Chapter 3 Investigation of the transcriptional profile of *S. coelicolor* biosynthetic genes: mRNA isolation and Optimization

3.1 Introduction to Chapter 3

In rod-shaped bacteria such as *E. coli* and *B. subtilis*, the division site usually occurs at the mid-cell of the rod, at right angles to the length of lateral walls (Adams & Errington, 2009, Flardh, 2003a). Following expression of the cytokinetic protein, FtsZ, this protein forms a ring at mid-cell. Constriction of the Z ring divides the mother cell into two equal daughter cells. This protein also recruits the downstream components of the divisome with which it collaborates to establish the location and shape of the division septum (Adams & Errington, 2009). In contrast, members of the genus *Streptomyces* differ from most bacteria by growing as branching hyphae and bear a superficial resemblance to filamentous fungi. Growth is exceptionally asymmetrical, and cell wall extension arises by assimilation of peptidoglycan precursors at the tips of hyphae (Flardh, 2003a). The key protein in streptomycete tip extension is DivIVA that is particularly localized at the tips of growing hyphae of the model organism, *S. coelicolor*. DivIVA from *S. coelicolor* is homologous to *B. subtilis* DivIVA (Angert, 2005, Flardh, 2003a, Flardh *et al.*, 2012, Hempel *et al.*, 2012, Hempel *et al*., 2008, Richards *et al.*, 2012). The MinCD complex is positioned by DivIVA in *B. subtilis* and by MinE in *E. coli*, consequently, although they are functional homologues, these proteins have no significant sequence or structural similarity. MinD works together with either DivIVA or MinE in *B. subtilis* or *E. coli* respectively to maintain the polar localization of MinC, which prevents the assembly of the Z ring near the poles (Angert, 2005, Barak & Wilkinson, 2007). In *E. coli*, MinD travels at regular intervals from one pole of the cell to the other. In contrast to *E. coli*, the MinD homologue in *B. subtilis* is consistently pole-

associated where it remains during the whole cell cycle through its interaction with DivIVA (Angert, 2005). There are no MinCDE homologues encoded by the genome of *S. coelicolor* and consequently it is unclear how rings of FtsZ are positioned in this organism during vegetative growth or sporulation. However in *B. subtilis*, spirals of the anionic PL PE are found within the membrane that coincide with the location of MinD and as at result it is possible that PL composition within the membrane play a role in the positioning of MinD (Barak *et al.*, 2008). As a result of this, it seems that membrane PL content indirectly affects the positioning of FtsZ, within the *B. subtilis* cell. DivIVA is recruited to the cell poles through an interaction with membrane negative curvature (Lenarcic *et al*., 2009, Oliva *et al*., 2010), although it was found that this interaction was mediated via the intrinsic negative curvature of the polar membrane rather than binding to a specific PL.

As it seems membrane PLs may be involved in the position of cytokinetic proteins in *B. subtilis*, we wondered if they might also play role PLs during the development of *S. coelicolor*. The first stage in this process was to identify genes whose products were predicted to encode the enzymes necessary to synthesise membrane PLs. Analysis of all the annotated streptomycetes genomes has shown that putative orthologues to many of the components of fatty acid biosynthesis (FAS) occur. The genes that encode the core enzymes involved in fatty acid biosynthesis are found in the fatty acid biosynthetic cluster and consist of the following genes: *fabD, fabH, acpP* and *fabF*. This cluster has been partially characterized in *S. coelicolor* and role in fatty acid biosynthesis pathway identified through genetic and biochemical experiments (Gago *et*

al., 2011). However as the fatty acid component of membrane PLs are found within the membrane bilayer we decided to concentrate on the enzymes responsible for synthesising PLs rather that their fatty acid constituent units.

Classification of the genes/enzymes involved in PL biosynthesis in *Streptomyces* has received little attention compared to other bacteria and most information available comes from the prediction of gene function form genome sequences. The proteins responsible for synthesising cytidine-diphosphatediacylglycerol (CDP-DAG), PE, PI, PG, PS and CL were previously predicted by analysis of the *S. coelicolor* genome sequence and are described in Fig. 1.6 (Borodina *et al*., 2005, Sandoval-Calderon *et al*., 2009). In 1947 CL, an anionic PL became the first-characterized PL and was proposed by Mary Pangborn who first isolated it from beef heart (Haines, 2009). CL consists of two phosphatidyl groups connected by a glycerol and has four hydrophobic acyl chains and a small hydrophilic head group, which is found in the membranes of mitochondrial cristae in bacteria and is responsible for generating the high curvature of polar membrane regions (Huang *et al.*, 2006, Jyothikumar *et al*., 2012).

In bacteria, the biosynthesic reaction for CL is a reversible transesterification reaction catalyzed by CL synthase containing two phospholipase D (PLD) super-family proteins that also includes poxvirus envelope proteins, type I PS synthases, phospholipases D as well as some nuclease. CL is synthesized from two molecules of PG in *E. coli* (Tian *et al.*, 2012) and in almost all bacteria synthesis proceeds from PA to CDP-DAG then via PG synthase to PGP, which is then dephosphorylated to PG (Lu *et al.*, 2011). The final step is a phosphatidyl transfer from one PG molecule to a second PG

molecule to form CL and glycerol (Hirschberg & Kennedy, 1972) catalyzed by a phospholipase D-type enzyme (ClsA) encoded by the *clsA* gene in *E. coli* (Pluschke *et al.*, 1978). CDP-DAG also provides the precursor to another major membrane PL PE. In eukaryotes CL synthase belongs to the CDP-alcohol phosphatidyltransferase superfamily (Tian *et al.*, 2012). Consequently in eukaryotic cells, CDP-DAG is the phosphatidyl donor to PG to yield CL and cytidine monophosphate (CMP). A gene encoding CL synthase found in *S. coelicolor* and in most actinobacteria possesses a CDP-DAG–dependent CL synthase activity (Sandoval-Calderon *et al*., 2009). Based solely on informatics analysis, *Trypanosoma brucei* was suggested to encode a bacterial-type CL synthase (Bogdanov *et al.*, 2008). The gene encoding CL synthase found in *S. coelicolor* and in most actinobacteria possesses a CDP-DAG–dependent Cl synthase (Tan *et al.*, 2012). *SCO1389 (clsA)* is the essential gene that encodes CL synthase activity and was also found to play a role in the erection of aerial hyphae (Jyothikumar *et al*., 2012). Using the CL specific staining NAO, CL could be localized at the polar and septal regions of the cytoplasmic membrane of *E. coli* and *B. subtilis* (Kawai *et al.*, 2004, Huang *et al.*, 2006).

Analysis of gene expression requires accurate determination of mRNA levels. PCR is based on amplification of DNA rather than RNA, mRNA is converted into DNA using reverse transcription, where RNA is first converted into DNA and a second reaction involving a PCR amplification is performed on the resulting complementary DNA (cDNA) (Owen, 2006). In the study reported in this thesis, RT-PCR was performed to analyse expression of the genes encoding PL biosynthetic enzymes in *S. coelicolor*. There

are many reports regarding RNA isolation from *Streptomyces* that demonstrate quantitative real-time reverse transcriptase PCR (qRT-PCR) as a means to analyse gene expression in this genus. For example, gene expression analysis demonstrated that the initiation by SapB of development of aerial hyphae and spores of *S. coelicolor* is inhibited by surfactin, a lipopeptide surfactant produced by *B. subtilis.* In this study plates of surfactin-treated or untreated *S. coelicolor* cultures were analysed for expression of genes diagnostic for aerial development. In order to determine if any displayed altered patterns of gene expression, primers for *chpA, chpH*, *rpoB* and *ramC* were used for RT-PCR analysis (Straight *et al*., 2006). The *whiB* sporulation gene of *S. coelicolor* was shown to encode cysteine rich putative transcription factor, which is homologus to a family of proteins encoded within the genome of *M. Tuberculosis*, *whmA–F* and potentially a seventh gene, *whmG*. Cloning and sequencing of these *whiB-l*ike (*wbl*) genes revealed likely orthologues of four of the *whm* genes of *M. tuberculosis* (Agarwal *et al.*, 2006, Chater & Chandra, 2006, Morris *et al.*, 2005, Raghunand & Bishai, 2006). In all, *S. coelicolor* contains several similar genes with at least five *wbl* genes in addition to *whiB* itself. All five were shown by RT-PCR to be transcribed (Soliveri *et al.*, 2000). A putative membrane protein gene *sarA* (*SCO4069*), sporulation and antibiotic production related gene A, was partially characterized in *S. coelicolor*. The *sarA* mutant showed rapids sporulation and greatly decreased the production of actinorhodin and undecylprodigiosin. RT-PCR analysis showed that SarA influenced antibiotic production by controlling the abundance of *actII-orf4* and *redZ* messenger RNA (Ou *et al.*, 2008).

Aims of this chapter

The aims of this chapter were to design RT-PCR primers that could be used to detect expression of PL biosynthetic genes. It was also necessary to optimize mRNA isolation from *S. coelicolor* and subsequently to optimize RT-PCRs on extracted mRNA in order to determine changes in PL gene expression during growth and development of this organism. This would allow us to determine if this pattern of expression changed during development and if this was reflected in alterations in the PL profile of the organisms and might shed light on the involvement of PLs during *S. coelicolor* development.

3.2 PCR optimization

3.2.1 Design of primers for detection of PL gene expression by RT-PCR

Whilst much is known about the proteins that are involved with growth and development in *S. coelicolor*, little is known about the changes in membrane components during these processes. A number of genes have been identified in the *S. coelicolor* genome that are thought to encode enzymes for synthesizing PLs (Table 2.4). In order to examine the role of these genes in development we decided to measure their expression by RT-PCR. PCR primers were designed using Primer 3 software (Rozen & Skaletsky, 2000) to generate PCR products of PL biosynthetic genes for *S. coelicolor* and listed in Table 2.4. *hrdB* was used as a control since it is expressed constitutively (Du *et al.*, 2013, Kieser *et al*., 2000)

PCR is not 100% efficient for a variety of reasons, but by increasing the number of cycles and optimizing conditions amplification is routinely achievable. One of the great advantages of PCR is its ability to amplify a defined region of DNA from a complex starting template such as genomic DNA. PCR uses two oligonucleotide primers that act as sites for initiation of DNA synthesis by the DNA polymerase and so these primers define the region of the template DNA that will be copied.

3.2.2 Genomic DNA isolation from *S. coelicolor* M145

In order to optimize the conditions necessary for optimal detection of RT-PCR products, PCR reactions were first carried out on genomic DNA thus, the expected PCR product was generated when genomic DNA served as the template (Fig. 3.1 and 3.2). When *S. coelicolor* M145 genomic DNA was isolated, a ten-fold dilution of DNA was quantified using a NanoDrop spectrophotometer (Table 3.1).

Table 3.1 DNA concentration of genomic DNA carried using the Nanodrop spectrophotmeter

Dilution of genomic DNA	DNA concentration (ng μl^{-1})
1X	888.2
10^{-1}X	129.7
10^{-2}X	14.1
10^{-3}X	2.3

gDNA quality at dilutions 10^{-1}X, 10^{-2}X and 10^{-3}X were also checked by agarose gel electrophoresis. Total gDNA was cut by SalI, PstI and XbaI restriction enzymes and λ HindIII digested DNA used as a marker. The results of this experiment are shown in Fig. 3.1. The DNA digested well with SalI and PstI which, due to the G+C rich nature of their target sites (G^TCGAC and CTGCA^G respectively), cut frequently in the G+C rich DNA of *S. coelicolor* DNA. XbaI (target site T^CTAGA) is known to cut infrequently in *S. coelicolo*r DNA and consequently DNA digested with this enzyme displays larger fragment sizes (Gagnat *et al.*, 1999). As a result, the best dilution for subsequent optimization of PCR conditions was considered be 10^{-1}X gDNA.

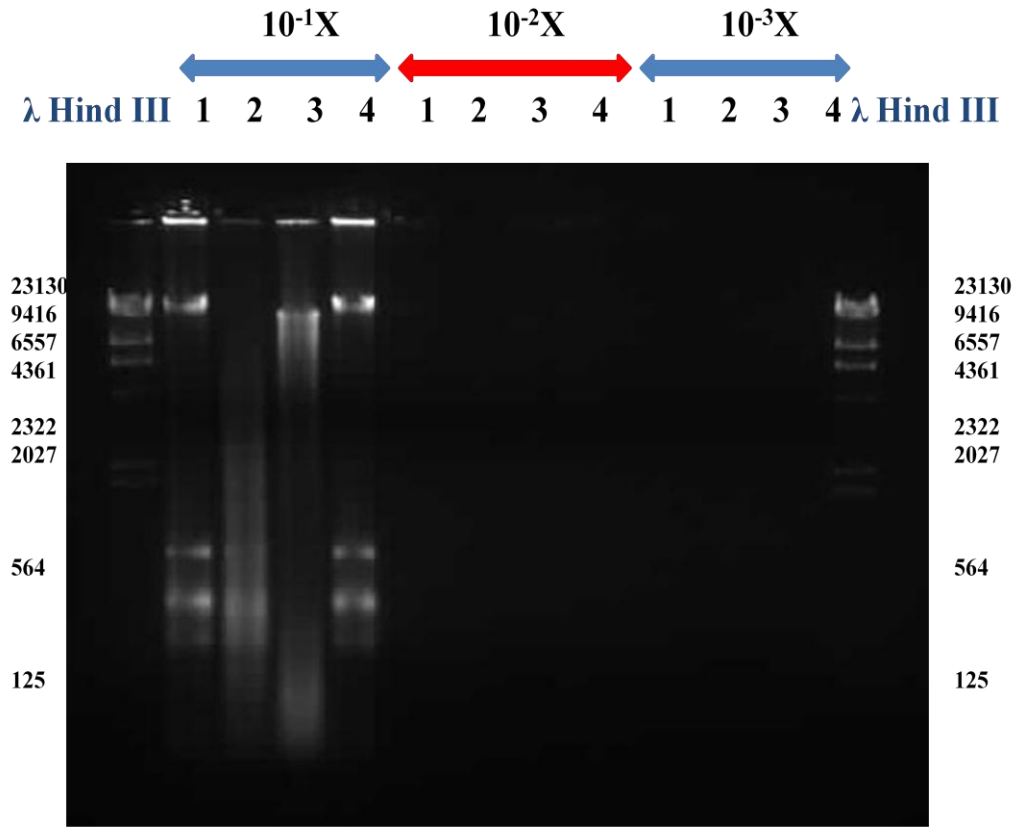


Fig. 3.1 Genomic DNA isolated from *S. coelicolor* for use as a PCR DNA template at dilution 10^{-1}, 10^{-2} and 10^{-3}X when digested with Sal I, Pst I and Xba I. Lane 1: Undigested; Lane 2: Sal I; Lane 3: Pst I and Lane 4: XbaI.

3.2.3 Optimization of $MgCl_2$ for amplification of *S. coelicolor* biosynthetic genes

Mg^{2+} ion is one of the most critical components in the PCR as its concentration can affect the specificity and efficiency of the reaction; therefore we set up an experiment to find the suitable Mg^{2+} concentration for PCR. *Taq* DNA polymerase is dependent upon the presence of Mg^{2+} and shows its highest activity at around 1.2–1.3 mM free Mg^{2+}, although 1.5 mM $MgCl_2$ is already present in Standard PCR buffers (Green & Minz, 2005). The free Mg^{2+} concentration is affected by the dNTP concentration as there is equimolar binding between dNTPs and Mg^{2+} (Owen, 2006). Suppliers may supply their enzymes with a buffer that lacks Mg^{2+} as well as an Mg^{2+} stock solution to allow the user to optimize the Mg^{2+} concentration most appropriate for their application.

The first step was to establish optimal conditions for PCR, therefore we set up the PCR (see Materials and Methods, section 2.9) using 1 µl (129.7 ng of (10^{-1}X) *S. coelicolor* gDNA (Table 3.1)) DNA template, final magnesium concentrations were adjusted by the addition of 25 mM $MgCl_2$ to 1.5, 2.0, 3.0 and 4.0 mM in reactions employing the primers for *hrdB* and *SCO1389* (see Table 3.2).

Table 3.2 Components of PCR reaction for final Mg^{2+} Concentration

Buffer	Final Mg^{2+} concentration in reaction (mM)	Required volume of 25 mM $MgCl_2$ per 50µl reaction (µl)
Promega Go Taq® 5x buffer (Contains 7.5 mM $MgCl_2$)	1.5 2.0 3.0 4.0	0 1 3 5
Promega Go Taq® Flexi 5x buffer (Do not contain Mg^{2+})	1.5 2.0 3.0 4.0	3 4 6 8
Qiagen HotStarTaq® 10X buffer (Contains 15 mM $MgCl_2$)	1.5 2.0 3.0 4.0	0 1 3 5

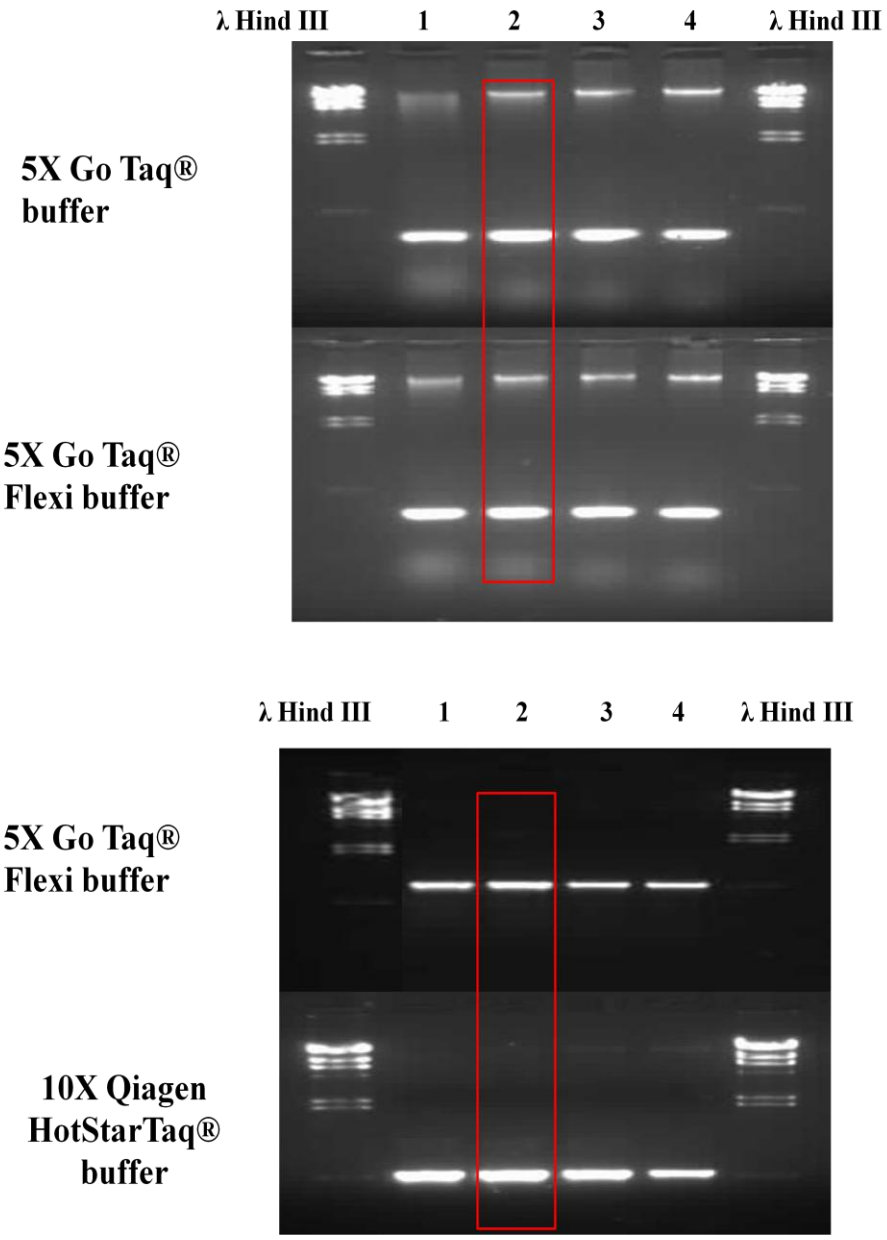


Fig. 3.2 $MgCl_2$ Optimization compared for 5x Go Taq® Reaction buffer, 5x Go Taq® Flexi buffer and 10X Qiagen HotStarTaq® buffer with *hrdB* and *SCO1389* primers respectively. The different Mg^{2+} concentration in each reaction are displayed. Lane 1: 1.5 mM; Lane 2: 2 mM; Lane 3: 3 mM and Lane 4: 4 mM.

The reactions contained 129.7 ng of *S. coelicolor* gDNA and were subjected to PCR amplification with various final Mg^{2+} concentrations that contained 5x Go Taq buffer, 5x Go Taq Flexi buffer and 10X Qiagen HotStarTaq buffer in the reaction with *hrdB* and *SCO1389* primers, respectively. The level of PCR amplification in reactions that contained 5x Go Taq buffer, 5x Go Taq Flexi buffer with *hrdB* primers were not different significantly. However, 10X Qiagen HotStarTaq buffer with *SCO1389* primers gave a best yield with 2 mM $MgCl_2$ and consequently this enzyme/buffer combination was chosen as that which gave the ideal concentration of Mg^{2+} ions to give the maximal yield, this is shown in Fig. 3.2.

3.2.4 Optimization of annealing temperature for amplification of *S. coelicolor* biosynthetic genes

In order to optimize PCRs in terms maximization of specificity, sensitivity and yield (Korbie & Mattick, 2008), we set up PCR reactions to employ a range of annealing temperatures of the primers being used over the course of successive cycles using *S. coelicolor* gDNA as a template. The PCR reaction was set up (see Materials and Methods, section 2.9) in the following way. For each reaction : Go Taq®, Go Taq® Flexi 25 µl of the mixture that includes 5 µl 5x Green or Colorless Go Taq® and Go Taq® Flexi Reaction Buffer, 2 µl 25 mM $MgCl_2$, 1 µl deoxyribonucleotide triphosphate (dNTP) mixture (each 10 mM) , 1.5 µl (10 pmol μl^{-1}) each of forward and reverse Primer, 1 µl (129.7 ng of (10^{-1}X) *S. coelicolor* gDNA (Table 3.1)) DNA template, nuclease-free water

was added to give a final volume of 25 µl and 1 µl (0.125 U) Promega Go Taq® DNA Polymerase .

For Qiagen HotStarTaq® reaction 25 µl of the mixture that included 2.5 µl 10X Qiagen HotStarTaq Buffer, 0.5 µl 25 mM $MgCL_2$, 5 µl 5XQ solution, 1 µl deoxyribonucleotide triphosphate (dNTP) mixture (each 10 mM) , 1.5 µl (10 pmol μl^{-1}) each of forward and reverse Primer, 1 µl (129.7 ng of (10^{-1}X) *S. coelicolor* genomic DNA (Table 3.1)) DNA template, nuclease-free water was added to give a final volume of 25 µl and 1 µl (0.5 U) Qiagen HotStarTaq® DNA Polymerase.

The PCR was carried out according to the Materials and Methods and *hrdB, SCO1389, SCO6467, SCO6468, SCO5628, SCO1527* and *SCO5753* amplified using appropriate primers (see Table 3.1). The Different annealing temperatures were used for amplification of each PCR products due to the constraints of the thermocycler. Annealing temperatures used for *hrdB* were 61.0, 62.5, 64.9, 66.4, 68.7 and 70.0^{o}C; for *SCO1389* 55.0, 56.3, 57.5, 61.4, 63.9 and 66.1^{o}C. For *SCO6467, SCO6468* and *SCO5628* annealing temperatures were 55.0, 55.9, 57.8, 61.0, 63.5 and 65.0^{o}C. For *SCO1527* annealing temperature were 52.8, 53.4, 54.2, 55.0, 55.7 and 56.9^{o}C. For *SCO5753* annealing temperature were 52.0, 52.4, 52.8, 53.4, 54.2 and 55.0^{o}C.

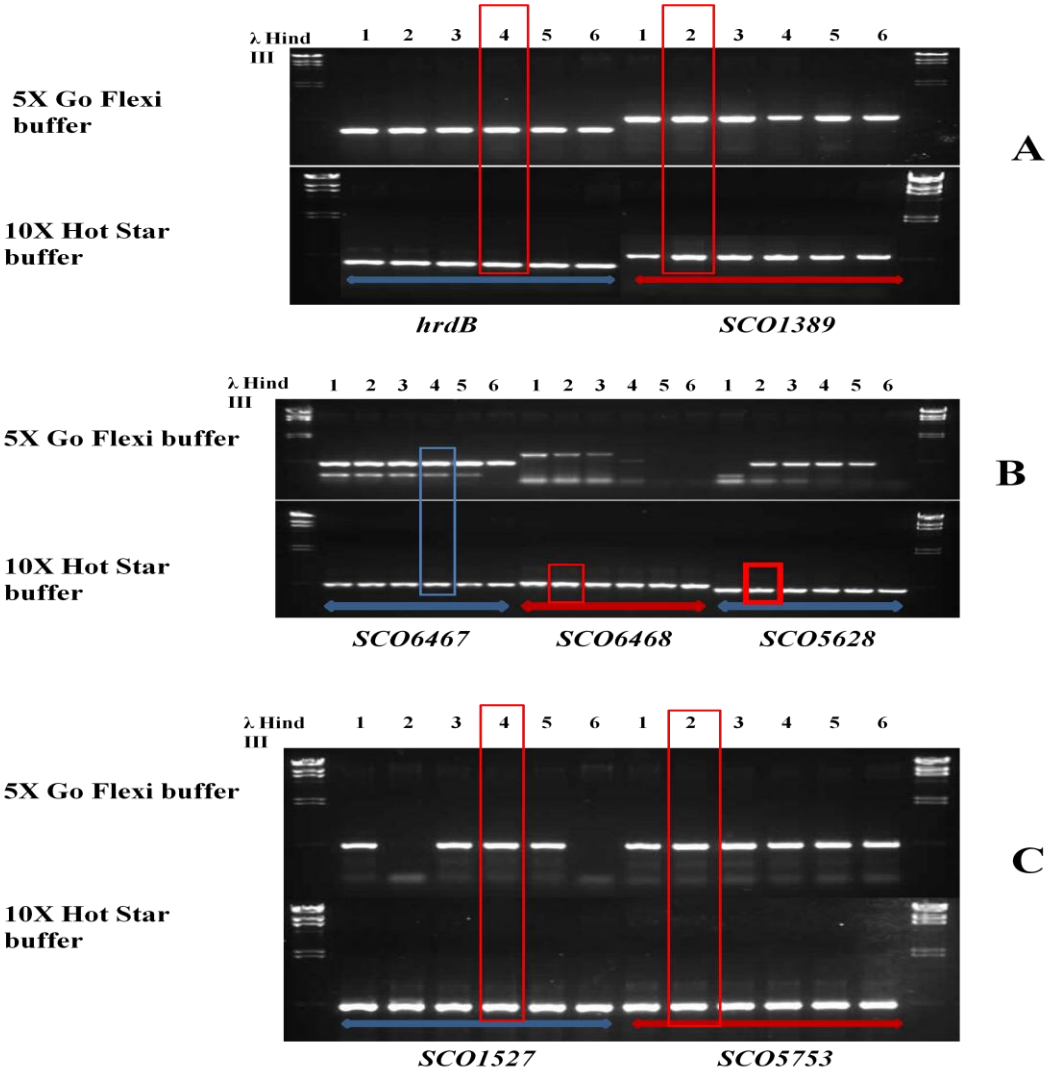


Fig. 3.3 Annealing temperature Optimization for amplification of *S. coelicolor* biosynthetic genes. Temperature gradients were compared between 5x Go Taq® Flexi Buffer and 10X Qiagen HotStarTaq® Buffer amplified with *hrdB, SCO1389, SCO6467, SCO6468, SCO5628, SCO1527* and *SCO5753* primers. **A:** different annealing temperatures used for *hrdB* at; Lane 1: 61.0, Lane 2: 62.5, Lane 3: 64.9, Lane 4: 66.4, Lane 5: 68.7 and Lane 6: 70.0^{o}C and *SCO1389* at; Lane 1: 55.0, Lane 2: 56.3, Lane 3: 57.5, Lane 4: 61.4, Lane 5: 63.9 and Lane 6: 66.1^{o}C. **B:** different annealing temperatures of *SCO6467*, *SCO6468* and *SCO5628* primers at; Lane 1: 55.0; Lane 2: 55.9; Lane 3: 57.8; Lane 4: 61.0; Lane 5: 63.5 and Lane 6: 65.0^{o}C. **C:** different annealing temperature of *SCO1527* at; Lane 1: 52.8, Lane 2: 53.4, Lane 3: 54.2, Lane 4: 55.0, Lane 5: 55.7 and Lane 6: 56.9^{o}C and *SCO5753* at; Lane 1: 52.0, Lane 2: 52.4, Lane 3: 52.8, Lane 4: 53.4, Lane 5: 54.2 and Lane 6: 55.0^{o}C.

As can be seen in Fig. 3.3, the optimum temperatures for amplification of *hrdB* was 66.4^{o}C, *SCO1389* 56.3^{o}C, *SCO1527* 55.0^{o}C, *SCO5753* 52.4^{o}C and *SCO6467* 61.0^{o}C in both 5x Go Taq® Flexi Buffer and 10X Qiagen HotStarTaq® Buffer, whilst both *SCO5628* and *SCO6468* had optimal annealing tempertures of 55.9^{o}C in 10X Qiagen HotStarTaq® Buffer only. Therefore, these optimal temperatures and 10X Qiagen HotStarTaq® Buffer were chosen as the appropriate conditions for further experiments.

3.3 Reverse Transcriptase PCR (RT-PCR)

3.3.1 RNA isolation from *S. coelicolor*

The appearance of RNA preparation on an agarose gel is the best way to assess the integrity of RNA prior to use for S1 mapping or primer extension experiments. Because total RNA is <5% mRNA, only stable RNA (16S, 23S and 5S rRNAs and tRNA) can be seen clearly on the gels. For a good preparation of RNA prepared by the Kirby mix procedure (Fig. 3.4) there should be a total absence of chromosomal DNA in the RNA preparation. In addition, the following bands should also be visible: a 23S rRNA band that is less intense than the 16S rRNA band. This is because the 23S rRNA is preferentially degraded, so in poor preparations of RNA the 16S band will be more intense than the 23S band and an absence of bands between the 23S and 16S bands and below the 16S band will be seen. These bands result from degradation of the 23S and 16S rRNAs which are cleaved preferentially at specific sites (Babitzke *et al.*, 1993). Depending on exactly how the tRNA/RNA is prepared, a ladder of one to three bands of decreasing intensity above the 23S rRNA band will also be seen; these are rRNA aggregation products and they are quite normal (Kieser *et al*., 2000).

3.3.1.1 Optimization of RNA yield from *S. coelicolor* for RT-PCR

In order to determine the optimal time to isolate RNA for RT-PCR experiments, we set up an experiment to determine the yield of RNA from liquid-grown *S. coelicolor* incubated for various lengths of time. RNA was isolated from *S. coelicolor* (see Materials and Methods, section 2.10.1) after incubation for 16, 24, 36, 48, 72 and 96 h.

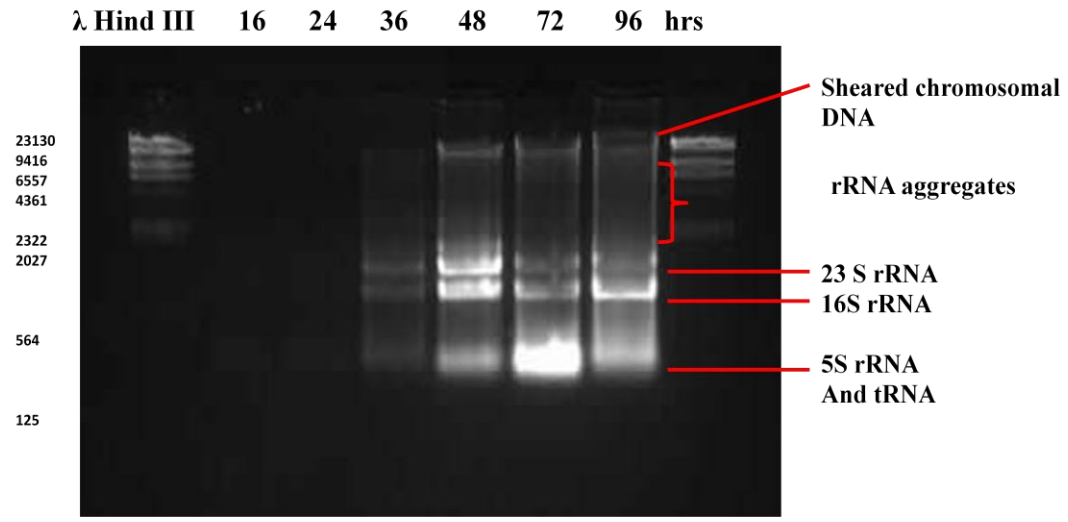


Fig. 3.4 Appearance of total nucleic acid (TNA) and RNA of *S. coelicolor* at different harvesting times (16, 24, 36, 48, 72 and 96 h).

Table 3.3 RNA yield determined using the NanoDrop RNA program. (Pure RNA : $A_{260}/A_{280} \geq 2.0$)

Harvesting times (h)	RNA concentration (ng µl $^{-1}$)	A_{260}/A_{280}
16	35.9	1.78
24	29.7	1.80
36	430.6	2.03
48	1,409.1	2.05
72	1,336.6	2.01
96	1,508.4	2.03

Appearance of RNA on an agarose gel was found at harvesting times of 36, 48, 72 and 96 h and RNA concentration and yield increased with time (Fig. 3.4 and Table 3.3). A_{260}/A_{280} values were also measured using the NanoDrop RNA program. We found that at 48 h incubation gave the clearest appearance on an agarose gel and gave the highest A_{260}/A_{280} value at 2.05. However, after 36h an A_{260}/A_{280} value at 2.03 was found. This was considered to be the optimal time point that, in conjunction with RNA yield and

appearance on an agarose gel, gave the best quality RNA at a sufficient quantity to detect PL gene expression by RT-PCR.

3.3.2 Removal of DNA from RNA templates

In order to ensure that RT-PCR products are derived from an mRNA template it is necessary to ensure that DNA is removed entirely from the RNA sample on which the RT-PCR is carried out. An RNAse-free DNAse I digestion step is therefore performed to remove contaminating DNA from RNA samples. Consequently, an appropriate control for any contaminating DNA in an RNA sample is a control PCR in which the reverse transcriptase step is omitted (Owen, 2006); any PCR products that are detected must therefore be derived from contaminating DNA.

RNA samples isolated from the 36 h time point (see 3.3.1.1) were treated with DNAse I before RT-PCR analysis in order to prevent PCR amplification from contaminating DNA. RT-PCR reactions were then carried out using the Qiagen® OneStep RT-PCR kit. Control reactions without RNA or using Qiagen® HotStarTaq DNA Polymerase were included in order to confirm that any amplified product is dependent on RNA and not contaminating DNA.

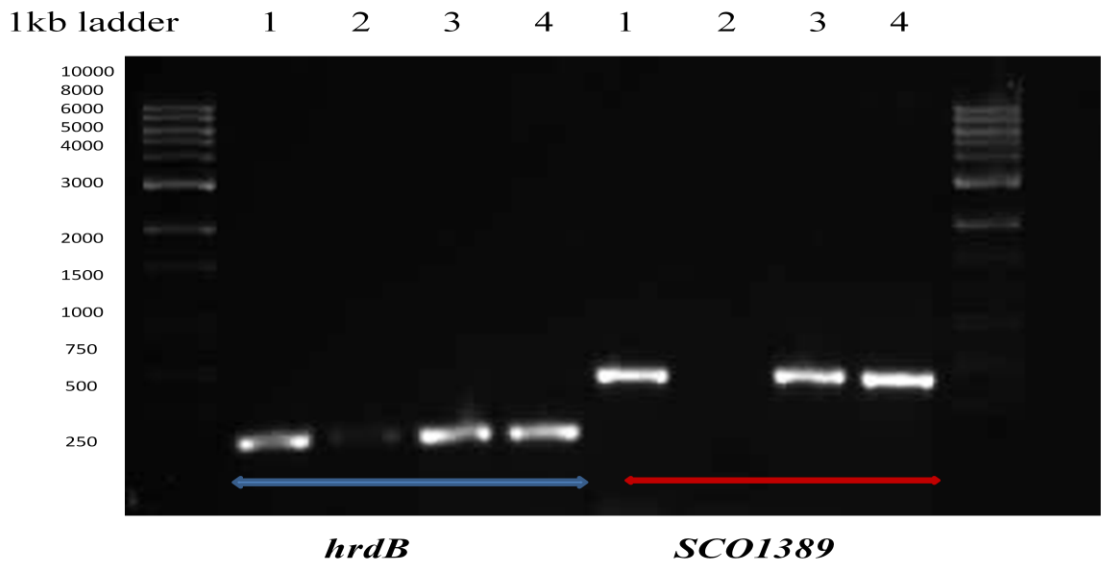


Fig. 3.5 RT-PCR products generated from RNA purified from *S. coelicolor* M145 cultures at 36 h amplified with *hrdB* and *SCO1389* primers. Lane 1: RT-PCR (+Dnase); 2: PCR with HotStarTaq DNA Polymerase (+Dnase); 3: PCR with HotStarTaq DNA Polymerase (-Dnase); 4: PCR with HotStarTaq DNA Polymerase using M145 gDNA as template instead of RNA (-DNase).

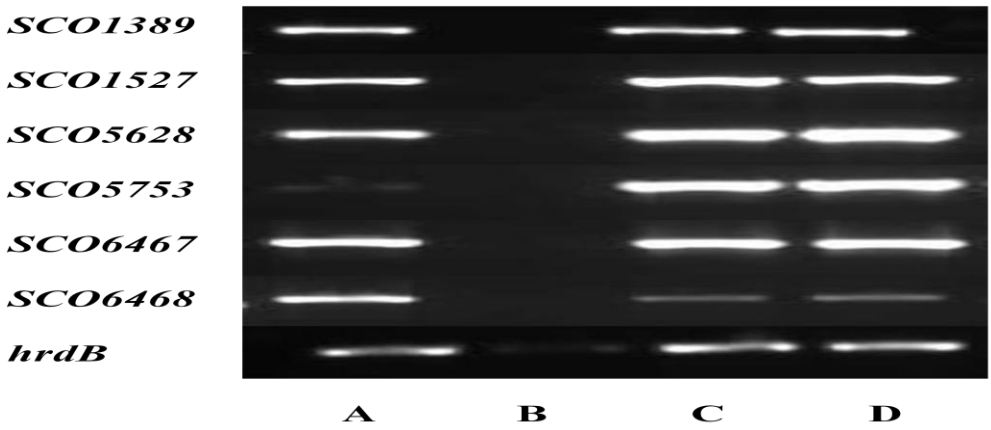


Fig. 3.6 Verification of RT-PCR products generated from RNA purified from *S. coelicolor* M145 cultures at 36 h and amplified with *hrdB, SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468* primers. Lane A: RT-PCR (+Dnase); B: PCR with HotStarTaq DNA Polymerase (+Dnase); C: PCR with HotStarTaq DNA Polymerase (-Dnase); D: PCR with HotStarTaq DNA Polymerase using M145 gDNA as template instead of RNA (-DNase).

Specificity of the primers was first checked by amplifying mRNA from these six genes and also a DNA template from *S. coelicolor* gDNA to produce products of predicted sizes that corresponded with RT-PCR products (see section 3.2.1 above). RT-PCR provided evidence for the presence of mRNA as a template (see Fig. 3.5 and 3.6 lane A). PCRs performed without RT served as controls to indicate the absence of contaminating DNA in the RT reaction mixtures (see Fig. 3.5 and 3.6 lane B). A PCR with HotStarTaq DNA Polymerase (-Dnase) was used as a negative control (see Fig. 3.5 and 3.6 lane C). PCR was performed with up to 100-fold dilutions of gDNA template to ensure that the RT-PCR products were of the same size as PCR products (see Fig. 3.5 and 3.6 lane D).

RT-PCR products of the six PL biosynthetic genes; *SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468* show that amplification occurred with all genes from RT-PCR with DNase treatment (see Fig. 3.5 and 3.6 lane A). There were no amplification on all genes RT-PCR products with PCRs performed with DNase treatment, but without RT indicated the absence of contaminating DNA in RNA sample (see Fig. 3.5 and 3.6 lane B). As a result we were satisfied that the primer set for the six PL biosynthetic genes could be used to detect mRNA of these genes by RT-PCR.

3.4. Expression of PL biosynthetic genes during growth of *S. coelicolor*

In this study, six genes encoding putatively involved with PL biosynthesis in *S. coelicolor* M145: *SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468* (as described in section 3.1) could potentially be expressed during development, therefore we extracted mRNA and analyzed their expression by RT-PCR to determine if they were expressed during development of *S. coelicolor* on agar plates

Fresh spores of wild-type *S. coelicolor* M145 were pre-germinated and then spread on cellophane membranes placed on 3MA plates and incubated at 30°C as described in materials and methods. Three sets of RNA were isolated, with mycelium harvested at 16, 24, 36, 48, 72 and 96 h after inoculation. RT-PCR carried out according to section 2.10.2. RNA isolation was carried out with One-Step RT-PCR kit (QIAGEN) following the procedures recommended by the manufacturer. Contaminating DNA was removed by digestion with DNase I (Qiagen) and verified by PCR analysis with the RNA as the template. The concentration and integrity of the RNA isolations were determined by both spectrophotometry at 260 nm and 280 nm using a NanoDrop spectrophotometer and agarose gel electrophoresis. *hrdB* was used as a control since it is expressed constitutively (Du *et al*., 2013, Kieser *et al*., 2000). No amplification products were detected in the absence of RNA confirming that amplification products are RNA dependent. Band intensity of each gene was quantified (Fig. 3.7) and compared with *hrdB* as internal standard before being normalized to the levels found at the first sample point. The data are displayed as a bar chart, error bars represent standard deviation (Fig. 3.8).

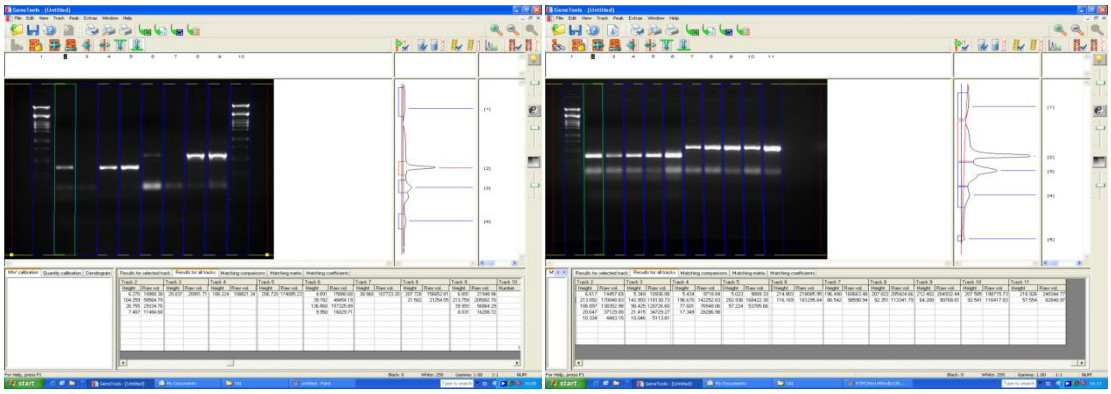
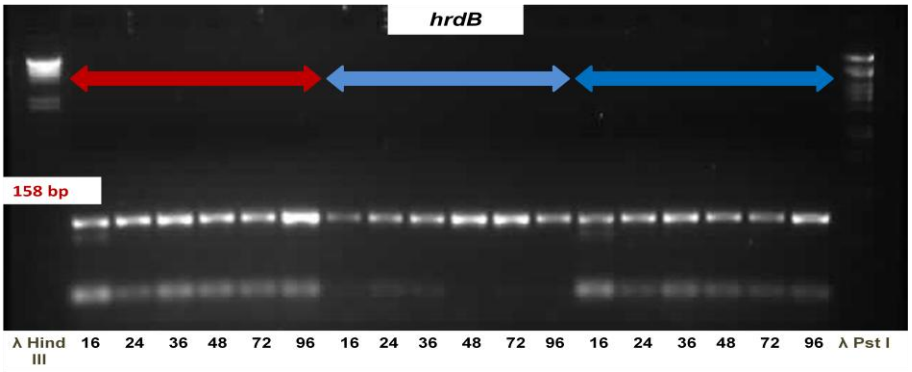
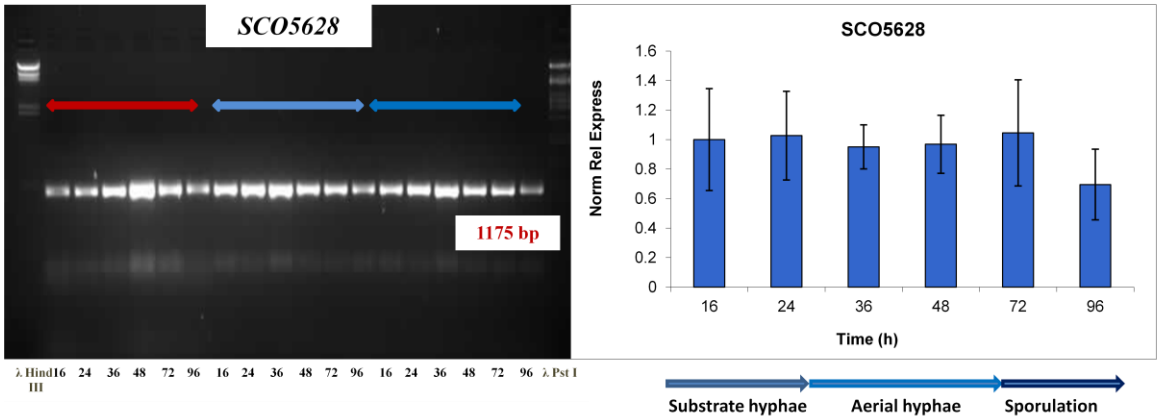
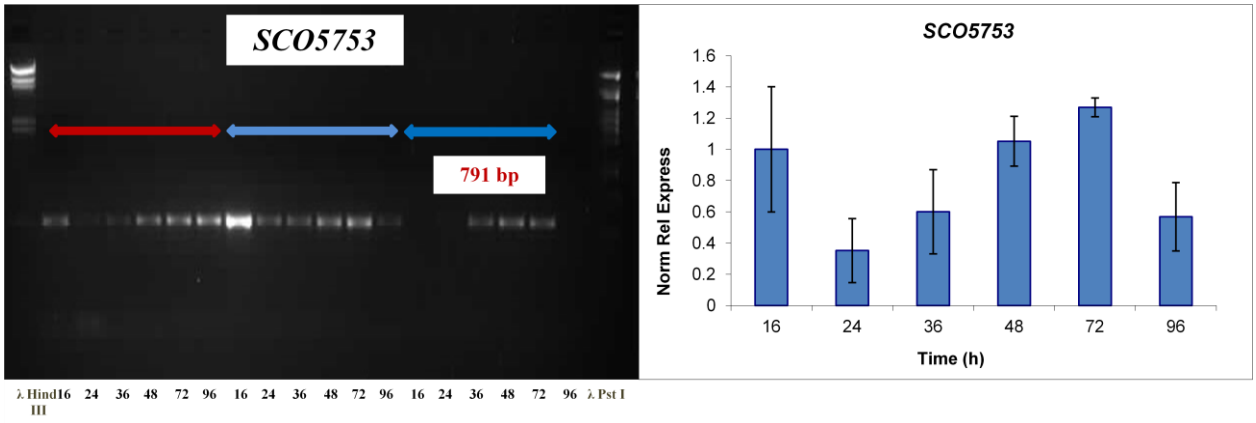
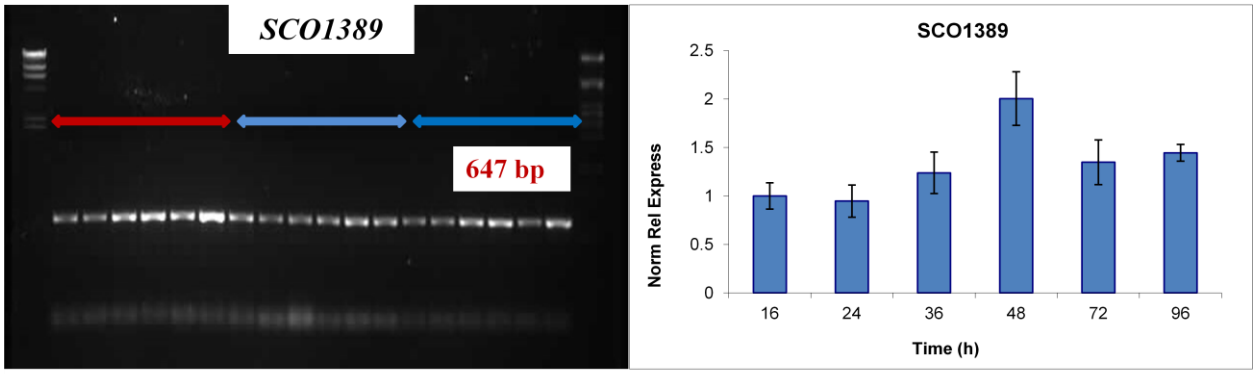


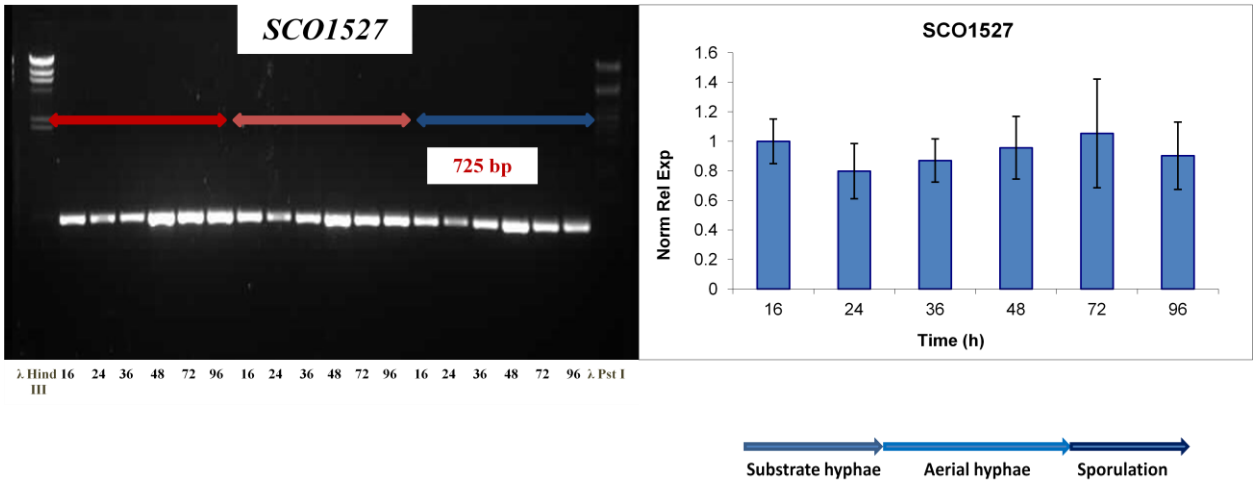
Fig 3.7 Quantification of PL biosynthetic gene expression by RT-PCR, agarose gel electrophoresis and densitometry. Bands were quantified by densitrometry using Genetools software (Syngene). Gene quantification was calculated according to the following formula. T_t = Average of gene intensity (pixels)/Average of *hrdB* intensity (pixels). Normalized relative Expression = T_t/T_{16} (t = time points at 16, 24, 36, 48, 72 and 96 h).






SCO5753
791 bp
λ Hind III 16 24 36 48 72 96 16 24 36 48 72 96 16 24 36 48 72 96 λ Pst I
SCO5753
Norm Rel Express
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16 24 36 48 72 96
Time (h)
Substrate hyphae
Aerial hyphae
Sporulation


SCO1389
647 bp
λ Hind III 16 24 36 48 72 96 16 24 36 48 72 96 16 24 36 48 72 96 λ Pst I
SCO1389
Norm Rel Express
2.5
2
1.5
1
0.5
0
16 24 36 48 72 96
Time (h)
Substrate hyphae
Aerial hyphae
Sporulation


SCO1527
725 bp
λ Hind III 16 24 36 48 72 96 16 24 36 48 72 96 16 24 36 48 72 96 λ Pst I
SCO1527
Norm Rel Exp
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16 24 36 48 72 96
Time (h)
Substrate hyphae
Aerial hyphae
Sporulation

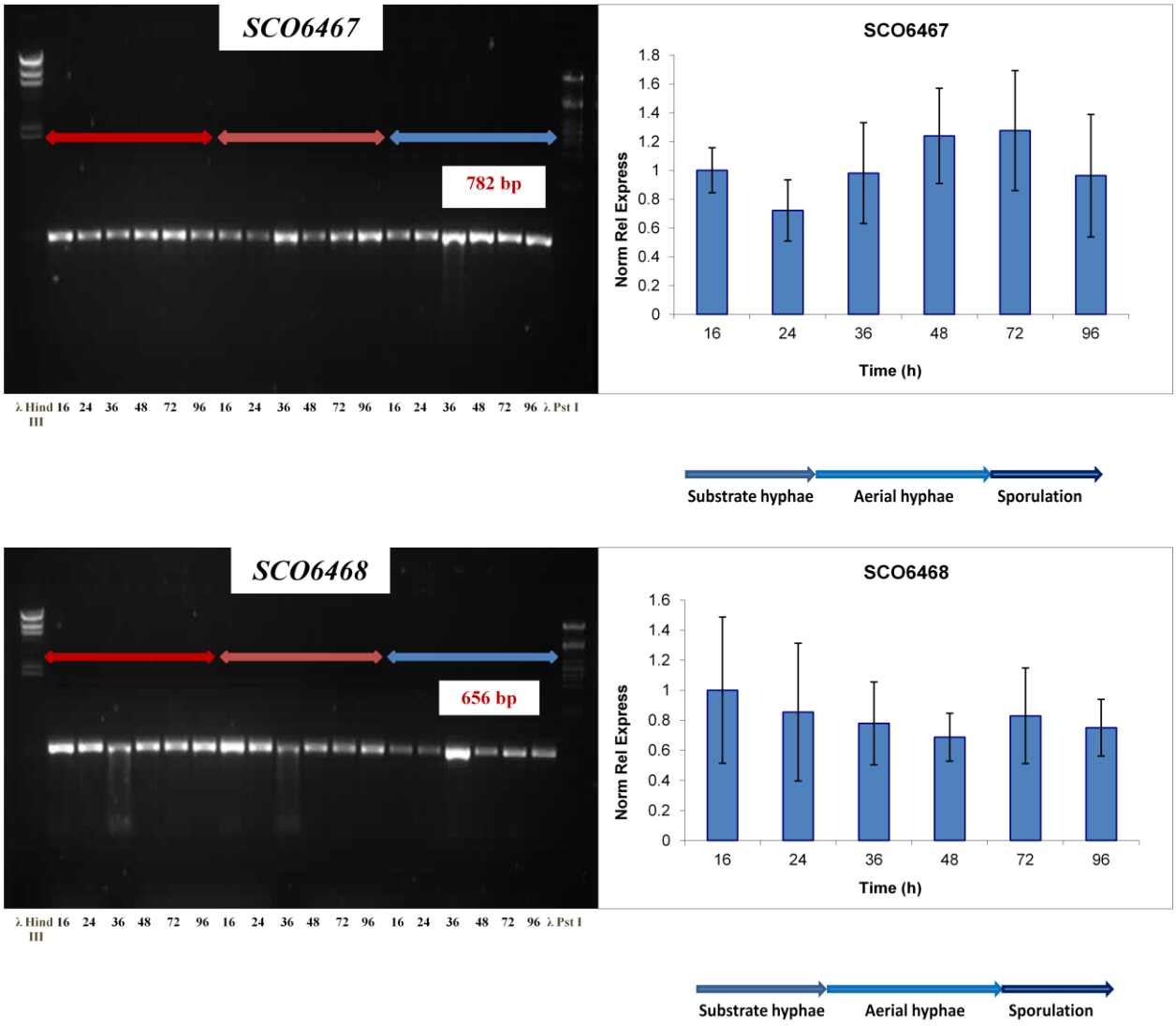


Fig. 3.8 Verification of RT-PCR products generated from three sets of RNA samples purified from *S. coelicolor* M145. A 1.4% agarose gel shows RT-PCR amplification products after 35 cycles, RNA were prepared from cell collected at 16, 24, 36,48,72 and 96 h amplified with *SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468* primers, respectively. The gene *hrdB* was used as a positive control and for normalization. The numbers of hours are shown along the bottom.

The RNA used in these assay was isolated from the 16, 24, 36, 48, 72 and 96 h after inoculation of 3MA agar covered with cellophane. The expression of *hrdB* gene is always expressed during time course since *hrdB* is express constitutively (Du *et al*., 2013, Kieser *et al*., 2000). *SCO5628* expression did not change significantly during the time course. This is to be expected as this gene is likely to encode phosphatidate cytidylyltransferase; since this protein is responsible for synthesis of CDP-DAG which is a precursor for all other membrane PLs it seems plausible that expression of this gene would be required at all stages of growth. *SCO5753* (Borodina *et al*., 2005; Sandoval-Calderon *et al*., 2009) has the highest expression of normalized relative expression at 72 h increasing during erection of aerial hypha. This protein is responsible for the synthesis of PG, the precursor for CL which is in turn synthesized by *SCO1389* (Borodina *et al*., 2005; Sandoval-Calderon *et al*., 2009). The normalized relative expression value increased during hyphal growth (from 24-36 h) and reached a maximal level around entry to the transition phase (48 h), then decreased during the later period of growth phase (72 h). As CL is known to be required for erection of aerial hyphae in *S. coelicolor* it is likely that elevated expression levels of *SCO5753* and *SCO1389* reflect the requirement of this PL and consequently its immediate precursor 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 vegetative growth (from 24-36 h) and reached a maximal level around entry to the transition phase (48 h), then decreased during sporulation phase (72 h). This is in contrast to *SCO6468* (PE synthase) where the

normalized relative expression value decreased during development (from 24-36 h) and reached a lowest level around entry to development (48 h), before decreasing during the later stages of development (72 h). Presumably, this is because *S. coelicolor* M145 produces less PE while producing CL. The normalized relative expression values for each gene show that during development of *S. coelicolor* on 3MA agar, some changes in PL biosynthetic gene mRNA could be detected in *S. coelicolor* M145 after inoculation, and might reflect altered patterns their expression during development of aerial mycelium production between the 24 and 72 h time points and spore formation occurring around 72 h after inoculation.

3.5 Determination of Changes in PL Profile during *S. coelicolor* Development

3.5.1 PL identification through comparison with known PL standards by TLC

All PL standards were developed by TLC and phosphorus-containing lipids identified with an ammonium molybdate spray as seen in Fig. 3.9. Two TLC plates are shown the first (Fig. 3.9 A standard TLC plate reproduced here as an exemplar (Tiong *et al*., unpublished data)), the second TLC plate (Fig. 3.9 B displays PLs developed and imaged as part of this study). It is worth noting that the PE standard consistently showed two spots and, by comparison with analogous solvent systems in the literature, the spot showing the furthest migration the furthest represents PE.

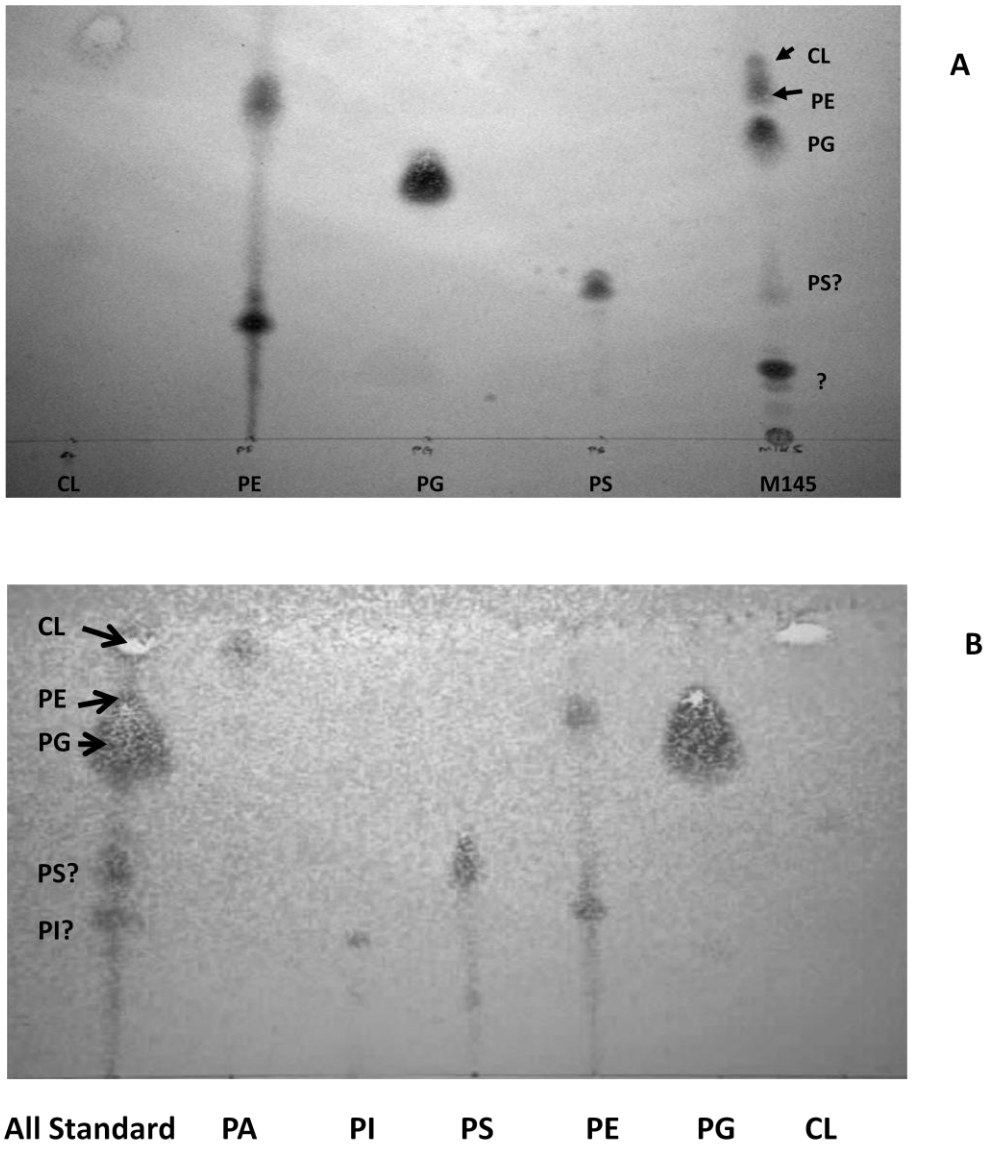


Fig. 3.9 TLC Analysis of PL standards. PLs were identified as phosphorus-containing lipids by spraying with ammonium molybdate. (A) Reference TLC analysis of PL standards and profile of mid-log phase *S. coelicolor* M145 developed in solvent system chloroform/methanol/acetic acid/water (80:12:15:4). CL migrated the furthest, followed by PE with some degree of overlap with PG, probable PS (faint spot) and finally an unknown PL spot. This TLC plate was reproduced from an exemplar (Tiong *et al*., unpublished data). (B) PL standards dissolved in ethanol (5 mg ml^{-1}) were also applied to a TLC plate to aid identification of unknown spots: phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG); phosphatidylethanolamine (PE) and cardiolipin (CL).

3.5.2 Development of PL biosynthetic genes during growth of *S. coelicolor*

To determine if production of PLs by *S. coelicolor* changed during development, in parallel with changes in expression of PL biosynthetic genes, PLs were also extracted from plate grown *S. coelicolor* and developed according to the Materials and Methods (Section 2.11) at the same time points that RT-PCR was carried out (16, 24, 36, 48, 72 and 96 h time points) (See section 3.4). Spots were provisionally identified on the basis of their migration in relation to the known standards. A spot, presumed to be CL (A), increased during vegetative growth (from 24-36 h) (Fig. 3.10 spot A) and reached a maximal level around the time that aerial hyphae were produced (72 h), then decreased during sporulation (96 h). It was not possible to identify spots B, C and D due to their lack of similarity in migration with the known standards. The abundance of spot B decreased from 16 to 24 h before increasing up to 72 h. It seems likely that the low recovery of PLs at 96 h reflects the difficulty in recovering PLs from spores rather than a decrease in PL content. Although it was not possible to conclusively identify spots B, C and D, a reduction in production of PG, PS and PE from 16 to 24 h followed by a subsequent increase in the abundance of these PLs would be consistent with the gene expression patterns displayed for the genes encoding the biosynthetic enzymes of these PLs (Fig. 3.8).

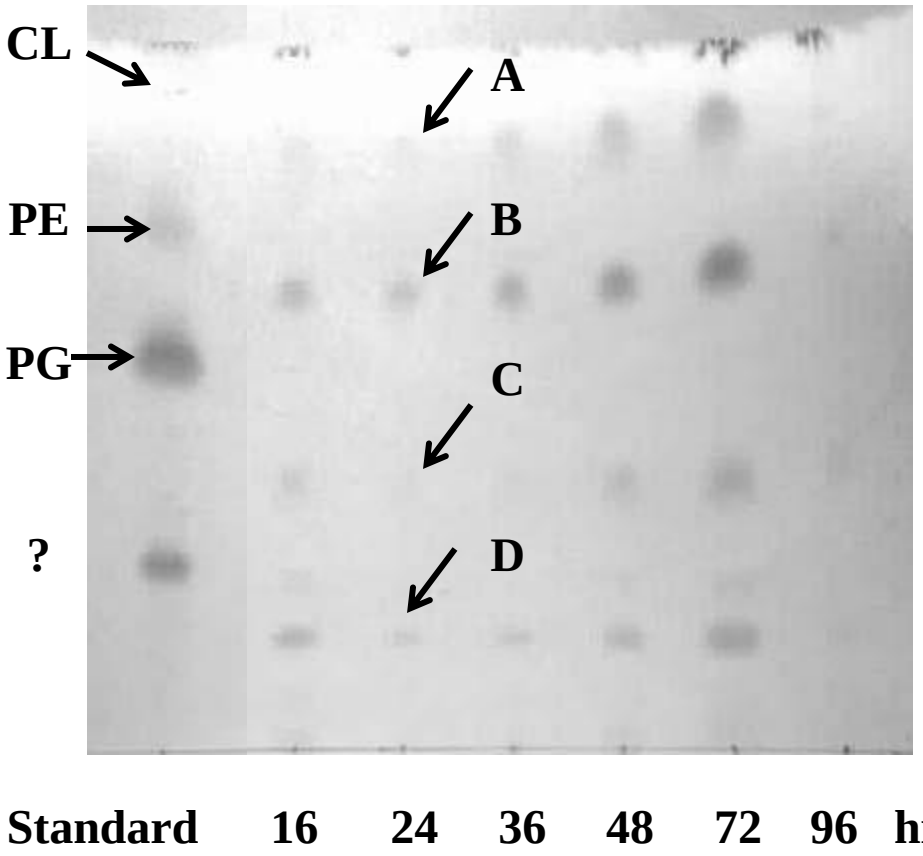


Fig.3.10 Total PLs extracted from 100 mg wet mass of plate grown *S. coelicolor* M145 during development. PLs were extracted at 16, 24, 36, 48,72 and 96 h were analyzed on silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). The plate was captured using Gene Snap densitometer and the bands were identified by their co-migration with standards where possible.

3.6 Conclusions to Chapter 3

The work presented in this chapter describes the optimization of RT-PCR conditions for the determination of the expression patterns of PL biosynthetic genes during growth and development of *S. coelicolor*. To establish optimal Mg^{2+} ion concentration for PCR, *S. coelicolor* gDNA was used as a template and following amplification of hrdB and *SCO1389* it was found that 2 mM $MgCl_2$ was the ideal concentration of Mg^{2+} ions to give the maximal yield. The optimum temperatures for PCR reaction were *hrdB* 66.4^{o}C, *SCO1389* 56.3^{o}C, *SCO1527* 55.0^{o}C, *SCO5753* 52.4^{o}C and *SCO6467* 61.0^{o}C in both 5x Go Taq® Flexi Buffer and 10X Qiagen HotStarTaq® Buffer, whilst *SCO5628* and *SCO6468* were 55.9^{o}C in 10X Qiagen HotStarTaq® Buffer only. RNA isolated from 36 h was chosen to be the optimal time point for RT-PCR. The amplification of these six genes by RT-PCR show that it was spossible to detect their expression in *S. coelicolor*. There was no amplification from all genes RT-PCR products with PCRs performed without RT that demonstrates the absence of contaminating DNA in RNA extracts.

Using the CL specific staining agent NAO to localize at the polar and septal regions of the cytoplasmic membrane of *E. coli* and *B. subtilis*, CL was shown to localize to these cellular locations (Kawai *et al.*, 2004, Huang *et al.*, 2006). The protein DivIVA, particularly localized at the tips of growing hyphae of the model organism *S. coelicolor*, is a homologue to *B. subtilis* DivIVA (Angert, 2005, Flardh, 2003a). While *S. coelicolor* DivIVA is localized at hyphal tips and is required during tip extension (Flardh, 2003a, Flardh, 2003b). It was proposed that curved membranes are involved in recruiting

DivIVA to the cell poles (Lenaric *et al*., 2009) so it is feasible DivIVA might be recruited to the cell poles via an interaction with CL (Jyothikumar *et al*., 2012). As a result, it is possible to predict that changes in PLs may play a role in development through changes in membrane biophysical properties allowing different proteins to associate with the cell membrane during tip extension and the erection of aerial hyphae. Clearly the *sapB* gene product is an example of a molecule that might associate with different PLs during aerial development (Capstick *et al*., 2007). The expression profile of most of the six biosynthetic genes did not change significantly development although minor changes in expression levels might generate altered PL content in the membrane during development (Fig. 3.8). *SCO5628* expression did not change significantly during the time course. This is to be expected as this gene is likely to encode phosphatidate cytidylyltransferase; since this protein is responsible for synthesis of CDP-DAG which is a precursor for all other membrane PLs, 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 increasing during erection of aerial hypha. This protein is responsible for the synthesis of the precursor for CL which is synthesized by *SCO1389* (Borodina *et al*., 2005; Sandoval-Calderon *et al*., 2009). The normalized relative expression value of *SCO1389* also increased during hyphal growth and reached a maximal level around the erection of aerial hyphae before decreasing during sporulation. *SCO1389* encodes a CL synthase (Borodina *et al*., 2005, Jyothikumar *et al*., 2012, Sandoval-Calderon *et al*., 2009). As CL is known to require for erection of aerial hyphae in *S. coelicolor* it is likely that elevated

expression levels of *SCO5753* and *SCO1389* reflect the requirement of this PL 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 the transition phase then decreased during this sporulation phase. This is in contrast to *SCO6468* (PE synthase) whose expression decreased during vegetative growth and reached a lowest level around entry to development (48 h), then increased during sporulation. Presumably, this is because *S. coelicolor* M145 produced less of 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; although clearly it is possible that these genes may also possess their own promoters.

Although it was not possible to conclusively identify the different PL spots by TLC when *S. coelicolor* PLs were extracted and resolved in parallel with the gene expression analysis described above some conclusions can be made. One spot, assumed to be CL (Fig. 3.10), increased during vegetative growth) and reached a maximal level around the time that aerial hyphae were produced (72 h). The intensity of this spot subsequently decreased during sporulation (96 h). This was consistent with the changes in expression of *SCO1389*, the gene that encodes CL synthase (Sandoval-Calderon *et al*., 2009). It was not possible to identify the other spots due to their lack of similarity in migration with the known standards, however if these PL spots did turn out to be PG, PS and PE, an initial reduction in their levels followed by a subsequent increase in the

abundance of these PLs is consistent with the gene expression patterns displayed for the genes encoding the biosynthetic enzymes of these PLs (Fig. 3.8).