Chapter 4 Characterization of mutants defective in CL biosynthesis of *S. coelicolor*

4.1 Introduction to Chapter 4

The fluid mosaic model proposed by Singer and Nicolson in 1972 assumed that cell membrane are homogeneous in terms of their PL content (Mileykovskaya & Dowhan, 2000). In rod-shaped bacterium, the PL, CL localizes at the polar and septal regions of the cytoplasmic membrane of *E. coli* and *B. subtilis* (Huang *et al*., 2006). In addition, this PL is enriched in the membrane of *E. coli* minicells and in the forespore membranes of *B. subtilis* during sporulation. In the latter organism, elevated levels of CL were found during spore engulfment (Huang *et al*., 2006, Kawai *et al*., 2004). However, its role in morphogenesis of the filamentous bacterium *S. coelicolor* is as yet an unanswered question. It was impossible to delete *SCO1389*, which encodes CL synthase (Jyothikumar *et al*., 2012), unless this gene was placed under the control of an inducible synthetic anhydrotetracycline (ATC) controllable promoter, tcp_{830} (Rodriguez-Garcia *et al.*, 2005). Morphogenesis then became dependent on the presence of inducer. Results described in the previous chapter indicated that levels of *SCO1389* expression increased during development in parallel with an increase in CL content during *S. coelicolor* development from vegetative to aerial hyphae. As a result we set out to determine the effects of altering expression of *SCO1389* on the PL content of *S. coelicolor*.

To determine the phenotypic consequences of its mutagenesis, *SCO1389* was disrupted by Tn*5062* mutagenesis (Herron *et al.*, 2004), but it was impossible to obtain any viable transconjugants that had undergone an allelic replacement (Jyothikumar *et al*., 2012). In addition, generation of a null mutant with *SC1A8AΔSCO1389* using PCR targeted mutagenesis (Gust *et al.*, 2003) was also impossible, as only transconjugants that

carried *SC1A8AΔSCO1389* integrated into the *S. coelicolor* chromosome via a single cross-over event were obtained. However when *S. coelicolor* was first complemented with a second copy of *SCO1389* it was possible to delete this gene resulting in *S. coelicolor* RJ114 that had undergone a deletion of the parental copy of *SCO1389*, but carried a second copy of this gene integrated at $attP_{\Phi BT1}$ attachment site (Fig. 4.1). pAV117B1 was also constructed that contained *SCO1389* under the control of the tcp_{830} cassette (Rodriguez-Garcia *et al*., 2005). When this plasmid was transferred via conjugation from ET12567 (pUZ8002) to RJ111, it was possible to obtain am^r, hyg^r and km^s transconjugants, but only when the agar was supplemented with ATC. This strain was termed RJ118b, had undergone a deletion of the parental copy of *SCO1389* (Fig. 4.1). This gene was also cloned in the opposite orientation into pAV11b (pAV11B2), so that antisense *SCO1389* would be expressed under the control of the tcp_{830} promoter upon addition of ATC. This construct was introduced into *S. coelicolor* M145 and termed RJ116 (Fig. 4.2). If *SCO1389* expression was required for normal growth of *S. coelicolor* the induction of anti-sense mRNA for this gene in RJ116 should retard growth of this strain in the presence of ATC through interference with translation of *SCO1389* (Jyothikumar *et al*., 2012)

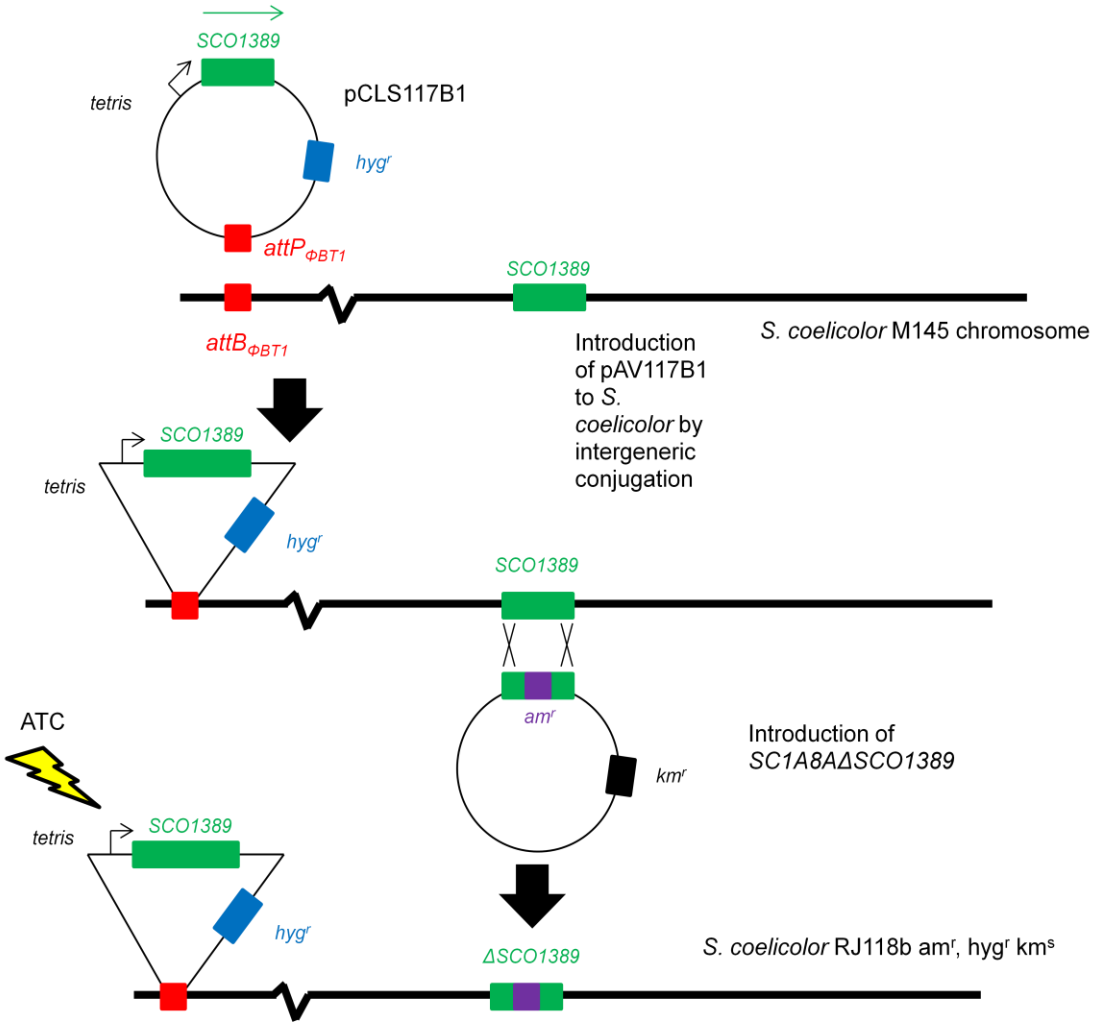


Fig. 4.1 Construction of depletion strain RJ118b. An extra copy of *SCO1389* was introduced to *S. coelicolor* M145 on pCLS117B1 where it integrated into the ΦBT1 phage attachment site. This strain carried two functional copies of *SCO1389*. The cosmid *SC1A8AΔSCO1389*, which carries a replacement of *SCO1389* with the apramycin resistance gene. This cosmid was introduced into the previous strain and colonies that had undergone an allelic replacement of *SCO1389* with the apramycin resistance gene identified. The conjugation was carried out in the presence of ATC as in the absence of the inducer viable colonies containing a double crossover of the replacement cassette could not be obtained in its absence; this is indicative of the essential nature of *SCO1389* (Jyothikumar *et al*., 2012).

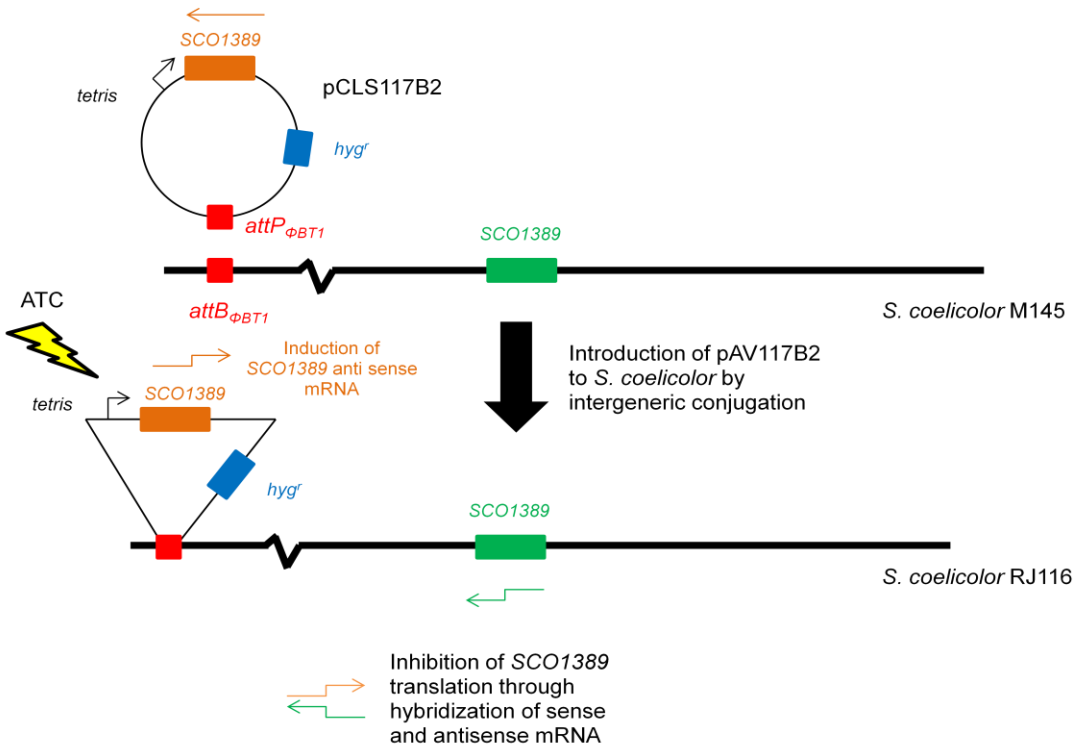


Fig. 4.2 Construction of depletion strain RJ116. pCLS117B2, which carries *SCO1389* in the opposite orientation, was conjugated into *S. coelicolor* M145 where it integrated into the ΦBT1 attachment site. This resulted in the strain, RJ116 that carries *SCO1389* in the opposite orientation, consequently antisense *SCO1389* mRNA should be expressed upon the addition of ATC. This antisense mRNA should hybridize with sense mRNA generated from the parental copy of *SCO1389* and subsequently block its translation. As a result addition of ATC to RJ116 should reduce the abundance of *SCO1389* (CL synthase) and therefore inhibit growth of this strain (Jyothikumar *et al*., 2012).

Aims of this chapter

The aims of this chapter were to confirm that *SCO1389* expression, and consequently CL, was required for normal growth and development of *S. coelicolor*. This was to be done through modulation of transcription of *SCO1389* by use of various strains of *S. coelicolor* where this gene was placed under the control of the tetracycline inducible promoter (Rodriguez-Garcia *et al*., 2005). We also set out to quantify *SCO1389* transcripts in the presence of the inducer of this promoter and determine if alterations in expression levels of this gene affected the PL profile of *S. coelicolor* on both solid agar and liquid cultures.

4.2 Effect of ATC concentration on *S. coelicolor* PL profile on solid cultures

4.2.1 Depletion of *SCO1389* affects the growth and sporulation in *S. coelicolor*

To confirm that *SCO1389* is an essential gene in *S. coelicolor* we used the strain RJ118b which has undergone a deletion of the parental copy of *SCO1389* and carries another copy of this gene under the control of the tetracycline inducible promoter, we cultured this strain on 3MA and MS agars containing different concentrations of $MgSO_4$ at 0, 0.2, 2 and 20 g L^{-1}. Each plate was also supplemented with ATC at concentrations of 0, 0.015, 0.15 and 1.5 μg ml^{-1}. Development of theses strains was then assessed visually and is shown in Fig. 4.3.

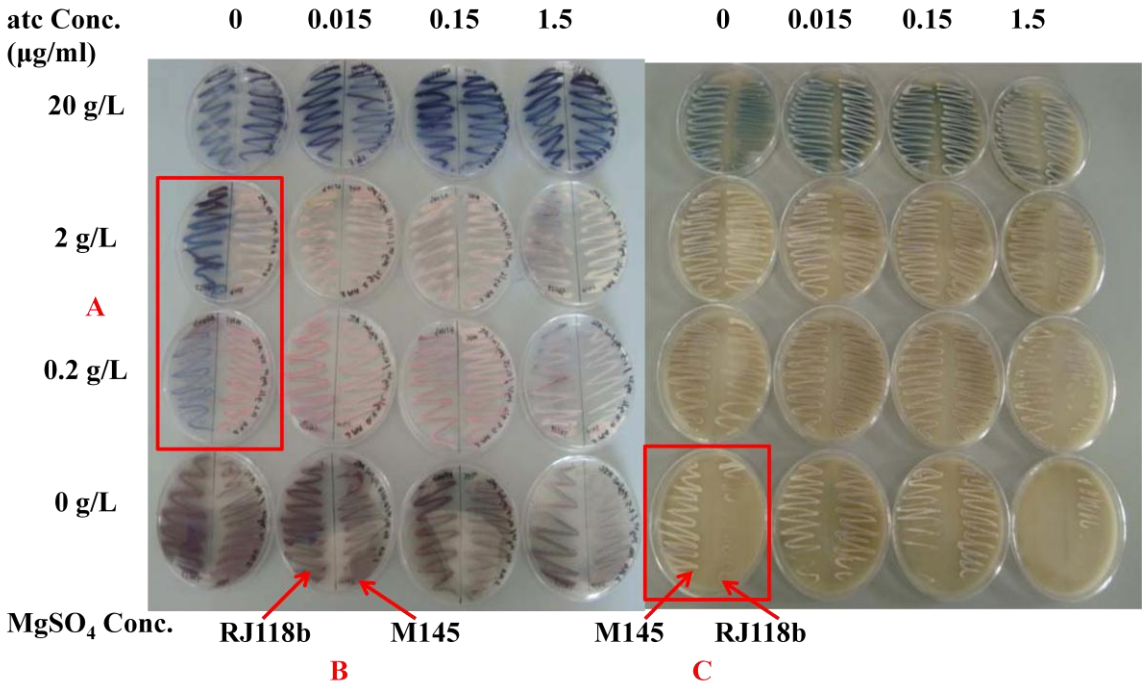


Fig. 4.3 Effect of $MgSO_4$ and ATC on *S. coelicolor* M145 and RJ118b development when cultured on 3MA and MS agar. Concentration of ATC at 0, 0.015, 0.15 and 1.5 µg ml^{-1} and concentrations of $MgSO_4$ at 0, 0.2, 2 and 20 g L^{-1} were used in both sets of agar.

In *E. coli*, an elevated concentration of Mg^{2+} ions can suppress effects of deletion of many of the PL biosynthetic genes (Mileykovskaya & Dowhan, 2005) and consequently we decided to investigate the effect of Mg^{2+} ion concentration on *S. coelicolor* development in conjunction with the effects of ATC induction of *SCO1389* expression. The erection of aerial hyphae was confirmed by the appearance of visible growth at different concentrations of ATC. The results show that RJ118b grew very poorly on both sets of agar and only showed weak erection of aerial hyphae unless the agar was supplemented with ATC (Fig. 4.3 A and C). It is thought that weak growth of substrate hyphae was observed on 3MA even in the absence of ATC due to the slight leakiness of the tcp_{830} promoter (Fig. 4.3 B).

4.2.2 Induction of *SCO1389* by ATC in *S. coelicolor* RJ118b

S. coelicolor M145 and RJ118b were grown on cellophane discs on 3MA in the presence of 0, 7.5, 15, 150 and 1500 ng ml^{-1} ATC for 48 h to observe that there was clear evidence that RJ118b was ATC dependent for its growth and development. After that, RNA was extracted and *SCO1389* expression analysed by semi quantitative RT-PCR using 100 ng RNA template. After RNA isolation and cDNA synthesis (see Materials and Methods, section 2.10.1 and 2.10.2), transcript levels were compared by RT-PCR with that of the internal control (*hrdB*) (Kieser *et al*., 2000). Three biological replicates of the RT-PCR were carried out. Bands were quantified by densitometry and related to the *hrdB* internal standard (Fig. 4.4 and 4.5). PLs were also extracted from 100 mg of M145 and RJ118b mycelia grown in the presence of 0, 7.5, 15, 150 and 1500 ng ml^{-1} atc for 48 h and scraped from 3MA plates and developed by TLC (Fig. 4.6).

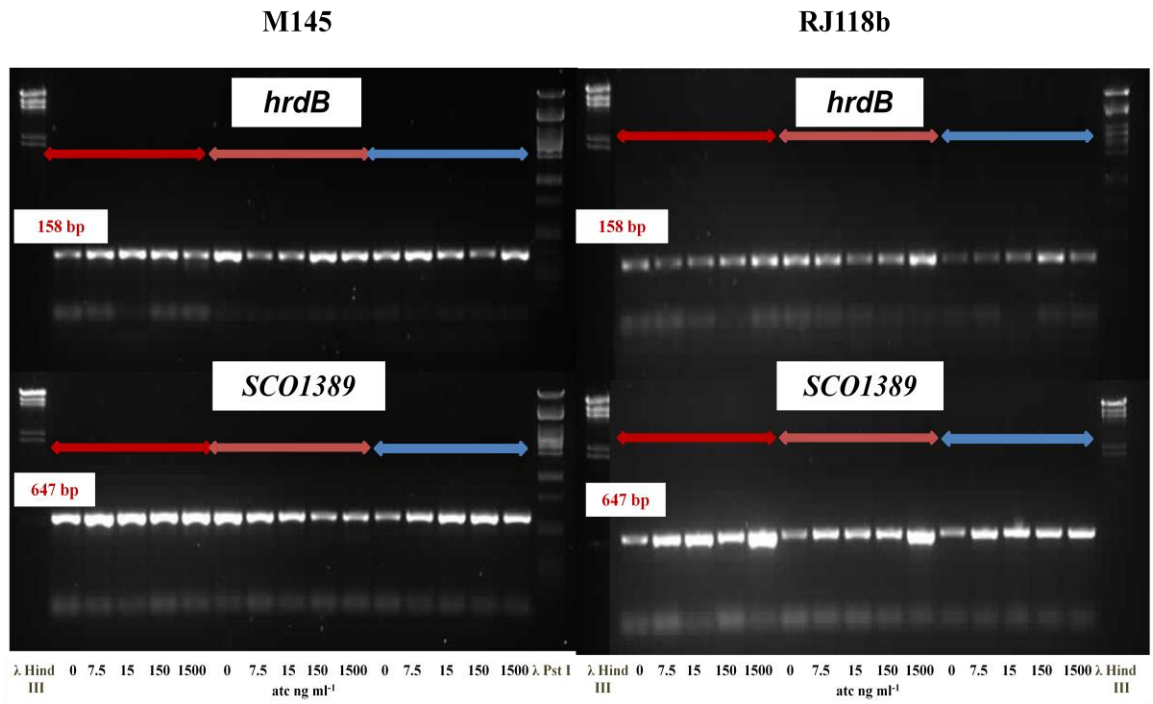


Fig. 4.4 Verification of RT-PCR products generated from three sets of RNA samples purified from *S. coelicolor* M145 and RJ118b. A 1.4% agarose gel shows RT-PCR amplification products after 35 cycles, RNA 100 ng were isolated from cultures grown on 3MA plates along a gradient of ATC at 0, 7.5, 15, 150 and 1500 ng ml^{-1}. Cells were collected at 48 h and amplified with *SCO1389* primers. The gene *hrdB* was used as a positive control and to normalize the RT-PCR. The concentrations of ATC are shown along the bottom.

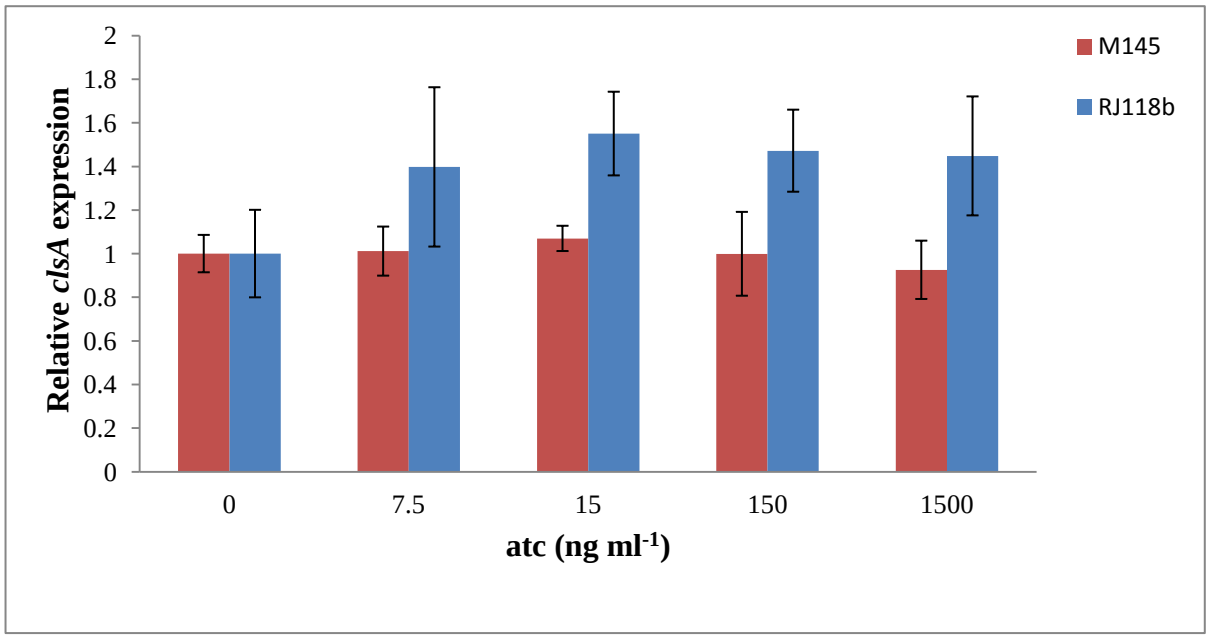


Fig. 4.5 Relative *SCO1389* expression by semi quantitative RT-PCR from 3 set of RNA samples purified from *S. coelicolor* M145 and RJ118b. RNA 100 ng were isolated from cultures grown on 3MA plates along a gradient of ATC at 0, 7.5, 15, 150 and 1500 ng ml^{-1}. Cell collected at 48 h amplified with *SCO1389* primers. The gene *hrdB* was used as a positive control to normalize the RT-PCR.

After RNA isolation and cDNA synthesis, transcript levels were compared by RT-PCR with that of the internal control (*hrdB)* (Fig. 4.5). Three biological replicates of the RT-PCR were carried out; all show similar patterns of *SCO1389* expression from *S. coelicolor* M145 and ATC-dependent *SCO1389* expression from RJ118b. Bands were quantified by densitometry and related to the *hrdB* internal standard before being normalized to the expression levels found in the absence of ATC; consequently the relative expression at 0 ng ml^{-1} for both *S. coelicolor* M145 and RJ118 was 1. The absolute level of expression of *SCO1389* was lower in uninduced RJ118b than for M145. These data are displayed as a histogram in Fig. 4.5; error bars represent standard deviation. Addition of ATC to *S. coelicolor* M145 did not cause any increase in *SCO1389*

transcription across the full range of concentrations. However when ATC was added to the media transcript levels were significantly increased in *S. coelicolor* RJ118b indicating that *SCO1389* expression was induced by the presence of ATC.

4.2.3 Effect of ATC concentration on *S. coelicolor* RJ118b PL profile

In addition to RT-PCR analysis of *SCO1389* expression we also extracted PLs according to (Bligh & Dyer, 1959, Iverson *et al.*, 2001) from 48 h old 3MA plates. These PL extracts were then developed by TLC (Fig. 4.6). A change in the PL profile was brought about by the addition of ATC to RJ118b (see Fig. 4.6, compare 0 ng ml^{-1} ATC with 7.5 ng ml^{-1} and higher), while the PL profile of M145 was not significantly affected by ATC. A PL spot that corresponded to the CL marker was absent from the profile of RJ118b grown without ATC (Fig. 4.6, A2). This spot was present at concentrations of ATC at levels at 7.5 ng ml^{-1} and higher, albeit at low levels. In addition another spot could be seen when RJ118b was grown in the absence of ATC (Fig. 4.6, C2), whilst another spot was not seen when RJ118b was grown without ATC (Fig. 4.6, B2). This spot approximately coincides with the position of PG marker. This is consistent with the accumulation of PG, the substrate for *SCO1389*, which might take place in the absence of *SCO1389* expression. In the presence of levels of ATC higher than 7.5 ng ml^{-1} no changes in the PL profile were observed from *S. coelicolor* RJ118b. This demonstrates that *SCO1389* is fully expressed when RJ118b was induced with 7.5 ng ml^{-1} of ATC.

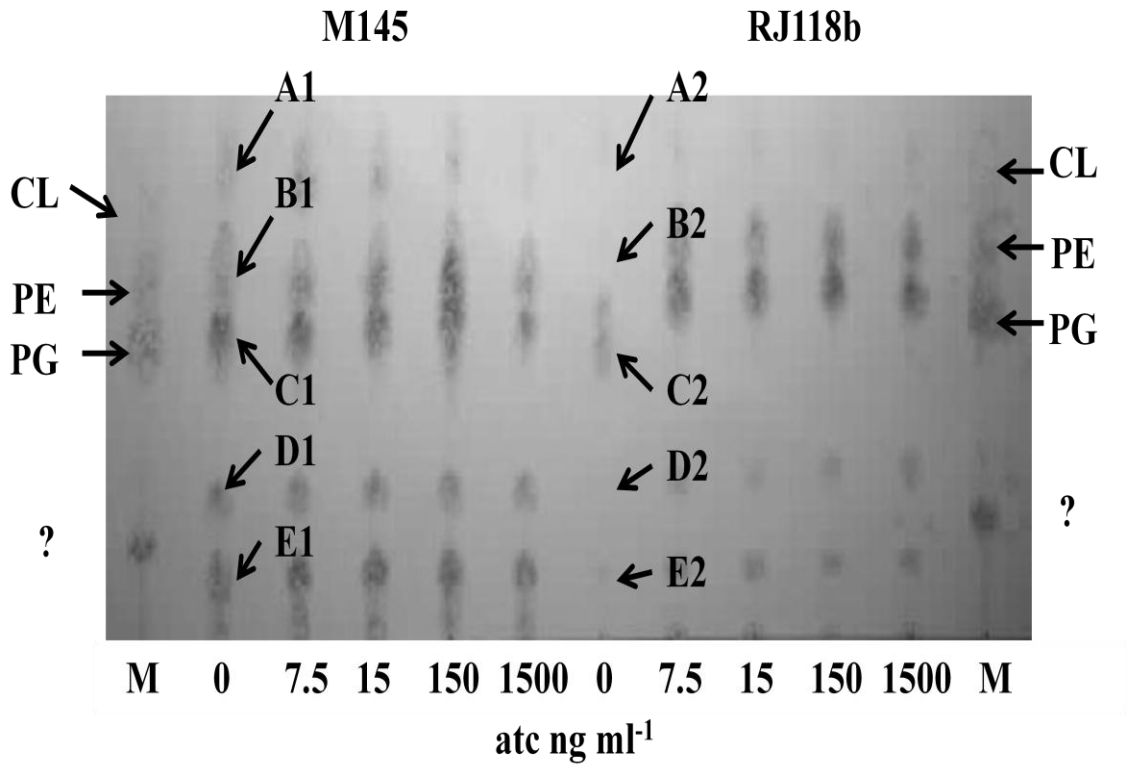


Fig. 4.6 Separation of PLs extracted from 100 mg wet mass of plate grown *S. coelicolor* M145 and RJ118b at different concentrations of ATC at 48 h. Agar plates were supplemented with at 0, 7.5, 15, 150 and 1500 ng ml^{-1} ATC**.** PLs were analyzed on silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). Staining was with molybdenum blue spray.

As 7.5 ng ml^{-1} fully induced *SCO1389* expression, we decided to investigate a narrower range of ATC concentrations. Consequently we set up another experiment that attempted to find the ATC concentration break point at which *SCO1389* transcription was induced. As before, PLs were extracted from 100 mg of *S. coelicolor* M145 and RJ118b mycelia except that they were grown in the presence of ATC 0, 1, 2, 4 and 6 ng ml^{-1} for 48 h and scraped from 3MA plates and developed by TLC (Fig.4.7).

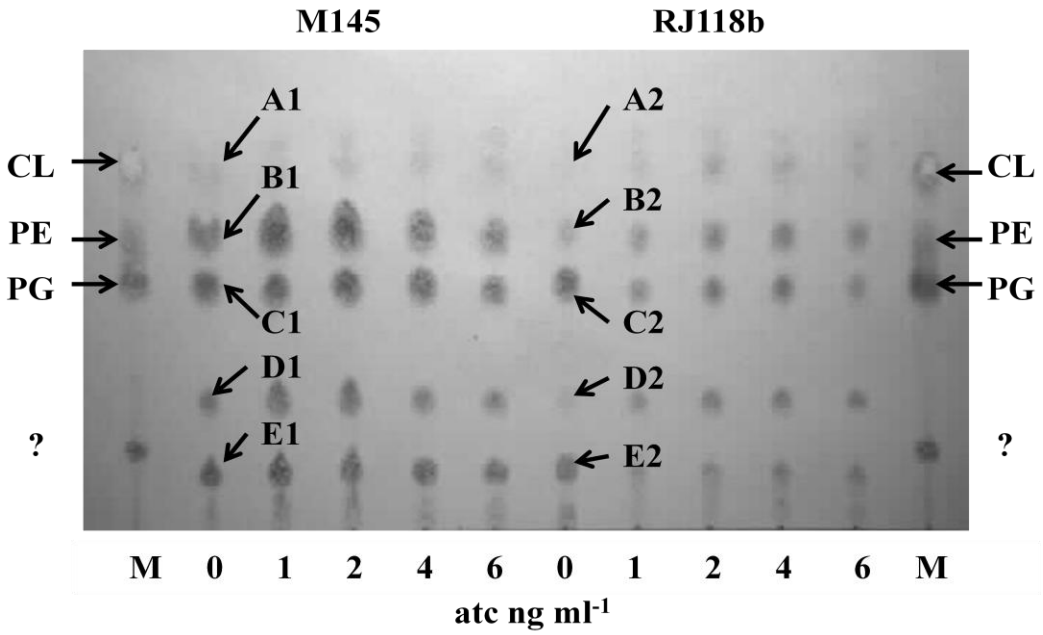


Fig. 4.7 Separation of PLs extracted from 100 mg wet mass of liquid grown *S. coelicolor* M145 and RJ118b at different concentrations of ATC at 48 h. Flasks were supplemented with 0, 1, 2, 4 and 6 ng ml^{-1} ATC**.** PLs were analyzed on silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). Staining was with molybdenum blue spray. Spot A is assumed to be CL; spot B, PE and spot C, PG. Two unidentified spots (D and E) were also visualised. 1 refers to *S. coelicolor* M145 and 2 to RJ118b.

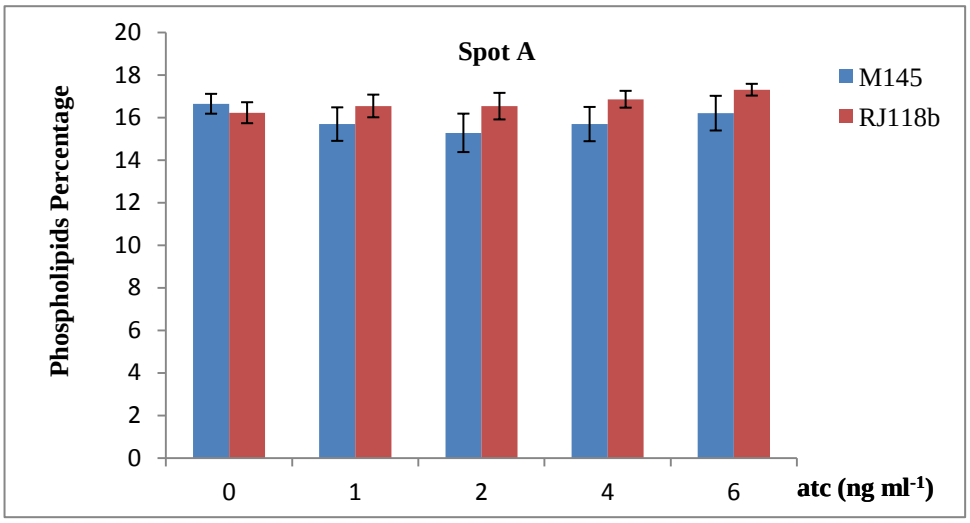
Three PL species could be identified on the basis of their similar migration to standards. These were CL (spot A), PG (spot B) and PE (spot C). Two additional spots (D and E) could also be seen, but it was not possible to identify them by similarity of migration with known standards. Changes in the PL profile brought about by the addition of ATC to RJ118b (see Fig. 4.7, compare 0 ng ml^{-1} ATC with 1 ng ml^{-1} and higher) could be clearly seen, while the PL profile of M145 was not significantly affected by ATC (Fig. 4.7, spot A-C). A PL spot (A2) that corresponded to the CL marker was absent from the profile of RJ118b grown without ATC, but appeared upon addition of ATC at

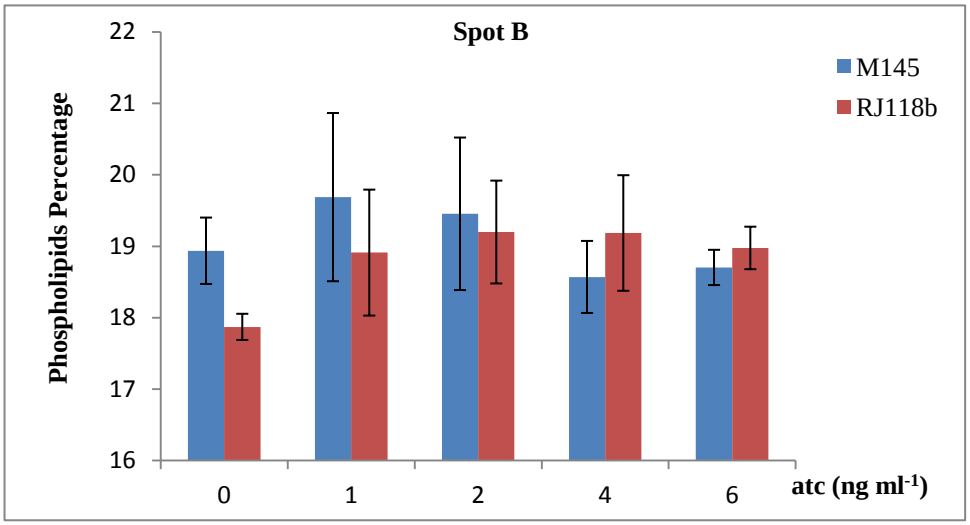
concentrations as low as 1 ng ml^{-1}. This strain, also displayed changes in other PL spots. For example, spots C2 (assumed to be PG) and E2 were more abundant when grown without ATC than in the presence of the inducer. This is consistent with the role of PG as a substrate for *SCO1389* and consequently, in the absence of expression of this enzyme it seems plausible that its substrate might accumulate. Less easy to explain is the fact that spots B2 (assumed to be PE) and D2 also displayed reduced abundance in the absence of ATC than in its presence. Although RJ118b grown with inducer did not display an identical PL profile to that of the wild-type strain in terms of spot intensity, the 5 major spots could be identified in both strains.

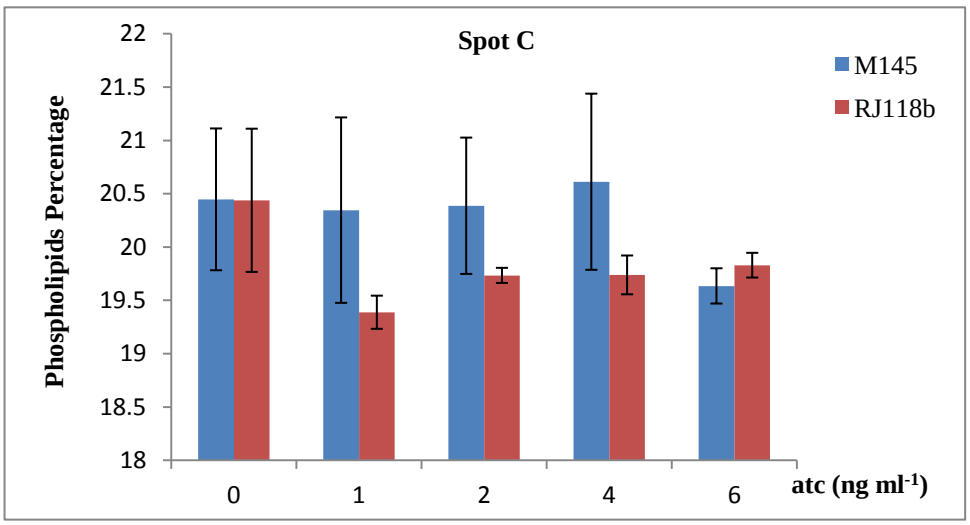
4.2.3.1 Quantification of PL spots induced in *S. coelicolor* RJ118b by densitometry

In order to analyse PL spot abundance in more detail we decided to quantify the five PL spots identified in the previous section. The PL extractions and TLC plates described in the previous section was repeated on a further two occasions (data not shown) to allow the generation of three biological replicates of quantitative data. Each of the five visible spots was quantified using densitometry. The total number of pixels for each treatment was then used to determine the percentage of each spot for that particular treatment. This was done for each of the two strains, *S. coelicolor* M145 and RJ118b, and the results are shown in Fig. 4.8. According to identification carried out in the previous section, spot A was assumed to be CL; spot B, PE and spot C, PG. Although many of the differences in spot intensities were not significant, some conclusions could be made from

this data. Induction of *SCO1389* by increasing ATC concentration caused the composition of CL (spot A) to be greater than in RJ118b than in M145; this was more pronounced and also evident in the case of PE (spot B). Increasing ATC concentration also reduced the percentage of spot C (PG) within the pool of five PLs in RJ118b. Again this is consistent with the role of PG as a substrate for *SCO1389*, as increased expression of this gene would likely result in increased utilization, and hence depletion, of PG. Although we were unable to identify spots D an E, increasing expression of *SCO1389* reduced the composition of these PLs in RJ118b with respect to M145.


Spot A
Phospholipids Percentage
20
18
16
14
12
10
8
6
4
2
0
0
1
2
4
6
atc (ng ml-1)
M145
RJ118b


Spot B
Phospholipids Percentage
22
21
20
19
18
17
16
0
1
2
4
6
atc (ng ml-1)
M145
RJ118b


Spot C
Phospholipids Percentage
22
21.5
21
20.5
20
19.5
19
18.5
18
0
1
2
4
6
atc (ng ml-1)
M145
RJ118b

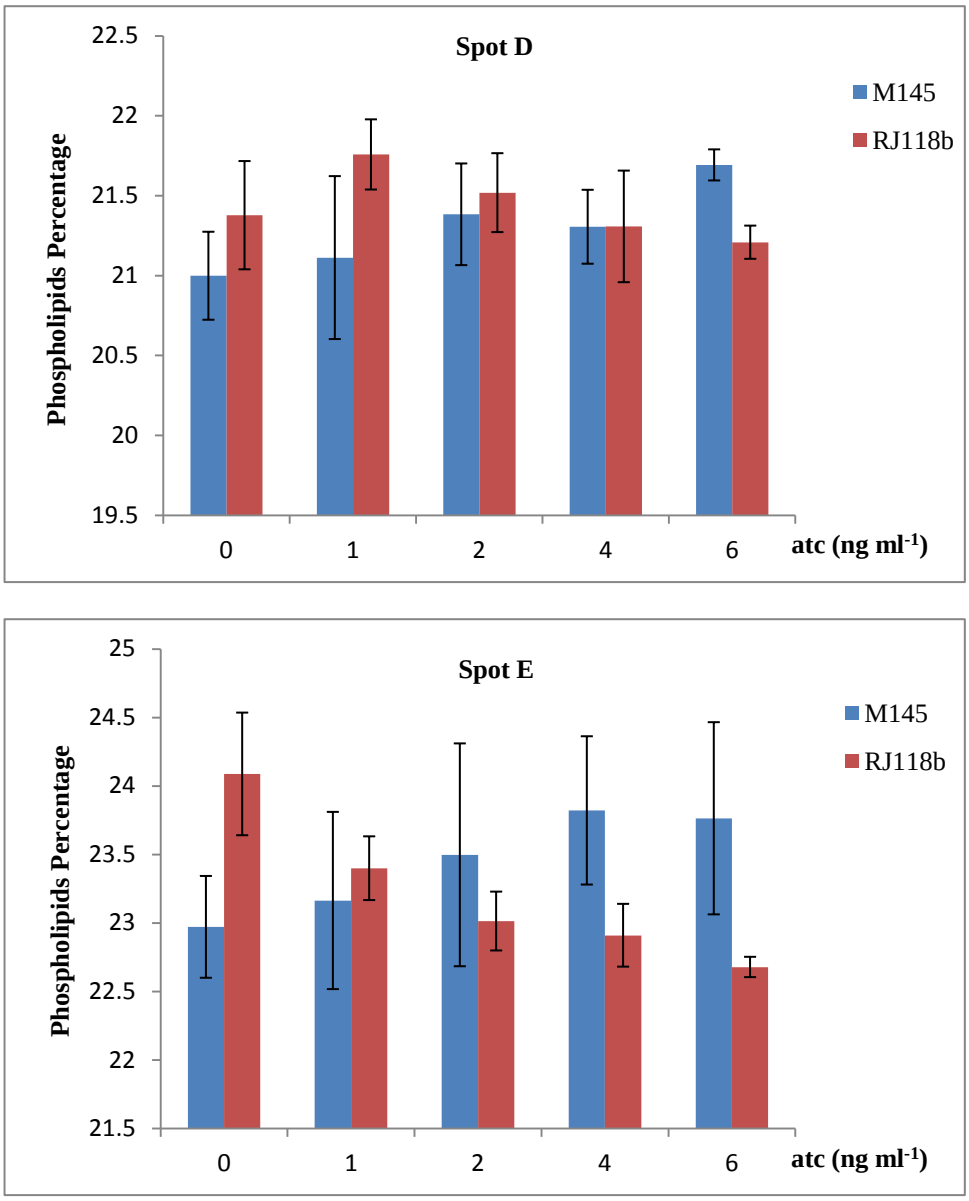


Fig. 4.8 The percentage PLs composition of *S. coelicolor* M145 and RJ118b at different ATC concentrations (0, 1, 2, 4 and 6 ng ml^{-1}). Spot A is assumed to be CL; spot B, PE and spot C, PG. Two unidentified spots (D and E) were also visualised. These data are displayed as a histogram. Error bars represent standard deviation.

4.3 Determination of induction and inhibition of *SCO1389* expression in liquid cultures

4.3.1 Analysis of *SCO1389* expression and PL profile in liquid cultures

RJ116 (Fig. 4.2) carries the copy of *SCO1389* in the antisense orientation under the control of the tcp_{830} promoter induced by ATC (Rodriguez-Garcia *et al*., 2005). However, it was observed that in solid media there was no effect on growth when plated on different concentrations of ATC (data not shown since plates grown agar used to make spore suspension only). This was surprising as induction of antisense *SCO1389* mRNA should block translation of parental *SCO1389* and cause a weakening of growth in relation to increased ATC concentration. As such we set out to verify if antisense *SCO1389* expression was induced by ATC in RJ116. In addition, we wished to determine if modulation of *SCO1389* expression by ATC was similar in liquid culture to that found on agar plates for RJ118b (see previous Section). This was done by growing *S. coelicolor* M145, RJ116, RJ117 and RJ118b in liquid YEME media after 100 µl of spore suspensions of each strain were inoculated into 25 ml of YEME medium in sterile 250 ml Erlenmeyer flasks. These flasks were fitted with stainless steel springs around the bottom to enhance dispersed growth in the presence of 0, 1 and 10 ng ml^{-1} ATC. RJ117 is the vector only control used to verify that our observations were not due to the integration of the delivery plasmid into the $AttB_{\Phi BT1}$ site on the *S. coelicolor* chromosome. This strain was constructed by the introduction of pAV11b into *S. coelicolor* M145; pAV11b is the vector into which *SCO1389* was cloned and used to construct pAV117B1 (Fig. 4.1) and Fig. pAV117B2 (Fig. 4.2 (Jyothikumar *et al*., 2012)) . The cultures were incubated at

30°C at 250 rpm for 24 h before extraction of RNA and analysis by semi quantitative RT-PCR using 100 ng RNA template (see Materials and Methods, section 2.10.1 and 2.10.2). Three biological replicates of the RT-PCR were carried out. In parallel PLs were extracted from 100 mg biomass and developed by TLC (Fig. 4.10).

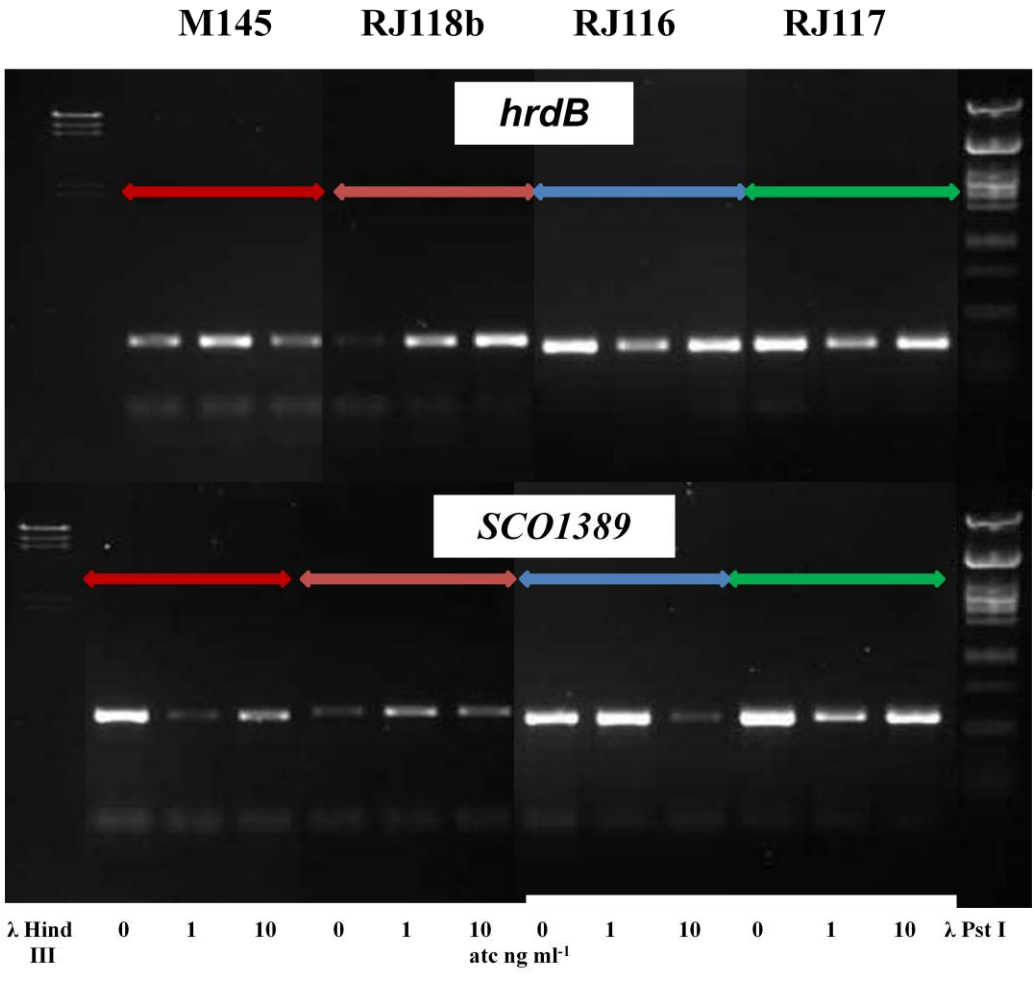
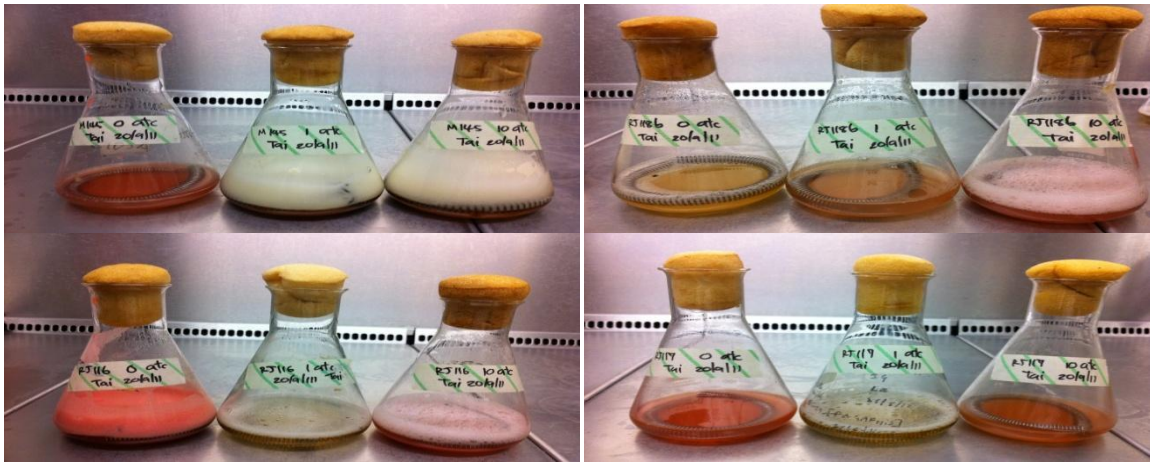


Fig. 4.9 Verification of RT-PCR products generated from RNA samples purified from *S. coelicolor* M145, RJ118b, RJ116 and RJ117. A 1.4% agarose gel shows RT-PCR amplification products after 35 cycles, RNA was isolated from cultures grown in 25 ml of YEME medium at different concentrations of ATC (0, 1 and 10 ng ml^{-1}). Cells were collected at 24 h and amplified with *SCO1389* primers. The gene *hrdB* was used as a positive control to normalize the RT-PCR. The concentrations of ATC are shown along the bottom.

Resolution of RT-PCR products by agarose gel electrophoresis (Fig. 4.9) showed that *SCO1389* expression could be detected in the four strains. However induction of *SCO1389* transcription by ATC was more difficult to interpret. *S. coelicolor* M145 and RJ117 carry *SCO1389* under the control of its native promoter, RJ118b under the control of tcp_{830} and RJ116 carries two copies of the gene; one under the control of the former promoter and one under the control of the latter. Whilst *SCO1389* expression in *S. coelicolor* M145 and RJ117 was unaffected by ATC, RJ118b, as an agar plates, showed slight amplification of *SCO1389* mRNA in the absence of ATC; presumably due to the slight leakiness of the tcp_{830} promoter. Nevertheless addition of ATC resulted in increased transcription of from this gene. The strain that carries an integrated copy of antisense *SCO1389*, RJ116, did not display an increase in expression upon ATC induction and we speculate that this might be due to the time difference in mRNA expression between the integrated copy of antisense *SCO1389* and the functional copy of *SCO1389* present within the chromosome. This was disappointing, as anti sense RNA is one of the recent approaches used to study gene function through the utilization of antisense RNA to manipulate the translation of target genes. This approach is especially useful for those genes that are involved in essential cell functions, such as *SCO1389*. It was also reported that size limitations on antisense RNA has some effects on inhibition of the translation of the target genes (Wang & Kuramitsu, 2005) and we speculate that the low inhibitory effect of *SCO1389* in RJ116 may be due to the size of the antisense RNA or that there is another activator gene which counter acts the effect of antisense mRNA during growth.

A



B

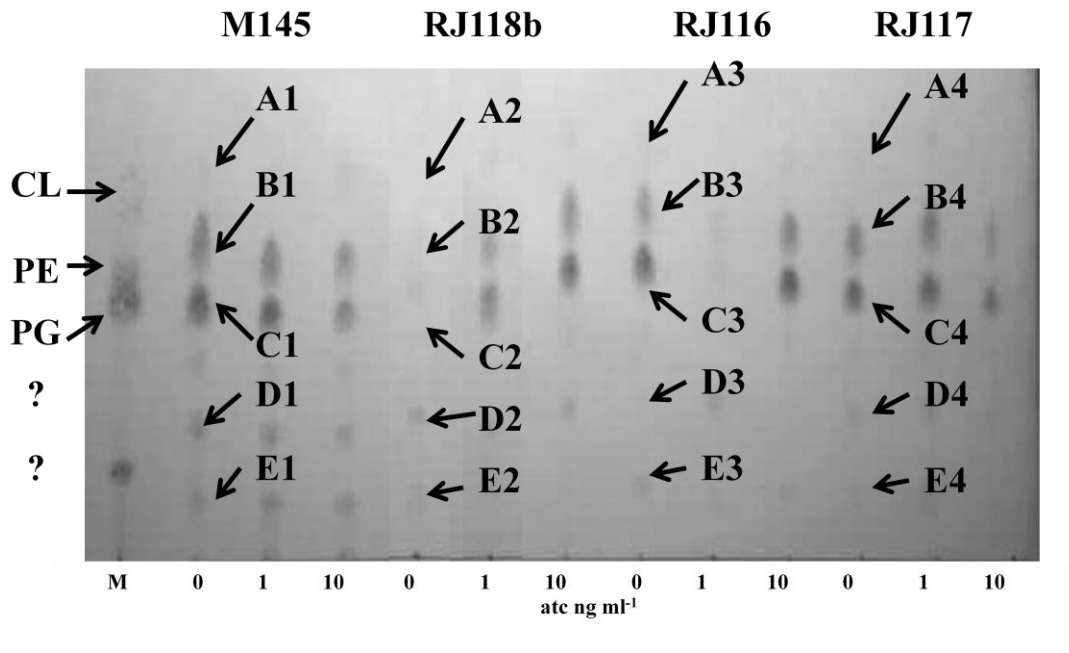


Fig. 4.10 Development of PLs extracted from 100 mg wet mass at 24 h of *S. coelicolor* M145, RJ118b RJ116 and RJ117 art different concentrations of ATC in liquid YEME. (A) Cultures of *S. coelicolor* M145, RJ118b, RJ116 and RJ117 (top left to right and buttom left to right, respectively) in liquid YEME media in the presence and absence of ATC. **(B)** PLs were analyzed on silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). Staining was with molybdenum blue spray.

To further analyse the PL profile of *S. coelicolor*, we investigated PLs produced by *S. coelicolor* during *SCO1389* depletion and over-expression following extraction from mid-log phase liquid cultures (24h). PLs from *S. coelicolor* M145, RJ116, RJ117 (vector only control) and RJ118b in the presence and absence of ATC were extracted from mycelial pellets and visualized by TLC (Fig. 4.10). *S. coelicolor* M145 and RJ117 did not show any change of PLs profile in response to ATC treatment. Liquid-grown RJ118b, grown without ATC, displayed a different TLC pattern to solid-grown cultures (see Fig.4.6 and 4.7, RJ118b, 0 ng ml^{-1} ATC), but still showed reduced production of PLs (Fig.4.10, spot A2 and B2) and increased production of another PL (Fig. 4.10, spot C2) when compared with *S. coelicolor* M145 that approximately corresponded to the CL, PE and PG markers respectively. The addition of ATC restored the PL profile of RJ118b to that of the wild-type strain. Interestingly addition of 1 ng ml^{-1} ATC to *S. coelicolor* RJ116 showed a similar PL profile to RJ118b grown without ATC. This suggests that induction of low levels of antisense *SCO1389* mRNA blocked CL production. Despite this, the addition of 10 ng ml^{-1} ATC caused RJ116 to display a wild-type PL profile. The reason for this is not clear, but perhaps the high levels of undecylprodigiosin produced by this strain might have affected resolution of PL spots by TLC. PL spots were also quantified by densitometry. The total number of pixels for each treatment was then used to determine the percentage of each spot for that particular treatment. This was done for each of the four strains, *S. coelicolor* M145, RJ118b, RJ116 and RJ117; the results are shown in Fig. 4.11. According to identification carried out in the previous section, spot A

was assumed to be CL; spot B, PE and spot C, PG. Although many of the differences in spot intensities were not significant some conclusions could not be made from this data.

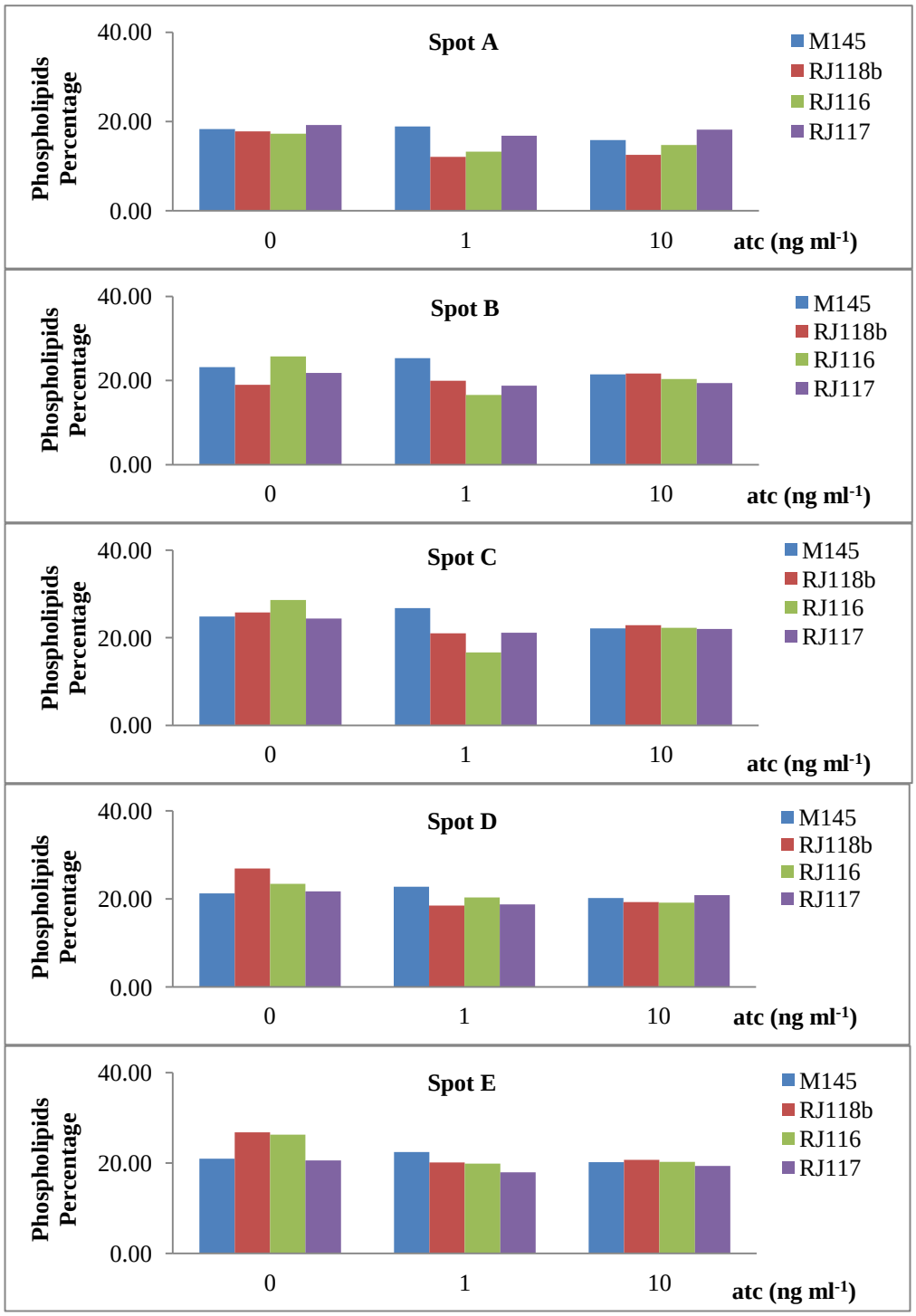


Fig. 4.11 The percentage PL composition of *S. coelicolor* M145, RJ118b, RJ116 and RJ117 from single sample at different concentrations of ATC (0, 1 and 10 ng ml^{-1}). Spot A is assumed to be CL; spot B, PE and spot C, PG. Two unidentified spots (D and E) were also visualised. These data are displayed as a histogram.

4.3.2 Optimization of RT-PCR for detection of antisense *SCO1389* mRNA produced by *S. coelicolor* RJ116

The induction of antisense *SCO1389* mRNA in *S. coelicolor* RJ116 did not display the expected response following induction with ATC (Fig. 4.9). We speculated that this could potentially be due problems in the order that the primers bound to the cDNA synthesised by reverse transcriptase from the *SCO1389* antisense transcript. For example, sense mRNA generated by RJ118b should generate cDNA that initially annealed to the primer *SCO1389_R* (See Table 2.4), whilst antisense mRNA generated by RJ116 should generate cDNA that initially annealed to the primer *SCO1389_F* (See Table 2.4). Consequently we set up an experiment where the RT reaction to generate the first cDNA strand was carried out without the addition of primers to determine if the order that the primers were added to the subsequent PCR reaction affected the outcome of the amplification.

The initial RT reaction was performed using 100 ng RNA isolation of M145, RJ116, RJ117 and RJ118b in the presence of 0, 1 and 10 ng ml^{-1} ATC were carried out with One-Step RT-PCR kit (QIAGEN) following the procedures recommended by the manufacturer (see Materials and Methods, section 2.10.1 and 2.10.2). After adding all the reaction components except *SCO1389_F, SCO1389_R, hrdB_F* or *hrdB_R* we first carried out the RT reaction to synthesise cDNA from mRNA transcripts. Next we denatured the RNA /cDNA hybrid molecule for 15 min at 95 $^{\circ}$C, before adding either the forward or reverse primer to the appropriate reaction. After one cycle of denaturation (1 min at 94°C), annealing (50-68°C, 1 min) and extension 72°C 1 min, we then added the

second primer to the appropriate reaction and continued the PCR for a further 34 cycles. *SCO1389*_F and *SCO1389*_R were used to amplify *SCO1389* whilst *hrdB*_F and *hrdB*_R were used to amplify the internal control. Subsequently, a final extension at 72°C for 10 min was carried out and the samples were subjected to electrophoresis on 1.4% agarose in 1X TAE buffer and stained with Etbr staining and UV illumination (Fig. 4.12).

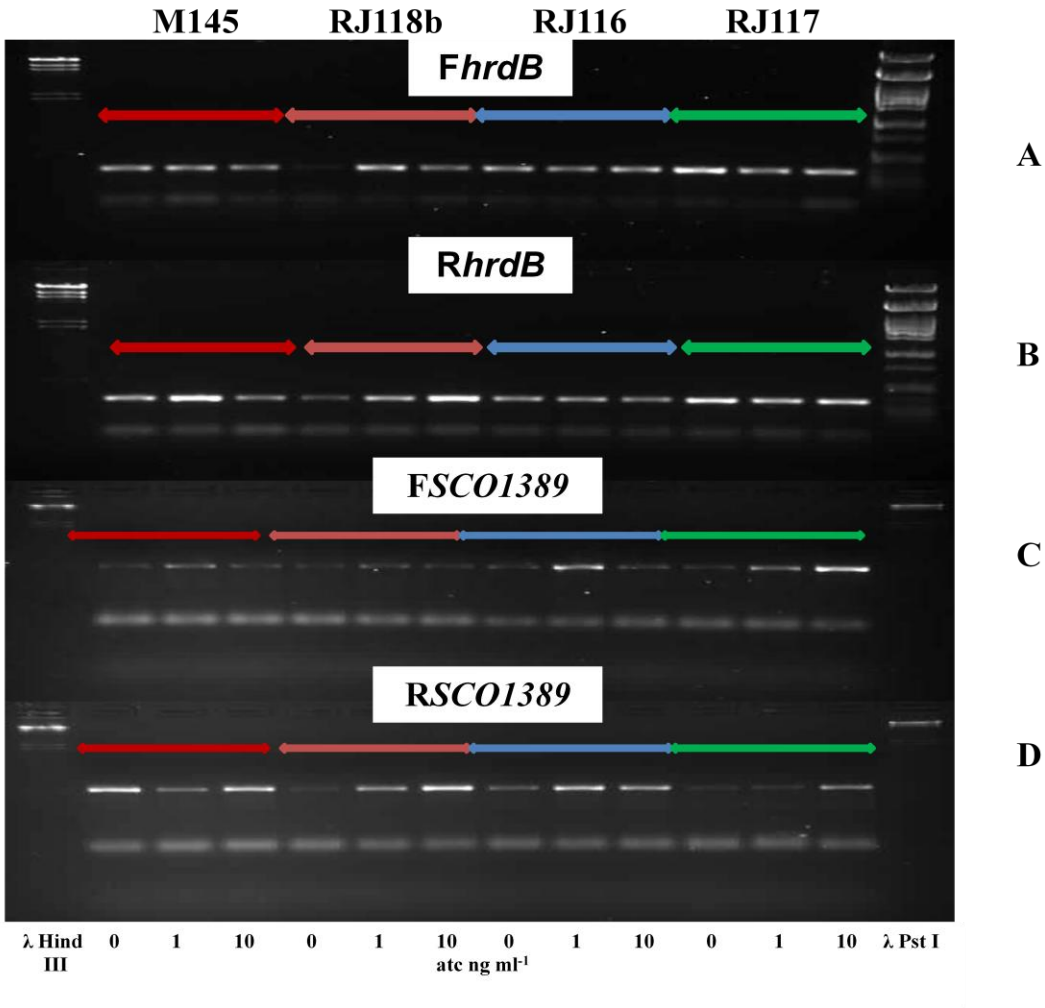


Fig. 4.12 Verification of RT-PCR products generated from RNA samples purified from *S. coelicolor* M145, RJ118b, RJ116 and RJ117. A 1.4% agarose gel shows RT-PCR amplification products after 35 cycles, 100 ng RNA was isolated from cultures grown in 25 ml of YEME medium at different concentrations of ATC at 0, 1 and 10 ng ml^{-1}. Cell collected at 24 h amplified with primers *SCO1389*_F and *SCO1389*_R were used to amplify *SCO1389*whilst *hrdB*_F and *hrdB*_R were used to amplify the internal control. The concentrations of ATC are shown along the bottom. F or R indicates the primer that was added to the first cycle of the PCR reaction following cDNA synthesis by RT.

Transcription of *hrdB* was always detected and in general, expression was independent of ATC concentration (Fig. 4.11) although some effect of ATC could be seen in RJ118b. Also in this strain, addition of *SCO1389_R* to the first PCR gave the best response to ATC treatment, although a similar response with the *hrdB* control suggests that this response is not entirely due to the order of primer addition. In the case of *S. coelicolor* RJ116, addition of ATC at 1 ng ml^{-1} and 10 ng ml^{-1} increased the level of *SCO1389* transcription when both *SCO1389_F* and *SCO1389_R* were added o the first PCR. In addition induction of *SCO1389* expression did not show an exact correlation with ATC concentration in either case. As a result we do not believe that our inability to demonstrate ATC induction of antisense mRNA was due to deficiencies contained with the RT-PCR protocol but was more likely due to a more fundamental problem with *S. coelicolor* RJ116. It may be that the essential nature of *SCO1389,* coupled with the lack of expression of the tcp_{830} promoter might have somehow selected for a mutations in the tcp_{830} promoter or *tetR* gene that prevented its proper operation.

4.4 Conclusions to Chapter 4

The work presented in this chapter demonstrates that *S. coelicolor* RJ118b was unable to grow well on MS agar unless supplemented with ATC and that weak growth of substrate hyphae was observed on 3MA even in the absence of the inducer. The ability of this strain to grow better in the presence of ATC is through the binding of the inducer to the TetR protein and causing a relief of repression of this promoter. As a result, RJ118b would be able to produce CL synthase and so synthesis CL even in the absence of ATC if this promoter was not completely silenced by TetR binding. 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 aerial hyphae or sporulation in the absence of ATC on 3MA. 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 induction of *SCO1389* expression. To some degree increasing concentrations of Mg^{2+} 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*, it occurs by a similar route in *S. coelicolor*; at least for *SCO1389.*

In order to determine the ATC dependence of tcp_{830}-*SCO1389* transcription in *S. coelicolor* RJ118b, which has undergone a deletion of parental copy of *SCO1389* (Jyothikumar *et al.*, 2012), 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. 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. Several PL spots changed in abundance upon inducer treatment; most notably CL (Fig. 4.7), which 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 seems plausible that its substrate might accumulate. Less easy to explain is the fact that a spots assumed to be PE also displayed reduced abundance in the absence of ATC than in its presence of ATC. However a recent paper (Tan *et al*., 2012) discovered 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 the inter-conversion of different PLs.

Resolution of RT-PCR products by agarose gel electrophoresis showed that *SCO1389* expression could be induced by ATC in *S. coelicolor* RJ118b. However, we were also keen to demonstrate the use of anti sense mRNA as a tool to block translation of an essential gene in *S. coelicolor* as this approach has not been successfully employed

in this organism to date. Despite, this we were unable to reliably demonstrate induction of anti sense *SCO1389* mRNA. The fact that increasing the ATC concentration at which *S. coelicolor* RJ116 was grown had no effect was rather disappointing. This suggests 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 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. This may have led to our inadvertent selection of mutants where anti sense *SCO1389* transcription was blocked.