Chapter 2

Materials and methods

2.1. Bacterial strains and plasmids used in this study

Table 2.1 Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
SC1A8A	Cosmid containing *SCO1389*; ap^r, km^r	(Redenbach *et al.*, 1996)
SC1A8AΔ*SCO1389*	SC1A8A; *SCO1389* replaced with am^r; ap^r, km^r, am^r	(Jyothikumar *et al.*, 2012)
E. coli JM109	General cloning host	(Kieser *et al.*, 2000)
S. coelicolor M145	Wild type, $SCP1^-$ $SCP2^-$ Pgl^+	(Kieser *et al*., 2000)
S. coelicolor RJ116	M145::pAV117B2; hyg^r	(Jyothikumar *et al*., 2012)
S. coelicolor RJ117	M145::pAV11b; hyg^r	(Jyothikumar *et al*., 2012)
S. coelicolor RJ118b	M145Δ*SCO1389*::pAV117B1; am^r, hyg^r (dxo)	(Jyothikumar *et al*., 2012, Stuttard, 1982)
S. venezuelae 10712	Wild type	
*S. coelicolor*Δ*bldA*	*bldA39 hisA1 uraA1 strA1* Pgl^-	M. J. Buttner
*S. coelicolor*Δ*whiD*	Δ*glkA119 whiD*::*hyg* Pgl^+ $SCP1^-$ $SCP2^-$	P.A. Hoskisson P.A. Hoskisson
*S. venezuelae*Δ*bldN*	Δ*bldN::apr*	
*S. venezuelae*Δ*whiA*	Δ*whiA::hyg*	M. J. Buttner M. J. Buttner

ap^r, km^r, am^r and hyg^r represent ampicillin, kanamycin, apramycin, thiostrepton and hygromycin resistant.
dxo: double cross over.

2.2 Media used for cultivation of bacterial strains

All strains of *Streptomyces* were grown on Mannitol salt agar (MS) or minimal medium plus mannitol at 30^{o}C for 3-4 days (Kieser *et al*., 2000). *E. coli* JM109 was grown at 37^{o}C on Luria–Bertani (LB) agar or broth (250 rpm) for 18 h. Media for growth of bacteria are listed below and the antibiotics used to supplement the media are shown in Table 2.3, unless stated otherwise all components were sterilized by autoclaving for 15 min, 121^{o}C, 15 psi.

Double strength Pre-germination medium (Hopwood *et al.*, 1985)

Yeast extract	0.1 g	(Sigma-Aldrich, St. Louise, USA)
Casamino acids	0.1 g	(Fisher Scientific, Leicester, UK)
dH_2O	1 L	
$CaCl_2$* (5 M)	2 ml	(BDH Laboratory Supplies, Poole, UK)

*Added after autoclaving

Luria –Bertani (LB)

Tryptone	10 g	(Oxoid LTD., Basingstoke, UK)
Yeast extract	5 g	(Oxoid LTD., Basingstoke, UK)
NaCl	5 g	(Sigma-Aldrich, St. Louise, USA)
Glucose*	1 g	(Fisher Scientific, Leicester, UK)
dH_2O	1 L	
adjust to pH 7.0 with NaOH		
Agar	10 g	(Oxoid LTD., Basingstoke, UK)

*Filter sterilized through 0.22 μm diameter filter paper

LS medium (Glazebrook *et al.*, 1990)

MOPS	21 g	(Fisher Scientific, Leicester, UK)
K_2HPO_4	10.5 g	(Sigma-Aldrich, St. Louise, USA)
KH_2PO_4	4.5 g	(Sigma-Aldrich, St. Louise, USA)
Galactose	50 g	(Fisher Scientific, Leicester, UK)
$NH_4(SO_4)_2$	20 g	(BDH Laboratory Supplies, Poole, UK)
dH_2O	1 L	

Mannitol salt agar (MS agar)

Soya bean flour	20 g	(Neal's Yard Wholefoods, Burton, UK)
Mannitol	20 g	(Fisher Scientific, Leicester, UK)
Agar	20 g	(Oxoid LTD., Basingstoke, UK)
Tap water up to 1 L		

Autoclaved for 30 min, 121^{o}C, 15 psi

Minimal medium plus mannitol (3MA)

L-asparagine	0.5 g	(BDH Laboratory Supplies, Poole, UK)
K_2HPO_4	0.5 g	(Sigma-Aldrich, St. Louise, USA)
$MgSO_4.7H_2O$	0.2 g	(Fisher Scientific, Leicester, UK)
$FeSo_4.7H_2O$	0.01 g	(BDH Laboratory Supplies, Poole, UK)
dH_2O	up to 1 L	
adjust to pH 7.2 with NaOH		
Agar	10 g	(Oxoid LTD., Basingstoke, UK)
D-Mannitol*	10 g	(Fisher Scientific, Leicester, UK)

*Added after autoclaving

Mannitol-Yeast extract-Malt extract (MYM) (Stuttard, 1982)

Maltose	4 g	(Fisher Scientific, New Jersy, USA)
Yeast extract	4 g	(Oxoid LTD., Basingstoke, UK)
Malt extract	10 g	(Oxoid LTD., Basingstoke, UK)
Agar	20 g	(Oxoid LTD., Basingstoke, UK)
dH_2O	1 L	

Yeast extract-malt extract medium (YEME)

Yeast extract	3 g	(Oxoid LTD., Basingstoke, UK)
Bacto-peptone	5 g	(Fisher Scientific, Leicester, UK)
Malt extract	3 g	(Oxoid LTD., Basingstoke, UK)
Glucose	10 g	(Fisher Scientific, Leicester, UK)
Sucrose	340 g	(Sigma-Aldrich, St. Louise, USA)
dH_2O up to	1 L	
After autoclaving add :		
$MgCL_2.6H_2O$ (2.5 M)	2 ml L^{-1} (5 mM final)	(Fisher, Leicester, UK)

For preparing protoplasts, also add :

Glycine (20%)	25 ml L^{-1} (0.5% final)	(Sigma, St. Louise, USA)

2xYT medium

Trytone	16 g	(Sigma-Aldrich, St. Louise, USA)
Yeast extract	10 g	(Oxoid LTD., Basingstoke, UK)
NaCl	5 g	(Sigma-Aldrich, St. Louise, USA)
dH_2O	1 L	

2.3 Preparation of chemicals and antibiotics

Table 2.2 Chemicals and reagents used

Reagent	Per litre
Alkaline lysis solution I	2M Glucose; Tris Cl (pH8); EDTA (pH8).
Alkaline lysis solution II	4M NaOH; 20% (w/v) SDS.
Alkaline lysis solution III	8M Potassium Acetate; Glacial acetic acid; Sterile distilled water
10xTE-buffer	100mM Tris (pH8); 10mM EDTA.
50xTAE-buffer	2M Tris; Glacial Acetic acid; 0.05M EDTA.
Agarose	0.8% (w/v) in 1x TAE buffer
DNA marker dye	50mM EDTA; 0.5% (w/v) SDS; 0.25% (w/v) Bromophenol Blue; 15% (v/v) Ficoll.
RNase A Solution	10mgs RNase A; 1 M Tris-HCl (pH7.8); 1M $MgCl_2$.
Lysozyme solutions	25mM Tris- pH8; lysozyme (2mg ml^{-1}); RNaseA (10mg ml^{-1}).
SDS solution	0.3 M NaCl; 20% SDS (w/v).
Phenol reagent	Phenol-chloroform in equal volume mixture.

Concentration of antibiotics

Table 2.3 Supplemented antibiotics

Antibiotics	stock concentration (mg ml^{-1})	working concentration (µg ml^{-1})
Ampicillin (ap)	50	50
Anhydrotetracycline (ATC)*,**	1.5	1.5
Apramycin (am)	100	100
Kanamycin (kan)	25	25
Hygromycin (hyg)**	25	25

*Dissolve in absolute ethanol
**Light sensitive
All antibiotics stored at -20°C unless otherwise stated.
All antibiotics from Sigma-Aldrich, St. Louise, USA supplier.

2.4 Preparation of *Streptomyces* spore suspensions (Queener & Capone, 1974, Shepherd *et al*., 2010, Kieser *et al*., 2000)

10 ml of sterile water was added on an MS agar plate and the spores suspended with an inoculating loop. Following this, the crude suspension was poured back into the container that held the sterile water and vortexed for a minute to break up the spore chains. Next the suspension was filtered through non-absorbent cotton-wool into a centrifuge tube before being centrifuged for 5-10 min at 4000 rpm to pellet the spores. The supernatant fraction was then removed and the pellet vortexed for a few seconds to disperse the spores in the drop of water remaining in the tube before being resuspended in 1 ml sterile 20% (w/v) glycerol. The spore suspension was then stored at -20^{o}C until use.

2.5 DNA isolation

2.5.1 Small scale isolation of plasmid DNA from *E. coli* (Birnboim & Doly, 1979, Ish-Horowicz & Burke, 1981)

Bacteria were cultivated in 5 ml of broth and incubated overnight at 37^{o}C, 250 rpm (with appropriate antibiotics). 1.5 ml of the overnight culture were poured into a sterile microcentrifuge tube and centrifuged at 14000 rpm for 30 s at 4^{o}C. The supernatant fraction was discarded and the tube thoroughly drained. Following this, the cell pellet was resuspended in 100 µl of ice-cold sterile solution I (50 mM Glucose, 25 mM Tris HCl pH8.0 and 10 mM EDTA.2H_2O pH8.0) by vigorous vortexing. 200 µl of freshly prepared solution II (0.2 N NaOH and 1 % (w/v) SDS) were then added to the tube and the contents mixed by inverting the tube rapidly five times. Next, 150 µl of ice-

cold sterile solution III (5M Potassium acetate 60 ml, Glacial acetic acid 11.5 ml and H_2O 28.5 ml) was added, the contents also mixed by inversion and the tube was stored on ice for 3-5 min. Following this, the tube was centrifuged at 14000 rpm for 5 min at 4^oC, the supernatant transferred to a fresh tube and an equal volume of phenol:chloroform (200 µl:200 µl) was added. This was mixed by vortexing and centrifuged at 14000 rpm for 2 min at 4^oC. The top layer was transferred to a fresh tube, double-stranded DNA precipitated with two volumes of ethanol and mixed by vortexing. After allowing the mixture to stand for 2 min at room temperature the tube was centrifuged at 14000 rpm for 10 min at 4^oC. The supernatant fraction was discarded, the excess gently removed with a pipette and the pellet dried using the speed vacuum concentrator for 30 min. Finally 25 µl of TE buffer (pH8.0) was added along with 1 µl of RNase, the mixture vortexed briefly and stored DNA at -20^oC.

2.5.2 Wizard®*Plus* SV Minipreps DNA Purification System (Promega)

Bacteria were cultivated in 5 ml of broth and incubated overnight at 37^oC, 250 rpm (with appropriate antibiotics). The culture was harvested by centrifugation at 12000 rpm for 5 min. Supernatant was discarded and resuspended cell pellet in 250 µl of Cell Resuspension Solution. 250 µl of Cell Lysis Solution was added to each sample; inverted 4 times to mix. Then added 350 µl of Neutralization Solution; inverted 4 times to mix and centrifuged at maximum speed for 10 min at room temperature.

The Spin column was inserted into the collection tube then the supernatant decanted into the provided spin column and centrifuged at maximum speed for 1 min at room temperature. The flow through was discarded from the collection tube and the

column reinserted into the collection tube. 750 µl of Column Wash Solution was added and centrifuged at maximum speed for 1 min at room temperature and the flow through discarded from the collection tube. 250 µl of Column Wash Solution was added again and centrifuged at maximum speed for 2 min at room temperature and the flow through was discarded from the collection tube. The spin column was transferred into a new sterile 1.5 ml microcentrifuge tube and the DNA eluted by adding 30 µl of Nuclease - Free Water to the spin column and centrifuged at maximum speed for 10 min at room temperature. Finally, the column was discarded and the extracted DNA stored at -20^{o}C.

2.6 DNA restriction digestion

Restriction digests were carried out by adding components from Promega, Invitrogen and New England Biolab (NEB). In the sterile microcentrifuge tube, 5 µl of DNA were added to each tube followed by the addition of 2 µl 10x buffer and the appropriate restriction enzyme 1 µl in each tube. Finally sterile distilled water up to 20 µl was added.

The components of the restriction digest were incubated at 37^{o}C for 2 h and 4 µl of 5x loading dye added. Digested DNA was analysed by loading on a 1% agarose gel, using 1xTAE buffer at 70 Volts for 2 h.

2.7 Agarose gel electrophoresis

A 1% (w/v) Agarose gel was prepared in 1XTris acetate EDTA (TAE) buffer stained with ethidium bromide (Etbr) (1 µl 100 ml^{-1}). An electrophoresis chamber was loaded in the gel tank with 1xTAE buffer. DNA samples were loaded with 6X loading dye and voltage applied at 70 Volts for 2 h. Then, the gel was removed for visualisation under UV light using a Syngene Ingenius Bioimaging system.

2.8 Isolation of genomic DNA from *S. coelicolor* M145 (Kieser *et al*., 2000)

200 µl of a *S. coelicolor* spore suspension was inoculated in 25 ml of YEME medium with 0.5% glycine final concentration and incubated for 36-48 h at 30^{o}C (250 rpm) until the media turned pink. The culture was centrifuged at 4000 rpm for 10 min at room temperature. The pellet was then resuspended in 25 ml 3M sucrose, vortexed and centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in 3 ml of 25 mM Tris pH 8.0 containing lysozyme (2 mg ml^{-1}) and RNaseA (10 mg ml^{-1}). The tube was incubated for 30 min at 37^{o}C. Next, 5 ml of 0.3 M NaCl with 20% SDS (w/v) was added and mixed by pipetting. After that 6 ml phenol:chloroform (3 ml:3 ml) was added and the solution mixed by inversion. After centrifugation at 4000 rpm for 10 min, the aqueous phase was transferred to a clean Oakridge tube using a cut blue tip. This process was repeated until the top aqueous layer became transparent. The aqueous phase was then transferred to a sterile tube. 10 ml of absolute ethanol was added and the tube was inverted 3 times. The DNA was spooled out

from the solution using a bench Pasteur pipette wiping away the drops of ethanol and dried at room temperature for 10 min. Finally the DNA was dissolved in 500 µl TE buffer and stored at -20^{o}C. Genomic DNA (gDNA) yield was quantified using a DNA NanoDrop spectrophotometer. Genomic DNA quality at dilution 1X, 10^{-1}X, 10^{-2}X and 10^{-3}X M145 gDNA was further checked on an agarose gel, 5 µl of DNA were added in each tube and analysed by restriction digestion (see 2.6 and 2.7).

2.9 Polymerase Chain Reaction (PCR)

PCR reactions were set up in the following way in a sterile, nuclease-free microcentrifuge tube. Using Go Taq®, Go Taq® Flexi kit and HotStarTaq® (QIAGEN) following the procedures recommended by the manufacturer. For each 50 µl reaction 10 µl 5x Green or Colorless Go Taq® and Go Taq® Flexi Reaction Buffer, 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 3.2), $MgCl_2$ (variable, see Table 3.3), 1 µl (0.125 U) Promega Go Taq® DNA Polymerase and nuclease-free water were added to give a final volume of 50 µl.

For Qiagen HotStarTaq® 50 µl reaction 5 µl 10X Qiagen HotStarTaq Buffer, 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 3.2) DNA template, $MgCl_2$ (variable, see Table 3.3), 1 µl (0.5 U) Qiagen HotStarTaq® DNA Polymerase and nuclease-free water were added to give a final volume of 50 µl.

The templates *hrdB* and *SCO1389*, *SCO6467*, *SCO6468*, *SCO5628*, *SCO1527* and *SCO5753* were amplified using the following conditions in a Biorad PCR machine (see

Table 3.1). Firstly, an initial denaturation of the DNA was carried out for 15 min at 95^{o}C. After this, and for each cycle, the DNA was denatured at 94^{o}C for 1 min, annealed at 50^{o}C – 70^{o}C for 1 min and extended at 72^{o}C for 1 min. These steps were repeated 25-40 times where appropriate. A final extension at 72^{o}C for 10 min was then carried out. The reaction samples were subjected to electrophoresis on 1.4% (w/v) agarose in TAE buffer and stained with Etbr staining and UV illumination.

2.10 Reverse Transcriptase PCR (RT-PCR)

2.10.1 RNA extraction from *Streptomyces*

Plasticware, glassware and autoclave reagents rendered RNase-free by autoclaving two or three times. Previously unopened plasticware (e.g. Bags of microcentrifuge tubes) or reagent (eg. ethanol) assumed to be RNase-free were used where possible. Pipettes and benches were cleaned thoroughly before beginning the protocol. Gloves were worn for all steps following biomass harvest and changed frequently, whilst tubes etc. were left open for as short a time as possible.

2.10.1.1 Growth on solid medium

Cellophane discs (Lakeland Jam Pot Covers) were boiled in distilled water for 10 min, drained and washed with fresh sterile dH_2O. Discs were placed in a glass dish and alternated with discs of filter paper dampened with dH_2O. The dish was wrapped in foil and autoclaved. Cellophane was laid on top of agar plate by sterile forceps. Any bubbles were excluded from plates in a laminar flow hood for a few minutes. Plates were used on the same day or stored in sealed plastic bag at 4^{o}C.

2.10.1.2 Spore pre-germination and inoculation

500 µl *Streptomyces* spore suspension was pre-germinated in 50 ml 2xYT in 250 ml Erlenmeyer flask for 8 h at 30^{o}C, 250 rpm. Cells were pelleted at 3000 rpm for 10 min and then resuspended in 10 ml sterile H_2O. Optical density was read at 450 nm. The concentration (C) and volume (V) required to inoculate $3x10^6$ pre-germinated spores (gs) was calculated according to the following formula.

$C = (OD_{450}/ 0.04) \times 4x10^6$ gs $ml^{-1} = OD_{450} \times 10^8$ gs ml^{-1}

$V = (3x\ 10^6/ C) \times 1000$ µl

3MA plates were overlaid with cellophane and inoculated with the required volume (V) of pre-germinated spores and incubate at 30^{o}C.

2.10.1.3 Harvesting biomass for RNA extraction

At each time point, biomass was harvested by scraping the surface of the cellophane with a sterile razor blade. This was transferred to an RNase-free tube and immediately 2 volumes of RNAprotect Bacteria Reagent was added to the tube, vortexed for 5 s and incubated for 5 min at room temperature before being centrifuged at 4000 rpm for 10 min. The supernatant was decanted and the residual supernatant was removed by inverting the tube onto a paper towel.

2.10.1.4 RNA extraction part 1

200 µl 1xTE containing 3 mg ml^{-1} lysozyme was added to the tube and vortexed for 10 s and incubated at room temperature for 30 min. 700 µl buffer RLT (containing β-mercaptoethanol) was added and vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the supernatant was transferred to a fresh tube. 1 volume phenol/chloroform

was added and the tube was inverted 5 times and centrifuged at max speed for 10 min at 4^{o}C. The upper (aqueous) phase was transferred to a fresh tube and the process repeated again. The supernatant was finally transferred to a fresh tube and ethanol (500 µl) was added.

2.10.1.5 RNA extraction part 2

The extract was added to an RNeasy mini column placed in a 2 ml collection tube and centrifuged at 10000 rpm for 30 s. The flow-through was discarded and 700 µl buffer RW1 added to the column. Next, the column was centrifuged at 10000 rpm for 30 s and the flow-through was discarded. The RNeasy column was transferred into a new 2 ml collection tube and 500 µl RPE buffer was added to the column following by centrifugation at 10000 rpm for 30 s and the flow-through was discarded. 500 µl RPE buffer was added again to the column and centrifuged at 10000 rpm for 2 min and the flow-through was discarded. To elute RNA the RNeasy column was transferred into a new 1.5 ml microcentrifuge tube. 50 µl of RNase-free water was added directly onto the membrane and centrifuged at 10000 rpm for 1 min in order to elute the RNA. This was aliquoted and stored at -80^{o}C.

2.10.1.6 Removal of DNA contamination from RNA samples

The isolated RNA was likely to contain some DNA contamination. This was removed by performing DNase digestion in solution followed by RNA cleanup. The RNA sample was treated with Qiagen DNase I before RT-PCR analysis in order to prevent PCR amplification from contaminating DNA. If there was a lot of DNA in the

sample it was necessary to repeat DNase treatmnet. DNase digestion in solution was found to be much more effective than on-column DNase treatment (data not shown).

2.10.1.7 Determination of RNA yield

RNA yield was checked with a NanoDrop ND-1000 (Thermo Fisher Scientific) for quantity and quality and stored at -80^{o}C in aliquots. Pure RNA gave a yield of $A_{260}/A_{280} \geq 2.0$. If the yield was too high (>1000 ng) samples were diluted by adding RNase-free water to give a 50 ng μl^{-1} RNA working concentration. RNA quality was also checked on an agarose gel. The gel, running buffer and loading buffer were autoclaved, and the tank, gel tray and comb were soaked in 0.1 M NaOH overnight before use. If the RNA had not degraded, clear bands were visible that corresponded to 23s, 16s and 5s rRNA.

2.10.2 Semi-quantitative RT-PCR

The RT-PCR was performed using the PCR method described previously with some modifications (Lu *et al.*, 2007, Ryding *et al.*, 2002). RNA isolation was carried out with a 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.

For each RT reaction (Tube A in Results Chapters Figs), 50-100 ng purified RNA was added and the following mixture prepared for DNase treatment 12 µl RNase- free water, 4 µl 5xRT buffer (contains 12.5 mM $MgCl_2$), 2.5 µl 5xQ-solution, 0.5 µl (5 U) DNase I (Qiagen) and 1 µl (50 ng μl^{-1}) RNA to give a total volume of 20 µl. Each reaction was placed in a Biorad PTC-100 DNA Engine thermal cycler at 37°C for 30

min. The reactions were stopped by adding 1 µl 25 mM EDTA and heating to 65°C for 10 min and cooled on ice. Following, this 30 µl of the mixture that included 11.5 µl RNase- free water, 6 µl 5xRT buffer, 2.5 µl 5xQ-solution, 2 µl deoxyribonucleotide triphosphate (dNTP) mixture (each 10 mM), 3 µl (10 pmol μl^{-1}) each of forward and reverse primer and 2 µl (5U) OneStep RT-PCR Enzyme Mix was added to the first mixture to give a final volume of 50 µl.

In order to detect DNA contamination, Qiagen HotStarTaq was used instead of OneStep RT-PCR Enzyme Mix. The following mixture was prepared: 2 µl 10X HotStarTaq buffer (contains 15 mM $MgCl_2$), 0.5 µl 25 mM $MgCl_2$, 2.5 µl 5xQ-solution, 1 µl (50 ng μl^{-1}) RNA and RNase- free water to give a final volume of 20 µl. To this mixture 0.5 µl (5 U) DNase I (Qiagen) was added to test for the absence of a PCR product (Tube B in Results Chapters Figs). In addition another control to test for the presence of a PCR product was included (Tube C in Results Chapters Figs) where DNase I was not added to this mix. Each reaction was placed in a Biorad thermal cycler as described before. Next 30 µl of a mixture containing 3 µl 10X HotStarTaq buffer, 0.5 µl 25 mM $MgCl_2$, 2.5 µl 5xQ-solution, 2 µl (10 mM) dNTP, 3 µl (10 pmol) each of Forward and reverse Primer and 1 µl (0.5U) HotStarTaqDNA Polymerase and RNase- free water was added to the first mixture. Another DNA template control (Tube D in Results Chapters Figs) that included ~100ng of *S. coelicolor* genomic DNA was also carried out (Table 3.2). This reaction contained 29 µl RNase- free water, 5 µl 10X HotStarTaq buffer, 1 µl 25 mM $MgCl_2$, 5 µl 5xQ-solution, 2 µl (10 mM) dNTP, 3 µl (10 pmol) each of Forward and reverse Primer and 1 µl (0.5 U) HotStarTaqDNA Polymerase.

The PCR program was as follows: Reverse transcription 30 min at 50^{o}C, Initial PCR activation step 15 min at 95^{o}C, followed by 35 cycles of 3-step cycling: Denaturation 1 min at 94°C, Annealing 50-68°C 1 min, and Extension 72°C 1 min, and Final extension completed by incubating at 72°C for 10 min. The following pairs of primers were used (Table 2.4). For semi-quantitative RT-PCR, to ensure that the DNA produced had not reached to a plateau, each set of PCR reactions for different tested transcript needed to be repeated using different cycles number (see Table 3.1). *hrdB* gene in *S. coelicolor* was used as positive internal control for the RT-PCR assay. The reaction samples were subjected to electrophoresis on a 1.4% agarose gel in 1XTAE buffer, stained with Etbr and photographed under UV illumination. Finally, bands were quantified by densitrometry using the Genetools software (Syngene). Gene expression quantification was carried out according to the following formula.

$$X_t = \frac{\text{Average intensity of band derived from PL gene (pixels)}}{\text{Average intensity of band derived from } \textit{hrdB} \text{ (pixel)}}$$

The relative expression of a gene at a given time point was then normalized by dividing X_t by X_0, where X_t is the relative expression at a given time point and X_0 is the relative expression at the first time point sampled.

Table 2.4 Predicted PL biosynthetic genes for *S. coelicolor*. Oligonucleotide primers used in PCR analysis and fragment size (bp) used in this study are shown.

PL biosynthetic enzyme	Primer name	Primer sequence (5´⟶3´)	Fragment size (bp)
	hrdB forward	GAGGCGACCGAGGAGCCGAA	158
	hrdB reverse	GCGGAGGTTGGCCTCCAGCA	
CL synthase	*SCO1389* forward	GAGCATCATTGGCTCGTTTC	647
	SCO1389 reverse	GAACACCTGCCCACCAATAC	
PI synthase	*SCO1527* forward	GAAGGCCATGCTGAACAAGT	725
	SCO1527 reverse	ATCGGCAGCAGGTACTGGAT	
Phosphatidate cytidyltransferases	*SCO5628* forward	GTCTTCGTCGGTGTGATCG	1175
	SCO5628 reverse	ATCAGGAACTGCATGCACAG	
PG synthase	*SCO5753* forward	CCAATCTGCTGACGATGCT	791
	SCO5753 reverse	AACTTCCGTCTCCTTCAACG	
PS synthase	*SCO6467* forward	CTTCATGGCGGTGTACTTCA	782
	SCO6467 reverse	GACAGGACGATCCAGGACAG	
PS decarboxylase	*SCO6468* forward	AGCCAAACCTCTGCACCTC	656
	SCO6468 reverse	CTCTACATCCACCTCGACACC	

2.11 Phospholipids Extraction

2.11.1 PLs Extraction from Plates

Total lipids of the *S. coelicolor* mycelium grown on cellophane discs were extracted according to published procedures (Bligh & Dyer, 1959, Iverson *et al.*, 2001) from 3MA plates and harvested with a sterile razor blade. 100 mg of wet mass, equivalent to 20 mg dry weight of mycelium, was transferred to a fresh microcentrifuge tube and resuspended in 100 µl chloroform, 200 µl methanol and 80 µl water by vortexing aggressively for 10 min. A further 100 µl of chloroform was added and the sample mixed aggressively for an additional minute. 100 µl of sterile distilled water was then added to the sample and mixed for 1 min. Centrifugation at 13000 rpm for 1 min caused the phases to separate, with the mycelial fragments forming a disc dividing the upper (aqueous) and lower (organic) phases. The lower phase was carefully removed and evaporated to dryness in a vacuum centrifuge. Each residue, was resuspended in 3 µl chloroform just prior to applying to thin-layer chromatography (TLC) on Kiesel Gel 60 F254 plates, 20x20 cm (Merck), placed in lined tanks containing chloroform/methanol/acetic acid/water (80:12:15:4 [vol/vol/vol/vol]) (Hoischen *et al.*, 1997). Vaporization of the solvent system was enhanced by lining the tank with chromatography paper (Whatman) for 30 min prior to addition of the TLC plate. PLs were identified by spraying with ammonium molybdate solution (Sigma-Aldrich Chemie). PL standards dissolved in ethanol (5 mg ml^{-1}) were also applied to the TLC plate to aid identification of unknown spots: L-α-Phosphatidyl-DL-glycerol sodium salt from egg yolk lecithin (PG); 3-sn Phosphatidylethanolamine from bovine brain (PE) and

Cardiolipin sodium salt from bovine heart (CL) (Sigma-Aldrich Chemie). After their development, the plates were captured using Gene Snap (SynGene Ingenious Bio imagine) densitometer. Spot quantification was calculated according to the following formula.

$$\text{Percentage of each PL spot} = \frac{\text{Spot intensity (pixels) X 100}}{\text{Total spot intensity for all PLs (pixels)}}$$

2.11.2 PLs extraction from Liquid culture

2.11.2.1 Pre-germination of *Streptomyces* spores (Hopwood *et al*., 1985)

5 ml of sterile water were added to the agar plate and the fresh spores resuspended with sterile cotton bud. Following this, the crude suspension was poured back into the container that held the sterile water and vortexed for a minute to break up the spore chains. Next the suspension was filtered through non-absorbent cotton-wool into a centrifuge tube before being centrifuged for 5-10 min at 4000 rpm to pellet the spores. The supernatant fraction was then removed and the pellet vortexed for a few seconds to disperse the spores before being resuspended in 5 ml sterile TES buffer. Then the spores were heat-shock at 50^{o}C for 10 min and cooled down under the cold tap and transferred into a centrifuge tube. An equal volume of double-strength pre-germination medium was added and incubated and the spores incubated at 37^{o}C, 220 rpm for 2-3 h. Next, the suspension was centrifuged for 5-10 min at 4000 rpm to pellet the spores and resuspended in 5 ml sterile TES buffer. Finally, the suspension was vortexed for a few seconds to disperse the clumps. The OD_{450} was measured and sufficient to the media to give a final OD_{450} 0.03-0.05.

2.11.2.2 Quantification of PL spots by densitometry

PL spots were quantified by densitometry using the Genetools software package (Syngene) and spots were abundance expressed as a percentage of the total number of pixels for all PL spots found in a given lane of a TLC plate

2.12 *Streptomyces venezuelae*

2.12.1 Strains and growth conditions

S. venezuelae 10712 is the wild-type strain and, along with, *S. venezuelae*Δ*bldN* and *S. venezuelae*Δ*whiD* were kindly provided by Professor Mark Buttner, John Innes Centre, Norwich. 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^{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 (A) 500 ml of LS medium (B) 500 ml of MYM medium. Cultures were incubated at 37^{o}C on a rotary shaker (220 rpm) for 3-5 days and visualized by phase-contrast microscopy.

2.12.2 Sporulation assay (Glazebrook *et al*., 1990)

Samples (1 ml) taken aseptically from cultures were diluted with 10 mM Tris-HCL buffer, pH 7.0 and the diluted sample subjected to lysozyme treatment (50 µg ml^{-1}) was added and the mixture incubated at 37^{o}C for 30 min to digest mycelial cell walls. After treatment, the samples were plated as a dilution series on MYM agar and incubated

for 24 h at 27^{o}C. The concentration of colony forming units (c.f.u.) surviving each treatment was compared with the concentration of c.f.u. in untreated culture samples similarly plated.

2.12.3 Phase contrast microscopy

Samples were studied using a Nikon TE2000S inverted microscope and observed with a CFI Plan Fluor DLL-100X oil N.A. 1.3 objective lens, and images were captured using a Hamamatsu Orca-285 Firewire digital charge-coupled device camera. Captured images were processed using IPlabs 3.7 image processing software (BD Biosciences Bioimaging, Rockville, MD). Briefly, 0.5 μm Z sections of phase-contrast image was captured and used to render three-dimensional images.