Chapter 7 General Discussion & Future work

7.1 General Discussion

Despite much interest in the proteins responsible for the development of streptomycetes over the last 50 years, there has been little work on the role of PLs in this process (Chater & Horinouchi, 2003). The role of PLs during streptomycete development has remained a poorly understood area of research. Through the use of the fluorescent dye, NAO, research workers have begun to explore how the role played by membrane PL heterogeneity in protein localization, division site selection and membrane engulfment in the model bacteria, *E. coli* and *B. subtilis*. CL-enriched membrane regions has been used to demonstrate CL localization to septal and the polar membrane regions of *E. coli* cells (Mileykovskaya & Dowhan, 2000). Also in *E. coli*, cells that had been genetically induced to form minicells also showed enrichment of CL (Koppelman *et al*., 2001). In addition CL was found in *B. subtilis* cells at the poles and mid cell, upon deletion of the gene encoding CL synthase, CL could no longer be detected at these sites (Kawai *et al.*, 2004). In sporulating cells, fluorescent domains were clearly seen in the membranes during prespore engulfment and subsequently in prespore membranes during the various stages during *B. subtilis* sporulation. Interestingly, spore membranes have a high CL content, although its localization in the membranes has not been observed (Kawai *et al.*, 2004). In the model streptomycetes, *S. coelicolor* and more recently, *S. venezuelae* little is known about the involvement of PLs in cell division and development in this genus. Recent work has showed that *SCO1389*, that encodes CL synthase, is an essential gene in and is required for development in *S. coelicolor*. By modulating the expression of this gene it was possible to inducibly-control development of this organism (Jyothikumar *et*

al., 2012). It is tempting to suggest that CL enrichment at the branches from which aerial hyphae emerge might act as a site for the recruitment of key proteins that drive development. Key to aerial growth is the recruitment of the small hydrophobic protein, SapB (the spore associated-protein) (Guijarro *et al*., 1988), which appears to be directly involved in erecting aerial hyphae (Willey *et al*., 1991). During growth on rich medium, such as a glucose carbon source SapB is required for morphological development (Champness, 1988, Merrick, 1976, Willey *et al.*, 1993). It is for this reason that we wondered whether the nutritional requirements for SapB dependent development may be mediated via the localization of PL, such as CL at the base of aerial hyphae. In *S. coelicolor* use of the fluorescent dye, NAO, showed that CL was found at tips and branch points in this organism (Jyothikumar *et al*., 2012). As a result, it is possible that CL plays a role in the recruitment of SapB to base of aerial hyphae. In this way, we speculate that CL might affect development through allowing SapB to break the surface tension of the air-water interface. In a similar way, the chaplins and rodlins, which are amphipathic proteins and make the aerial hyphal surface hydrophobic, might also require CL for localization during development (Claessen *et al*., 2003, Claessen *et al*., 2002, Elliot *et al*., 2003). The induction or depletion of *SCO1389* also affects the mycelial architecture of *S. coelicolor*; increasing the expression of this gene results in the formation of greater numbers of sites of CL enrichment and also increases the frequency at which this organism branches and changes direction (Jyothikumar *et al*., 2012). In *S. coelicolor*, the coiled-coil protein DivIVA is essential for growth and is localised at growing hyphal tips and branch points (Flardh, 2003a) . Partial depletion and over expression of this protein

has dramatic effects on tip extension so it may be enhanced localization of CL at tips and branch point directly or indirectly affects the localization of DivIVA and so alteration of *SCO1389* expression levels affects tip extension and branching through DivIVA recruitment to regions of CL enrichment. In *B. subtilis*, DivIVA is recruited to the cell poles through an interaction with membrane negative curvature (Lenarcic *et al*., 2009, Oliva *et al*., 2010). It was found that this interaction of DivIVA and the inner leaflet of the polar membrane was mediated via the intrinsic negative curvature of the polar membrane rather than binding to CL or another specific PL (Lenarcic *et al*., 2009). As it is energetically favourable for CL to form clusters at regions of curved membranes (Huang *et al*., 2006), it may be that there is an indirect association of CL with DivIVA with other proteins of the tip-organizing centre (Holmes *et al.*, 2012), such as FilP (Bagchi *et al.*, 2008), Scy (Ditkowski *et al.*, 2013, Holmes *et al.*, 2012) or CslA (Xu *et al.*, 2008).

As branching and tip extension were dependent on the addition of the inducer, ATC, for the tetracycline inducible promoter that was fused to *SCO1389* (Jyothikumar *et al*. 2012), it was necessary to demonstrate that transcription of this gene was also dependent on the addition of ATC to *S. coelicolor* RJ118b. This gene carries a deletion of *SCO1389* as well as an additional copy of this gene under the control of the tetracycline inducible promoter, tcp_{830} (Rodriguez-Garcia *et al*., 2005). As such, we attempted to demonstrate that transcription of *SCO1389* was ATC dependent in this strain. We also wished to determine if transcription of other genes predicted to encode the other enzymes responsible for PL biosynthesis in this organism were developmentally regulated

(Borodina *et al*., 2005, Sandoval-Calderon *et al*., 2009). As a result, we set out to optimize PCR conditions for the amplification of these genes with the eventual aim of carrying out RT-PCR experiments to monitor transcription of the PL biosynthetic genes. Optimal Mg^{2+} ion concentration for PCR reactions (see Fig. 3.2) and PCR annealing temperatures (see Fig. 3.3) were first determined through PCR and were found to be: 66.4^{o}C, *hrdB*; 56.3^{o}C, *SCO1389*; 55.0^{o}C, *SCO1527*; 52.4^{o}C, *SCO5753* and 61.0^{o}C, *SCO6467*. Both *SCO5628* and *SCO6468* had an optimal PCR annealing temperature of 55.9^{o}C. In addition we also optimized RNA isolation from *S. coelicolor* and amplified the RT-PCR products of six PL biosynthetic genes (*SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468*) as well as the control gene, *hrdB*. We showed that there was an amplification on all genes following RT-PCR and there was no amplification from all genes following DNase treatment and PCR without RT This demonstrates that there was no DNA in RNA sample and the bands seen after agarose gel electrophoresis were derived from mRNA of each gene and not contaminating DNA (see Fig. 3.5 and 3.6).

We next investigated how the transcription of the set of PL biosynthetic genes changed during the development of *S. coelicolor* on minimal agar plus mannitol (see Fig. 3.8). *SCO5628* expression did not change significantly during the course of the experiment. This is unsurprising as this gene is predicted to encode phosphatidate cytidylyltransferase. If this prediction is correct, SCO5628 is responsible for the synthesis of CDP-DAG (Borodina *et al*., 2005; Sandoval-Calderon *et al*., 2009). This PL is a precursor from which all other membrane PLs are made. Consequently, it seems likely

that expression of this gene would be required at all stages of growth. *SCO5753* (PG synthase) (Borodina *et al*., 2005, Sandoval-Calderon *et al*., 2009) showed the highest expression of normalized relative expression at 72 h and increased during development. This protein is responsible for the synthesis of the precursor for CL which is synthesized by *SCO1389* (Borodino *et al*., 2005; Sandoval-Calderon *et al*., 2009). The normalized relative expression value of *SCO1389* also increased during hyphal growth (from 24-36 h) and reached its highest level around the onset of aerial hyphae formation, before decreasing during stationary phase. *SCO1389* was shown to encode a CL synthase (Borodina *et al*., 2005, Jyothikumar *et al*., 2012, Sandoval-Calderon *et al*., 2009). As CL is required for the erection of aerial hyphae in *S. coelicolor* (Jyothikumar *et al*., 2012), it is likely that elevated expression levels of *SCO5753* and *SCO1389* reflect the requirement of CL and, consequently, its immediate precursor, PG, for development. *SCO1527* (PI synthase) expression changed little during the whole of the equivalent time courses, whilst *SCO6467* (PS synthase) had the same pattern of the normalized relative expression as *SCO1389* which increased during hyphal growth and reached a maximal level around entry to development, then decreased during the sporulation phase. This is in contrast to *SCO6468* (PE synthase), whose expression decreased during hyphal growth before reaching its lowest level around the onset of development before decreasing during development. This may be due to the fact that *S. coelicolor* produces less PE while producing CL. *SCO6468* and *SCO6467* are putatively located in a two gene operon and it seems likely that they would be subject to co-regulation and it seems unlikely that their

expression would display different patterns during development. Clearly it is also possible that expression of these genes may be driven from their own promoters.

It was not possible to conclusively identify the different PL spots resolved by TLC when *S. coelicolor* PLs were extracted and resolved in parallel with the gene expression analysis describe above. In order to do this it would be necessary to carry out mass-spectrometry on each individual PL spot (Smith & Butikofer, 2010). Clearly, this is technically challenging and requires advanced equipment that was unavailable during the course of this study. Nevertheless this approach was successfully used to identify two species of CL found in *S. coelicolor* that differ in the length of at least one of their acyl chains (Jyothikumar *et al*., 2012). However it is possible to make some conclusions about the identity of PL spots resolved by TLC in this experiment. For example the abundance of a spot assumed to be CL (Fig. 3.10) was found to increase during growth of substrate hyphae and peaked at the time that aerial hyphae were produced. Subsequently the abundance of this spot decreased as the organism began to sporulate before decreasing during sporulation. *SCO1389*, the gene that encodes CL synthase (Sandoval-Calderon *et al*., 2009), expression also increased during the early stages of growth supporting the involvement of CL in *S. coelicolor* development. Also in this experiment, gene expression of *SCO5753*, *SCO6468* and *SCO6467* increased during vegetative growth. When analysed by TLC, the abundance of the PL spots that we believe corresponded to PG, and PE and an unidentified PL displayed similar changes to the expression of their biosynthetic genes

S. coelicolor RJ118b carries a single copy of *SCO1389* that is under the control of the tetracycline inducible promoter. As a result it was only able to grow weakly without supplementation of the growth medium with the inducer ATC. The tcp_{830} promoter displays weak uninduced activity in *S. coelicolor* when used in supplemented minimal medium (Rodriguez-Garcia *et al*., 2005) and this low level of activity might be sufficient for the weak growth of RJ118b substrate hyphae, but not allow for the production of enough *SCO1389* to generate sufficient CL to allow the erection of aerial hyphae in the absence of ATC. In *E. coli*, an elevated concentration of Mg^{2+} ions can suppress effects of mutations in many of the PL biosynthetic genes through an unknown mechanism (Mileykovskaya & Dowhan, 2009) and it was interesting to observe the effects of Mg^{2+} ion concentration on *S. coelicolor* development in conjunction with the effects of ATC on induction of *SCO1389* expression (see Fig. 4.3). To some degree increasing concentrations of Mg2+ ions also relieved the effects of the absence of inducer. This suggests that whatever the mechanism of the suppression of mutations in PL biosynthetic genes in *E. coli*, by Mg2+ ions, suppression of CL depletion occurs by a similar mechanism in *S. coelicolor* and *E. coli.*

In order to determine the ATC dependence of tcp_{830}-*SCO1389* transcription in *S. coelicolor* RJ118b, we used semi-quantitative RT-PCR to analyse the relative expression levels of this gene when placed under the control of the tcp_{830} promoter (see Fig. 4.4 and 4.5). Whilst *SCO1389* expression in M145 was unaffected by ATC concentration, *S. coelicolor* RJ118b showed a clear induction upon inducer addition, despite the leaky expression of this promoter. In parallel experiments, the PL profile of *S. coelicolor* M145

and RJ118b were analysed by TLC in an attempt to identify the inducer break-point at which concentration the PL profile of ATC was induced (see Fig. 4.6 and 4.7). Several PL spots changed in abundance upon inducer treatment, the most notable of these was the spot that we assumed to be CL. This spot could only be visualised in the presence of ATC. In addition, RJ118b also displayed changes in other PL spots. For example, a spot assumed to be PG was more abundant when grown without ATC than in the presence of the inducer. This is consistent with the role of PG as a substrate for *SCO1389* and consequently, in the absence of expression of this enzyme it is possible that its substrate might accumulate. Less easy to explain is the fact that a spot assumed to be PE also displayed reduced abundance in the absence of ATC than in its presence. Despite this, a recent paper (Tan *et al*., 2012) identified a third gene encoding CL synthase from *E. coli*, *clsC*. The protein encoded by this gene is able to synthesise CL from PE and PG in a hitherto unknown mechanism and it might be that the *S. coelicolor* genome encodes unknown proteins responsible for PL biosynthesis. Certainly there are many several genes encoded in the *S. coelicolor* genome that display similarity to other the PL biosynthetic genes predicted in the literature (Borodina *et al*., 2005, Sandoval-Calderon *et al*., 2009)

Quantification of RT-PCR products by agarose gel electrophoresis and densitometry showed that *SCO1389* expression could be induced by ATC in *S. coelicolor* RJ118b. However, we also wished to use anti sense mRNA as a vehicle to block translation *SCO1389* in *S. coelicolor* (see Fig. 4.9). This approach to block expression of an essential gene has not yet been successfully employed in streptomycetes to the best of

our knowledge. Despite our best attempts we were unable to reliably demonstrate induction of anti sense *SCO1389* mRNA. To do this we used *S. coelicolor* RJ116 that carried *SCO1389* cloned in the antisense orientation and subject to control by the ATC inducible tcp_{830} promoter, The fact that increasing the ATC concentration at which *S. coelicolor* RJ116 was grown had no effect suggested that induction of anti-sense *SCO1389* was no longer subject to control by the tcp_{830} promoter. Similarly disappointing results of ATC inducible anti sense *SCO1389* mRNA expression and changes to the PL profile of *S. coelicolor* RJ116 in response to ATC treatment pointed to the fact that perhaps the leaky expression of the tcp_{830} promoter generated sufficient anti sense transcript to block efficient growth of this strain (see Fig. 4.10). This may have led to our inadvertent selection of mutants where anti sense *SCO1389* transcription was blocked. If this was the case, we believe it is most likely to have happened through a mutation in *tetR* resulting in the loss of ability of TetR to bind ATC and so relieve repression. Cloning and sequencing of this gene from *S. coelicolor* RJ116 would allow us to test this hypothesis.

In order to extend our earlier studies on the changes in the PL profile of *S. coelicolor* during development in solid agar we decided to move our studies to liquid cultures. There were several reasons for this; firstly that it was difficult to obtain sufficient quantities of PLs for determination of spot abundance without the use of large numbers of agar plates. Our earlier work on development was carried out on minimal medium containing mannitol, which is a is a good medium for the study of morphological development, but is a poor medium for the growth of this organism in liquid culture (Kieser *et al*., 2000). The reason for this is that it is almost impossible to obtain dispersed

growth of *S. coelicolor* when grown in minimal medium. As a result, we moved to YEME based medium; this medium, when supplemented with agar, supports morphological development of *S. coelicolor*. In addition, when used in its liquid form, YEME can support dispersed growth of *S. coelicolor* and has been routinely used for preparation of DNA and antibiotics from this organism. Consequently, we first investigated the changes in the *S. coelicolor* PL profile on YEME agar by extracting PLs at time points designed to coincide with vegetative and aerial growth (see Fig. 5.1). In this experiment we also investigated the effect of glucose concentration in order to discover if this important nutrient source played a role in the determination of the PL content of the membrane. Another reason for supplementation with glucose was that SapB dependent aerial growth is only operative in the presence of a rich nutrient source such as glucose (Capstick *et al*., 2007), so we wondered if the development effect of glucose on SapB-dependent development might be mediated through CL. In general, the percentage composition of the PL profile did not change in response to changes in glucose concentration with the spots that we believe corresponded to CL, PG and an unidentified PL being the most abundant PLs (Fig. 5.2). The most noticeable change however was a depression of two PL spots in the presence of 20g glucose at 48 h with respect to spot A. This might be due to a requirement of CL for development under these conditions and is consistent with genetic evidence for the requirement of *SCO1389* for aerial development (Jyothikumar *et al*., 2012). Sucrose is often used as a supplement for YEME in order to encourage dispersed growth of *S. coelicolor* in liquid (Kieser *et al*., 2000). However addition of 34% (w/v) sucrose is likely to present an osmotic challenge

to the organism (Bishop *et al*., 2004) that might affect membrane composition. Consequently we omitted sucrose from plates that were supplemented with glucose. However, we also set up a control plate that was supplemented with 34% (w/v) sucrose in addition to 10g l^{-1} glucose. Interestingly a spot corresponding to CL was more intense in the presence of sucrose with respect to PG at 36 h than in the equivalent lane grown without sucrose (Fig. 5.1). This position was reversed at 48h and might reflect changes in membrane composition due to osmotic stress. This change in spot intensity was borne out by densitometric analysis that showed that the addition of sucrose to YEME agar exhibited an apparent increase in the proportion of a spot corresponding to CL at the expense of a spot corresponding to PG at 36h and might reflect a requirement for CL under hyperosmotic conditions. In *E. coli* CL is known to control the response of the cell to osmotic stress through its role in the localization of ProP (Romantsov *et al.*, 2007, Romantsov *et al.*, 2008). The fact that the CL content of *S. coelicolor* is enhanced by the presence of high levels of sucrose on YEME agar suggests that the CL content of this organism may also be involved to in adaptation to osmotic stress.

We also carried out an experiment where attempted to recreate the previous experiment in liquid culture for the purpose of comparing the PL profiles of *S. coelicolor* in both solid and liquid grown YEME cultures. Disappointingly, it was impossible to resolve PL spots in all samples with the exception of cultures grown in the absence of glucose (Fig. 5.5-5.9). The reason for this is unclear, but we believe the appearance of the red pigment (undecylprodigiosin) produced by *S. coelicolor* affected the migration of PLs on the TLC plate. Although several PL spots could be resolved in lanes loaded with

extracts from YEME containing no glucose it was impossible to visualise a spot that equated to CL As it was impossible to visualise PL spots on TLC plates from any extracts except those that were taken from the culture grown in the absence of glucose, it was only possible to analyse changes in the PL profile over time from this culture. The spot assumed to be CL, could not be visualised in this experiment and, indeed, the proportion of all PL spots did not change a great deal. The major change that could be visualised over the course of this experiment was that the proportion of an unidentified spot increased as *S. coelicolor* biomass increased. Although it was not possible to identify this PL spot, the fact that it increased in relation with respect to the major membrane PLs, PE and PG suggests that this spot maybe a precursor molecule such as PA that might be expected to increase as the organism shifted from exponential to stationary phase and consequently growth slows. As mycelial growth slows, it is likely that membrane synthesis will also slow, presumably resulting in a reduced requirement for PG or PE. It might be expected therefore that this would result in a precursor PL such as PA (Sandoval-Calderon *et al*., 2009).

As antibiotic fermentations are often carried out as fed-batch fermentations we wished to discover if the PL profile of *S. coelicolor* changed after feeding with a glucose nutrient source at the end of exponential phase in a simulation of a fed-batch antibiotic fermentation (Fig. 5.12-5.16). During this experiment, it was not possible to identify spots grow in the presence of 20 g l^{-1} glucose due to the poor PL resolution of extracts taken from these cultures. This was especially true of the later time points where high levels of undecylprodigiosin in the PL extracts interfered with PL migration and it was

impossible to visualise spots on TLC plates from cultures grown at a glucose concentration of 20 g l^{-1}. Consequently we were only able to quantify spots by densitometry for the cultures supplemented with 10 g l^{-1} glucose (Fig. 5.17). During the early part of the experiments a spot, assumed to be PG was the dominant PL, but as time increased a spot that was assumed to be PE, replaced the former spot as the dominant PL. Another spot assumed to be CL also increased as a proportion of total PLs during the course of the experiment. Interestingly the changes in PL abundance took place during exponential phase and there were no significant changes after 36 h. This is important as the culture 10gFB was fed with glucose at 36h; this pulse of glucose did not therefore cause any changes in the PL content of the cell. This suggests that glucose is not the determining factor in controlling the relative PL abundance in the cell membrane of *S. coelicolor*. Although we only investigated the effect of a glucose feed, our results also suggest that the PL content of the cell membrane does not change after feeding. Clearly feeding with other nutrient sources might affect membrane composition, but this will depend on their biochemical nature. Previous results showed that alteration of CL content affected the mycelial architecture of *S. coelicolor* (Jyothikumar *et al*., 2012), in that an increased CL content generated additional branching. At least on the basis of the results presented here, it seems unlikely that feeding with glucose is likely to affect membrane CL content, the mycelial architecture and, as a result, the behaviour in industrial fermentations through reduced pellet formation (Manteca *et al*., 2008, van Wezel *et al.*, 2005).

In order to further study the involvement of PLs in development of streptomycetes, we investigated the expression patterns of PL biosynthetic genes and their PL profile in developmental mutants of *S. coelicolor* that carried mutations in *bldA* and *whiD* (see Fig. 6.2). Despite the same amount of RNA being included in each RT-PCR reaction, *hrdB* RT-PCR products were consistently lower when isolated from mid-log phase cultures than stationary phase cultures. This effect was more pronounced in *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD.* It was also noticeable that these mutants grew more slowly in liquid culture than the wild-type strain (Fig. 6.1). Although *SCO5753* expression could be weakly detected at 30 h in *S. coelicolor*Δ*bldA*, expression of all other PL biosynthetic genes was undetectable in *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD*. It may be possible that *SCO1527, SCO6468* or *SCO6467* were not expressed in the mutant strains. However, as S*CO1389* was expressed, it seems unlikely that *SCO5628* or *SCO5753* expression would be repressed in the mutant strains as the PL synthesised by the products of these genes (CDP-DAG and PG respectively) are precursors for the synthesis of CL. CDP-DAG is the product of *SCO5628* producing PG and both these PLs are substrate for *SCO1389*, which synthesises CL by the eukaryotic mechanism (see Fig. 6.3). Perhaps these transcripts are more susceptible to degradation by nucleases that are perhaps more strongly expressed in developmental mutants. For example, RNase III regulates antibiotic production in *S. coelicolor*, and it may be that this gene is subject to developmental regulation by the *bldA* or *whiD* products. Perhaps mutation of these genes leads to enhanced RNase activity that targets the transcripts of certain PL gene

preferentially (Gravenbeek & Jones, 2008). However, it is clearly more likely that our failure to detect transcription of these genes was due to inhibition of the RT-PCR.

In parallel with RT-PCR analysis we also analysed the PL profile of *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* (Fig. 6.5)**.** Although there were considerable differences between PL profiles extracted at mid-log phase and in stationary phase there was no discernible difference between the patterns displayed by the wild type and mutant strains. Presumably this reflects the fact that the products of *bldA* and *whiD* are not responsible for the regulation of PL biosynthetic genes during exponential phase and their effects are only displayed during the erection of aerial hyphae and sporulation. If our assumption that PL spots B and C correspond to PE and PG respectively is correct, then it seems unlikely that our inability to detect *SCO6467*/*SCO6468* and *SCO5753* expression is correct as the proteins encoded by these genes are responsible for the synthesis of PE and PG respectively. Despite this, it has recently been shown that *E. coli* possesses a third CL synthase gene, *clsC*, that can synthesise CL from PE (Tan *et al*., 2012). There are many uncharacterised genes encoded within the *S. coelicolor* genome that contain domains that are highly similar to the to the PL biosynthetic proteins described in Fig. 1.6, so it is not inconceivable that this organism possess novel PL biosynthetic pathways that have so far escaped discovery.

As it was difficult to obtain good quality PL extracts from these developmental mutants of S. *coelicolor,* we decided to shift our investigations to analysing the PL profile of *S. venezuelae.* This organism is able to undergo synchronous sporulation in liquid culture and therefore offered the opportunity to isolate large amounts of PLs at different

stages of submerged sporulation. In this organism, strains mutated in *bldN* and *whiA* were investigated. Shaken cultures of *S. venezuelae* 10712, *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiA* were treated with lysozyme in order to determine the efficacy of sporulation in liquid culture (see Fig. 6.6 and Table 6.1). No colonies were detectable after treatment of the development mutants with lysozyme since both these strains are able to sporulate in liquid culture due to their developmental blockage. Consequently the vegetative hyphae at which stage these developmental mutants were held were susceptible to lysozyme treatment. In contrast, *S. venezuelae* 10712, after treated with lysozyme, still allowed the isolation of viable spores. The lysozyme treatment gave values for sporulation that correlated well with microscopic observations.

We also analysed the PLs extracted from the three *S. venezuelae* strains by TLC (see Fig. 6.10). Four PL spots could be separated and, on the basis of their similarity, with the standard PLs they were identified as CL, PE, PG and an unidentified PL respectively. As *S. venezuelae* 10712 is able to sporulate in liquid culture, whilst *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* cannot, we expected difference in the PL profile to reflect the inability of the latter strains to sporulate. Small differences could be seen between the three strains; firstly a spot equivalent to CL could not be visualised in *S. venezuelae* 10712, but was present in the two developmental mutants. Interestingly, the two developmental mutants displayed a reduced intensity of the spot assumed to be PG. This PL is the substrate for CL synthase and this increase in CL coupled with a decrease in PG content might reflect the continued synthesis of CL in *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* as a result of their developmental blockage. This is consistent with

previous work in *S. coelicolor*, where CL is involved in the erection of aerial hyphae (Jyothikumar *et al*., 2012).

7.1 Future work

We provisionally identified PL spots on the basis of their similarity with known standards on TLC plates. Clearly this does not allow us to conclusively identify these spots given the many different PL that are made by bacteria. As a result the key analysis necessary to confirm many of our findings would be to purify PL spots from TLC plates, extract the PL from the plate silica and subject it to lipidomic analysis by mass spectrometry. If MS/MS were employed, not only would it be possible to identify the molecular weight of the PL it would also be possible to identify the head group and length of each individual acyl chains. Although it was used not for the purpose of identifying spots on TLC plates, this approach was used in *S. coelicolor* to carry out a provisional characterisation of the lipidome of this organism. (Jyothikumar *et al*., 2012). This research demonstrated that the PLs predicted in earlier studies (Borodina *et al*., 2005, Sandoval-Calderon *et al*., 2009) were found *in S. coelicolor*. These PLs included CL, PG, PE, PS and PI.

During the course of this study, limitations in the use of *S. coelicolor* became apparent. Key to this was the inability of this organism to sporulate in liquid culture. It was only possible to extract sufficient quantities of RNA and PLs in particular by employing large numbers of agar plates. Despite, shifting our studies to liquid culture, with the aim of improving levels of PL recovery from larger volume cultures, the

pigmented antibiotics actinorhodin and undecylprodigiosin were extracted along with PLs and prevented good resolution of PLs by TLC. *S. venezuelae,* although producing the antibiotic chloramphenicol, does not produce highly pigmented compounds. As a result we believe that this species offers the possibility of better PL resolution by TLC. In addition *S. venezuelae* can sporulate in liquid culture and although some limited studies were carried out in this organism, it is clear that the ability to recover large amounts of PLs. or indeed a protein through the use of submerged sporulation provides a more amenable system to study streptomycete development than *S. coelicolor*. Development of this organism is restricted to agar plates and, although there is great availability of mutant strains, genetic tools and available literature, it seems likely that the improving availability of these resources in *S. venezuelae* will result in the supplanting of *S. coelicolor* as the model streptomycete. As such, a promising avenue for studying change in PLs during streptomycete development would be to transfer the constructs made in *S. coelicolor* to *S. venezuelae*. This would also offer the possibility to extend the findings carried out in this study to *S. venezuelae* for the purpose of more accurately tracking the role of PLs in development. The increasing availability of genetic tools for the latter organism means that it would also be possible to determine changes in PL content in different *bld* and *whi* mutants.

Whilst it was difficult to interpret changes in PL biosynthetic gene expression and PL abundance during growth in liquid culture, the fact that modulation of the mycelial architecture in *S. coelicolor* in response to induction of *SCO1389* (Jyothikumar *et al*., 2012) suggests that changing the membrane components of streptomycetes may be a

route to change branching patterns in antibiotic fermentations with the aim of increasing antibiotic yield or reduced fermentation time.

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