{50} (nm)	D_{90} (nm)	STD DEV.
Drug 1	**230**	**76**	**65**	**139**	**582**	**202**
Drug 2	**145**	**96**	**72**	**116**	**260**	**87**
Drug 3	**249**	**82**	**69**	**187**	**565**	**190**
Empty 1	**147**	**74**	**54**	**101**	**284**	**110**
Empty 2	**247**	**197**	**125**	**232**	**397**	**100**
Empty 3	**154**	**76**	**63**	**138**	**272**	**82**

Table 6.2

Table summarising the size distribution measured for triplicate batches of both empty and ceftazidime loaded MPPA: nano-vesicle formulation using the Nano-Sight NTA particle tracking system.

SECTION 6.3.3

BIOFILM ENDPOINT MIC DETERMINATIONS AND TIME-KILL CURVES

It was deemed to be of interest to establish whether or not encapsulation of ceftazidime in MPPA vesicles enhanced its antibiotic activity against 6 test strains of *P. aeruginosa* grown in biofilm. To answer this question, biofilm MIC was determined for each strain using the resazurin reduction method and bactericidal activity was assessed over time using viable CFU counts taken from disrupted biofilms after 1, 2, 3, 4, 5, 6 and 24 hours exposure to either free or encapsulated drug. Encapsulation of ceftazidime variably increased ceftazidime activity as evidenced by a 4 to 32 fold reduction in MIC depending on the test strain and the attainment of bactericidal activity within 24 hours for all test strains with encapsulated drug versus only 2 of 6 strains with free drug.

SECTION 6.3.3.1

C1426

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 512 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded MPPA nano-vesicle formulation was determined to be 8 fold lower at 64 µg/ml (Figure 6.4.1). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 512 µg/ml, free ceftazidime reached its maximal effect against triplicate C1426 biofilms after 4 hours of exposure, reducing viable cell density from a starting count at 0 hours of 4.6×10^7 down to 2×10^5 cfu/ml. After the 4 hour time-point, growth of biofilm cells was observed to resume and a mean viable cfu/ml count of 8.64×10^7 was reached after 24 hours of free drug exposure, in contrast, encapsulated ceftazidime at the same concentration – equivalent to 8 times its own MIC value, continued to reduce cell viability in biofilms after the 4 hour time-point, reducing viable cell density to 6.6×10^3 cfu/ml after 6 hours and 0 cfu/ml after 24 hours (Figure 6.4.2).

SECTION 6.3.3.2

C1433

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 128 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded nano-vesicle formulation was determined to be 4 fold lower at 32 µg/ml (Figure 6.4.3). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 128 µg/ml, free ceftazidime reached its maximal effect against triplicate C1433 biofilms after 4 hours of exposure, reducing viable cell density from a starting count at 0 hours of 9.6×10^6 down to 1.2×10^5 cfu/ml. After the 4 hour time-point, growth of biofilm cells was observed to resume and a mean viable cfu/ml count of 3.72×10^6 was reached after 24 hours of free drug exposure, in contrast, encapsulated ceftazidime at the same concentration – equivalent to 4 times its own MIC value, continued to reduce cell viability in biofilms after the 4 hour time-point, reducing viable cell density to 3.3×10^3 cfu/ml after 6 hours and 0 cfu/ml after 24 hours (Figure 6.4.4).

SECTION 6.3.3.3

J1385

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 64 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded nano-vesicle formulation was determined to be 4 fold lower at 16 µg/ml (Figure 6.4.5). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 64 µg/ml, free ceftazidime reached its maximal effect against triplicate J1385 biofilms after 3 hours of exposure, reducing viable cell density from a starting count at 0 hours of 1.4×10^7 down to 1.67×10^5 cfu/ml. After the 3 hour time-point, growth of biofilm cells was observed to resume and a mean viable cfu/ml count of 3.2×10^6 was reached after 24 hours of free drug exposure, in contrast, encapsulated ceftazidime at the same concentration – equivalent to 4 times

its own MIC value, continued to reduce cell viability in biofilms after the 4 hour time-point, reducing viable cell density to 1.8 x 10^4 cfu/ml after 6 hours and 5.6 x 10^1 cfu/ml after 24 hours (Figure 6.4.6).

SECTION 6.3.3.4

J1532

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 512 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded MPPA formulation was determined to be 8 fold lower at 64 µg/ml (Figure 6.4.7). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 512 µg/ml, free ceftazidime reached its maximal effect against triplicate J1532 biofilms after 24 hours of exposure, reducing viable cell density from a starting count at 0 hours of 2.0 x 10^6 down to 1.34 x 10^4 cfu/ml, in contrast, encapsulated ceftazidime at the same concentration – equivalent to 4 times its own MIC value, was found to reduce cell viability in biofilms more rapidly, reducing viable cell density to 0 cfu/ml after 24 hours (Figure 6.4.8).

SECTION 6.3.3.5

PAO1

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 2048 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded MPPA nano-vesicle formulation was determined to be 32 fold lower at 64 µg/ml (Figure 6.4.9). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 2048 µg/ml, free ceftazidime reached its maximal effect against triplicate PAO1 biofilms after 24 hours of exposure, reducing viable cell density from a starting count at 0 hours of 1.38 x 10^7 down to 9.3 x 10^2 cfu/ml, in contrast, encapsulated ceftazidime at a concentration of 512 µg/ml – equivalent to 4 times its own MIC value, reduced viable cell density more rapidly, reducing viable cell density to 6.67 cfu/ml after 24

hours and beginning to kill cells exponentially after only 2 hours and reducing viable cell density to 1.0 x 10^5 cfu/ml by the 6 hour time-point, whilst free drug at 2048 µg/ml took longer than 6 hours to reduce cell density by a single order of magnitude (Figure 6.4.10).

SECTION 6.3.3.6

PA14

The biofilm endpoint MIC of free ceftazidime for this strain was determined to be 128 µg/ml whereas the biofilm endpoint MIC of the ceftazidime loaded MPPA:cholesterol 5:1 w/w nano-vesicle formulation was determined to be 8 fold lower at 16 µg/ml (Figure 6.4.11). This constituted a significant reduction in endpoint biofilm MIC for encapsulated vs. free ceftazidime ($p < 0.05$). At its MIC of 128 µg/ml, free ceftazidime reached its maximal effect against triplicate PA14 biofilms after 24 hours of exposure, reducing viable cell density from a starting count at 0 hours of 1.69 x 10^7 down to 5.86 x 10^3 cfu/ml, in contrast, encapsulated ceftazidime at half this concentration – equivalent to 4 times its own MIC value, reduced cell viability in biofilms at a more rapid rate, reducing viable cell density to 0 cfu/ml after 24 hours (Figure 6.4.12).

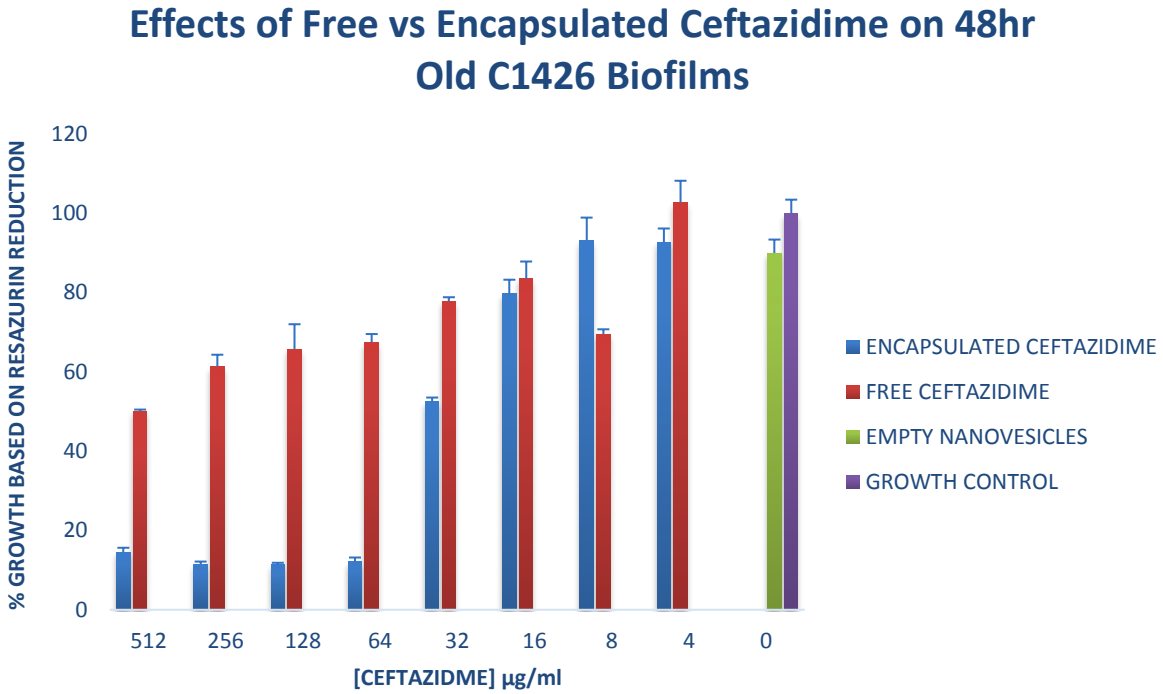


Figure 6.4.1
Endpoint MIC assay for strain C1426.

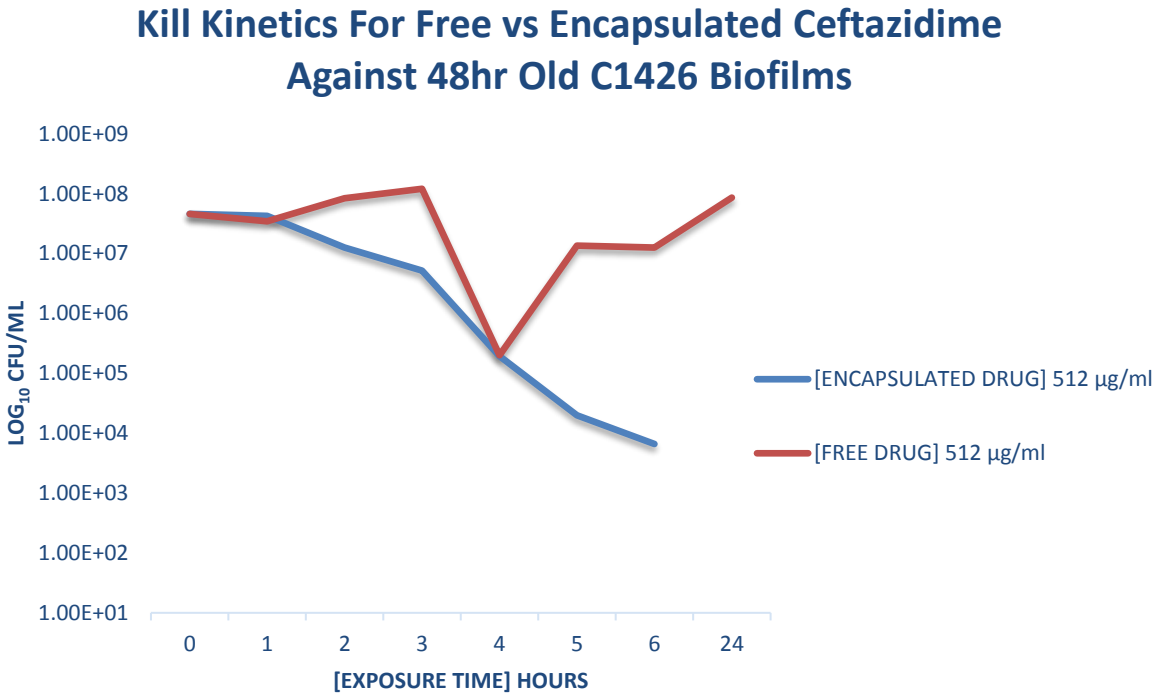


Figure 6.4.2
Time-kill curves for strain C1426. Error bars omitted for clarity.

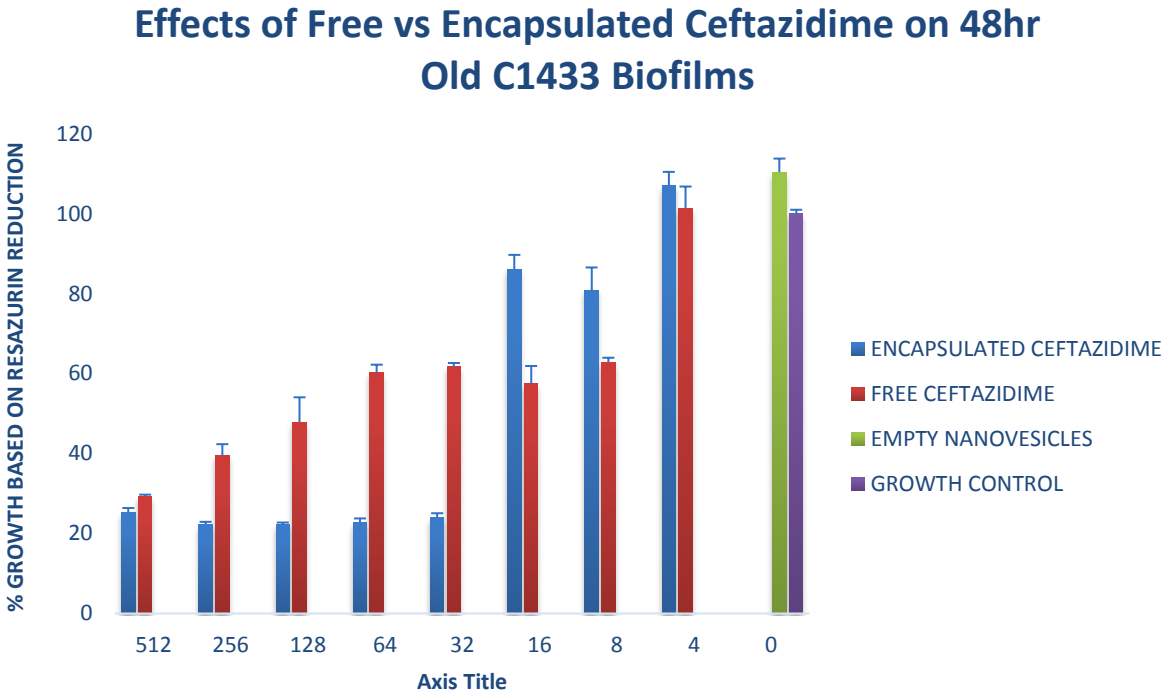


Figure 6.5.1
Endpoint MIC assay for strain C1433.

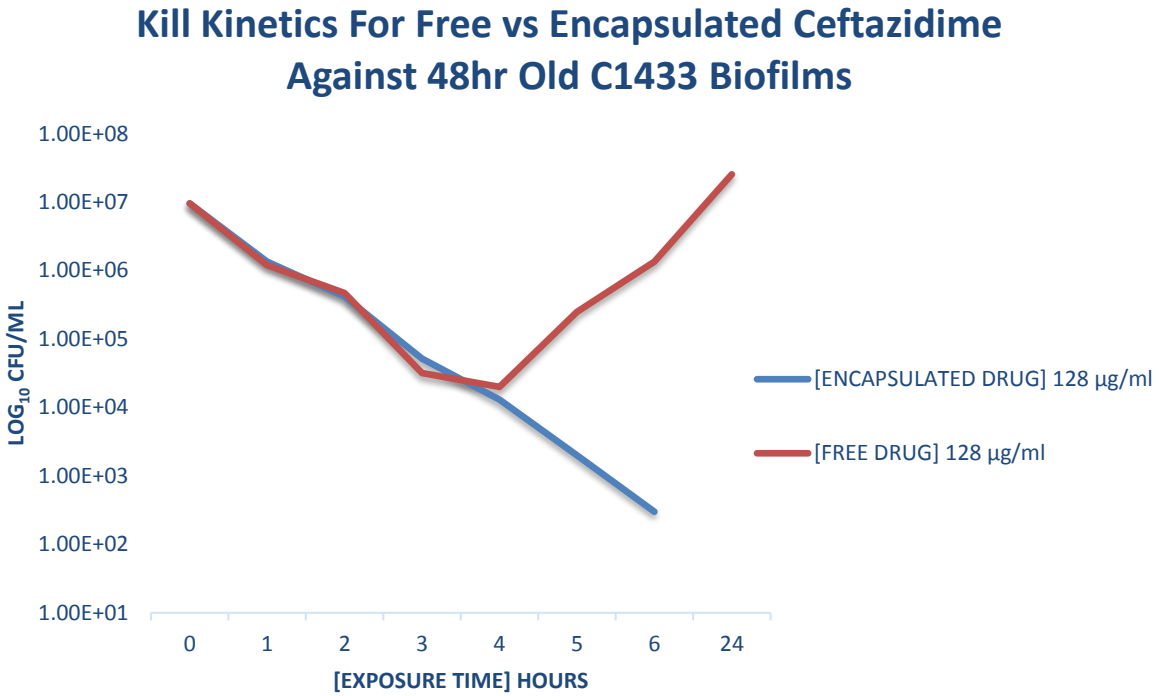


Figure 6.5.2
Time-kill curves for strain C1433. Error bars omitted for clarity.

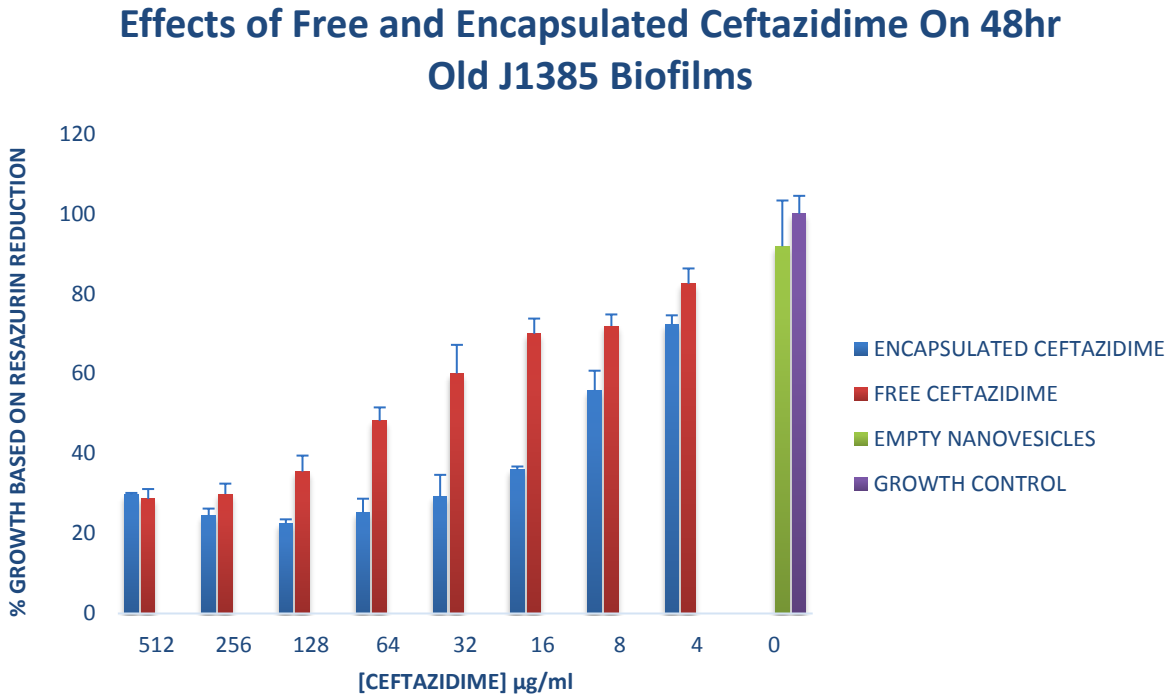


Figure 6.6.1
Endpoint MIC assay for strain J1385.

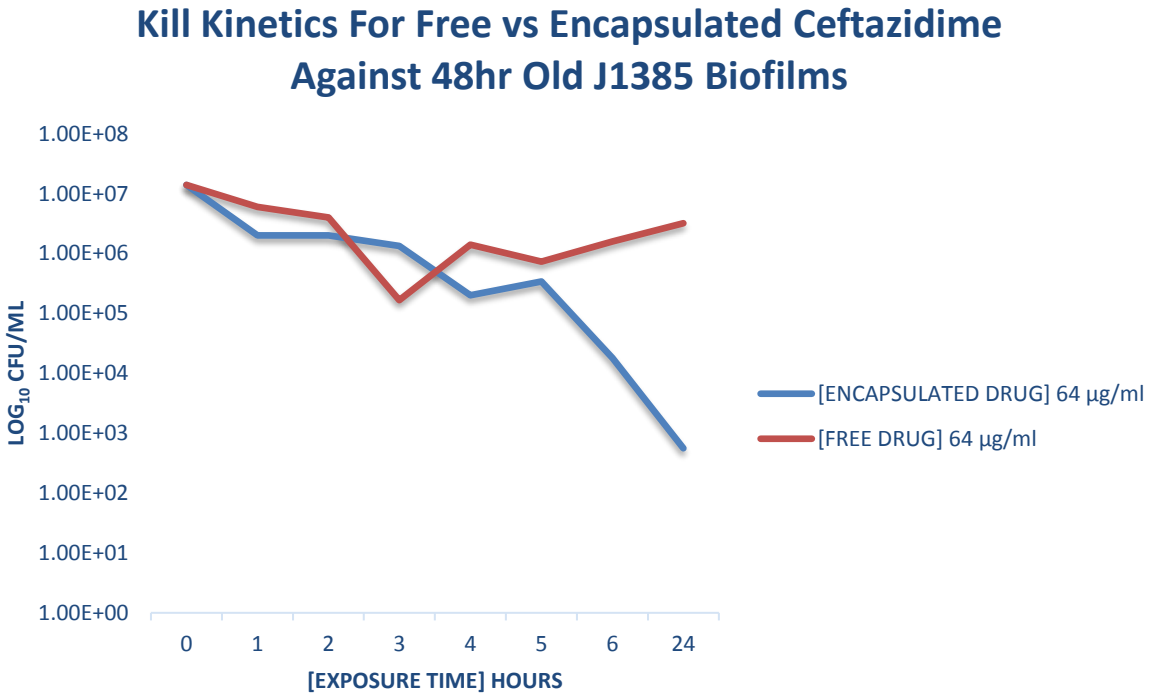


Figure 6.6.2
Time-kill curves for strain J1385. Error bars omitted for clarity.

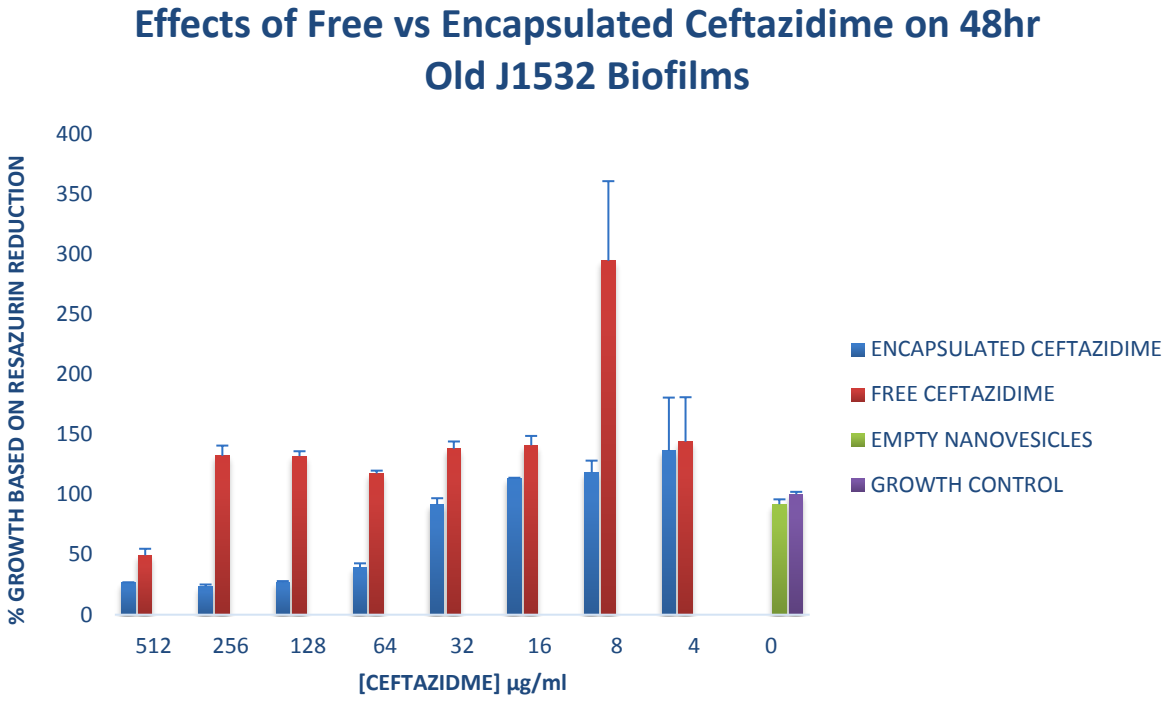


Figure 6.7.1
Endpoint MIC assay for strain J1532.

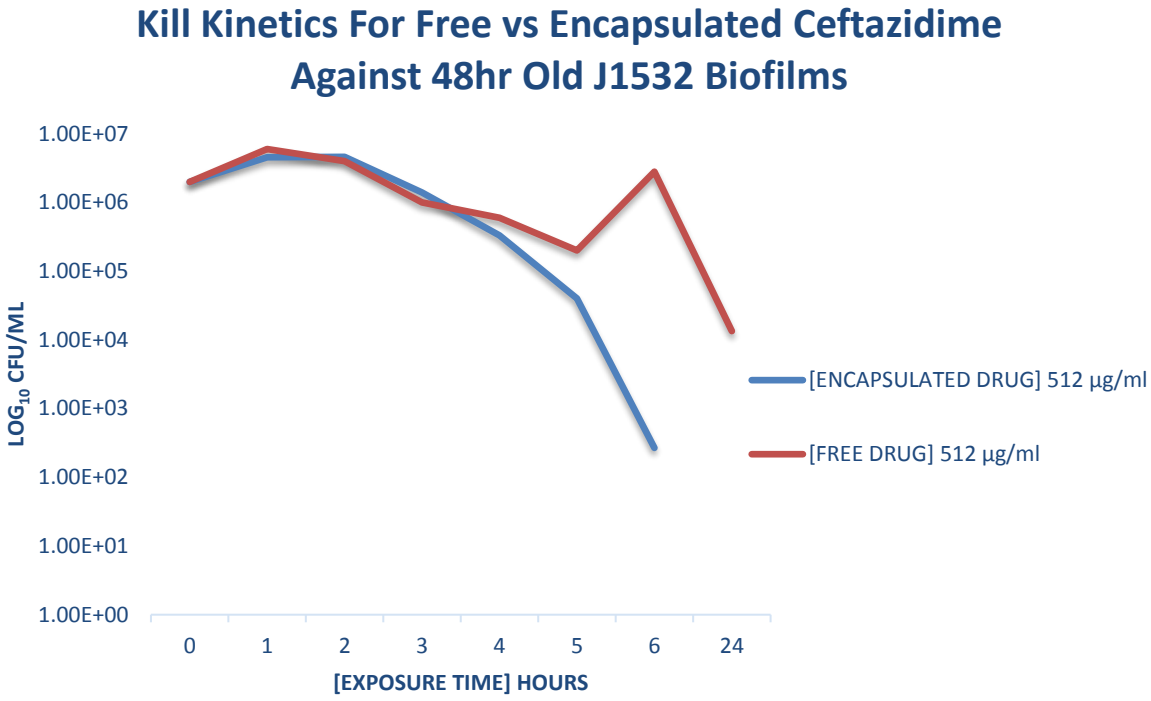


Figure 6.7.2
Time-kill curves for strain J1532. Error bars omitted for clarity.

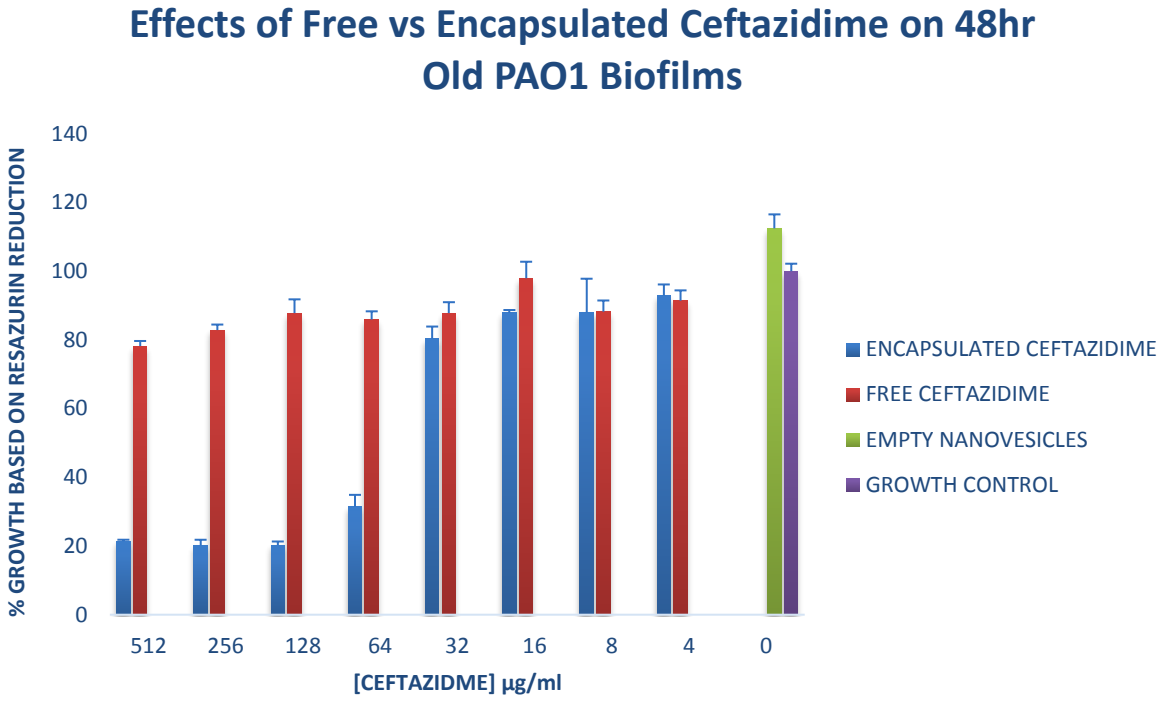


Figure 6.8.1
Endpoint MIC assay for strain PAO1.

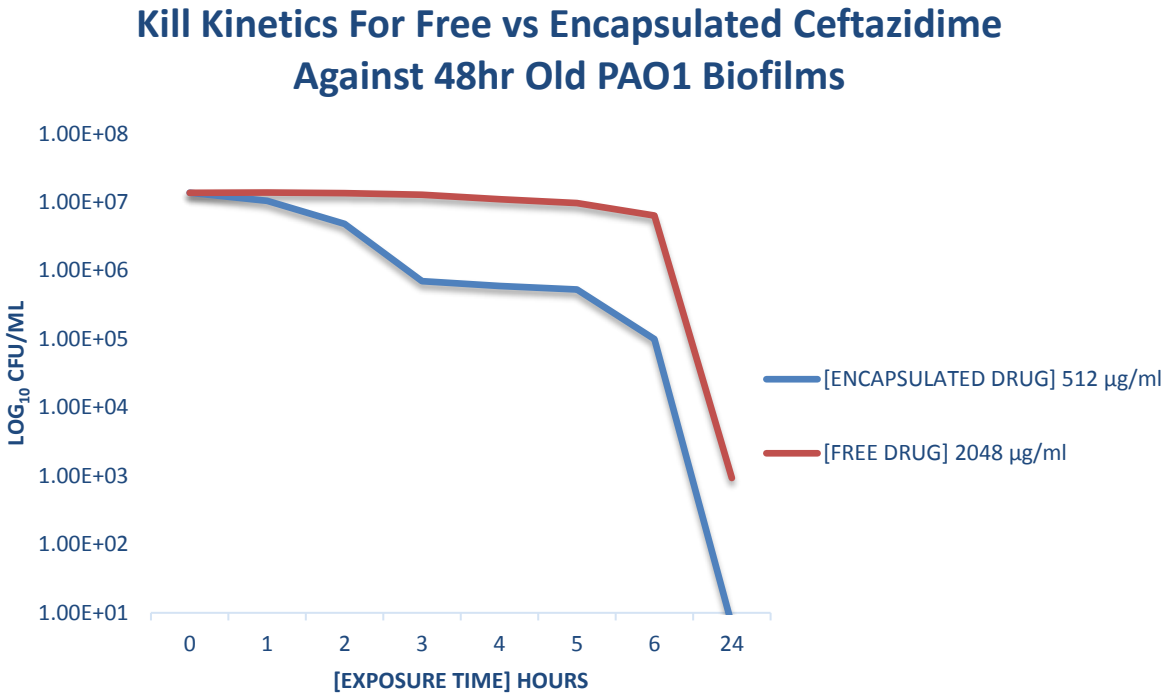


Figure 6.8.2
Time-kill curves for strain PAO1. Error bars omitted for clarity.

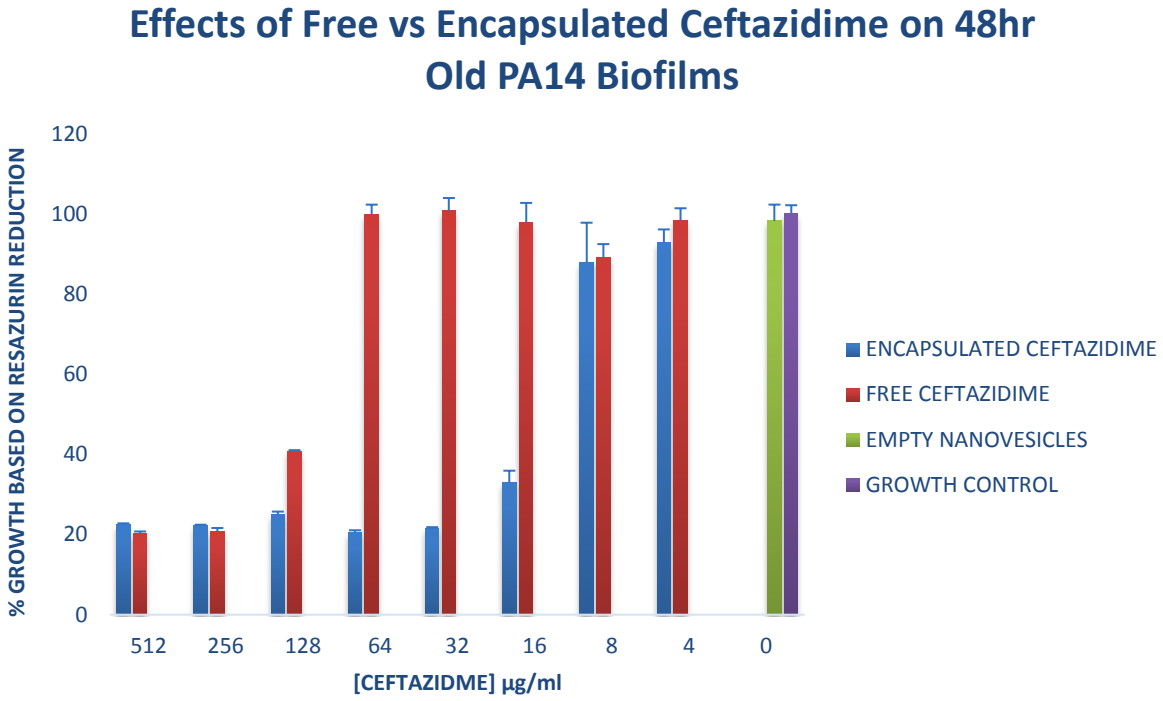


Figure 6.9.1
Endpoint MIC assay for strain PA14.

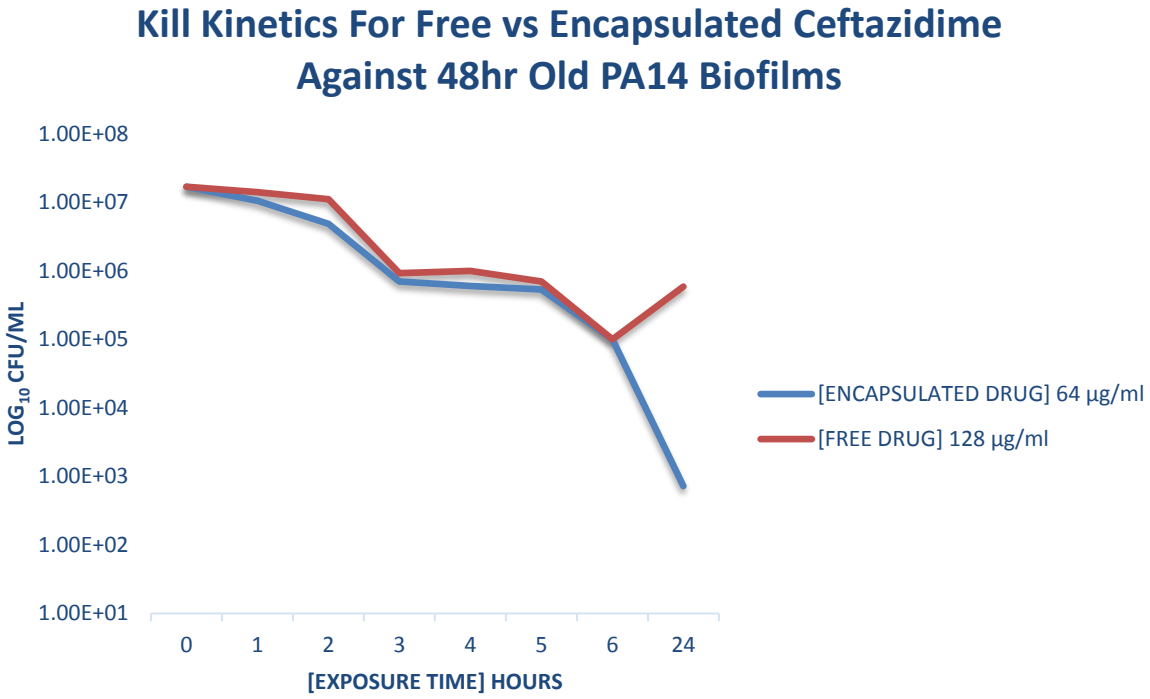


Figure 6.9.2
Time-kill curves for strain PA14. Error bars omitted for clarity.

SECTION 6.4

DISCUSSION

SECTION 6.4.1

FORMULATION – SIZE DISTRIBUTION ANALYSES AND ENCAPSULATION EFFICIENCIES

6 batches of ceftazidime loaded MPPA formulation exhibited minimal variation in encapsulation efficiency between them, which varied from 4.34 to 4.95 % (Table 6.1). Although this encapsulation efficiency is low, it is very similar to values reported in the literature for previous liposomal formulations of hydrophilic antibiotics prepared using the film hydration method, although these may not be strictly comparable given the different physicochemical composition of the vesicles (Torres *et al*, 2012 ; Rukholm *et al*, 1996). Nevertheless, poor encapsulation efficiency of hydrophilic low molecular weight drugs has been a longstanding problem in most lipid vesicle based drug delivery systems regardless of the specific lipid make-up (Eloy *et al*, 2014). Particle size distribution analyses of empty (Figures 6.2.1 – 6.2.3) and ceftazidime encapsulating (Figures 6.3.1 – 6.3.3) MPPA formulation conducted using the Nanosight NTA particle tracking system revealed a uniformly poldisperse distribution with few obvious differences in mean and modal size distribution or range (Table 6.2). Lysophospholipids such as MPPA possess comparatively large polar phosphate head groups relative to their single nonpolar acyl tail groups and do not spontaneously form bilayers in aqueous media but instead exhibit rapid curvature to form spherical micelles (Fuller & Rand, 2001 : Krogfelt *et al*, 2000). These micelles are relatively soluble in aqueous media and therefore a constant but undefined proportion of MPPA would have been lost in our experiments, discarded with the loading solution rather than being pelleted by centrifugation. Although supernatants initially appeared clear, giving the impression that all lipid had been pelleted, they became hazy with a white precipitate after refrigeration for a few days, suggesting that pelleting was incomplete. Few attempts to incorporate lysophospholipids such as MPPA into liposomes or other lipidic nano-

formulations have been reported, probably due largely to the amphiphilic and inherently less stable nature of their monoacylated structures, nonetheless, there is an expanding body of evidence to imply that sterols can be co-assembled with lysolipids to form liposomal structures with a relatively stable bilayer (Koklic & Trancar, 2012 : Khandelia *et al*, 2014). A pilot experiment incorporating cholesterol into the lipid film in a 2:1 w/w MPPA:cholesterol ratio was therefore performed to test the possibility that this would enable the formation of liposomes rather than micelles and prevent loss of MPPA in the supernatants. This was found to prevent the appearance of a haze in the supernatants, therefore implying that MPPA likely associated with the cholesterol to formed liposomes with a bilayer structure, preventing loss of MPPA to the supernatants, at least in part and encapsulation efficiency was found to be comparable with this formulation to that previously attained for the formulation prepared without cholesterol, work with this formulation, however, was not pursued further as a test of endpoint biofilm MIC with strain PAO1 at this stage, revealed that it was not bioactive.

SECTION 6.4.2

FORMULATION – BIOACTIVITY

Encapsulation of ceftazidime in MPPA vesicles has shown an impressive potential to increase its activity against biofilms of all tested *P. aeruginosa* strains from 4 to 32 fold depending on the specific strain. However, in no case was this sufficient to cause a decrease in MIC to levels of ≤ 8 μg/ml, equivalent to the breakpoint for clinical susceptibility. It should be noted however, that breakpoints such as these derive from data pertaining to simulated inhibition of planktonic growth at the maximum drug concentrations safely achievable at the site of infection following intravenous administration of the drug. It is likely that administration of the drug locally, either in free or encapsulated form, via nebulisation, could achieve much higher levels in the infected airway (Lu *et al*, 2011). Moreover, MPPA encapsulated ceftazidime achieved a bactericidal effect as defined by Pankey and Sabath as being

equal to a 3-$\log_{10}$ reduction in viable cell count (Pankey & Sabath, 2004) against biofilms of all 6 test strains within 24 hours whilst ceftazidime alone was merely bacteriostatic for all but 2 tested strains – J1532 and PAO1. Conventional and biofilm sensitivity testing of planktonically grown sputum isolates has been shown to be an inadequate predictor of clinical and bacteriological response to therapy and personalised antibiotic regimens tailored to sputum isolates based on the results of such tests does not generally lead to better outcomes when compared to blind treatment, clinical improvements are commonly encountered in spite of apparent resistance and conversely, treatment failures frequently occur despite apparent sensitivity *in vitro* (Hurley *et al*, 2012 ; Waters & Ratjen, 2015). In the present work, MPPA encapsulated and free ceftazidime were compared for effect against polystyrene grown biofilms in a 96 well microtitre plate format and the encapsulated drug invariably exhibited greater activity, with at least a 4 fold drop in MIC relative to the free drug at an equal or higher concentration. The clinical relevance of this is not clear. Whilst the 96 well microtitre plate model of biofilm formation used in the present work may not be truly representative of conditions in the CF airway, the fact that encapsulation in MPPA nanovesicles invariably improved ceftazidime activity by a factor of at least 4 fold regardless of the specific strain and its baseline sensitivity nevertheless suggests that this formulation may be of value in enhancing drug efficacy in the airways, especially given the fact that MPPA has also been shown to markedly enhance the activity of ceftazidime and other antibiotics in planktonic cultures and reduce accumulation of virulence associated exoproducts (Figures 4.14 – 4.19, Table 4.1). The mechanism by which MPPA encapsulation enhances the effect of ceftazidime against polystyrene bound biofilms is not clear. It is likely that MPPA enhances the outer membrane permeability of biofilm cells as well as planktonic cells, allowing increased access of ceftazidime into the periplasm where it can bind to its target ligand, penicillin binding protein 3 (Krogfelt *et al*, 2000 ; Tavio *et al*, 2014). It is also possible that increased permeability of the outer membrane results in leakage of chromosomal β-lactamase that would otherwise have intercepted ceftazidime in the periplasm, hydrolysing the drug before it could reach its target. Encapsulation of β-lactams in lipid vesicles has also previously been shown to protect them from β-lactamases present in the extracellular milieu, and this

is yet another possibility which deserves consideration (Naccuchio *et al*, 1985). In experiments with planktonically growing cultures, MPPA appeared to increase growth rate and oxidative respiration, conversely, in these experiments, empty MPPA vesicles appeared to slow biofilm growth of some strains even in the absence of antibiotic, this effect was not observed in planktonic cultures of these strains treated with MPPA and may be due to cation supplementation of the MHB used in biofilm studies or to particular aspects of the biofilm phenotype, such as altered LPS expression and reduced metabolic rate (Ciornei *et al*, 2010). Future work in this direction could aim to test the effects of this formulation in an *in vivo* background of CF airway infection such as the murine model reported by Carter and colleagues (Carter *et al*, 2010)

CHAPTER 7

CONCLUSIONS & FUTURE DIRECTIONS

SECTION 7.1

CONCLUSIONS AND FUTURE DIRECTIONS

The transcriptomic data in chapter 5 provide clues to the genetic basis underlying phenotypes reported in chapter 4. Reductions in pyocyanin production brought about by MPPA appear likely to result from carbon catabolite repression of genes involved in aromatic amino-acid metabolism but may also be linked to downregulation of the *phzB1* gene. Several genes related to chemotaxis and the biogenesis of type IV pili and flagellae are upregulated, explaining the increases in swimming and twitching motility observed in chapter 4. Increased biofilm formation does not appear to be related to the Pel operon, suggesting that other polysaccharides (Psl) absent in PA14 may be involved, which makes sense given that this was the only strain found not to increase biofilm formation in response to MPPA. Upregulation of the *phoPQ-oprH*, *pmrAB* and *arn* operons as well as genes involved in LPS biosynthesis strongly suggest that membrane damage is responsible for the potentiation of antibiotics, other than polymyxins and aminoglycosides, with MPPA. Downregulation of the *dsbA2* gene may be partly responsible for aberrant production of elastase and other exoproteases. Quorum sensing genes do not appear to be dysregulated although this could be due to the time-point at which RNA was extracted. Several genes related to type III secretion are upregulated and future work may endeavour to confirm or reject this finding at the phenotypic level. Upregulation of type III secretion may have the potential to render organisms more virulent but also more immunogenic. MPPA was unexpectedly found to increase biofilm formation by most strains tested. It is unclear overall whether or not MPPA would be useful or detrimental in the

treatment of CF airway disease and in future this may be tested in an animal model. The role of efflux genes in the synergistic interactios between MPPA and antibiotics could be confirmed or rejected using non-redundant mutants as this has not been resolved in the present work. The MPPA concentration of 250 µg/ml used here was arbitrarily chosen and preliminary experiments with colistin and tobramycin suggest that much lower concentrations close to those naturally present in inflammatory exudates may be effective and this merits further investigation.

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APPENDIX A

MEDIA, BUFFERS & BACTERIAL STRAINS

LURIA-BERTANI MEDIUM

10g tryptone

5g yeast extract

5g sodium chloride

1l distilled water

These ingredients in the stated ratio were mixed thoroughly in a clean, rinsed Duran bottle. pH was then verified after dissolution using a probe and, where necessary, was adjusted via dropwise addition of 1M hydrochloric acid or 5M sodium hydroxide solutions until a value of 7.4 ± 0.2 was attained. The medium could be solidified at this point if required by adding 20g/l agar granules (Melford Laboratories, Ipswich, UK) and microwaving until melted and well mixed. The medium was then sterilised by autoclaving at 121˚C for 15 minutes.

MUELLER-HINTON BROTH

300g/l dehydrated beef infusion

17.5g/l casein hydrolysate

1.5g/l starch

This medium was purchased as a ready mixed powder from Oxoid, Thermo-Fisher Scientific, Basingstoke, UK. 21g of powder per litre of distilled water were dissolved to prepare the broth medium in a clean, rinsed Duran bottle and its pH (7.3 ± 0.1) was verified using a probe and adjusted by dropwise addition of 1M hydrochloric acid or 5M sodium hydroxide, if necessary. The medium could be solidified at this point if required by adding 20g/l agar granules (Melford Laboratories Ltd, Ipswich, UK) and microwaving until melted and well mixed. The medium was then sterilised by autoclaving at 121˚C for 15 minutes. Broth media prepared in this manner contained 78 µM (3.126 mg/l) of Ca^{2+} and 225 µM (5.468625 mg/l) Mg^{2+}, respectively, as indicated in the batch information. Where necessary, the medium was supplemented to obtain final concentrations of 594 µM Ca^{2+} (23.8 mg/l) and 509 µM Mg^{2+} (12.37 mg/l), respectively, by adding 57.27 mg/l calcium chloride and 70 mg/l magnesium sulphate (as heptahydrate), prior to pH adjustment.

CITRATE BUFFER (pH 6.0)

0.1 M citric acid solution (19.2 g citric acid/ litre distilled water)

0.1 M sodium citrate solution (25.8 g trisodium citrate dehydrate/litre distilled water)

9.5 ml of 0.1M citric acid solution were added to 41.5 ml of sodium citrate solution and the volume was completed to 100 ml by adding 49 ml of distilled water. The pH was then verified using a pH probe. The buffer was then sterilised by filtration with a 0.2 µm pore size filter.

ELASTASE BUFFER (pH 7.5)

40.078 mg calcium chloride

12.114 g tris

1 l distilled water

Tris and calcium chloride are dissolved in distilled water in the above ratios. The pH is verified as 7.5 with a pH probe and the buffer is sterilised by autoclaving at 121˚C for 15 minutes.

TOTAL PROTEASE BUFFER (pH 7.8)

80.156 mg calcium chloride

4.8456 g tris

1 l distilled water

Tris and calcium chloride are dissolved in distilled water in the above ratios. The pH is verified as 7.8 with a pH probe and the buffer is sterilised by autoclaving at 121˚C for 15 minutes.

CACODYLATE BUFFER (pH 7.2)

1.379 g sodium cacodylate

100 ml distilled water

Dissolve 1.379 g sodium cacodylate in 100 ml water and verify pH using a probe. Add 1/10 volume of 25 % electron microscopy grade glutaraldehyde immediately prior to use to give a final concentration of 2.5 % v/v glutaraldehyde for use as a fixative.

STRAIN	ORIGIN
A36	Sputum isolate previously identified as LES by PCR in laboratory of Prof. C. Winstanley. Obtained during acute exacerbation. Non-mucoid.
A38	Non-pigmented sputum isolate previously identified as LES by PCR in laboratory of Prof. C. Winstanley. Obtained during acute exacerbation. Non-mucoid.
C1426	Sequenced sputum isolate, non-mucoid. Obtained from CF patient attending Edinburgh Royal Infirmary and catalogued by Prof. J.R.W Govan. (Stewart *et al*, 2014)
C1433	Isogenic mucoid variant of C1426, obtained from same patient as C1426 ~1 month later and catalogued by Prof. J.R.W Govan. (Stewart *et al*, 2014) .
J1385	Sputum isolate, non-mucoid. Obtained from patient attending Edinburgh Royal Infirmary and catalogued by Prof. J.R.W Govan. (Stewart *et al*, 2014) .
J1532	Isogenic mucoid variant of J1385, obtained from same patient as J1385 ~3 months later and catalogued by Prof J.R.W Govan. (Stewart *et al*, 2014) .
LES400	Sequenced LES strain from laboratory of Prof C. Winstanley (Salunkhe *et al*, 2005 ; Jeukens *et al*, 2014).
LES431	Sequenced hypervirulent LES strain which caused pneumonia in the non CF parent of a CF patient, catalogued by Prof. C Winstanley (Salunkhe *et al*, 2005 ; Jeukens *et al*, 2014)
MUC28	Mucoid strain from laboratory of Prof. C. Winstanley.
PAO1	'Type strain'. Originally catalogued by Prof. B.W Holloway as a chloramphenicol resistant variant of a wound isolate from a patient in Melbourne, Australia in the 1950s and first to be sequenced in 2000. (Holloway, 1955 ; Stover *et al*, 2000).
PA14	Highly virulent reference strain. Sequenced in 2004. (He *et al*, 2004)
S1	Sputum isolate previously identified as LES by PCR in laboratory of Prof. C. Winstanley. Obtained during clinically stable period. Non-mucoid.
S2	Sputum isolate previously identified as LES by PCR in laboratory of Prof. C. Winstanley. Obtained during clinically stable period. Non-mucoid.
S38	Sputum isolate previously identified as LES by PCR in laboratory of Prof. C. Winstanley. Obtained during clinically stable period. Non-mucoid.

APPENDIX B

LAMELLAR BIOMEDICAL INVESTIGATOR'S BROCHURE