Chapter 6 Analysis of PL profile

of *Streptomyces bld* and *whi* mutants

6.1 Introduction to Chapter 6

Mutations in *bld* (bald) genes generate mutants that are blocked at the earliest stages of differentiation and allow growth as a substrate mycelium. However they are unable to erect aerial hyphae. Colonies of *bld* mutants have a shiny and bald appearance and more details on the erection of aerial hyphae are provided in Section 1.3.2.2. *bldA* encodes a tRNA that distinguishes the rare leucine codon UUA. This is not often found in *Streptomyces* DNA (Lawlor *et al.*, 1987) and permits TTA codon-containing genes to require *bldA* tRNA to be expressed so that mRNA can be translated. As a result, *bldA* expression is required for the erection of aerial growth (Nguyen *et al.*, 2002, Takano *et al.*, 2003). The chaplin and rodlin genes (See Section 1.3.2.2) were found to be highly induced during submerged sporulation of *S. venezuelae* in a *bldN*-dependent manner through the application of techniques like chromatin immunoprecipitation-microarray (ChIP-chip). Using microarrays, σ^{BldN} ChIP-chip analysis showed that the chaplin and rodlin genes are direct biochemical targets of σ^{BldN}. The mutant *sven3186* (named *rsbN* for regulator of sigma BldN), the gene lying directly downstream of *bldN*, was also identified as a target of σ^{BldN}. Disruption of *rsbN* demonstrate that RsbN functions as a σ^{BldN}-specific anti-sigma factor (Bibb *et al*., 2012).

White mutants are unable to differentiate from aerial hyphae to spores and more details sporulation are provided in Section 1.3.2.3. Two early regulatory *whi* genes, expressed during sporulation include *whiA* and *whiB* (Ainsa *et al*., 2000, Chater, 2001, Flardh *et al*., 1999). The WhiA protein contains both a low-level constitutive upstream promoter and a second promoter that is strongly transcribed during growth of the aerial

mycelium. Other sporulation factors, including *whiB*, *parAB* and *ftsZ* also contain multiple promoters, some of which are sporulation-specific. WhiA and WhiB influence each other expression by an unknown mechanism and WhiA is required for its own sporulation-specific transcription (Kaiser & Stoddard, 2011). Each of these two genes has a constitutive promoter that is transcribed at a low level, while a second promoter is strongly transcribed at the onset of aerial hyphal formation. As such, it is thought that the WhiG protein could indirectly promote *whiA* transcription as *whiA* expression was not dependent on WhiG (Ainsa *et al*., 2000). The WhiA protein is comprised of two separate structural regions (an N-terminal domain containing two LAGLIDADG motifs and a C-terminal helix-turn-helix DBA binding domain) that possess highly specific DNA recognition and binding activity (Kaiser & Stoddard, 2011). Along with spore pigment synthesis (*whiE*), *sigF* transcription requires *whiA* and *whi*B (Kelemen *et al.*, 1996). Both WhiB and WhiD belong to a family of proteins only found in actinomycetes (Molle & Buttner, 2000). Furthermore, evidence for the control of the expression of downstream genes by WhiB were provided by the biochemical characterization of the WhiB-homologue, WhiD (Jakimowicz *et al.*, 2005). It was proposed that WhiB could sense a temporary oxidative stress during growth of aerial hyphae, leading to changes in the expression of the WhiB-regulated genes and increase termination of aerial hyphal growth (Chater, 2001). In contrast to the phenotype of a *whiB* mutant, a *whiD* mutant formed normal aerial hyphae that reduced sporulation (Molle & Buttner, 2000). This suggests that WhiD activates the processes that take place during the later stages of spore maturation (Fig. 1.4).

Although the advent of ChIP-chip is increasing our knowledge of the regulons governed by various *bld* and *whi* loci, the developmental regulation of PL biosynthesis has not been investigated. Following the discovery that CL is required for morphological development in *S. coelicolor* (Jyothikumar *et al*., 2012), we set out to determine if PL biosynthesis was subject to regulation by the products of selected *bld* and *whi* loci.

Aims of this chapter

In order to 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*, namely strains that carried mutations in *bldA* and *whiD*. As it was difficult to obtain good quality PL extracts from these mutants, we also investigated the PL profile of *S. venezuelae* that is able to undergo synchronous sporulation in liquid culture. In this organism, strains mutated in *bldN* and *whiA* were investigated.

6.2 Expression of PL biosynthetic genes during growth of *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD*

6.2.1 Growth of *S. coelicolor* M145, *bldA* and *whiD* in Liquid cultures

*S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* are unable to erect aerial hyphae or sporulate respectively. As such, it was our aim to identify if any genes responsible for PL biosynthesis (Table 2.4) were subject to regulation by the *bldA* and *whiD* gene products. Despite repeated attempts it was impossible to extract sufficient quantities of PLs for visualization by TLC from plate grown cultures from *S. coelicolor* developmental mutants. As a result we decided to investigate changes in PL biosynthetic gene expression and PL abundance using large volumes of liquid cultures. To determine whether these strains exhibited a similar growth curve to wild type *S. coelicolor* M145 in submerged culture, we inoculated pre-germinated spores (50 µl) into 500 ml of YEME medium with 10 g l^{-1} glucose in sterile baffled 2 L flasks with 0.5% glycine and without sucrose. Flasks were incubated at 30^{o}C and shaken at 220 rpm. It was possible to generate spores of these mutants through the capacity of MS agar to suppress the *bld* phenotype of the *bldA* mutant and aerial hyphal fragments were used to inoculate cultures for the *whiD* mutant. Standard growth curve experiments were performed using *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD*. 5 ml samples were collected from each flask every 3h until the stationary phase was reached. The cells were filtered through pre weighed Whatman filter paper, and the filtrate was washed with sterile distilled water three times. The filter paper was dried at 60°C for 14h and subsequently weighed. The data were processed and using the average dry cell biomass,

the growth curve was plotted against time (hrs) (Fig. 6.1). For *S. coelicolor* M145 the mid log phase was identified as occurring after 9h and the stationary phase after 30 h. For *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* the mid log phase was identified as occurring after 15 h and the stationary phase after 36 h. This was done to account for the slower growth of the mutant and ensure that samples were at taken equivalent stages in their growth curve. 400 ml of cultures were also collected at the time indicated and centrifuged at 4000 rpm for 10 min at room temperature for RNA extraction.

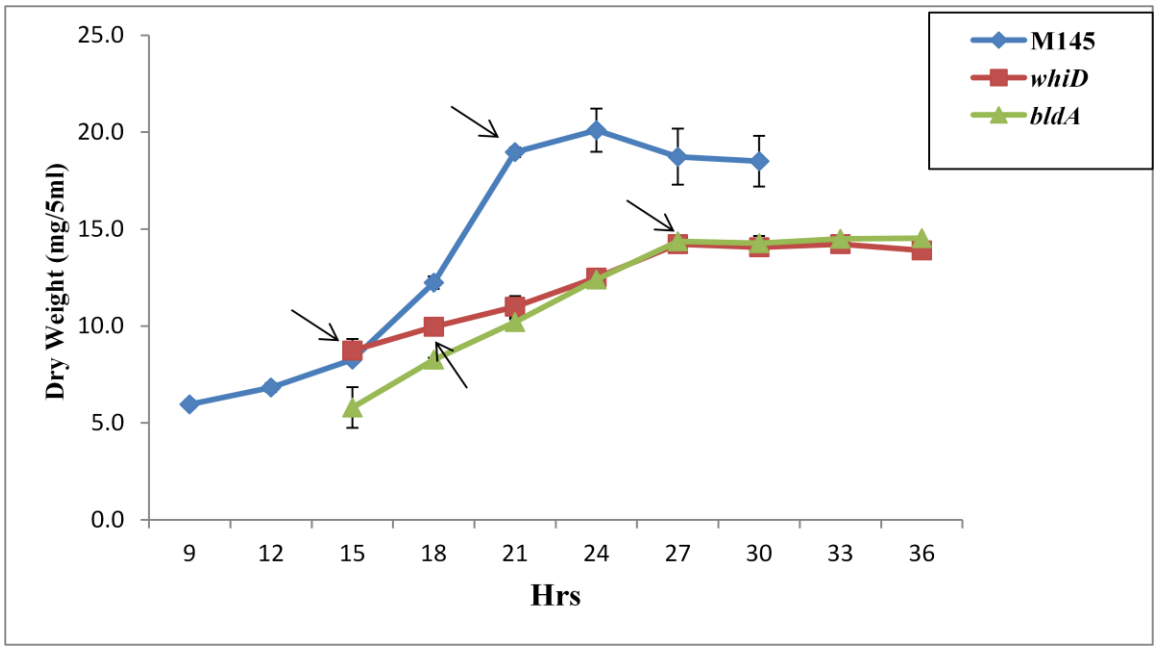

Fig. 6.1 Dry weight (mg 5 ml^{-1}) of *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* in YEME medium without sucrose and measured every 3 h. The numbers of h are shown along the bottom. The growth curve was measured by cell dry weight in triplicates. Arrows represent the time points at which cultures were sacrificed for mRNA isolation.

*S. coelicolor*Δ*bldA* and *whiD* mutants grew more slowly than wild type *S. coelicolor* M145. *S. coelicolor* M145 grew rapidly during exponential phase (from 15-21 h) and reached the highest cell density around the transition phase (21-24 h), before decreasing slightly in stationary phase (after 24 h). *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* grew slower during exponential phase (from 18-27 h) and reached a maximal level of biomass around entry to the transition phase (27 h). This was surprising as the developmental phenotype of these mutants should only manifest itself during aerial growth. Nevertheless, these results suggest that vegetative growth of these mutants is also impaired to some degree. From this growth curve in Fig. 6.1, we choose two time points at mid-log phase and mid-stationary phase from *S. coelicolor* M145 at 18 and 24 h and 24 and 30 h cultures of *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* to isolate RNA for analysis of PL biosynthetic gene expression by RT-PCR.

6.2.2 Expression of PL biosynthetic genes during growth of *S. coelicolor*, *S. coelicolorΔbldA* and *S. coelicolorΔwhiD* by semi quantitative RT-PCR

The six genes predicted to encode PL biosynthesis in *S. coelicolor*, *SCO1389, SCO1527, SCO5628, SCO5753, SCO6467* and *SCO6468* (as described in Chapter 3) demonstrated changes in expression levels during development of *S. coelicolor*, therefore we extracted mRNA and for analysis by RT-PCR to identify if these genes displayed altered patterns of gene expression in the mutant strains when compared to wild type *S. coelicolor*. This was done in liquid culture as it proved difficult to obtain sufficient quantities of PLs for visualization by TLC from agar grown cultures of the developmental mutants in parallel experiments (data not shown).

The RT-PCR was carried out using 100 ng RNA isolated from 18 (mid-log phase) and 24 h (stationary phase) cultures of *S. coelicolor* M145 and 24 (mid-log phase) and 30 h (stationary phase) cultures of *S. coelicolorΔbldA* and *S. coelicolorΔwhiD* (Fig. 6.2) amplified with *hrdB, SCO1389, SCO6467, SCO6468, SCO5628, SCO1527* and *SCO5753* primers. The RT-PCR was carried out according to section 2.10.2 and RNA isolation was carried out with the 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 260/280 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).

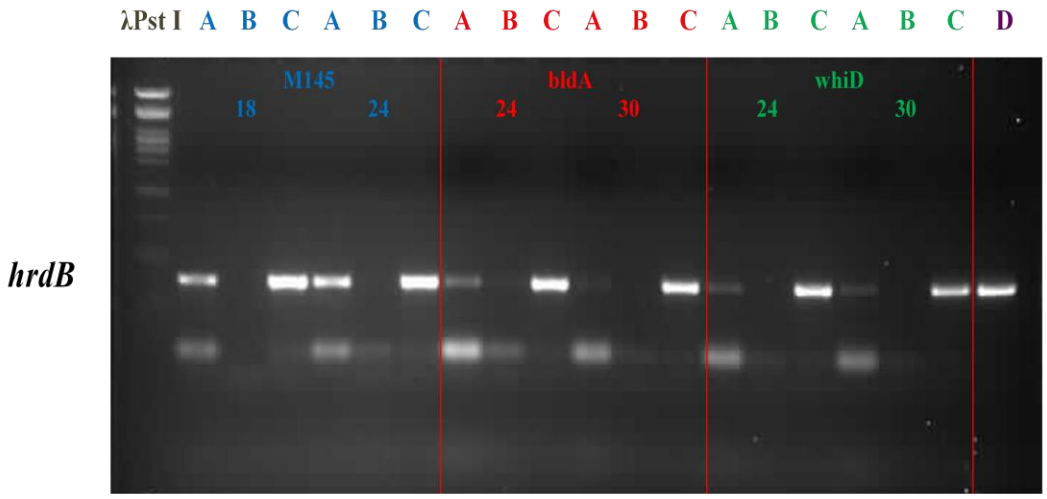

Fig. 6.2 RT-PCR products generated from RNA purified from *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* cultures at 18, 24 and 30 h using *hrdB* primers. Lane A: RT-PCR (+Dnase); B: PCR with HotStarTaq DNA Polymerase (+Dnase); C: PCR with HotStarTaq DNA Polymerase (-Dnase); D: PCR with HotStarTaq DNA Polymerase using M145 gDNA as template instead of RNA (-DNase).

The amplification of the control gene, *hrdB* mRNA from all strains show that DNase treatment was successful (Fig. 6.2) and that the RT-PCR products (Fig. 6.2) were derived from mRNA (lane A). There was no amplification of *hrdB* when PCRs were performed without RT indicated the absence of contaminating DNA in RNA sample (see Fig. 6.2 lane B). A PCR with HotStarTaq DNA Polymerase (-Dnase) was used as a further control (see Fig. 6.2 lane C). PCR was performed with up to 100-fold dilutions of gDNA template to ensure that the RT-PCR matched the size of this product (see Fig. 6.2 lane D). Despite the same amount of RNA (100ng) 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, although this effect was more pronounced in the mutant strains than the wild type.

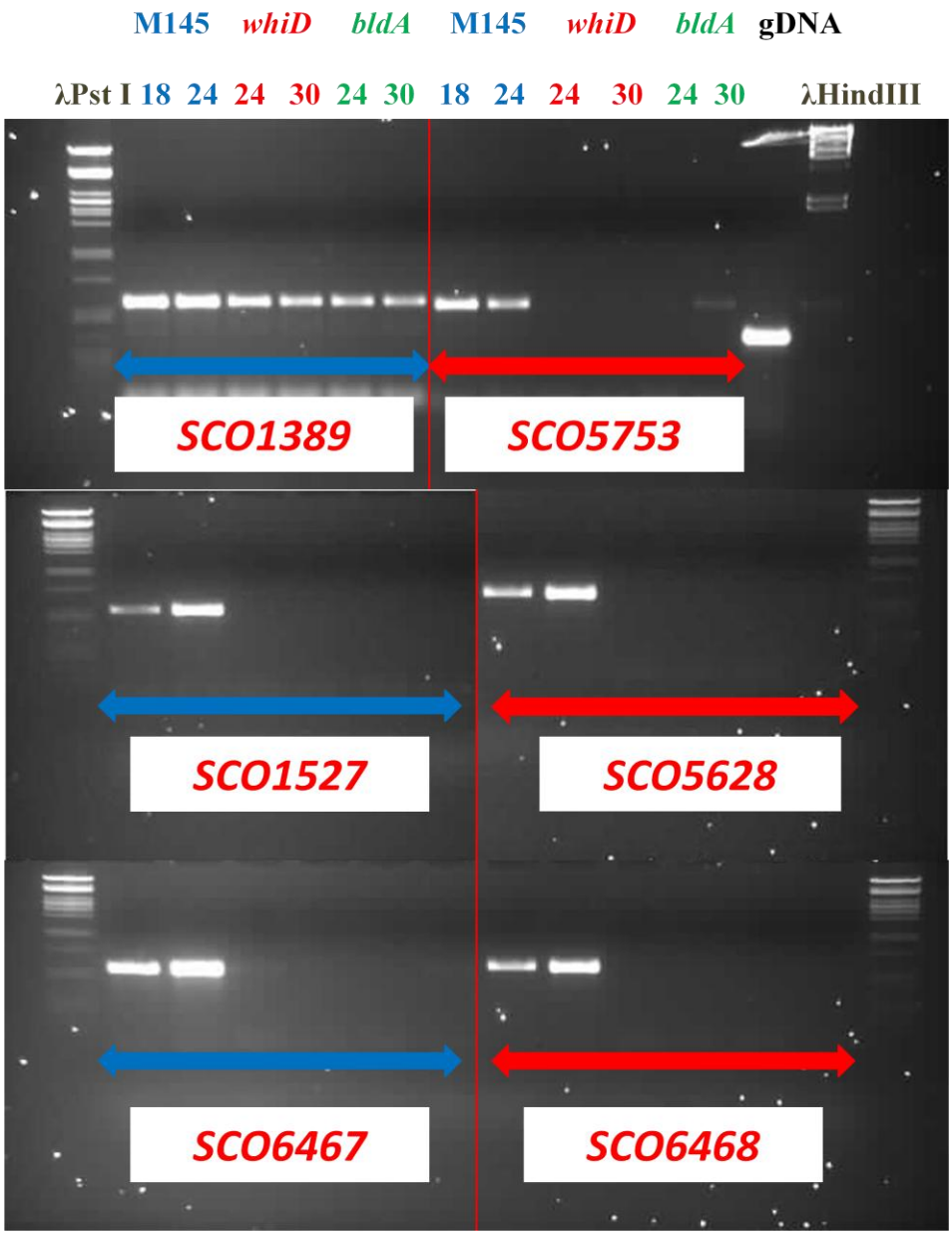

Fig. 6.3 Verification of RT-PCR products generated from PL biosynthetic genes from *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* cultures. A 1.4% agarose gel shows RT-PCR amplification products after 35 cycles, RNA 100 ng was isolated from cultures grown in YEME medium. Cells were collected at 18, 24 and 30 h amplified with *SCO1389*, *SCO5753*, *SCO1527*, *SCO5628*, *SCO6467* and *SCO6468* primers. The gene *hrdB* was used as a positive control to normalize the RT-PCR (Fig. 6.2).

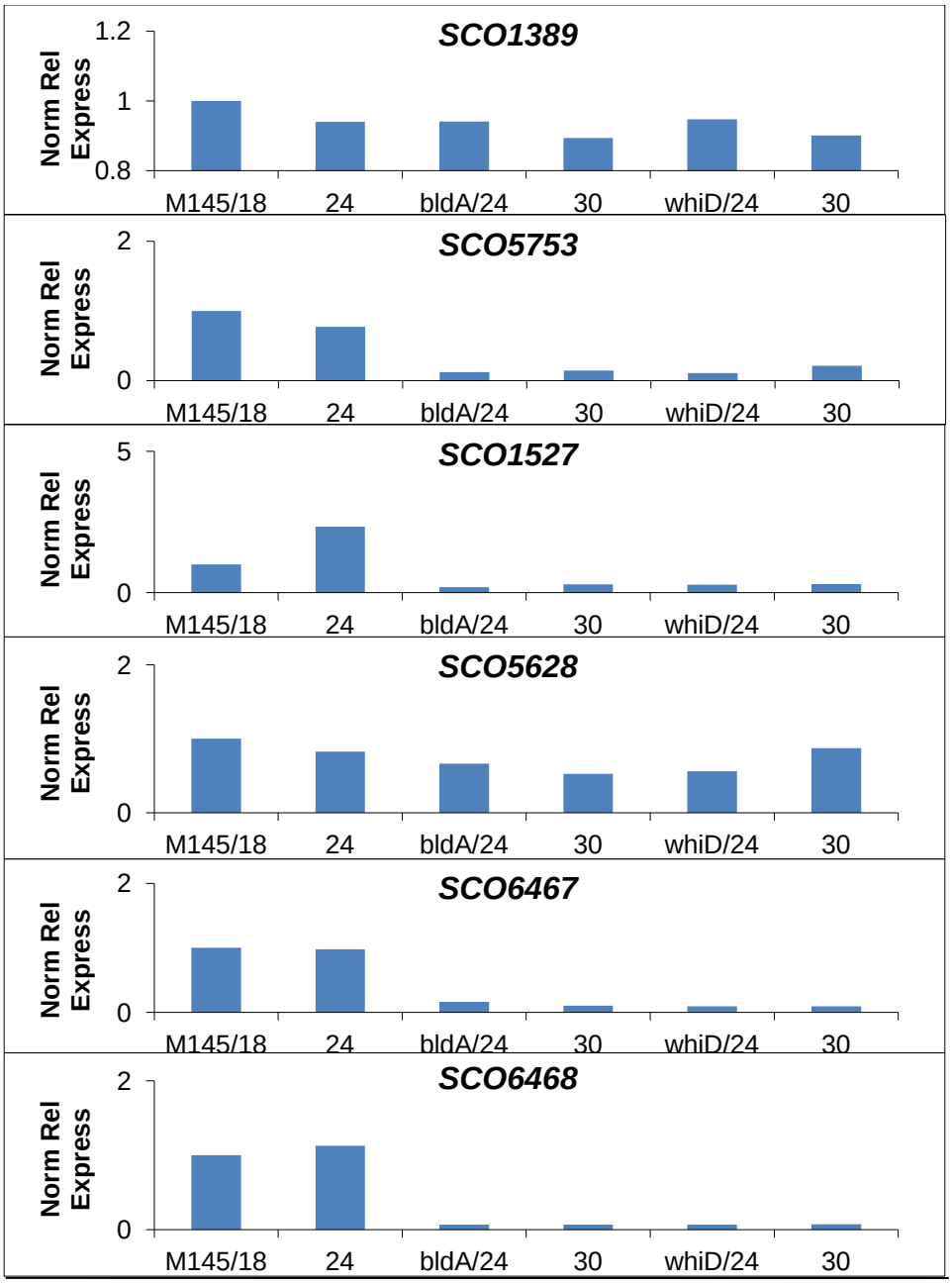

Fig. 6.4 Relative expression by semi quantitative RT-PCR from RNA samples generated from *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* cultures from single sample. RNA 100 ng was isolated from cultures grown in YEME medium. Cells were collected at 18, 24 and 30 h amplified and with *SCO1389, SCO5753, SCO1527, SCO5628, SCO6467* and *SCO6468* primers. The gene *hrdB* was used as a positive control to normalize the RT-PCR.

The expression of the *hrdB* gene was always detected during growth of *S. coelicolor* since *hrdB* is expressed constitutively (Du *et al*., 2013, Kieser *et al*., 2000) and we can confirm that there was no DNA contamination in our RNA samples (Fig. 6.2). *SCO1389* was expressed in all strains indicating that CL synthase was produced during growth. 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. It seems more likely that 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 leading to enhanced RNase activity against certain genes (Gravenbeek & Jones, 2008)

6.2.3 PL profiles from *S. coelicolor* M145, *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD*

In parallel with RT-PCR analysis (See section 6.2.2), mycelial pellets were collected at 16 and 24 and 48 h cultures of *S. coelicolor* M145 and 24 and 48 h cultures of *S. coelicolor*Δ*bldA* and *S. coelicolor*Δ*whiD* and PLs extracted according to (Bligh & Dyer, 1959, Iverson *et al.*, 2001) and developed by TLC (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 vegetative growth and their effects are only displayed during the erection of aerial hyphae and sporulation. We have assumed that PL spots B and C correspond to PE and PG respectively; these spots are the products of the proteins encoded by *SCO6467*/*SCO6468* and *SCO5753* respectively and consequently it seems likely that our inability to detect expression of these genes is an artefact. 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 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.

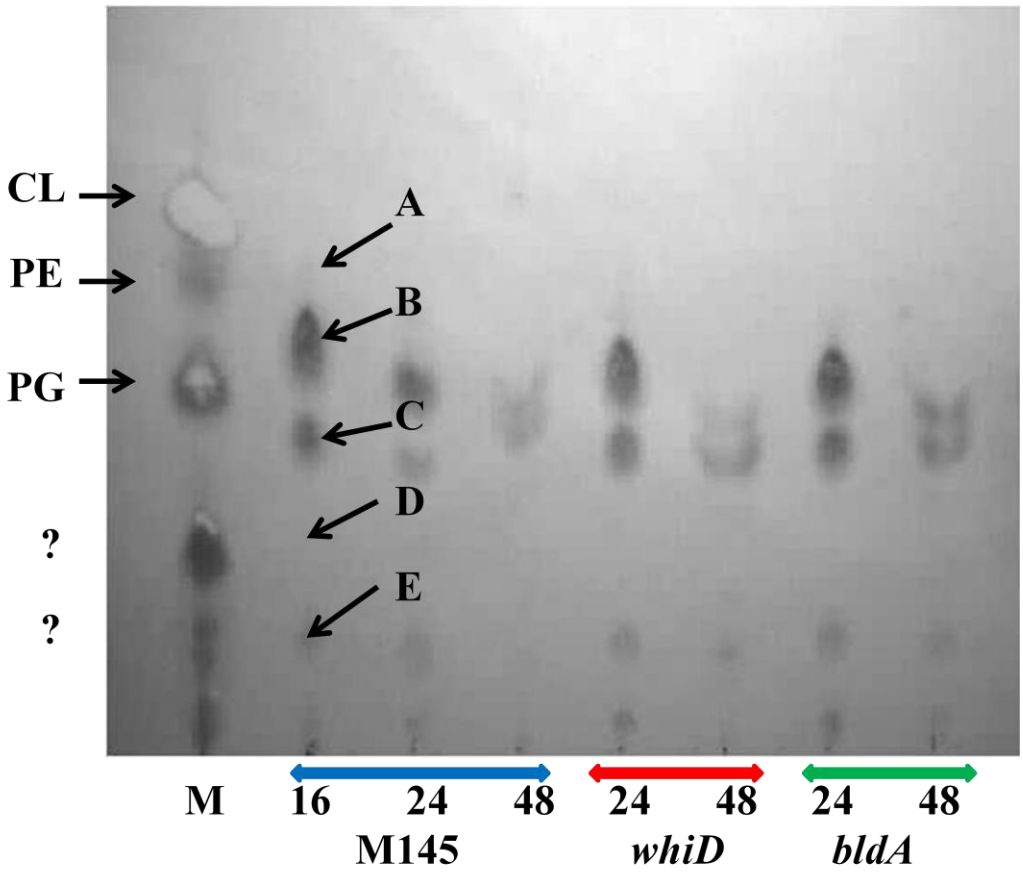

Fig. 6.5 Total PLs extracted from 100 mg wet mass of *S. coelicolor* M145, *S. coelicolor*Δ*whiD* and *S. coelicolor*Δ*bldA* at 16, 24 and 48 h. PLs were analyzed on a silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). The plate was captured using a Gene Snap densitometer, and the bands were identified by their co-migration with standards.

6.3 Sporulation of *S. venezuelae* in submerged cultures

6.3.1 Determination of *S. venezuelae* sporulation in liquid culture

In contrast to *S. coelicolor*, *S. venezuelae* sporulates in liquid culture (Glazebrook *et al*., 1990) and > 90% total biomass is converted into spores, whilst *S. coelicolor* sporulates only on solid medium and the differentiating part of the colony (the aerial mycelium) constitutes only ~ 5% of the total biomass. Thus, we choose *S. venezuelae* 10712 to study PL biosynthesis because of the ability of this organism to sporulate in liquid culture. We also grew *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* with the aim of identifying any PLs in these developmental mutants that showed altered spot intensity when visualised by TLC. In this way, we hoped to take advantage of the ability of liquid sporulation to isolate sufficient PLs for visualisation by TLC in the *S. venezuelae* developmental mutants.

We first wished to verify the ability of *S. venezuelae* to sporulate in liquid culture and that the two developmental mutants were unable to produce viable spores. This was done using the sporulation assay described by Glazebrook *et al.* (1990). *S. venezuelae* 10712 spores were obtained from a culture grown on MYM agar for 5 days, the spores were collected by centrifugation and resuspended in sterile water to give approximately 10^{10} c.f.u ml^{-1}. Mycelial fragments of *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* were taken directly from cultures grown for 5 days on MYM medium (approximately 10^8 c.f.u. ml^{-1}). Submerged sporulation in the three strains was analysed by first inoculating *S. venezuelae* 10712 spores and *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* mycelial fragments (approximately 10^8 c.f.u. ml^{-1}) into liquid MYM medium. After 24 h growth

(30°C, 220 rpm), mycelium was collected by filtration and centrifugation, before inoculation to LS (Liquid Sporulation) medium with galactose and ammonium sulphate as the carbon and nitrogen sources respectively (Fig. 6.6). After five days growth the percentage survival after treatment with lysozyme (Glazebrook *et al*., 1990) was used to determine percentage sporulation for each strain (Table 6.1).

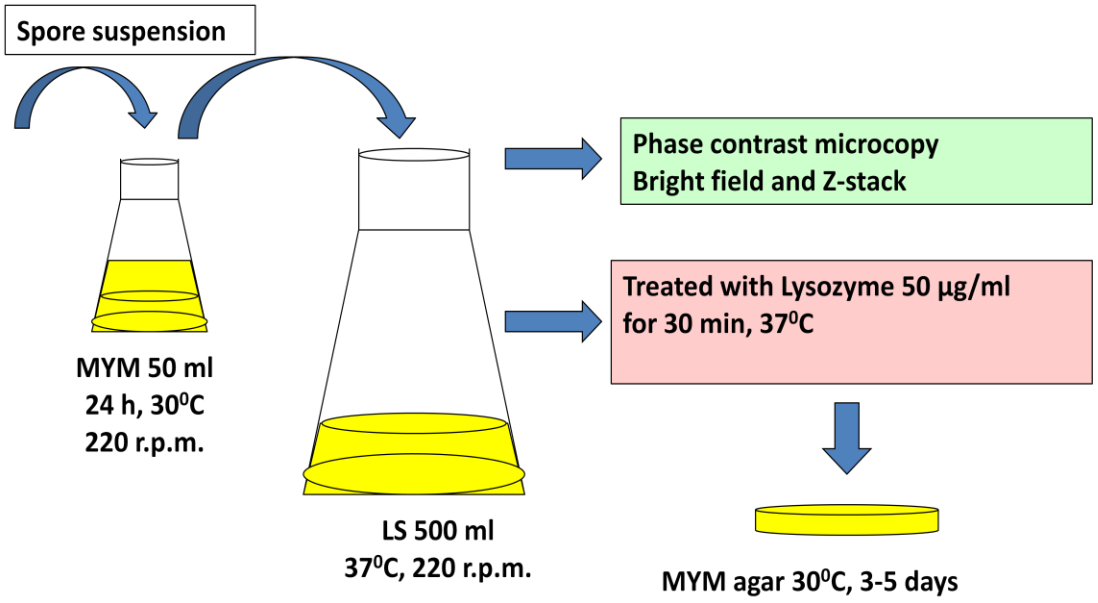

Fig. 6.6 Measurement of *S. venezuelae* submerged sporulation by lysozyme resistance. Vegetative mycelial suspensions used as inocula were prepared by transferring 50 µl portions from a stock suspension of spores with an OD_{600} of 0.4 in 20% (v/v) aqueous glycerol to 50 ml of MYM medium (Stuttard, 1982) and incubating the culture for 24 h at 27°C on a rotary shaker (220 rpm). Shaken cultures in which sporulation was investigated were grown from a 1% (v/v) vegetative inoculum in 2 l Erlenmeyer flasks, each containing 500 ml of LS medium. Then treated sample with lysozyme and visualized by phase contrast microscopy.

Table 6.1 Survival (%) **of *S. venezuelae* strains after treatment with lysozyme**

	Survival (%)	
	(+) Lysozyme	(-) Lysozyme
S. venezuelae 10712	30	100
*S. venezuelae*Δ*bldN*	0	100
*S. venezuelae*Δ*whiA*	0	100

Shaken LS medium cultures of *S. venezuelae* 10712, *S. venezuelae⊿bldN* and *S. venezuelae⊿whiA* were treated with lysozyme (50 μg ml^{-1}) for 30 min at 37^{o}C and plated on MYM agar for 3-5 days. The percentage survival after treatment was estimated from the numbers of colonies obtained by plating treated and untreated samples (Table 6.1). No colonies were detectable after treatment of *S. venezuelae⊿bldN* and *S. venezuelae⊿whiA* with lysozyme. This was because these strains are unable to sporulate and so are susceptible to lysozyme treatment. In contrast, *S. venezuelae* 10712, after treatment with lysozyme, showed that around 30% of c.f.u could survive lysozyme treatment indicating that around 30% of colonies in the LS medium were derived from spores. The lysozyme treatment gave values for spore content that correlated well with microscopic observations.

6.3.2 Analysis of submerged *S. venezuelae* sporulation by phase contrast and Z-stack microscopy.

In order to confirm the results of lysozyme treatment and demonstrate that submerged sporulation took place when *S. venezuelae* 10712 was grown in LS medium and *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* were unable to sporulate in the same medium, we also analysed submerged sporulation by phase contrast microscopy. Cultures were grown in LS medium for 6 days and 8 µl samples collected and visualized by phase contrast microscopy. Sporulation was studied using a Nikon TE2000S inverted microscope at 1000x magnification. Mycelial fragments were often deeper than the focal depth of the microscopes objective lens. As a result, 0.5-µm Z sections of phase-contrast images were captured and used to flatten three-dimensional images. It was found that after 4 days only *S. venezuelae* 10712 could sporulate in liquid culture, whilst no detectable spores were observed in *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* (Fig. 6.7).

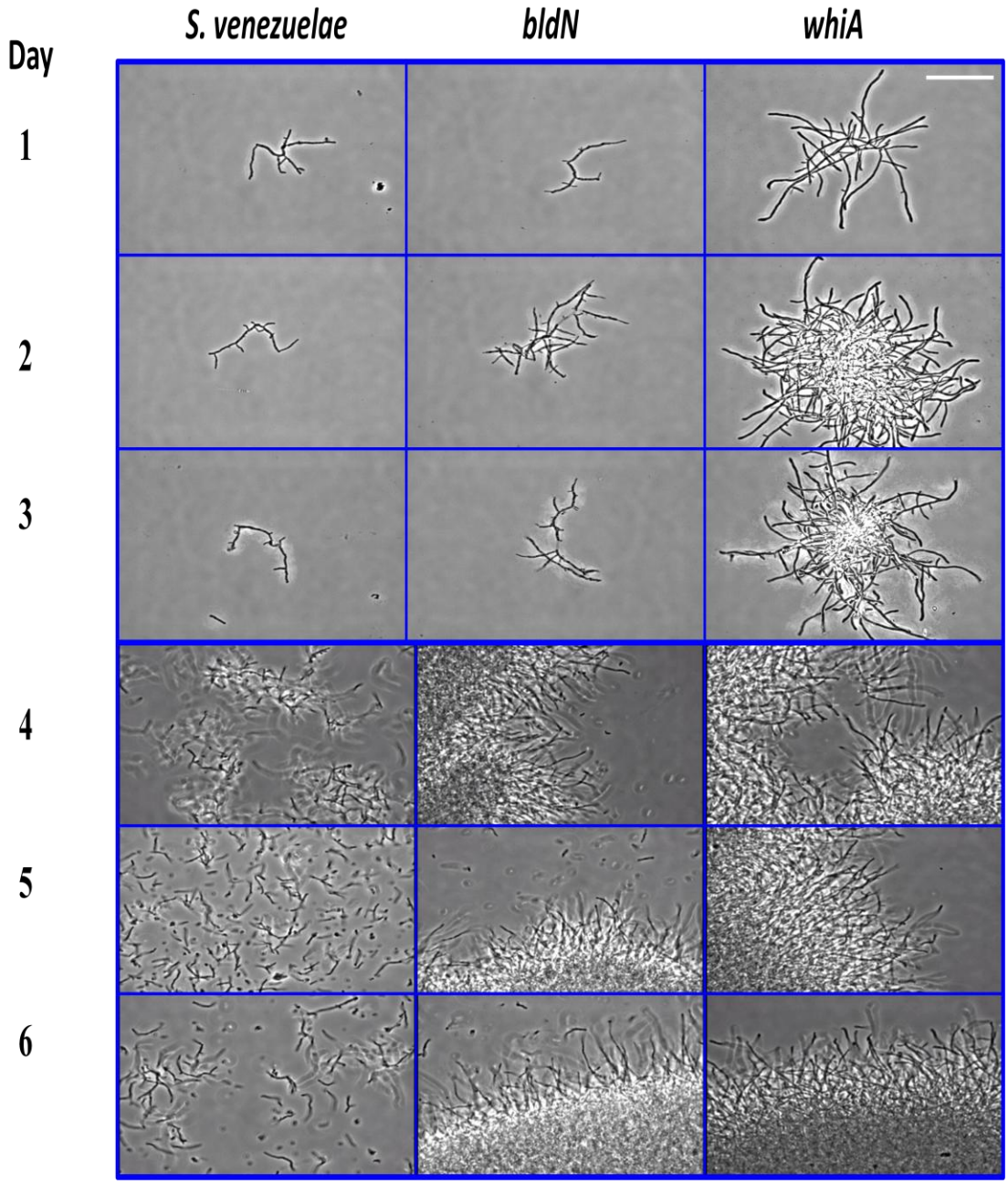

Fig. 6.7 Phase contrast and Z-stack microscopy of *S. venezuelae* 10712, *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* grown in LS medium with galactose (5%) and ammonium sulphate (2 g l^{-1}). Samples were collected on days 1, 2, 3, 4, 5 and 6. Horizontal white bar represent 5 μm.

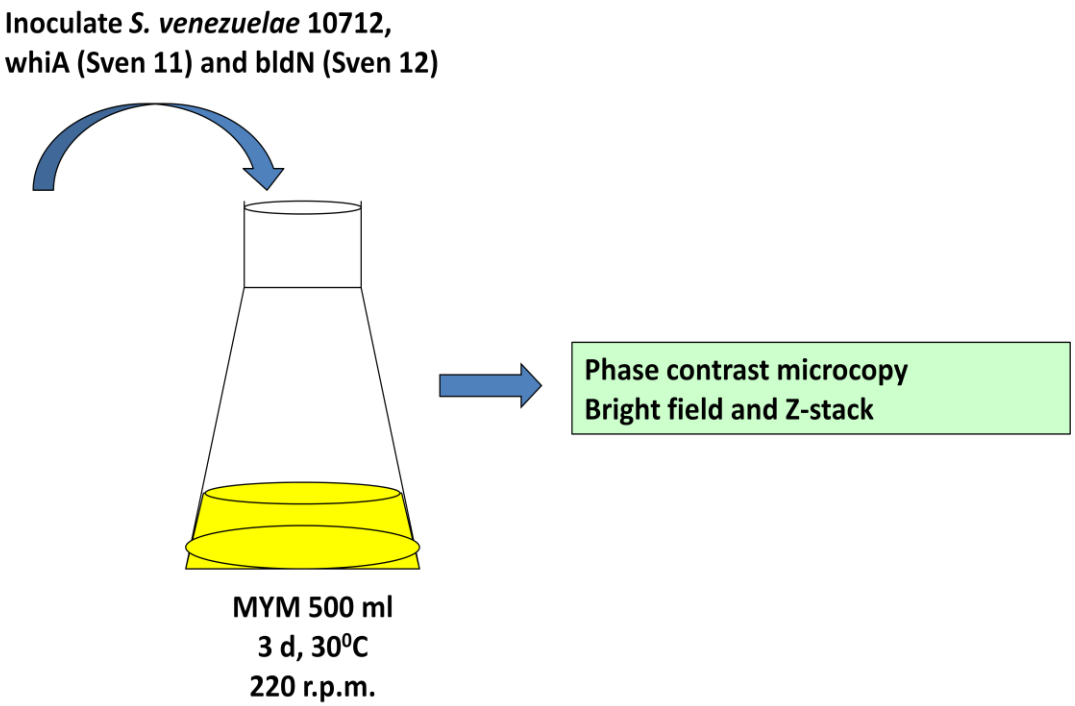

Fig. 6.8 Preparation of *S. venezuelae* inoculums for submerged sporulation. Vegetative mycelial suspensions used as inocula were prepared by transferring 50 µl portions from a stock suspension of spores with an OD of 0.4 in 20% (v/v) aqueous glycerol to 50 ml of MYM medium (Stuttard, 1982) and incubating the culture for 24 h at 30^{o}C on a rotary shaker (220 rpm). Shaken cultures in which sporulation was investigated were grown from a 1 % (v/v) vegetative inoculum in 2 l Erlenmeyer flasks, each containing 500 ml of MYM medium and were then visualized by phase contrast microscopy.

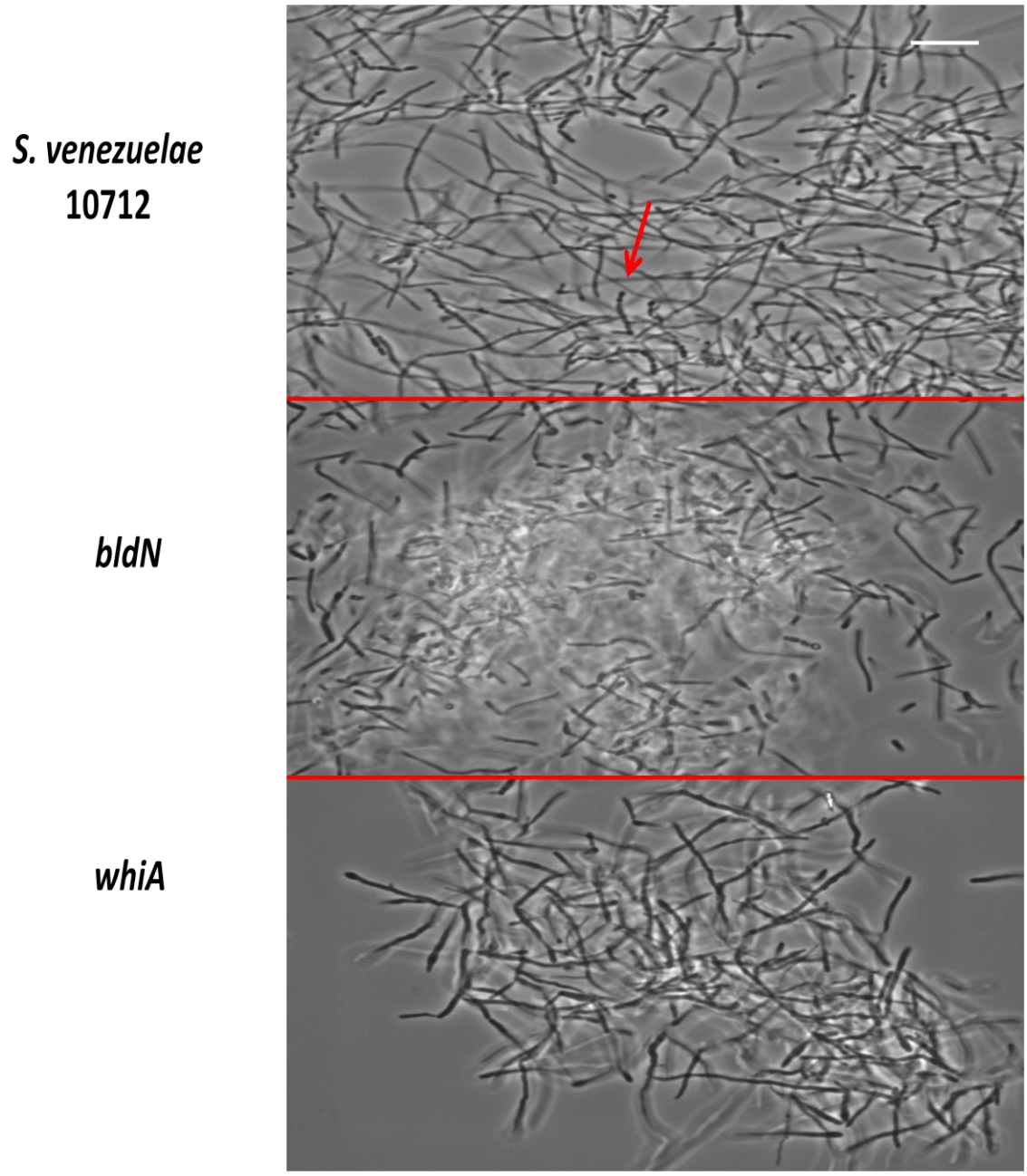

Fig. 6.9 Phase contrast microscopy of S. *venezuelae* 10712, *S. venezuelaeΔbldN* and *S. venezuelaeΔwhiA* grown in MYM medium with galactose (5%) and ammonium sulphate (2 g l^{-1}). Samples were collected on day 3 (Red arrow indicate spore chain). Horizontal white bar represent 5 μm.

Cell material from cultures of *S. venezuelae* in LS medium were examined at daily intervals during a 6 days growth period (Fig. 6.7) however growth in this medium resulted in pellet formation, especially for *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD*. As a result we decided to generate submerged spores in MYM medium (Fig. 6.8) as this broth not only gave a higher biomass, but also showed a progressive increase in the number of spore chains present (Fig. 6.9), at least for *S. venezuelae* 10712. After three days growth in MYM medium, this strain contained large numbers of spores. This contrasted with the absence of free spores at comparable times in *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* that are unable to sporulate (Fig. 6.9).

6.3.3 Analysis of PLs during submerged sporulation of *S. venezuelae*

In order to identify if there were any differences in the PL profile of *S. venezuelae* 10712 and *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* during submerged sporulation, these strains were grown in 500 ml of MYM medium, incubated at 30^{o}C on a rotary shaker (220 rpm) for 3 days. At this stage, the maximum numbers of spores was reached (Fig. 6.9). The entire cultures were collected at the time indicated and centrifuged at 4000 rpm for 10 min at room temperature before extracting PLs (see Section 2.11). TLC was carried out on Kieselgel (60H) TLC plates and the amount loaded onto the TLC plate was equalised so that PLs isolated from 500 mg of wet mass to carry out a preliminary identification of PLs (see Section 2.11) (Fig. 6.10).

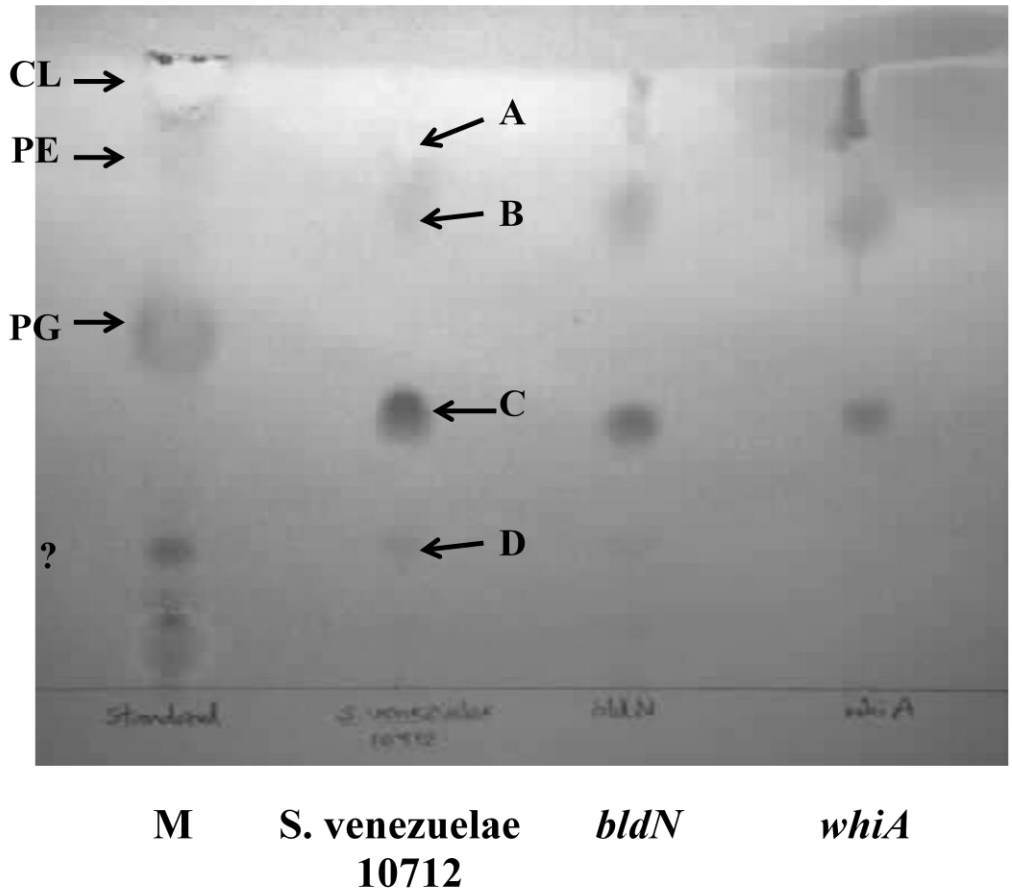

Fig. 6.10 Total PLs extracted from 200 mg wet mass of *S. venezuelae* 10712, *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* grown in MYM medium with galactose (5%) and ammonium sulphate (2 g l^{-1}). Samples were collected on day 3, analyzed on a silica gel TLC plate and developed in chloroform/methanol/acetic acid/water (80:12:15:4 vol/vol/vol/vol). The PL spots were identified by their co-migration with standards.

When the PL fractions were analyzed by TLC, four components (spots A- D) could be separated from the three *S. venezuelae* strains. On the basis of their similarity with the standard PLs they were identified as CL, PE, PG and an unidentified PL respectively (Fig. 6.10). 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). In contrast, an unidentified PL spot (D) displayed a reduced abundance in the two developmental mutants.

6.4 Conclusions to Chapter 6

In order to 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*. 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, although 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. 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 (CDP-DAG and PG respectively) are precursors for the synthesis of CL. It seems more likely 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 leading to enhanced RNase activity against certain genes (Gravenbeek & Jones, 2008).

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***.** 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* respectively 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. 1.5, 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 mutants, we also investigated the PL profile of *S. venezuelae* that is able to undergo synchronous sporulation in liquid culture. 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. No colony was 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. 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.

When the PL fractions were analyzed by TLC, four PL spots could be separated from the three *S. venezuelae* strains. 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)