Analysis of secondary metabolite gene and protein expression profiles in Streptomyces coelicolor grown under environmental conditions.

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Analysis of Secondary Metabolite Gene and Protein Expression Profiles in *Streptomyces coelicolor* Grown Under Environmental Conditions

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For Mam and Dad

Abstract

Streptomycetes are Gram positive, soil dwelling filamentous bacteria, known for their production of secondary metabolites. Genome sequencing of *Streptomyces coelicolor* identified 26 known or predicted secondary metabolite gene clusters ranging from antibiotics to siderophores, lipids, pigments and lantibiotics. Most studies investigating secondary metabolite production in *Streptomyces*, as well as other bacteria, are undertaken in liquid or on solid media. There is little gene expression data available from *in situ* studies. This study determined expression in a totally different growth medium, soil, to gain insight into growth and adaptation under more 'normal' habitat conditions and the effect changing environmental factors has on the expression of secondary metabolite gene clusters.

In order to do this, *S. coelicolor* was grown in soil microcosms from which RNA was extracted and amplified using an optimised T7 polymerase-based RNA amplification protocol. The amplified RNA was used to determine gene expression profiles via endpoint RT-PCR and RT-qPCR. In a complementary approach, *S. coelicolor* sand microcosms were subjected to a novel protein extraction procedure to determine protein expression profiles in soil.

This study elucidated how carbon, nitrogen and metal availability, the environmentally bio-active entomopathogenic fungus *Metarhizium anisopliae* and nematode *Steinernema kraussei* affect secondary metabolite gene expression in soil.

In contrast to the consensus of secondary metabolism commencing after reduction or cessation of growth, this study revealed expression of secondary metabolite biosynthetic genes and proteins related to secondary metabolism before the onset of exponential growth. Some secondary metabolite genes/proteins were even expressed constitutively.

Soil microcosms have been shown to be an important tool for gene expression analysis. The results of these novel transcriptomic and proteomic approaches therefore have given new insight into secondary metabolism and its role under natural habitat conditions.

DECLARATIONS

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Date 10 Oct 12

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

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Abbreviations

1D	one dimension
2D	two dimension
A	adenylation
ACCase	acetyl-CoA carboxylase
ACN	acetonitrile
ACP	acyl carrier protein
Act	actinorhodin
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
API	atmospheric pressure ionisation
aRNA	amplified ribonucleic acid
AT	acyl transferase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
b	number of bacteria at the end of the time interval
B	number of bacteria at the beginning of a time interval
BSA	bovine serum albumin
C	condensation
Ca	calcium
CCR	carbon catabolite repression
CDA	calcium-dependent antibiotic
cDNA	copy deoxyribonucleic acid
CE	capillary electrophoresis
cfu	colony forming units
CHB	chitin binding protein
CLF	chain length factor
cm	centimetre
CP	carrier protein
cT	critical threshold
CTP	cytosine triphosphate
DH	dehydratase
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dscDNA	double stranded copy deoxyribonucleic acid
DTT	dithiothreitol
E	epimerization
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ER	enoylreductase
ESI	electrospray ionisation
FA	formaldehyde agarose
FACS	fluorescence-activated cell sorting

FAS	fatty acid synthase
FPP	Farnesyl pyrophosphate
g	gram
GAP	glyceraldehyde-3-phosphate
GC	guanine cytosine
gDNA	genomic deoxyribonucleic acid
GelCMS	gel separation and mass spectrometry
GFP	green fluorescent protein
GGPP	geranylgeranyl-pyrophosphate
GlcNAc	*N*-acetylglucosamine
GOI	gene of interest
GPP	geranyl pyrophosphate
GS	glutamine synthetase
GS-GOGAT	the glutamine synthetase-glutamate synthase
GTP	guanosine triphosphate
h	hour
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
ID	identification
IPP	isopentenyl pyrophosphate
IPTG	isopropyl β –D-1-thiogalactopyranoside
K_2HPO_4	dipotassium phosphate
kb	kilobyte
kD	kilodalton
KR	ketoreductase
KS	β-ketosynthase
kV	kilovolts
L	litre
LC	liquid chromatography
M. anisopliae	*Metarhizium anisopliae*
mA	milliamp
MALDI	matrix assisted laser desorption ionisation
MEP	methylerythritol pathway
mg	milligram
min	minute
ml	millilitre
mM	millimole
mRNA	messenger ribonucleic acid
MS	mass spectrometry
N	nitrogen
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate

NaH2PO4	sodium bicarbonate
NaOH	sodium hydroxide
nm	nanometre
nM	nanomole
nr	number
NRPS	non-ribosomal peptide synthase
NTC	no template control
OD	optical density
ORF	open reading frame
P	peptide probability
PCP	peptidyl carrier protein
PCR	polymerase chain reaction
PHC	petroleum hydrocarbon
PKS	polyketide synthase
PMF	peptide mass fingerprinting
ppGpp	guanosine pentaphosphate
PPP	pentose phosphate pathway
PTS	phosphotransferase system
qPCR	quantitative polymerase chain reaction
Red	undecylprodigiosin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
RTA	relative transcript abundance
rtPCR	reverse transcription polymerase chain reaction
S. clavuligerus	*Streptomyces clavuligerus*
S. coelicolor	*Streptomyces coelicolor*
S. exfoliates	*Streptomyces exfoliates*
S. filivissimus	*Streptomyces filivissimus*
S. glaucescens	*Streptomyces glaucescens*
S. griseus	*Streptomyces griseus*
S. lividans	*Streptomyces lividans*
S. peucetius	*Streptomyces peucetius*
S. pilosus	*Streptomyces pilosus*
S. rimosus	*Streptomyces rimosus*
S. venezuelae	*Streptomyces venezuelae*
SARP	Streptomyces antibiotic regulatory proteins
SCB	*Streptomyces coelicolor* butanolides
SDS	Sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SEM	standard error of mean
SFM	soya four mannitol
ST	seryl transferase

St. kraussei	*Steinernema kraussei*
t	time
TBE buffer	Tris/Borate/EDTA
TCA cycle	tricarboxylic acid cycle
TE	thioesterase
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
THNS	1,3,6,8-tetrahydroxynaphthalene synthase
TOC	total organic carbon
TOF	time of flight
tRNA	transfer ribonucleic acid
UTP	thioesterase
V	volt
v/v	volume/volume
w/v	weight/volume
WPDT	water penetration droplet test
WPT	water penetration time
ZnSO4	zinc sulphate
µg	microgram
µl	microlitre
µmol	micromole

Chapter 1 Introduction

1.1. Actinomycetes

Actinobacteria is one of the largest phyla recognised in the bacterial domain (Stackebrandt, Rainey et al. 1997). The phylum includes bacteria such as *Bifidobacterium, Mycobacterium,* and *Corynebacterium* with an extensive range of morphologies from the coccoid *Micrococcus* to the highly differentiated *Streptomyces*. Divergence analysis has been unsuccessful in identifying the bacterial group most closely related to Actinobacteria due to its ancient divergence (Ventura, Canchaya et al. 2007). Actinomycetes are gram-positive bacteria with high guanine-cytosine content within the actinobacteria phylum ranging from 51% in *Corynebacterium* to 72% in *Streptomyces* and *Frankia.* Through all their differences, actinomycetes share a specific molecular character within the 23s rRNA gene. This is an insertion of circa 100 nucleotides between helices 54 and 55 (Ventura, Canchaya et al. 2007). Actinomycetes inhabit a range of ecosystems. Actinomycetes play an important role in the carbon and nitrogen cycles in soil by recycling of organic matter through decomposition of a range of organic materials including cellulose, starch and chitin. They are also contributing to the formation of humus (Dou and Wang 2011). Some also inhabit other organisms as pathogens and commensals including *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* and a few species of *Streptomyces* (Kieser, Bibb et al. 2000).

Actinomycetes are of great pharmacological and commercial importance due to a particular trait, their ability to produces antibiotics. Over two thirds of all clinically used antibiotics are produced by Actinomycetes (Kieser, Bibb et al. 2000). Antibiotic production in Actinomycetes was first discovered in 1944 by Selman Waksman's research group who discovered that the soil bacterium *Streptomyces griseus* produced streptomycin: the first antibiotic found to have activity against tuberculosis (Schatz and Waksman 1944). Waksman received a Nobel Prize in 1952 for both its discovery and the developments of methods and techniques, which lead to its discovery (Waksman 1952). This discovery led to extensive screening of actinomycetes for novel bioactive compounds. Actinomycetes have been found to produce a large variety of secondary metabolites especially from the genus *Streptomyces* (Berdy 1995).

1.2 Streptomycetes

Streptomycetes are some of the most numerous and ubiquitous soil dwelling bacteria. They have a complex life cycle (Fig 1.1). After a suitable germination trigger, a germ tube emerges from the spore as its cell wall grows from the tip. During this early stage, the growth at the tip doubles with every cycle until after a few DNA replication cycles when it begins to stall as new tips are produced through lateral branching. Septa are formed away from the tips producing apical and sub-apical compartments. In the latter, there is no new cell wall synthesis but DNA replication continues to take place. After two to three days, due to nutrient limitation or other physiological stress, aerial hyphae form (regulated by a number of *bld* genes) and the hyphae undergo synchronous cell division producing pre-spore compartments where glycogen deposits transiently (Kieser, Bibb et al. 2000). A spiral syncytium, containing many tens of genome copies, is formed when the cell compartments round up and become grey in colour due to the production of a polyketide-derived aromatic compound expressed by the *whiE* gene cluster (Shen, Yoon et al. 1999). Mature spores are formed as the spore wall thickens before their release.

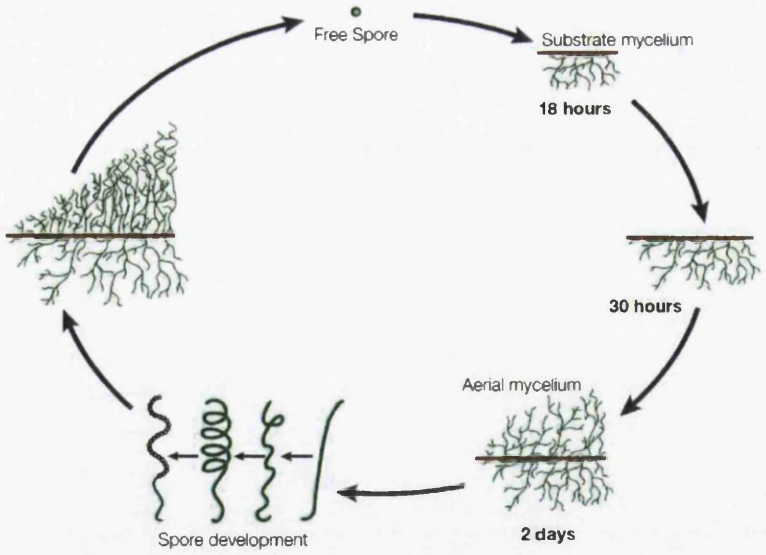


Fig. 1.1 *Schematic representation of the life cycle of* S. coelicolor *(Angert 2005)*

The immobile substrate mycelia allow for high local concentrations of secreted hydrolytic enzymes, which aid them in the biodegradation of insoluble organic debris, particularly from

plants and fungi. Their ability to breakdown these polymers, including protein, starch, xylan, chitin and cellulose make them important agents in carbon and nitrogen cycling (Kieser, Bibb et al. 2000).

Streptomycetes belong to the sporoactinomycete group along with *Sprichthya*, *Streptoverticillum*, *Kineosporia* and *Intrasporangium*. The ability to differentiate in spores assists in their distribution and persistence of the genus. Whilst hyphae are susceptible to drought and nutrient shortage, the spores are semi-dormant and can survive for extended periods of time before germinating when conditions are agreeable. For germination to occur the spore requires water, Ca^{2+} and exogenous nutrients (Ensign 1978). More recently, studies on *Streptomyces* germination revealed that spore germination is also stimulated by electromagnetic microwaves (Komarova, Likhacheva et al. 2010). The importance of hydrolytic enzymes, which cleave *S. coelicolor* cell walls, was highlighted with altered vegetative growth and reduced germination with the deletion of hydrolytic enzymes RpfA, Sw1A, Sw1B and Sw1C (Haiser, Yousef et al. 2009). Humidity is also important in *Streptomyces* spore germination and development. At low humidity germination is delayed, while lateral branching does not appear until day five, compared to day two in mid humidity (Zvyagintsev, Zenova et al. 2009). Germination rates are limited by the production of inhibitory substances (such as germicidin and hypnosin). These have been suggested to be produced to inhibit mass germination in situ in spore chains (Grund and Ensign 1985; Petersen, Zahner et al. 1993; Aoki, Yoshida et al. 2007).

1.2.1 Regulation by *bld* genes

Bld mutant strains produce an aerial hyphae-deficient phenotype and have therefore been studied to gain insight into aerial hyphae formation. Whilst early studies concentrated on six *bld* genes A-D (Merrick 1976) and G-H (Champness 1988), more recent studies have identified more than 20 genes whose inactivation results in the Bld phenotype (Elliot, Buttner et al. 2008). *bld* gene products transmit information relevant to the decision to differentiate. There is evidence of the transmission of extracellular signals by cross-stimulation of aerial growth when different mutants are grown together (Willey, Willems et

al. 2006). Genes controlled by *bldH* include both pathway-specific activator genes for antibiotic production and genes important for morphological development (Nguyen, Tenor et al. 2003; Takano, Tao et al. 2003). *bldA* mutant strains have reduced ability to produce antibiotics form sporulating mycelium. This is due to defective translation of *adpA* mRNA and of UUA codon-containing mRNAs for pathway associated regulators of antibiotic pathway genes (Chandra and Chater 2008) and for components of an extracellular protease cascade of developmental importance. The *bldG* gene product interacts with ApgA plus another partner to regulate σ^B sigma factors, which influence development (Parashar, Colvin et al. 2009). BldD is an autoregulatory DNA-binding protein, which represses several developmental regulatory genes. In *S. coelicolor*, these include *whiG*, *bldN* and *sigH* (Elliot, Bibb et al. 2001; Kelemen, Viollier et al. 2001). The *bldC* gene product contains a DNA-binding domain of a transcriptional regulator. The target promoter of BldC is unknown but it is required for maximum transcription of Act and Red biosynthesis regulatory genes (Hunt, Servan-Gonzalez et al. 2005). BldB has 18 paralogues in the *S. coelicolor* genome Chater (Chater 2011), most of which are located next to DNA-binding proteins (Gehring, Nodwell et al. 2000; Ainsa, Bird et al. 2010). BldB-like proteins are thought to counteract the repression of development and antibiotic production by their neighbouring DNA-binding protein. BldB itself however, is not adjacent to such a gene, although it is thought to interact with unidentified partner proteins for the same function and to form an asymmetric homodimer (Eccleston, Willems et al. 2006).

1.2.2 *Streptomyces coelicolor*

S. coelicolor A3 (2) is the model organism for the *Streptomyces* genus as it exhibits the primary features of the genus. This species originated in the former Royal College of Technology in Glasgow in the 1950's. It was named by Sir David Hopwood due to its ability to produce the blue pigment later discovered to be actinorhodin (Act) (Hopwood 1999). Although *S. coelicolor* does not produce any medically important antibiotics, two pigmented antibiotics are studied as models for antibiotic production and regulation. These are the aromatic polyketide Act (an easily diffusible pigment with pH indicator properties) and the red-pigmented tripyrrole undecylprodigiosin (Red), which is a mycelium-bound pigment

(Chater 1998). Two other well studied *S. coelicolor* antibiotics include calcium-dependent antibiotic (CDA) and methylenomycin (Wright and Hopwood 1976; Rudd 1978).

The genome sequence of *S. coelicolor* was published in 2002 (Bentley, Chater et al. 2002). The 8,667,507 base pair linear chromosome was the largest known bacterial genome at that time, which encoded for 7,825 predicted genes. The GC content was calculated to be 72.1% and contained 7,846 open reading frames. The genome sequence highlighted a strong emphasis on the regulation importance of *S. coelicolor* with 12.3% (965 proteins) associated with regulatory function. The genome codes for 65 sigma factors (which direct selective transcription), which is further evidence for regulatory control. Evidence of evolution to exploit the natural soil habitat was also evident with 819 predicted secreted proteins (10.5% of the genome) with 60 protease/peptidases hydrolases, 13 chitinases/chitosanases, eight cellulases and three amylases (Bentley, Chater et al. 2002). The genome sequence also revealed the presence of 23 known or predicted secondary metabolite gene clusters, which covered 4.5% of the genome, including the four known antibiotics Act, Red, CDA and methylenomycin. Many of the gene clusters were cryptic and predicted to encode for biosynthetic enzymes for catalyzing the production of a wide range of secondary metabolites e.g. *cpk*. Since the publication of the genome sequence, other clusters have been highlighted as secondary metabolite genes, including those related to methylisorborneol synthesis (Wang and Cane 2008) and lantibiotic production (Foulston and Bibb 2010). The role of secondary metabolism and the advantages of the extensive diversity found and predicted in the genome is still unclear. Proteomic analysis of *S. coelicolor* has confirmed that *S. coelicolor* excrete a large number of proteins (Kim, Chater et al. 2005; Widdick, Dilks et al. 2006).

1.3.1 Primary metabolism

For most Streptomycetes their natural environment is soil. Plants provide one of the main nutrient inputs into soil, therefore nutrient level is intrinsically linked to plant productivity (Hodgson 2000). Due to the influence of plants on the soil, soil tends to be carbon-rich but is very limited in nitrogen and phosphorus (see 1.5.1 Soil and soil ecology). Under standard soil

growth conditions, *Streptomyces* face acute nutrient limitations in a competitive environment (Hodgson 2000). Streptomycetes have adapted to the excess in carbon by producing a plethora of carbon catabolic systems (Gorke and Stulke 2008). Streptomyces have been shown to grow exclusively under oligotrophic conditions on low-C agar medium (Senechkin, Speksnijder et al. 2010).

Streptomyces possess two systems to assimilate nitrogen. The first converts ammonium to glutamate via glutamase dehydrogenase, the second produces glutamate via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. The process of nitrogen assimilation via GS-GOGAT expenses a lot of energy but is functional at low ammonia levels and is therefore better suited to the soil environment where nitrogen is limited (Geisseler, Doane et al. 2009). There are two GSs found in *Streptomyces*. GSI is similar to other GSs found in prokaryotes and is encoded by the *glnA* gene (Wray Jr and Fisher 1988; Hillemann, Dammann et al. 1993). GSII is more similar to a eukaryotic GS (Carlson and Chelm 1986). Both GSI and GSII are regulated by adenylation similar to Gram-negative bacteria (Streicher and Tyler 1981). Enzyme assays in liquid media revealed that in *S. coelicolor* GSI is responsible for most GS activity (95%) (Fink, Weissschuh et al. 2002).

Due to the limited availability of amino acids in soil (Jones, Owen et al. 2002), *Streptomyces* synthesise their amino acids. This system is similar to enteric bacteria with a few exceptions, firstly with the production of tyrosine. In *Streptomyces* tyrosine is synthesised via arogenate instead of hydroxyphenylpyruvate (Keller, Keller et al. 1985). Secondly, a fungal pathway (the trans-sulpharase pathway) is present in *Streptomyces* to transfer sulphur bi-directionally between methionine and cysteine. In most bacteria, regulation of amino acid biosynthesis is via amino acid feedback inhibition of the biosynthetic enzyme genes. In *Streptomyces*, these biosynthetic enzymes are instead expressed constitutively at low levels (Hood, Heidstra et al. 1992). Amino acid catabolism is also constitutive (Hodgson 2000). This may be explained by the fact, unlike enteric bacteria, *Streptomyces* live in environments low in nitrogen metabolites. Therefore, there is not such need for feedback regulation as if there are no amino acids available, the bacteria will synthesise them. The evolution of a mechanism to shut down amino acid metabolism has not evolved or has been lost (Hodgson 2000).

Chitin metabolism

Chitin, a nitrogen-containing polysaccharide, is a major nutrient source for both carbon and nitrogen for *Streptomyces* (Schrempf 2001) and is one of the most abundant polysaccharides in nature with over $1x10^{10}$ tonnes being produced annually (Chater, Biro et al. 2010). It consists of chains of β-1,4-linked *N*-acetylglucosamine (GlcNAc) forming high molecular weight molecules, which are stabilised by hydrogen bonds and van der Waal's (Chater, Biro et al. 2010). Alpha-chitin is extremely strong in crystalline form and is therefore an important component of fungal cell walls, and arthropod exoskeletons. Chitin formation is dependent on a range of chitin synthases. Some streptomycetes produce inhibitors of these chitin synthases presumably to aid in competition of chitin-containing organisms in their environment (Rogg, Fortwendel et al. 2011) (Chaudhary, Chavan et al. 2009).

Streptomyces use specialised chitin-binding proteins (CHBs), such as CHB1 and CHB2, to bind chitin for degradation. CHB1 binds crab shell chitin, potworm chitin and the spores and hyphae of many chitin-containing fungi (Zeltins and Schrempf 1997). *S. coelicolor* contains a third CHB, CHB3, which targets alpha and beta chitin (Saito, Miyashita et al. 2001). *S. coelicolor* encodes 11 deduced family 18 chitinase and two family 19 chitinases (Chater, Biro et al. 2010). These enzymes cleave the glycosidic linkages between the sugar units. Uptake of the β-1,4-linked *N*-acetylglucosamine dimer, chitobiose, in *S. coelicolor,* is via the chitobiose-specific ABC transporter Ngc (Wang, Xiao et al. 2002; Xiao, Wang et al. 2002). This is encoded by the *das*ABC operon, which also requires the ATP-hydrolysing protein MsiK (Chater, Biro et al. 2010). The *das*ABC operon is regulated by DasR (Saito, Shinya et al. 2007). The degraded chitin substrates also interact with DasR exerting carbon catabolite repression, affecting morphological differentiation and secondary metabolism (Rigali, Titgemeyer et al. 2008). Once in the cell, the dimer is monomerised and phosphorylated by NagK (Rigali, Nothaft et al. 2006). *N*-acetylglucosamine metabolisms is linked with the sugar phosphotransferase system (PTS) as a *N*-acetylglucosamine monomer from outside the cell is internalised via a transporter encoded by the IIC component of PTS (Nothaft, Dresel et al. 2003). IIA then activates the *N*-acetylglucosamine-specific permease IIB and *N*-acetylglucosamine is phosphorylated (Nothaft, Dresel et al. 2003). Phosphorylated *N*-acetylglucosamine is deacylated to glucosamine-6-phosphate by NagA and isomerised to fructose-6-phosphate by NagB (Rigali, Nothaft et al. 2006). Fructose-6-phosphate is central

to primary carbon metabolism as an intermediate of glycoloysis, which feeds into a variety of different carbon metabolic pathways such as the TCA cycle and peptidoglycan precursor synthesis (Barreteau, Kovač et al. 2008).

1.3.2 Secondary metabolism

1.3.2.1 Link with primary metabolism

The production of secondary metabolites depends highly on the availability of biosynthetic building blocks, most of which are products of primary carbon metabolism (Olano, Lombo et al. 2008; Keulen, Siebring et al. 2011). Changes in primary metabolism can have knock on effects on secondary metabolism. For example in *S. coelicolor*, malonyl-CoA is a precursor in Act biosynthesis. When the malonyl-CoA-producing acetyl-CoA carboxylase encoding gene (*acc*) is overexpressed, production of Act is increased (Ryu, Butler et al. 2006). Also in *S. coelicolor,* deletion of the important TCA cycle genes *citA* and *acoA* has a negative effect on secondary metabolite production (Viollier, Minas et al. 2001; Viollier, Nguyen et al. 2001). The production of clavulanic acid in *S. clavuligerus* was increased by deletion of *gap1*, which directly increased the supply of the precursor molecule glyceraldehyde-3-phosphate (GAP) in glycolysis (Li and Townsend 2006). In *S. lividans*, where Act production is usually silenced, the resulting accumulation of polyphosphates from *ppk* gene inactivation resulted in Act production. Changes in pentose phosphate pathway (PPP) flux can also affect secondary metabolism. In *S. coelicolor,* Zwf catalyses the first step in the oxidative phase of PPP producing NADPH. A deletion of one of the *zwf* genes (*zwf2*) resulted in increased mycelial growth, and as a result increased Act production (which is attributed to the increase in growth) (Ryu, Butler et al. 2006). However, the deletion of the *zwf2* gene overall results in a decrease in specific Act production rate (Ryu, Butler et al. 2006). During growth of *S. coelicolor* there is a change in flux ratio between PPP and glycolysis. The production of methylenomycin increases the flux through PPP (Obanye, Hobbs et al. 1996). An increase in flux through PPP also resulted in an increase in production of Act and Red in a *pfkA2* mutant strain. Furthermore, NADPH levels increased and therefore the biosynthesis of NADPH-dependent pigmented antibiotics (Borodina, Siebring et al. 2008). For the biosynthesis of

Act, 50% of the acetate units are derived from branched amino acid by nutrient dependent pathways. It has been suggested that for secondary metabolite production amino acid catabolism pathways are most relevant due to dependency on nutrients from the lysing of substrate mycelia after the cessation of vegetative growth. From the substrate mycelia, protein is the major carbon source and branched chain amino acids are the most prevalent in protein (Stirrett, Denoya et al. 2009).

1.3.2.2 Regulation of secondary metabolism

The majority of secondary metabolite gene clusters contain pathway-specific regulatory genes. Expression of the regulator genes often depends on genes that are required for the production of several secondary metabolites (Bibb 2005).

The concept that the onset of secondary metabolism is related to a reduction in growth rate is consistent with much of the published literature. Highly phosphorylated guanosine nucleotide (ppGpp), due to its function in growth rate control of gene expression, is thought to trigger secondary metabolism. When *S. coelicolor* is grown under nitrogen-limited conditions the ribosome-associated ppGpp (RelA) is required for the production of Act and Red (Chakraburtty and Bibb 1997). Studies suggest that ppGpp has a direct role in activation of transcription of secondary metabolite biosynthetic genes. When ppGpp synthesis was induced using a modified *relA* gene, which does not cause a reduction in growth rate, the pathway-specific regulator of the Act cluster (*actII-ORF4*) (open reading frame; ORF) was transcribed (Hesketh, Sun et al. 2001). The transcription of secondary metabolite biosynthetic genes in *S. rimosus* is repressed under excessive levels of inorganic phosphate (Martin 2004). At low phosphate levels, a mutation in the PhoR-PhoP regulatory systems reduced alkaline phosphatase activity and phosphate transport while Act and Red production increased (Sola-Landa, Moura et al. 2003).

γ-Butyrolactones are further signalling molecules in antibiotic production, as well as in morphological differentiation in *Streptomyces sp.* (Takano 2006). Bioassays have predicted at least six γ-butyrolactones in *S. coelicolor* (Anisova, Blinova et al. 1984; Kawabuchi, Hara et al. 1997). The structure of three *S. coelicolor* butanolides (SCB) have been elucidated

(Takano, Nihira et al. 2000). Unlike other *Streptomyces sp.*, none of these three SCBs are required for or influence morphological differentiation in *S. coelicolor*; they are devoted to the regulation of secondary metabolism (Bibb 2005). SCBs are produced in the late transition stage and are dependent on ScbA (Takano, Kinoshita et al. 2005). SCBs bind to ScbR and prevent DNA binding of the transcriptional regulator. ScbR targets include *scbR*, *scbA* and *cpkO* (Takano, Chakraburtty et al. 2001; Takano, Kinoshita et al. 2005). Therefore, ScbR regulates its own expression, as well as signalling molecules of the *S. coelicolor* butanolide system and the secondary metabolite biosynthesis gene cluster, *cpk* (Gottelt, Kol et al. 2011). Both Act and Red production are also affected by ScbR (Takano, Chakraburtty et al. 2001). ScbR binds to the pathway-specific regulatory gene CpkO, repressing expression of *cpkO* but does not bind to pathway-specific genes in the Act or Red biosynthetic clusters (Fig 1.2). It is theorised that Act and Red production is controlled by ScbR either by cross-talk with other secondary metabolism pathways and/or regulators or the repression of *cpkO* resulting indirectly in more of the precursor metabolite malonyl-CoA.

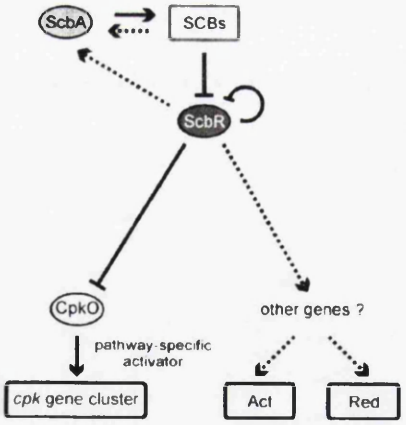


***Fig 1.2 Schematic representation of the butanolide system of* S. coelicolor.** *Arrows denote activation, lines with bars denote repression. Dotted lines denote effects currently being investigated (Gottelt, Kol et al. 2011).*

Streptomyces antibiotic regulatory proteins (SARPs) are pathway-specific proteins controlling secondary metabolism in many streptomycetes (Wietzorrek and Bibb 1997). In *S. coelicolor*, they are associated with secondary metabolite gene clusters that encode type I polyketides (Takano, Kinoshita et al. 2005), non-ribosomal peptide synthases (NRPS) (Ryding, Anderson et al. 2002) and undecylprodiginines (Cerdeño, Bibb et al. 2001).

Along with pathway-specific regulators such as the SARP family of proteins, *S. coelicolor* has a pleiotrophic regulator, AfsR, which is a transcriptional activator with ATPase activity (Lee, Umeyama et al. 2002). This protein serves as an integrator of multiple signals, both physiologically and environmentally sensed by serine/threonine or tyrosine kinases. One of these kinases, AsfK, autophosphorylates upon sensing an environmental condition (presumably), which enhances kinase activity. This leads to the phosphorylation of threonine and serine residues on AfsR, enhancing DNA binding activity. AfsR-P activates transcription of *afsS* that increases Act, Red and CDA production (Bibb 2005).

S. coelicolor contains over 13 type I polyketides and two glycopeptides gene clusters that include genes for large ATP-binding regulators of the LuxR family (LAL), however, little is known of their role in regulation of secondary metabolism (Bibb 2005). However, deletion or mutation of *pikD* (a LAL gene) ceased production of pikromycin in *S. venezuelae* (Wilson, Xue et al. 2001). A further pleiotrophic regulator in *S. coelicolor* is *abs*A1A2, a two-component histidine kinase response regulator pair. Phosphorylated AbsA2 is a negative regulator of the biosynthesis of multiple antibiotics, primarily CDA, but also Act and Red (Anderson, Brian et al. 2001).

1.3.2.3 Secondary metabolite classes and biosynthesis

There are several classes of secondary metabolite molecules. These include: polyketides and fatty acids, terpenoids and steroids, phenylpropanoids, alkaloids, specialised amino acids and peptides and finally specialised carbohydrates (Hanson 2003).

Polyketides are formed by a stepwise condensation of acetate units, although starter and extender units can be from acetate to other units. The product is a carbon chain with

alternate carbon atoms from the carboxyl and methyl groups of the reactant acetate. Polyketide chain growth is initiated by the condensation of a starter unit with an extender unit, which tend to be coenzyme A-thioesters. Common starter units include acetyl-CoA and propionyl-CoA, whilst common extender units include malonyl-CoA and methylmalonyl-CoA. Other derivatives of carboxylic acids are also used. Type I polyketide synthase (PKS) gene cluster products biosynthesise complex polyketides with multi-functional enzymes, which have a modular construction. Each module is accountable for a single carbon chain extension. Type I PKSs can be subdivided into iterative and non-iterative types. Iterative PKSs use functional domains repeatedly whereas non-iterative PKSs have a distinct active site for each enzyme catalysed step. In type I PKS biosynthesis, the PKS contains a number of modules within which lay domains. Each module stipulates a chemical structure to be added to the growing polyketide. A minimal module contains acyl transferase (AT), acyl carrier protein (ACP) and β-ketosynthase (KS). Firstly, at the loading module, an AT transfers the acyl/propionyl group from the starter unit to the ACP. The acyl/propionyl group is then transferred to a KS domain. The polyketide chain is condensed with the extender substrate (either malonyl or methylmalonyl-CoA), which is preloaded on the ACP domain on the extension module. The polyketide continues to grow as it passes through other modules, which may contain β-ketoreductase (KR), dehydratase (DH) and enoylreductase (ER), which reduce each carbonyl group. The polyketide is released from the PKS by a thioesterase (TE) domain. The TE may also be used for circularisation (Smith and Tsai 2007).

Type II PKS gene clusters encode for many mono- or bifunctional proteins whose active sites are used iteratively for the construction of the carbon chain (Shen 2000). Type II minimal PKS contains at least a KS, ACP and chain length factor (CLF). An unreduced polyketide is formed from a unit of the starter substrate and several units of the extender substrate. Further proteins reduce and tailor to produce the final product (Shen 2000).

Chalcone is an example of a type III PKS. It is hybridised with a phenylpropanoid, which is a secondary metabolite of a different class. The biosynthesis of a Type III polyketide results from sequential condensation of the starter unit coumaroyl-CoA (phenylpropanoid) with for example three extender units. The extender unit is frequently malonyl-CoA. This is decarboxylated to give rise to C_2 unit extension. This is then condensed and cyclised into a

hydroxylated aromatic ring system (Staunton and Weissman 2001). The hybrid molecule is termed a chalcone.

Fatty acid synthesis is similar in many aspects to PKS synthesis. However, in contrast to PKS synthesis commences specifically with malonyl-CoA. This is synthesised from acetyl-CoA and CO_2, a reaction catalysed by biotin carboxylase (Alberts and Vagelos 1972; Guchhait, Polakis et al. 1974; Marini, Li et al. 1995). The malonyl units are transferred to the 4-phosphopantetheine of the *holo*-ACP by malonyl-CoA:ACP transacylase (Harder, Ladenson et al. 1974). The chain is elongated by condensation of beta-ketoacyl-ACP synthase III with malonyl-ACP which results in an ACP-bound acyl chain that is extended by C_2 (D'Agnolo, Rosenfeld et al. 1973; Davies, Heath et al. 2000). The ACP tethered beta-carbon is consequently reduced by KR and dehydrated by DH. ER catalyses the reduction of the beta-carbon to CH_2. Further rounds of elongation by the acyl-ACP involving additional KSs result in a complete fatty acid synthase (FAS) (D'Agnolo, Rosenfeld et al. 1975; Garwin, Klages et al. 1980).

Terpenoids have a wide variety of structures. They are composed of a chain of C_5 isoprene units. Activated forms of isoprene are components of the biosynthetic pathway, isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP). IPP is produced in two ways, either formed from acetyl CoA (with the intermediate mevalonic acid) or derived from C_5 sugars via the methylerythritol phosphate MEP pathway (Daum, Herrmann et al. 2009). Isopentenyl pyrophosphate isomerase isomerises IPP to DMAPP. Linear prenyl diphosphate precursors (GPP, FPP and GGPP) are produced from the head to tail condensation of IPP with a DMAPP starter unit. Terpene synthases catalyse cyclisation of this isoprenoid multimer or rearrange to the parent carbon skeleton with the loss of pyrophosphate (Chang and Keasling 2006). Terpene synthases are responsible for the vast diversity of terpenoids (Withers and Keasling 2007). The final product is generated by tailoring enzymes, which modify the parental backbone (Chang and Keasling 2006). Steroids are derived from tetracyclic triterpenes with a backbone of cyclopentaperhydrophenethrene (Nes 2011).

Phenylpropanoids are compounds with a three carbon chain attached to an aromatic ring, which function as both structural and signalling molecules. They are derived from phenylalanine, which is converted to cinnamic acid by deamination (phenylalanine

ammonia-lyase) before hydroxylation (and often methylation) to produce acids with a phenylpropane unit (Weisshaar and Jenkins 1998). Phenylpropanoid metabolism branches to produce a wide variety of different compounds, such as chalcones, aurones, isoflavonoids, flavones, flavonols, and flavandiols, anthocyanins, condensed tannins and phlobaphene pigments (Winkel-Shirley 2001) . Chalcone synthases are responsible for the first commitment step in the pathway to produce these compounds, including flavanoids. Chalcone (a Type III PKS) is produced by the condensation of a phenylpropanoid (p-coumaroyl-CoA) with three acetate units unit (Weisshaar and Jenkins 1998).

Alkaloids were some of the first natural products isolated for medicinal purposes. They are nitrogen-containing bases (from a non-peptidic origin), which on addition of acid form salts. Alkaloids tend to be neuroactive. The biosynthetic origin of most alkaloids are from amino acids, which are modified by decarboxylation, aldol condensation, reductive amination or methylation to form the alkaloid (De Luca and St Pierre 2000).

NRPSs, like Type I PKSs, have a modular organisation. A minimal NRPS contains an adenylation domain (A domain), a peptidyl carrier protein domain (PCP) and a condensation domain (C domain). The A domain controls entry of substrates into nonribosomal peptide synthesis by selection of the amino (or carboxy) acid substrates. The linear sequences of the of the modules correspond to the generated amino acid sequences, therefore selection of (carboxy) amino acids can be predicted from primary sequence (Lautru and Challis 2004). The A domain activates a specific amino acid as an aminoacyl adenylate at the expense of ATP, which is then transferred to the PCP domain. The PCP domain accepts the activated amino acid, forming an aminoacyl thioester. The growing peptide chain is connected to the enzyme through its thiol group being attached to a 'phosphopantetheinyl arm', which is bound to the NRPS. During the chain assembly process, the phosphopantetheinyl arm allows different active sites on the NRPS to be reached. The cofactor is post-translationally transferred to a conserved serine of the carrier protein (CP). The C domain is the central entity of nonribosomal peptide synthesis. The C domain catalyses the nucleophillic attack of the amino acyl bound to PCPs of adjacent modules resulting in peptide bond formation. The resulting dipeptide subsequently acts as the electrophile in the active site of the following C-domain. The chain is terminated in the TE, which liberates the chain. The product is released by macrocyclisation yielding a cyclic peptide or by hydrolysis yielding a linear peptide.

Macrocyclic release is the favored method (Finking and Marahiel 2004), which is a two step process. Firstly, the peptide chain is transferred from the PCP domain to the active serine site on the TE domain by acylation, then the TE domain is deacylated by an internal nucleophille of the peptide chain (either the N-terminal amino group or a functional side chain) (Finking and Marahiel 2004).

There are two classes of siderophore, polypeptides and non polypeptides. Polypeptide siderophores are biosynthesized by members of the NRPS family and often contain a peptide backbone heterocycles (Crosa and Walsh 2002). Many bacterial siderophores are not polypeptides. These are assembled from alternating dicarboxylic acid and diamine or amine alcohol building blocks linked by amide or ester bonds formed by siderophore synthases (Challis 2005). The first stage in polypeptide siderophore synthesis is PCP activation where the PCP chains are converted from apo to holo form bearing the phosphopantetheinyl arm by a phosphopantetheinyl transferase (PPTase). This enzyme is responsible for the transfer of phosphopantathenate from coenzyme A producing a phosphodiester linkage to the PCP serine side chain. Next, the A domain selects and activates the substrates for incorporation into the chain. The selected amino acid is converted to aminoacyl-AMP and is transferred to the -PCP domain where the aminoacyl moiety and thiol are covalently linked producing an aminoacyl thioester. Chain elongation takes place at the C domain (peptide synthetase catalytic domain). A downstream aminoacyl-S-PCP acts as an acceptor substrate to an upstream peptidyl-S-PCP donor, producing a peptide bond. The chain then acts as the upstream donor for the next downstream module as the chain extends. When the full length chain reaches the most downstream PCP domain, it requires cleavage from the assembly line. This is achieved at the TE domain, where the chain is transferred from the active site of the PCP domain to the serine side chain the TE yielding an acyl-O-TE intermediate. The intermediate either undergoes hydrolysis or cyclisation to release the siderophore (Crosa and Walsh 2002). Compared to the NRPS pathway, little is known of the enzymatic pathways of non-polypeptide siderophore biosynthesis, however, it is theorised that amide bonds link alternating dicarboxylic acid and diamine or amino alcohol units. Bonds between these units are formed by NRPS- independent synthases (Challis 2005; Kadi, Oves-Costales et al. 2007). These pathways utilise siderophore synthetases to produce siderophores containing

hydroxamic acid and alpha-hydroxy acid chelating ligands for the binding of ferric (Challis 2005).

1.4.1 *Streptomyces* secondary metabolism

Streptomyces are known to produce a wide range of secondary metabolites. Due to their interest to the pharmaceutical industry they have been studied in great detail. *Streptomyces* natural products include antibiotics such as chloramphenicol (*S. venezuelae*), streptomycin (*S. griseus* and others), and clavulanic acid (*S. clavuligerus*). *Streptomyces* also produce immunosuppressants such as rapamycin and tacrolimus (both from *S. tsukabiensis*) along with antitumor agents bleomycin and phleomycin (*S. verticullus*) and daunomycin (*S. peucetius*). Other secondary metabolites produced include antiparasitic compounds, herbicides and the iron chelator desferrioxamine B (*S. pilosus*) used for purging iron overdoses (Kieser, Bibb et al. 2000).

Whilst the production of secondary metabolites has a major benefit in the pharmaceutical industry, there is still debate as to the role of secondary metabolism for the producing organism. Streptomycetes have extensive and energy costing systems for the production of secondary metabolites and the advantage of having such a system is under debate. It was suggested that due to the lysing of substrate mycelia for the provision of nutrients during aerial hyphae formation, secondary metabolites reduce competition from other microbials (Chater and Merrick 1976). This may be further explained by the synergistic effect of certain secondary metabolites, for example, cephamycin C and clavuligerus by *S. clavuligerus* (Jensen and Paradkar 1999). The activity of these molecules against other organisms is greater than cumulative, i.e. the activity of both together is greater than the sum of activity when alone. The benefit of non-antibiotic secondary metabolites is witnessed by the production of siderophores. These may be produced as part of a systematic approach in response to low iron levels (Challis and Hopwood 2003).

Research to date suggests the onset of secondary metabolism coincides (or slightly precedes) the development of aerial hyphae (Challis and Hopwood 2003; Bibb 2005). During

this growth stage there is a metabolic shift and activation secondary metabolism (Obanye, Hobbs et al. 1996; Borodina, Krabben et al. 2005; Ryu, Butler et al. 2006).

Before the publication of the complete genome sequence of *S. coelicolor* (Bentley, Chater et al. 2002), gene clusters for several secondary metabolites had already been identified. These included the clusters for Act (Bystrykh, Fernandez-Moreno et al. 1996), Red (Feitelson (Feitelson, Sinha et al. 1986), CDA (Hopwood and Wright 1983) and the *whiE* cluster (Yu and Hopwood 1995). Complete genome sequence identified a further 18 clusters with probable secondary metabolite function including PKSs, NRPSs, chalcone synthases amongst others (Bentley, Chater et al. 2002). The probable function/structure of gene products of some of the clusters has been determined with comparisons to similar clusters of other organisms (Bentley, Chater et al. 2002). With use of predictive models for substrate amino acid recognition two clusters (*sco0489-0492* and *sco7681-7683*) were identified to catalyse novel (putative) siderophores coelichelin and coelibactin. Desferrioxamines G1 and E are probably biosynthesised by products of *sco2782-2785* (Barona-Gómez, Lautru et al. 2006). With similarity to a type I PKS/FAS, two open reading frames are predicted to have products which catalyse eicosapentaenoic acid biosynthesis. In the same way, the cluster *sco6759-6771* is predicted to be associated with hopanoid biosynthesis, *sco6073* involved in the biosynthesis of geosmin and *sco0185-0191* implicated in carotenoid isoreneriatine biosynthesis (Bentley, Chater et al. 2002). More recently, the earthy odorant methylisoborneol biosynthetic gene cluster *sco7700-7701* (Wang and Cane 2008) was characterised along with two putative lantibiotic clusters, *sco0268-0270* and *sco6929-6931*.

Table 1.1 shows a summary of known/putative secondary metabolite gene clusters in *S. coelicolor* to date. Each cluster will be discussed in turn.

Table 1 **S. coelicolor** ***secondary metabolite classes, gene clusters, GOI and GOI function.***

Class	Subclass	Metabolite	Gene Cluster sco number	GOI sco	GOI function	Reference
Polyketide	Type I	cpk	6273-6288	*sco6273*	type I polyketide synthase	Pawlik *et al* 2006
	Type I	unknown polyketide	6826-6827	*sco6827*	polyketide synthase	Bentley *et al* 2002
	Type II	Actinorhodin (Act)	5076-5092	*sco5087*	polyketide synthase	Bystrykh *et al* 1996
	Type II	Tw95a, WhiE	5314-5350	*sco5318*	ketoacyl synthase	Davis NK & Chater KF 1990
	Type III	flaviolin	1206-1208	*sco1206*	type III polyketide synthase	Zhao *et al* 1995
	Type III	germicidin	7221	*sco7221*	type III polyketide synthase	Song *et al* 2006
	Type III	putative chalcone	7669-7671	*sco7671*	type III polyketide synthase	Bentley *et al* 2002
Fatty Acid	Type I/II	eicosapentaenoic acid	0124-0129	*sco0126*	FAS ketoacyl synthase	Bentley *et al* 2002
	Type II	putative fatty acid	1265-1273	*sco1271*	FAS oxoacyl synthase	Bentley *et al* 2002
Terpenoid	Triterpene	isorenieratene (carotenoid),	0185-0191	*sco 0185*	Geranylgeranyl pyrophosphate synthase	Takano *et al* 2005
	Sesquiterpene	albaflavenone	5222-5223	*sco 5222*	terpene synthase	Lin *et al* 2006
	Sesquiterpene	geosmin	6073	*sco 6073*	cyclase/synthase	Gust *et al* 2003
	Triterpenoid	hopanoids	6759-6713	*sco 6759*	phyotene synthase	Poralla *et al* 2000
	Sesquiterpene/diterpe	methylisoborneol	7700-7701	*sco 7700*	terpene synthase	Wang and Cane 2008
Peptide	Ribosomal peptide	lantibiotic 1	0268-0270	*sco 0270*	cyclase	Foulston and Bibb 2010
	Non-ribosomal	coelichelin	0484-0499	*sco 0492*	NRPS	Lautru *et al* 2005
	Lipopeptide	calcium-dependent antibiotic	3230-3232	*sco 3230*	NRPS	Chong *et al* 1998
	Non-ribosomal	Peptide	6429-6288	*sco 6431*	NRPS	Bentley *et al* 2002
	Ribosomal peptide	lantibiotic II	6929-6931	*sco 6929*	cyclase	Foulston and Bibb 2010
	Non-ribosomal	coelibactin	7676-7692	*sco 7682*	NRPS	Hesketh et al 2009
Miscellaneous	Carbohydrate	deoxysugar	0381-0401	*sco 0381*	glycosyl transferase	Bentley *et al* 2002
	Hydroxamate	desferrioxamine	2782-2785	*sco 2785*	cyclase	Barona-Gómez *et al* 2006
	Putative peptide	putative siderophore	5799-5801	*sco 5800*	conserved hypothetical	Bentley *et al* 2002
	Oligopyrrole	Undecylprodigiosin (Red)	5877-5898	*sco 5892*	polyketide synthase	Cerdeno *et al* 2001
	Butanolide	SCB1	6266	*sco 6266*	scbA: regulatory protein	Hsiao *et al* 2007,
	Cyclopentanone	methylenomycin,	SCP1	*SCP1.233*	ketoacyl synthase	Corre and Challis 2005

1.4.2.1 Polyketides

The genome sequence of *S. coelicolor* revealed a 54-kb cluster encoding a cryptic type I PKS containing three genes, *cpkA*, *cpkB* and *cpkC*, each encoding a PKS. The complete synthase consists of a loading module, five extension modules and instead of a usual thioesterase terminal module, there is a unique reductase module (Pawlik, Kotowska et al. 2007). CpkA contains the loading module plus extension modules 1 and 2, CpkB contains extension modules 3 and 4, while CpkC contains extension module 5 and the reductase module. Studies to date indicate CpkC (SCO6273) is expressed during transition stage (Pawlik, Kotowska et al. 2007). The *cpk* genes are regulated by butyrolactones (see secondary metabolism regulation section 1.3.2.2).

The genome sequence also revealed a cluster (*sco6826-6827*), which products putatively biosynthesise an unknown polyketide (Bentley, Chater et al. 2002). *Sco6827* encodes a polyketide synthase.

The cluster gene *sco5314-sco5350* (*whiE* cluster) encodes the type II polyketide spore pigment TW95a (Shen, Yoon et al. 1999). The *whiE* gene cluster is usually expressed shortly before sporulation in *S. coelicolor* aerial mycelium, leading to the production of the grey spore pigment (Yu and Hopwood 1995). The cluster contains ORF I-VIII. *whiE*-ORFI plays a role in retaining or targeting the spore pigment to the appropriate site in the spore, whilst *whiE*-ORFII-VIII control steps in biosynthesis (Yu and Hopwood 1995). *whiE*-ORFII is a cyclase, ORFII a ketosynthase, ORFIV a chain length factor, ORF V an acyl carrier protein, ORFVI an aromatase, ORFVII a cyclase and ORFVIII a hydrolase. The minimal PKS is encoded by ORFIII-IV (Kelemen, Brian et al. 1998).

The type III PKS biosynthetic machinery for flaviolin production is encoded by *sco1206-1208*. *sco1206* putatively encodes a 1,3,6,8-tetrahydroxynaphthalene synthase (THNS), which produces 1,3,6,8-tetrahydroxynaphthalene. This is auto-oxidated to produce flaviolin (Fujii, Mori et al. 1999). *sco1206* was not expressed under nutrient-rich growth conditions on LB plates and LB/TB liquid medium, and is thought to be silent under these conditions (Izumikawa, Shipley et al. 2003).

Germicidin is a type III PK. The PKS Gcs, encoded by *sco7221,* is required for germicidin biosynthesis. Germicidin production was noted after wildtype strain M145 was grown in minimal media for five days (Song, Barona-Gomez et al. 2006). Gcs catalyzes elongation of a specific beta-ketoacyl ACP thioester utilising ethylmalonyl-CoA as an extender unit. This is unusual for a type III PKS, where malonyl-CoA is normally exclusively an extender unit except for the plant type III PKS PstCHS2 (Song, Barona-Gomez et al. 2006).

The final type III PKS is from the putative chalcone biosynthesis cluster. The cluster *sco7669-sco7671* was first identified in the complete genome sequence (Bentley, Chater et al. 2002). SCO7671 is a predicted type III PKS. The product of this pathway is unknown (Nett, Ikeda et al. 2009).

1.4.2.2 Fatty acids

The gene cluster *sco0124-0129* was elucidated in the genome sequence, although eicosapentaenoic acid has not been shown to be produced in *S. coelicolor.* SCO0126, a beta-ketoacyl synthase, is likely involved in fatty acid synthesis. Similarly, a putative fatty acid secondary metabolite (*sco1265-sco1273*) was discovered when the genome was sequenced. In this cluster, *sco1271* encodes a beta-ketoacyl synthase, putatively involved in acetoacyl production in fatty acid biosynthesis.

1.4.2.4 Terpenoids

The isorenieratene (a carotenoid) biosynthesis gene cluster consists of two convergent operons, *crtEIBV* and *crtYTU* (Takano, Kinoshita et al. 2005). The *S. coelicolor* A3(2) *crt* gene cluster is flanked by a putative regulatory region that consists of two divergent operons, *litR*Q and *litSAB*, for light-induced transcription (Takano, Kinoshita et al. 2005). CrtE is a trans-isoprenyl diphosphate synthase that catalyses the successive 1'-4 condensation of the 5-carbon IPP to allylic substrates geranyl-, farnesyl-, or geranylgeranyl-diphosphate (Altschul, Madden et al. 1997).

The biosynthesis of albaflavenone in *S. coelicolor* A3(2) is under the control of a two-gene cluster, *sco5222* and *sco5223*. Farnesyl diphosphate (FPP) is cyclised to epi-isozizaene catalyzed by a terpene synthase (SCO5222) (Lin and Cane 2009; Aaron, Lin et al. 2010). SCO5223, a cytochrome P450 monooxygenase, catalyses the two-step allylic oxidation of epi-isozizaene to albaflavenone (Zhao, Lin et al. 2008).

The earthy odorant geosmin is biosynthesised by *sco6073* (Darriet et al 2000). This germacradienol synthase catalyses Mg^{2+} dependent conversion of FFP to the intermediate germacrene D (Cane and Watt 2003). Studies show that the conversion of FFP to geosmin is catalysed by the single enzyme, without intervention of any additional enzymes or the requirement for redox cofactors (Gust, Challis et al. 2003; Jiang, He et al. 2006).

The hopanoid biosynthetic gene cluster (*sco6759-6767*) encodes a range of biosynthetic enzymes including a phytoene synthases, dehydrogenases, a farnesyl diphophate synthase, a hopene cyclase, a lipoprotein and also a DNA-binding protein. SCO6759 is a member of a superfamily (pfam00494) of phytoene and squalene synthases. These catalyse the head-to-tail condensation of polyisoprene pyrophosphates. Hopanoids are produced when grown on solid R2YE (and left to differentiate) but are limited to trace amounts in liquid R2YE media (Poralla, Muth et al. 2000).

Methylisoborneol is an earthy odorant which is biosynthesised by the products of the gene cluster *sco7700-7701*. The terpene synthase, SCO7700 works in combination with the *C*-methyl transferase SCO7701 to catalyse the two-step conversion of GPP to 2-methyl-isoborneol with the intermediate 2-methylgeranyl diphosphate (Wang and Cane 2008).

1.4.2.4 Peptides

Lantibiotics are ribosomally synthesised and post-translationally modified peptides (Jack and Jung 2000). They contain thioether cross–links (lanthionines or methyl-lanthionines) along with the amino acids 2,3-dihydroalanine and 2,3-dihydrobutyrine. In *S. coelicolor* homologues to genes *lanA-C* have been found. *lanA* encodes a precursor peptide, while *lanB* encodes a dehydratase and *lanC* a cyclase. LanB is responsible for the dehydration of serine

resides on LanA forming 2,3-dihyrdoalanine, and the threonine resides on LanA producing 2,3-dihydrobutyrine. LanC catalyses the addition of cysteine thiols to the amino acid compounds resulting in thioether formation, generating lanthionine or methyl-lanthionine (Patton and van der Donk 2005). *S. coelicolor* contains at least two putative lantibiotic clusters; *sco0267-sco0270* and *sco6927-6932*. *sco0268, sco6931* and *sco6932* are putative *lanA* genes, *sco0269* and *sco6930* are putative *lanB* genes whilst *sco0270* and *sco6929* are putative *lanC* genes (Marsh, O'Sullivan et al. 2010).

The partial genome sequencing of *S. coelicolor* revealed a cluster of 11 genes (*cch*A-K) encoding for a novel NRPS system. One gene, *cchH* encoded a protein similar to several well characterised NRPSs (Challis and Ravel 2000). The structure of the novel iron-chelating compound, coelichelin, was elucidated in 2005 (Lautru, Deeth et al. 2005). *cchH* has ten catalytic domain separated into three modules. Module one contains an A domain followed by a PCP and epimerization (E) domain, module two contains a C domain, A domain, PCP and E and module three contains a C domain, A domain and PCP. This differs significantly from other NRPSs as it lacks a TE domain (Challis and Ravel 2000).

Calcium-dependent antibiotic is a lipopeptide with a fatty acid side chain and several non-proteogenic amino acid residues. The cluster contains three large genes encoding for NRPS (*cdaPS1-3*) with typical modular organisation of A, C, PCP and E domains (Hojati, Milne et al. 2002).

The genome sequence unearthed a putative NRPS secondary metabolite cluster, *sco6429-6438*. The structure of the product of these genes is unknown (Bentley, Chater et al. 2002). *sco6431* encodes a putative peptide synthase. The cluster also contains a putative oxidoreductase, SCO6434, and a putative diaminopimelate decarboxylase, SCO6438, which catalyses a reaction producing L-lysine.

Coelibactin is an uncharacterised siderophore-related non-ribosomal peptide (Kallifidas, Pascoe et al. 2010). *sco7676-7692* is predicted to be involved in the biosynthesis of coelibactin (Bentley, Chater et al. 2002). Within the 17 gene cluster, there are at least three transcriptional units and genes encoding ferredoxin (*sco7676*), a putative AMP binding ligase (*sco7681*) and an NRPS (*sco7682*). Expression of coelibactin biosynthetic genes has

only been observed in mutant strains grown on solid media, but not in wild type (Kallifidas, Pascoe et al. 2010). AbsC controls expression of the gene cluster encoding production of coelibactin (Hesketh, Kock et al. 2009).

1.4.2.5 Miscellaneous

The genome sequence also exposed a putative deoxysugar secondary metabolite cluster, *sco0381-0401*. The structure of the product of these genes is unknown (Bentley, Chater et al. 2002). *sco0381* encodes a putative glycosyl transferase. The cluster also contains a putative asparaginine synthase (SCO0386), a putative oxidoreductase (SCO0387), a putative lipoprotein (SCO0389), putative transferases (SCO0391 and SCO0393) and methyltransferase (SCO0392).

Desferrioxamine is a hydroxamate siderophore, containing hydroxamic acid-chelating groups consisting of alternating diamine and dicarboxylic acid linked by amide bonds (Baróna-Gomez, Wong et al. 2004). In *S. coelicolor*, a four gene cluster, *desA-D* (*sco2782-sco2785*) has been implicated in desferrioxamine production (Baróna-Gomez, Wong et al. 2004). *desA* encodes an amino acid carboxylase, which yields cadaverine via the decarboxylation of L-lysine and desB encodes an amine monoxygenase, which hydroxylates cadaverine to N-hydroxycadaverine. DesC is an acetyltransferase, which catalyses the acylation of N-hydroxycadaverine producing hydroxamic acid. DesD, a cyclase, catalyses the oligomerisation of hydroxamic acid followed by cyclisation producing a macromolecule of desferrioxamine G and E (Baróna-Gomez, Wong et al. 2004; Barona-Gómez, Lautru et al. 2006). *S. coelicolor* produces desferrioxamine G and E in iron-limited Muller and Raymond medium (Imbert, Bechet et al. 1995).

A putative siderophore synthetase secondary metabolite cluster, *sco5799-sco5801* was unearthed by genome sequencing (Bentley, Chater et al. 2002). The three gene cluster contains a putative aminotransferase gene, *sco5799*, a gene similar to *rhsC*, a *Rhizobium* siderophore biosynthesis gene, sco*5800,* and a putative malonyl-CoA decarboxylase gene, *sco5801*. The structure of the product of this gene cluster is unknown.

The *red* cluster, encoding the biosynthetical machinery for production of the oligopyrrole Red contains 23 genes (*sco5877-sco5892*) (Bentley, Chater et al. 2002). Two genes encode pathway-specific regulators, eight genes are assigned to 2-undecylpyrrole biosynthesis, six genes to 4-methoxy-2,2'-bipyrrole-5-carboxaldehydes and two housekeeping genes (Cerdeño, Bibb et al. 2001). The remaining five genes in the cluster have unknown function. Red is produced via the condensation of 2-undecylpyrrole biosynthesis and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde via to-electron oxidation (Cerdeño, Bibb et al. 2001). RedL is a polyketide synthase containing an A domain, KS domain, AT domain followed by a seryl transferase (ST) domain (Cerdeño, Bibb et al. 2001).

As discussed in Section 1.3.2.2 Secondary metabolism regulation, SCBs are butanolides involved in the regulation of secondary metabolism (Takano, Kinoshita et al. 2005). *Sco6266* encodes ScbA which along with regulatory properties has homology to fatty acid synthases and is able to synthesise SCB1, although the biosynthetic pathway is not known (Hsiao, Soding et al. 2007).

Methylenomycin is a complex secondary metabolite, whose biosynthetic machinery is encoded by *mmy* genes on the SCP1 linear cluster (Kirby and Hopwood 1977). *mmyA* codes for an acyl carrier protein and *mmyC* a type III PKS. MmyC catalyses the decarboxylative condensation of malonyl-MmyA with acetyl-CoA to form acetoacetyl-MmyA, the mid stage in methylenomycin production (Corre and Challis 2005; O'Rourke, Wietzorrek et al. 2009).

1.5.1 Soil and soil ecology

Soil consists of solid, liquid and gas components, which are heterogeneous with regards to their distribution (Smiles 1988). The solid phase of soil is composed of both organic (e.g. humic) and inorganic (e.g. sand, silt and clay) substances, which are complexed in aggregates. Complexes between the organic and inorganic matter have been shown to affect bacterial survival and conjugation due to negatively charged surfaces (Hattori and Hattori 1976).

Soil has a very important ecological function in both the anabolic and catabolic cycles of carbon, nitrogen and sulphur. The soil biota as a whole plays a crucial role in the decomposition of soil organic matter and in nutrient cycling (Coleman, Crossley et al. 2004; Wardle, Bardgett et al. 2004). These processes are key in the determination of soil fertility, productivity as well as global biogeochemical cycling (Nazir, Warmink et al. 2010). Soil is one of the most diverse habitats known for microorganisms (Dance 2008), with a significantly higher diversity of prokaryotes compared to any other natural environment (Gans, Wolinsky et al. 2005). The main factor driving the biodiversity of soil is its heterogeneity, providing a range of microhabitats which differentially select bacterial or fungal types (Standing and Killham 2007).

The soil pore network is contained within the soils matrix, containing a large number of pores. Bacterial communities (in the form of single cells or microcolonies) are often found within the soil pores in close association with soil surfaces. Bacterial communities are often limited in movement to under a micrometer (Trevors, Van Elsas et al. 1990) through the soil column without the aid of a transporting agent, such as water flow, growing roots or burrowing organisms. The fate and activity of these organisms is therefore largely dependent on their site of origin (Foster 1988). Kohlmeier et al demonstrated the movement of bacteria through soil by occupying the thin water layer surrounding fungal hyphae as microhabitats (Kohlmeier, Smits et al. 2005). Bacteria have been shown to migrate from the spot of inoculation (on a *Lyophyllum karsten* hyphal growth front) to a distant spot by the formation of a biofilm around the hyphae (Warmink and Van Elsas 2009). The soil matrix acts as a migratory barrier to non-hyphal bacteria. However hyphal bacteria may overcome this constraint by crossing the air-filled spaces by hyphal/mycelial growth (Schafer, Ustohal et al. 1998). Filamentous organisms also benefit from their ability to transfer carbonaceous compounds over the whole distance of the organism providing resources across the whole hyphal matrix, thus allowing them to cross nutrient-poor sites (Nazir, Warmink et al. 2010).

The growth and metabolism of microorganisms in soil can lead to changes in pH, redox potential and the ionic strength of soil (Haferburg and Kothe 2007). Soil microorganisms also have a major influence on particle aggregation as well as soil texture and the availability of nutrients for plants (Barto, Alt et al. 2010). The effect of microorganisms on soil composition

is due to the production of compounds that change the microenvironment, e.g. by the solubilisation of minerals (Cole 1979), or by the modification of soil structure, e.g. by the biogeochemical cycling of elements such as sulphur (Schippers, Von Rege et al. 1996).

1.5.2 Soil metals

Soils are naturally low in metal content. Heavy metals, such as arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin, and thallium, occur naturally but rarely in toxic concentrations (Ross 1994). However, heavy metal contamination of vast areas of urban and agricultural soils has resulted from anthropogenic sources, i.e. metalliferous mining and smelting, industry, atmospheric deposition, agriculture and waste disposal (Ross 1994). Metals in soil, which do not have biological function, are usually tolerated only in minute concentrations. Biologically important metals, however, tend to be tolerated in much higher concentrations (Haferburg and Kothe 2007). An excess of heavy metal accumulation in soil is toxic to humans and other organisms.

Swansea, also referred to as Copperopolis, has a long history of heavy metal contamination. Over 250 years of metal smelting has left metal-rich slag across the local area and aerial metal pollution over the wider landscape (Morley and Ferguson 2001; Walsh, Blake et al. 2007). Elevated heavy metal concentrations can remain a problem for long periods after mining and smelting activities have ceased (Hudson-Edwards, Macklin et al. 1999). Heavy metal concentrations around the Swansea area have steadily decreased over recent years in stream waters (Walsh, Thornton et al. 2000) due to landscape rehabilitation since the 1960s (Bridges and Morgan 1990) and also industrial decline. Storm events remain problematic however (Blake, Walsh et al. 2003).

The impairment of biological activity in soils due to metal loading can lead to a reduction in decomposition and turnover rates of organic matter (Debosz, Babich et al. 1985), causing a reduction in primary production (Ruhling and Tyler 1973). A high concentration of heavy metal in soil will increase the intracellular concentration within a microbe (Lopez-Maury, Garcia-Dominguez et al. 2002). The consequences of this may include inhibition of enzymes or DNA damage via the production of reactive oxygen species or irreversible binding to the

active centres of enzymes (Lopez-Maury, Garcia-Dominguez et al. 2002). As discussed previously, microbiological activity has a major affect on soil composition. For example, *Acidithiobacillus spp.* oxidises pyrite, which results in a drastic decline in soil pH. This subsequently results in a higher mobility of heavy metals (Haferburg and Kothe 2007).

Total soil metal concentrations are poor indicators of the true concentration in the soil solution to which soil microorganisms are exposed. Bioavailability is the proportion of total metals that are available for incorporation into biota (John and Leventhal 1995). Bioavailabilty is not directly correlated to total metal concentrations. For example, sulphide minerals, although in high concentrations, may be encapsulated in quartz or other chemically inert minerals and therefore would not be readily available to microorganisms (Davis, Drexler et al. 1993). The bioavailability of metals in soil is influenced by many factors, these include: the constitution of the soil matrix, climatic conditions, microbial activity, and especially the water flow (Haferburg and Kothe 2007). Factors also include pH and soil texture, which can strongly influence metal bioavailability. These factors are sometimes taken into account when establishing permissible limits for soil metal concentrations (Giller, Witter et al. 2009).

A major influence on the solubility of metals in soil comes from microbial metabolism and growth (Haferburg and Kothe 2007). The relationship between microbes, the environment and metal solubility is complex due to the amount of factors involved (Fig 1.3). Metals are solubilised by microbes by, for example, heterotrophic leaching, metabolic excretion and redox reactions. Redox reactions are important in controlling the chemical speciation and toxicity of a number of contaminant metals, notably As, Se, Cr, Pu, Co, Pb, Ni, and Cu (Sparks 2001). Soluble metal compounds effect microbes by biosorption, transport, intracellular sequestration and/or precipitation. The effects of insoluble metal compounds on microbes include particulate adsorption and entrapment by mycelial networks. Microbes may immobilise metals via precipitation or reduction (Haferburg and Kothe 2007).

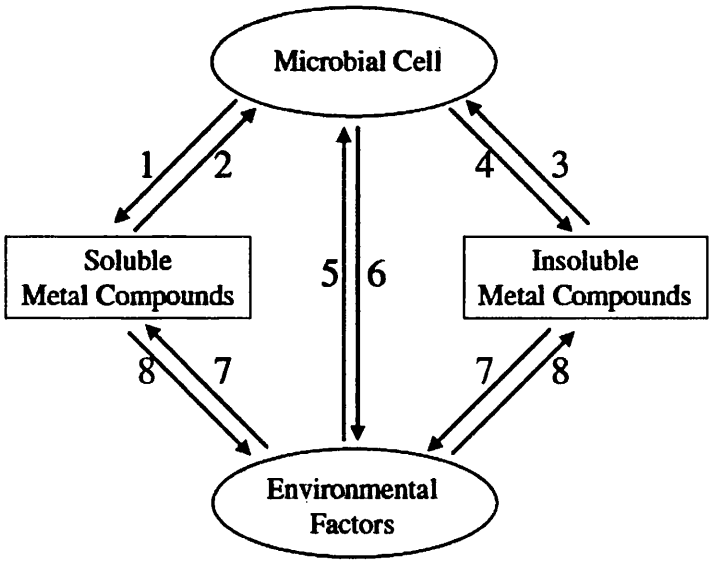


Figure 1.3 *Interaction of metal mobilization and fixation. (1) Metal solubilisation, (2) Effects of soluble metal compounds on microbe, (3) Effects of insoluble metal species on microbes, (4) Metal immobilization, (5) Influence of environmental factors, (6) Influence of microbial activities on the environment, (7) and (8) Environmental factors, which direct the equilibrium between soluble and insoluble metal species (Adapted from Haferburg and Kothe 2007).*

Metals can influence secondary metabolism in actinobacteria and other prokaryotic taxa as well as fungi (Chakrabarty and Roy 1964; Weinberg 1990). Metals can have a poorly understood effect on secondary metabolite production. For example, in *S. galbus*, an increase in the production of an antifungal antibiotic was noted upon the addition of copper, zinc and iron to the media, whereas production slowed with the addition of nickel and cadmium (Paul and Banerjee 1983; Raytapadar, Datta et al. 1995). Chromium has a stimulatory affect on the production of actinorhodin in *S. coelicolor* in SY liquid media (Abbas and Edwards 1990) as does cobalt on coumermycin A1 production in *S. rishiriensis* in liquid media (Claridge, Rossomano et al. 1966). Secondary metabolite production (of a reddish pigment with napthoquinone-like structure) is induced by the rare earth element ytterbium in *Streptomyces* YB-1 (Kamijo, Suzuki et al. 1999).

Kawai et al reported in 2007 the overproduction of antibiotics in *S. coelicolor*, *S. antibioticus* and *S. griseus* upon addition of rare earth metals to the media. A rare earth metal may be

one of a group of 17 elements including scandium, yttrium and the lanthanides. These metals are important in the chemical industry for the production of permanent magnets, fluorescent materials and in many electronics (Kawai, Wang et al. 2007). In GYM and SPY media, scandium caused antibiotic overproduction by 2–25-fold when added at low concentrations (10–100 mM) to cultures of *S. coelicolor* (Act), *S. antibioticus* (actinomycin) and *S. griseus* (streptomycin). Scandium also induced production of actinorhodin (a usually dormant system) in *S. lividans*. The effects of scandium were identified at transcription level with a marked up-regulation of actII-ORF4 in *S. coelicolor* (Kawai, Wang et al. 2007).

1.5.3 Siderophores

A siderophore, literally meaning 'iron carrier', is a ferric iron (FeIII) specific chelating agent produced by bacteria and fungi growing under low iron conditions (Neilands 1995). Their role is to scavenge iron from the local environment (in the case of saprophytes) or host (in the case of commensals and pathogens) and to make it available to the microbial cell (Neilands 1995). Siderophore production is exclusive to microbes; they are not produced by plant/animal metabolism (Neilands 1995). Plants have evolved two strategies to take up Fe from the soil. Upon Fe starvation, non-grasses activate a reduction-based strategy (proton release, FeIII chelate reduction or Fe^{2+} transport), whereas grasses activate a chelation-based strategy, a strategy similar to that of siderophore production (Kim and Guerinot 2007).

Iron has a variety of functions in the growth of a microorganism, for example for the formation of haem, for the reduction of ribotide precursors of DNA amongst others (Miethke and Marahiel 2007). Over 100 enzymes acting in primary and secondary metabolism possess iron-containing cofactors, for example, iron-sulphur clusters or heme groups (Miethke and Marahiel 2007). A total iron concentration of at least 1μM is needed for optimum growth (Neilands 1995). However, even though iron is one of the earth's most abundant elements, it is usually in an unavailable state. Atmospheric oxygen reacts with surface iron converting it to oxyhydroxide polymers with very low solubility making it unusable for most microorganisms. At best, the amount of ferric ion in bioavailable form in

biological systems (at neutral pH) is 10^{-18} M (Neilands 1987). A siderophore can form a soluble Fe(III) complex by binding to it with a high affinity and is shuttled back into the microbial cell. Once Fe(III) is complexed with the secreted siderophore molecule, it becomes accessible for cellular uptake. The iron is then either released by reduction from the siderophore at the extracellular surface and is taken up as a single ion or the whole complex is internalised (Miethke and Marahiel 2007). Bacterial uptake of Fe-siderophores complexes depends on ABC-type transporters (Sutcliffe and Russell 1995). For gram-negative bacteria, the Fe-siderophore complex is first recognised by outer membrane receptors. The delivery of the iron-siderophore complex into the cytoplasm depends on ABC-type uptake systems that generally possess extracytoplasmic substrate-binding proteins, which are usually located in the periplasm in gram-negative bacteria and are present as lipoproteins tethered to the external surface of the cytoplasmic membrane in gram-positive bacteria (Sutcliffe and Russell 1995).

It has been noted that almost all aerobic and facultative anaerobic microorganisms produce at least one siderophore (Neilands 1995). Without the use of siderophores, iron may be assimilated by the reduction of other ferric species at the membrane surface, a reduction in pH or extraction of protein-complexed metal (Neilands 1987). Genome sequencing of *S. coelicolor* identified four clusters for putative siderophore biosynthesis. Two clusters (*sco0482-0499* and *sco7681-7683*) were indicated to produce compounds that catalyse novel siderophores coelichelin and coelibactin. Desferrioxamines G1 and E are biosynthesised products of *sco2782-2785* whilst *sco5799-5801* is suspected to produce the biosynthetic machinery to synthesise a siderophore synthase. Grown under iron-limitation conditions, *S. coelicolor* excretes desferrioxamine B, desferrioxamine E and coelichelin (Baróna-Gomez, Wong et al. 2004; Lautru, Deeth et al. 2005).

Three siderophore complex uptake systems have been identified in *Streptomyces coelicolor*, DesEF, CchCDEF and an uncharacterised system. *desE* encodes a ferric-siderophore lipoprotein receptor and *desF* a ferric-siderophore hydrolase. The four *cch* genes encode a ferric-siderophore uptake system similar to those found in other Gram-positive bacteria, consisting of a lipoprotein receptor (CchF), an ATPase (CchE) and two permeases (CchC and CchD). The third system is uncharacterised but is identified due to desferrioxamine B stimulating significant growth in a mutant lacking both the *cch* and *des* clusters (Barona-

Gómez, Lautru et al. 2006). Coelibactin is an uncharacterised NRPS-generated peptide with predicted siderophore activity. The coelibactin gene cluster is controlled by Zur, a zinc-responsive transcriptional repressor. Studies with mutant strains indicated that deregulation of coelibactin biosynthetic genes inhibited sporulation by suppressing Zur (Kallifidas, Pascoe et al. 2010).

1.5.4 Study of soil microbial ecology

To study soil ecology, mesocosms and microcosms have long been used as powerful research tools due to the complex nature of soil (Moore, de Ruiter et al. 1996). With use of these cosms, soil and soil organisms can easily be manipulated and studied under controlled laboratory conditions. A soil microcosm is a simplified laboratory system, typically 1-100 gram of soil, set up to emulate soil in the environment but in an artificial way. Mesocosms are cosms on a larger scale, designed to bridge the gap between laboratory studies and field studies. Mesocosms are usually taken directly from the field, containing a full complement of organisms from the soil community (Elliott 1986).

Microcosms have been used to study soil organisms as most soil organisms are very small (microbes to small arthropods of <1mm) and so microcosms are an effective tool to studying soils under controlled conditions. Whole communities of these can be brought into the laboratory to study with the use of microcosms, due to the scale of the habitats (spore spaces to m^2) and their generation times of minutes to weeks (Moore, de Ruiter et al. 1996). Soil ecologists have also adopted a systems approach to study complex interactions of communities of species and their associated biological, chemical, and physical processes with the aid of models. These models have been developed with the use of microcosms to provide information on how soil organisms interact under a variety of environmental conditions (Elliott 1986).

Although microcosms are vital in soil ecology research, they are simplified systems and are limited with regards to soil composition (deficient in the mineral component), biotic composition (lacking in a root system and reduced biodiversity) and altered microclimate. The reliability of microcosms was discussed by Taylor and Parkinson 1988. Attempts have

been made to standardise microcosms (Van Straalen and Van Gestel 1993), producing more reliable and reproducible results for further studies.

1.1.5 Soil transcriptomics

Soil transcriptomics enables the study of compositions and dynamics of diverse microbial communities and the RNA level (Lu, Rosencrantz et al. 2006; Wang, Shimodaira et al. 2008). However, low extraction levels and the co-extraction of contaminants that inhibit downstream reactions make the process difficult. The analysis of rRNA provides little information on the functional status of the soil community yet the study of mRNA can elucidate the expressional profile of functional genes (Handelsman, Tiedje et al. 2007). However the amount of mRNA in a total extraction equates to only 1-5% (Neidhardt and Umbarger 1996). Furthermore, these valuable molecules are more susceptible to degradation by RNases than the more robust rRNAs (Andersson, Isaksson et al. 2006). Methods have been described which evaluate the effectiveness of contaminant removal, yield and integrity of total RNA and in particular the enrichment of mRNA (Mettel, Kim et al. 2010; Gilbert and Hughes 2011).

1.5.6 *S. coelicolor* in soil studies

Even though most studies involving *Streptomyces* have been performed using pure laboratory cultures and molecular tools, some studies have been performed on *Streptomyces* in soil. *Streptomyces* have been implemented in enhanced degradation of oil for bioremediation in sediment microcosms (Bachoon, Araujo et al. 2001). The study used phylogenetically nested probes (16S rRNA) in hybridization assays to monitor changes in the microbial communities of petroleum hydrocarbon (PHC)-contaminated sediment amended with bioremediation products. The relatively high proportion of *Streptomyces* in treatment N (a mineral salt mixture), compared to other bioremediation treatments along with enhanced PHC degradation in the N-treated microcosm suggested that *Streptomyces* adapt well to oil contamination (Bachoon, Araujo et al. 2001).

S. rimosus microcosm studies have resulted in development of a biomarker for detection of the antibiotic oxytetracycline and an approach developed to study interactions between antibiotic producers and their hosts (Hansen, Ferrari et al. 2001). The biosensor contained a plasmid containing a transcriptional fusion between the *tetR*-regulated P*tet* promoter from Tn*10* and a FACS-optimized *gfp* gene. The plasmid produces green fluorescent protein (GFP) in the presence of tetracycline (Hansen, Ferrari et al. 2001). This tetracycline biosensor was used to detect the production of oxytetracycline by *S. rimosus* in sterile soil microcosms (Hansen, Ferrari et al. 2001). The tetracycline-induced GFP-producing biosensors were detected by fluorescence-activated cell sorting (FACS) analysis (Hansen, Ferrari et al. 2001).

Metabolic activities of two strains of *S. griseus*, CAG17 and 26K, have been determined in sterile soil microcosms (Katsifas, Koraki et al. 2000). The strains were isolated from an agricultural soil and a forest soil, respectively. The fitness of each strain was studies by inoculation in a microcosm of soil different to its origin. Respiration rates and percentage germination were determined to be at maximum level within the first 48hr (Katsifas, Koraki et al. 2000). The study also revealed the effect of temperature on life cycle in relation to soil type where lower temperatures favour extended germination but less spore numbers in foreign soil compared to soil of origin (Katsifas, Koraki et al. 2000).

Soil microcosm studies have also lead to the discovery that giant plasmids are important in gene transfer between *Streptomyces* and its environment (Ravel, Wellington et al. 2000). The plasmids pRJ3L and pRJ28, which encode mercury resistance, were transferred in amended microcosms from the isolates CHR3 and CHR28, their streptomycete hosts, to a mercury-sensitive strain of *S. lividans* (TK24). Pulsed-field gel electrophoresis and restriction digests confirmed the plasmid identity. Hybridisation analysis revealed the functional presence of the mercury resistance gene in the transconjugants (Ravel, Wellington et al. 2000).

Microcosm studies also lead to the discovery that *S. exfoliatus* can be used as a biocontrol agent for cyanobacteria (Sigee, Glenn et al. 1999). Cyanobacterial blooms can have an adverse effect on water quality, lake ecology, livestock as well as contamination of human water supplies. Typically used algicides can be expensive and damaging to the environment. In this study, Sigee et al investigated the characterisation of anti-cyanobacterial activity of *S.*

exfoliatus and the protozoans *Nuclearia delicatula* and *Nassula tumida* in microcosms and optimised their effectiveness as biocontrol agents to produce a lake management strategy (Sigee, Glenn et al. 1999).

1.6 Proteomics

The proteome can be described as the proteins expressed by a genome. It is not a fixed feature in an organism, like a genome. It changes with every step in development and in response to environmental conditions (Wilkins 1997). The proteome has also been described as the molecular phenotype of an organism (Biron, Loxdale et al. 2006). Transcriptomic studies measure message abundances rather than the functioning end product molecule, whilst proteomic studies reveal the end products of the genome. Most activities in a living cell are performed by proteins; therefore the study of metabolism and other functions within a cell proteomics holds the key (Wasinger, Cordwell et al. 1995). Knowledge of genome sequences aids protein identification and protein characterisation in studied organisms (Westermeier, Naven et al. 2002).

Proteomic studies can be divided into three categories: expression proteomics, functional proteomics and proteomic-related bioinformatics (Westermeier, Naven et al. 2002). This study focused on expression proteomics, where a complex mixture of proteins is studied simultaneously to look for qualitative and/or quantitative changes in expression levels. Expression studies require the separation of complex protein mixtures followed by some sort of relative quantification and identification of proteins of interest. Separation techniques include 1/2D gel electrophoresis or chromatography (usually liquid chromatography for protein separation) (Twyman 2004). The scheme of work for this study included GelCMS analysis with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) separation, trypsin digestion and liquid- chromatography linked mass spectrometry.

A cell's proteome is extremely complex, consisting of thousands of proteins (Westermeier, Naven et al. 2002). Analysis is therefore totally dependent on an efficient separation technique. 1D SDS PAGE separates proteins on a gel matrix according to their molecular

weights. Gel electrophoresis can be used quantitatively using gel analysis software where spots/bands are quantified by intensity.

1.6.1 Soil proteomics

As explained in section 1.5 Soil and soil ecology, soil is a diverse and dynamic system in terms of its biochemical characteristics and the interaction between biological, physical and chemical components. Total protein fractions in soil originate from a variety of sources including microorganisms (both prokaryotic and eukaryotic) as well as multicellular organisms and anthropogenic waste (Ogunseitan 2006). The extensive diversity or microorganisms in soil along with its heterogeneity and hydrophobic nature makes the study of total proteins difficult (Van Elsas, Tam et al. 2006), therefore methods are often optimised to recover proteins of each soil fraction to different degrees. Exhaustive recovery often compromises protein activity or secondary structure whereas a more gentle approach to maintain or preserve function may not achieve complete recovery. Proteins can be absorbed to soil particles (particularly clay) and soil organic matter deeming them immobile and hampering their recovery. The remaining proteins are located in one of three major categories. Firstly, intracellular proteins are found within the cell membranes of microorganisms or plant and animal cells. Secondly, extracellular proteins that are from lysed cells, secreted enzymes or other bioactive proteins that are free in soil solution. Thirdly, proteins may be located on cell surfaces acting as exoenzymes or for the active excretion of toxic metabolites out of the cell (Ogunseitan 2006).

Metaproteomics is an invaluable tool for understanding the complex microbial community in soil. For successful metaproteomic analysis of soil microbes, cells must be lysed in the soil sample and proteins extracted without introducing bias (by loss of proteins) but it is also important to limit the co-extraction of contaminants that inhibit downstream processes. Humic substances, for example, have been shown to degrade proteins (Solaiman, Kashem et al. 2007) and can also suppress peptide signals in LC-MS (Chourey, Jansson et al. 2010). To overcome interfering substances sequential extraction methods have been devised to

obtain clear protein profiles including the use of citrate and SDS buffers followed by phenol extraction (Chen, Rillig et al. 2009).

The extraction method is highly influential in the success of soil metaproteomic analysis (Nannipieri and Smalla 2006; Ogunseitan 2006; Benndorf, Balcke et al. 2007). Protein extraction from soil may be direct from the soil or indirect where the microorganisms are first extracted from the soil matrix. Chourey et al developed both direct and indirect methods resulting in the identification of greater than 500 unique intracellular, extracellular and cell surface proteins from the inoculated organisms, *P. putida* (Chourey, Jansson et al. 2010).

1.7 Introduction to methods

1.7.1 RNA amplification

The method of RNA isolation holds an important key to the RNA quality and quantity. The quality and quantity of RNA can be greatly improved with careful sample handing, reducing the risk of denaturing RNases. Commercial kits offer a range of products and procedures to ensure the highest quality and quantity of RNA recovery, although RNA yield is often low.

RNA amplification is an important tool when starting material is scarce as is frequently the case in environmental samples (Gao, Yang et al. 2007). When the starting quantity of RNA is low in a sample, RNA amplification is needed to provide sufficient material for gene expression analysis (Gao, Yang et al. 2007). Random polymerase chain reaction-based amplification may not be appropriate due to amplification bias and therefore the loss of reliable information (Gao, Yang et al. 2007). For eukaryotic gene expression profiling, a T7 polymerase-based linear amplification procedure is frequently used to generate copy DNA (cDNA) or amplified RNA (aRNA) producing accurate relative mRNA levels from low starting quantities (Xiao, Wang et al. 2002; Li, Adams et al. 2003; Xiang, Chen et al. 2003). This method, however cannot be applied directly to prokaryotic mRNAs as prokaryotic mRNAs generally lack a poly(A) sequence. Studies have shown a T7 polymerase-based amplification procedure to produce reliable quantitative data for a range of mRNA species (Gao, Yang et

al. 2007) making it an appealing option for prokaryotic studies. The amended procedure involves either attaching a T7 RNA promoter sequence to poly(dT) oligonucleotides (emulating eukaryotic mRNA by producing polyadenylated bacterial RNA with polyA polymerase) or attaching T7 promoter sequences to random hexamers for use in reverse transcription of the prokaryotic mRNAs. The resulting DNA acts as a template for amplification with T7 RNA polymerase for linear amplification. The outline of each procedure is demonstrated in Fig 1.4.

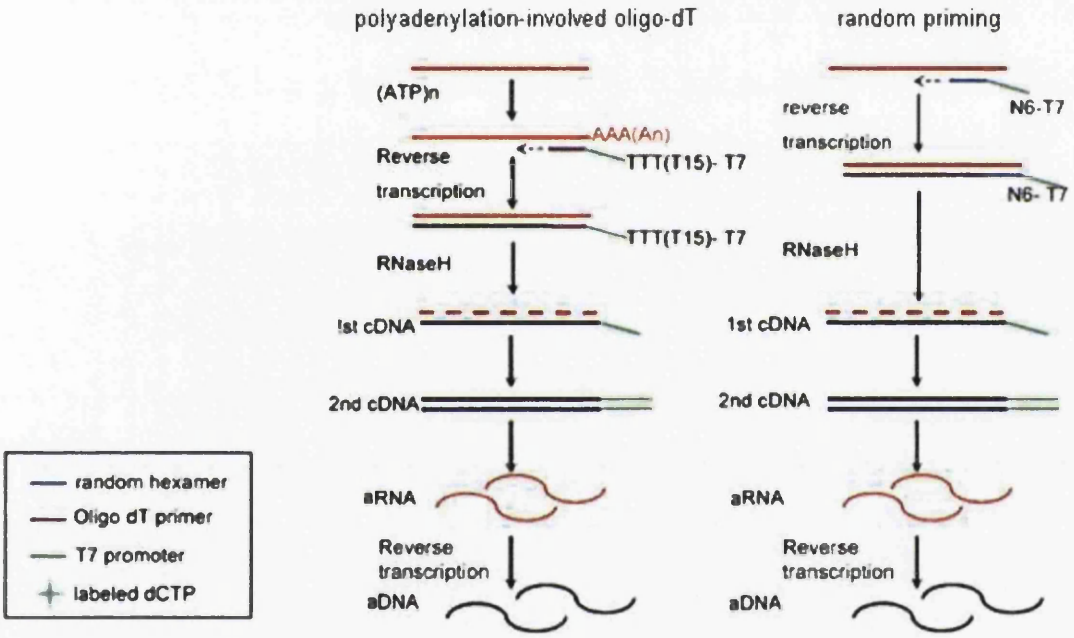


Fig. 1.4 *Schematic representation of RNA target preparation using polyadenylation involved oligo-dT method and random-priming amplification approach. RNA is shown in red, and DNA is shown in black Adapted from Cao et al 2010 (Cao, Liu et al. 2010).*

The generation of double stranded cDNA (ds-cDNA) is a critical step in RNA amplification. Generated ds-cDNA is used as a template for *in vitro* transcription. The process was first based on the work of Gubler-Hoffman (Gubler and Hoffman 1983) but was later optimised

by Van Gelder and Eberwine (Van Gelder, von Zastrow et al. 1990; Eberwine, Yeh et al. 1992). RNase H digestion creates short fragments of RNA from the RNA-cDNA hybrid. These fragments act as primers for DNA polymerase I to initiate second strand cDNA elongation. The fragments of ds-cDNA are ligated together using *E. coli* DNA ligase. The ds-cDNA is polished using T4 DNA polymerase, which eliminates loops and form blunt ends. This process may introduce bias via 5' under-representation (Wang 2005), although this under-representation may be overcome by hairpin loop second strand synthesis (Kacharmina, Crino et al. 1999). The low temperature at which the ds-cDNA synthesis takes place has been suggested to introduce additional bias (Phillips and Eberwine 1996). Overall, however, the fidelity and reproducibility of this method have been extensively validated from a range of samples (Eberwine, Yeh et al. 1992; Phillips and Eberwine 1996; Li, Adams et al. 2003; Li, Li et al. 2004; Park, Cao et al. 2004; Rudnicki, Eder et al. 2004).

The desire to amplify RNA without skewing relative transcript abundance has led to the development of linear amplification methods. These maintain the proportionality of each RNA species between the sample RNA and the amplified RNA. The ds-cDNA with attached T7 promoter sequence is transcribed *in vitro* producing large amounts of amplified RNA. The amplified RNA can be either sense or antisense according to the position of the promoter sequence. Oligo-dT attachments during first strand synthesis lead to the promoter sequence being at the 5' end of the cDNA (3' end of the gene) resulting in amplification of antisense RNA. However, the use of random primers for first strand synthesis position the promoter at the 3' end of the copy DNA (cDNA) (5' end of the gene) generating sense RNA (Marko, Frank et al. 2005).

1.7.2 Reverse transcription polymerase chain reaction

Reverse transcription is the process by which cDNA is produced from RNA by a reverse transcriptase. Polymerase Chain Reaction (PCR) is a molecular technique based on the amplification of nucleic acids with a low limit of detection. PCR was first developed in 1983 by Kary Mullis. Ten years later Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for their work in the development and application of the procedure. It is an

indispensible tool in molecular research with a variety of applications including DNA cloning and sequencing, gene expression analysis and DNA-based phylogeny. It is used medically for diagnosing hereditary disease, whilst DNA fingerprinting has revolutionised police work.

PCR consists of three temperature-dependent stages. Firstly, when exposed to high temperatures the DNA is denatured. This separates the double stranded sequences into single strands. Next, oligonucleotides (primers) anneal to complimentary regions of the DNA at specific temperatures. The temperature is primer specific and must be must be optimised for increased stringency (specificity). At higher temperature the primers will not anneal, at lower temperatures the primers may anneal to aspecific regions of the DNA sequence. With 100% efficiency, the amount of DNA is double the quantity of the preceding cycle. However, successful amplification of DNA also requires the optimisation of other factors which affect the efficiency of each PCR cycle. These include (along with annealing temperature) the template and primer concentrations, the concentration of magnesium ions (essential for optimal DNA polymerase activity) and the number of PCR cycles. PCR products are separated by DNA (agarose) gel electrophoresis and visualised by staining. Most commonly, ethidium bromide is used as a fluorescent tag. Ethidium bromide is aromatic compound which intercalates with DNA, which intensifies its ultraviolet fluorescence by 20 fold (Singer, Lawlor et al. 1999), which can be visualised under an ultraviolet light source. Due to its properties, ethidium bromide acts as a mutagen and therefore poses a health risk (Waring 1965). Consequently, alternative dyes have been developed, namely SYBR based dyes, which pose a lower risk to human health (Singer, Lawlor et al. 1999).

1.7.3 Quantitative PCR

Quantitative PCR (qPCR) measures the amount of PCR product after each cycle using fluorescent dyes, in contrast to end point PCR, which is analysed after a fixed (user defined) number of cycles (Higuchi, Dollinger et al. 1992). The theory behind the method is that the relationship between the amount of starting DNA and the amount of product produced during the process is quantitative. In comparison to end point PCR, qPCR is high throughput as there is no manipulation of product for analysis. This is a benefit also to reduce

laboratory contamination for post-PCR detection methods. Also, the highly sensitive fluorescent dyes reduce variability between reactions significantly (Heid, Stevens et al. 1996).

After each cycle the change in concentrations of DNA is detected using fluorescent molecules that interact with the DNA. This can be done in two ways, either by using fluorescently tagged probes or fluorescent dyes that intercalate with the double stranded DNA. As the DNA amplification is achieved, the signal density increases in direct correlation with how much product is being formed (Heid, Stevens et al. 1996).

During the first cycles the fluorescent signal is so low that it cannot be distinguished from the background levels. After several cycles the signal becomes greater than background and signal levels can be seen to increase exponentially. During the late exponential stage and early in the plateau stage the signal levels off and is no longer proportional to the starting template copy number (Kubista, Andrade et al. 2006).

The critical threshold (Ct) represents the number of cycles it takes to reach a threshold level of fluorescence above background levels. qPCR analysis uses the Ct value as a representation of starting template copy number as the lower the starting copy number the more cycles it will take to reach the threshold value (Fig 1.5). The threshold value is usually determined automatically but can be user defined to ensure it is in the exponential phase and the same value for all samples in the run (Kubista, Andrade et al. 2006).

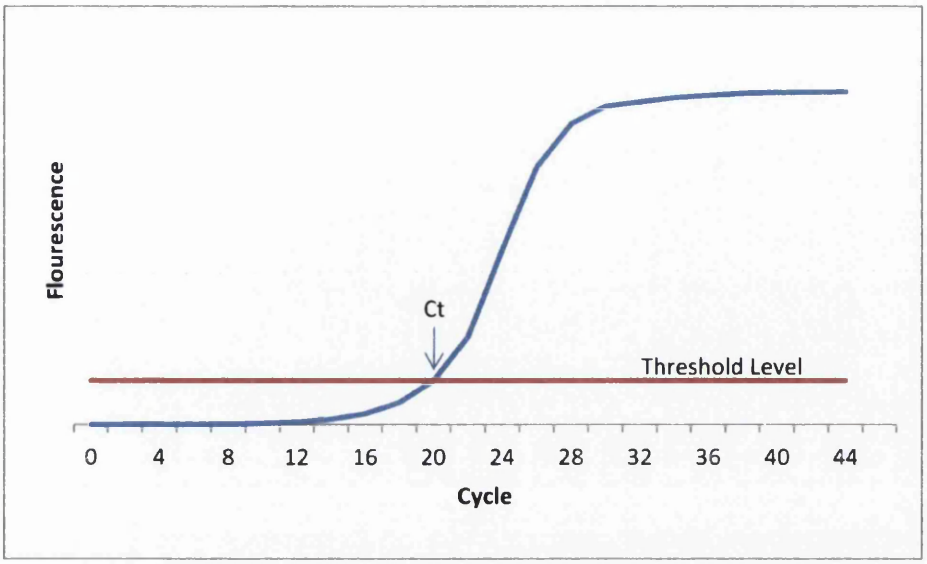


Fig 1.5 *Graphical representation of the increase in fluorescence over 45 cycles of qPCR. The Ct value and threshold level for this run are indicated.*

When performing reverse transcription-PCR, the process of formation of cDNA is critical as the amount of cDNA should be proportional to the starting RNA amount. Reverse transcription can be primed using oligo(dT) primers (for eukaryotes), sequence specific primers or random primers. Oligo(dT) primers anneal to the poly(A) tail of mRNA and so are not directly suitable for *S. coelicolor* gene expression studies. Sequence-specific primers enable specific generation of cDNA and can reduce background priming. However, they reduce the number of mRNA that can be analysed from the sample. Random primers (usually hexamers or nonamers) anneal at multiple points on any RNA (tRNA, rRNA or mRNA). They allow for total reverse transcription and are ideal for prokaryotic studies. The combination of random primers for reverse transcription to cDNA with a second stage of PCR specific PCR primers are used to create specific expression data.

There are two groups of detection chemistries for qPCR. The use of DNA-binding fluorophores is the cheapest and easiest way to perform qPCR. The binding of the fluorophore to DNA is nonspecific, making it ideal for work where there is little information known about the target sequence, due to non-specificity the procedure also requires less optimisation. The fluorophores have low fluorescence when unbound in the qPCR mix.

When associated with DNA they start to fluoresce brightly when exposed to a particular wavelength. This is usually detected by the machine after the extension step in each cycle to monitor DNA amplification. SYBR Green is the most common fluorophore used in qPCR (Ginzinger 2002). A disadvantage to using these fluorophores is that they fluoresce in the presence of any dsDNA including primer dimers and which may lead to false positive results.

To detect the presence of primer dimers or other aspecific PCR products a method called 'melt curve analysis' is used. A melt curve is produced at the end of a qPCR run by measuring fluorescence over a temperature gradient. When the dsDNA is denatured there is a sudden drop in fluorescence as the fluorophore is released. A melting peak is produced by plotting dF/dT (where F is fluorescence and T is time) against temperature. A peak is seen at the melting temperature of the dsDNA. If the PCR is specific and has no primer-dimers, only one peak should be seen. If other dsDNA products are present then more peaks will be showing (Rasmussen, Morrison et al. 1998; Mackay 2004; Espy, Uhl et al. 2006).

Primer design is very important to ensure good qPCR. There are several rules that allow for optimal qPCR including; 15-25 base pair length, a low G+C content (as G/C have stronger bonds and may anneal aspecifically), a closely matched melting temperature (Tm) of forward and reverse primers with no more than 2 G/C bases in the last 5 bases at the 3' end of the primer. Also, primers should be checked to avoid secondary structures because they adversely affect primer-template annealing.

qPCR is a valuable tool for both DNA detection and mRNA analysis in soil although the carryover of humic acid from extraction inhibits both reverse transcriptase and DNA polymerase (Mendum, Sockett et al. 1998). qPCR assays have quantified the abundance of dominant groups of bacteria and fungi in soil, assess the soil microbial community structure (Fierer, Jackson et al. 2005). Application of qPCR techniques have also included disease risk assessments for the economically important potato blemish disease causing fungus *Helminthosporium solani* (Cullen, Lees et al. 2001) and for the detection of *Burkholderia* (Trung, Hetzer et al. 2011; Bergmark, Poulsen et al. 2012) and *Pseudomonas* in soil samples (Bergmark, Poulsen et al. 2012), both of which can cause a potential bio-threat. Due to the challenges with isolating RNA from soil, relatively few microbial gene expression studies have been attempted (Saleh-Lakha, Shannon et al. 2011). Some studies focus on indigenous

microbial communities whereas others use sterile soil and inoculate their microorganism of interest. For example, Baelum et al focussed on bioremediation and characterised expression of *tfdA* gene involved in phenoxyacetic acid degradation by indigenous bacteria in agricultural soil (Bælum, Nicolaisen et al. 2008). Henderson et al utilised qPCR to elucidate the effect of red clover, soybean, barley plant residues and glucose on denitrification gene mRNA levels also on indigenous bacteria (Henderson, Dandie et al. 2010). Using sterile soil inoculated with *Rhodococcus*, Wang et al detected gene expression of *bphAa*, a gene involved in biphenyl degradation (Wang, Shimodaira et al. 2008).

1.7.4 Separation of proteins

In expression studies, the study of the proteome requires the separation of complex protein mixtures extracted from a sample followed by relative (or less commonly absolute) quantification and identification of proteins of interest. Separation is usually achieved via 1/2D gel electrophoresis or chromatography (usually liquid chromatography with proteomics) (Twyman 2004).

Gel electrophoresis has been the principle technique for high resolution protein separation (Rabilloud 2002). 1D gel electrophoresis (native or SDS PAGE) separates proteins on a gel matrix according to molecular mass. O'Farrell in 1975 introduced the addition of a second dimension, proteins may also be separated according to their isoelectric point (O'Farrell 1975).

For SDS-PAGE, the sample to be analysed is first mixed with SDS. This acts as an anionic detergent to denature secondary and non–disulfide linked tertiary structures while also applying a negative charge to each protein in proportion to its mass. The samples are boiled to further promote protein denaturation, and to aid with the binding to SDS. A tracking dye may also be added at this step, usually bromophenol blue, to track the front through the gel. For SDS-PAGE, two gels are generally used: a top stacking gel and a lower separating or resolving gel. The stacking gel is slightly acidic (pH6.8) with a low acrylamide concentration (around 5%). This makes the gel more porous, making separation poor but allowing the proteins to form thin, sharp bands. The separating gel is more basic, with a higher

acrylamide concentration (10-16%). The narrower pores allow smaller proteins to travel quicker and more easily than the larger proteins, therefore separating them by mass (Berg, Tymoczko et al. 2002).

Gel electrophoresis can be used quantitatively using image analysis software that quantifies spots/bands by intensity, which is related to quantity. For mass spectrometry (MS) analysis, liquid chromatography (LC) is also linked for further separation after SDS-PAGE.

1.7.5 Peptide generation and cleanup

For most applications of protein identification by mass spectrometry, the proteins of interest must first be cleaved into constituent peptides. Proteolysis is the routine method for protein cleavage in proteomic studies, with a wide range of enzymes available, including endoproteinase, Glu-C, chymotypsin, elastase, pepsin and the most commonly used enzyme, trypsin (Westermeier, Naven et al. 2002). Trypsin is highly specific, cleaving at the C-terminus of arginine and lysine, except when followed by proline, yielding tryptic peptides. The restriction of basic residues to the C-terminal end enables more efficient fragmentation during MS/MS. In-gel digestion is common practise yielding best results with proteins in concentrations of above 10pmol, with the number and yield of peptides dropping significantly below 5pmol (Staudenmann, Hatt et al. 1998).

After peptide generation, the peptide solutions need to be cleaned and desalted. This is achieved by reverse phase C18 chemistries in the form of bonded silica. When passing over the column, peptides in aqueous solution bind to the C18-derivatised surfaces by hydrophobic interaction. This allows contaminating/interfering molecules such as salts and buffers to be washed away (Bagshaw, Callahan et al. 2000). The peptides are eluted from the column using an appropriate solution, usually a dilution of acetonitrile.

1.7.6.1 Mass spectrometry

Mass spectrometry is an analytical technique that determines the mass of an ion as a mass to charge ratio. The mass/charge ratio can be used to determine the elemental composition of a molecule and for the elucidation of chemical structures (Sparkman 2006). The development of instrumentation and understanding has lead to mass spectrometry becoming an invaluable analytical tool for many industries including biotechnology (analysis of proteins, peptides and oligonucleotides), pharmaceutical (drug discovery and metabolism, biomarkers, pharmacokinetics), clinical (drug testing, neonatal screening, haemoglobin analysis), environmental (water quality, food contamination) and geological (oil composition). Mass spectrometry can be used to produce accurate molecular weight measurements, reaction monitoring, amino acid sequencing, oligonucleotide sequencing and protein structure elucidation.

A mass spectrometer consists of four sections. Each section can have many variations depending on which mass spectrometer being used. Firstly there is sample introduction, followed by ionisation, then a mass analyser and finally a detector. A computer software program is used to produce and interpret the results.

There are many ways in which the compound of interest can be introduced into the mass spectrometer. A direct insertion probe or infusion pump inserts sample directly. However, if coupled to a chromatography device the samples can be separated into components before entering the mass spectrometer for individual analysis. This is performed via high-pressure liquid-chromatography (HPLC) (Arpino, Baldwin et al. 1974), gas chromatography (GC) (Harrison 1987) or capillary electrophoresis (CE) (Smith, Wahl et al. 1993) separation.

The ionisation source produces either a positive or negatively charge ion from the sample. The ionisation method of choice is dependent on the type of sample for analysis. Examples of ion sources include matrix-assisted laser desorption ionisation (MALDI), electron ionisation, chemical ionisation, atmospheric pressure chemical ionisation, fast atom bombardment, electrospray ionisation (ESI) and field desorption/field ionisation.

After ionisation, the ions travel to the mass analyser which may be a quadrupole, time of flight tube, an ion trap or a magnetic sector. The mass analyser separates (resolves) ions

formed in the ionisation source according to their mass-to-charge (m/z) ratios. The detector then monitors the ion current and transmits the data to a computer system where mass spectra are produced. Detectors include a photomultiplier, an electron multiplier and a micro-channel plate detector. The spectra are produced when the m/z values are plotted against its abundance compared to the other ionised particles in the sample.

Further discussion of mass spectrometry will be confined to methods used in this study.

1.7.6.2 LC ESI MS/MS

Liquid chromatography was first coupled with mass spectrometry in 1974 by Aprino permitting the separation of molecules by LC followed by the detection and identification of all components by mass spectrometry (Arpino, Baldwin et al. 1974).

The hyphenation of liquid separations such as HPLC and mass spectrometer was further developed into the modern day technique, electrospray, by Fenn *et al* (Fenn, Mann et al. 1989). The technique involves dissolving the sample in a polar volatile solvent. A strong electric field is applied to the liquid under atmospheric pressure as it is pumped through a narrow stainless steel capillary. The sample is dispersed into an aerosol of highly charged droplets aided by a flow of nitrogen gas flowing outside the capillary. The nitrogen flow also aids to direct flow of the particles towards the mass spectrometer. The droplet size shrinks as the solvent evaporates increasing the charge at the droplet surface. A series of small, lower charged particles are formed as the at the Rayleigh limit the Coulombic repulsion overcomes the droplet's surface tension and the droplet explodes. Eventually, individually charged analyte ions are formed which are fed into the mass spectrometer (Mann, Ong et al. 2002).

1.7.6.3 MS/MS Quadrupole-Ion trap

Quadrupole ion traps have an advantage in that they allow for multiple stages of mass spectrometry (MS^n). The performance characteristics of a quadrupole ion trap make them

highly attractive in proteomic applications interfaced with nanospray (Ogueta, Rogado et al. 2000), LC-MS/MS (Go, Prenni et al. 2003) or MALDI (Qin and Chait 1996).

The ions generated from the ionisation source are transferred to the first MS analyser (MS1), the quadrupole. The ions pass from the ESI probe to the atmospheric pressure ionisation (API) stack. The API stack includes an ion transfer tube, a tube lens and a skimmer. The ion transfer tube is used to separate the capillary from the heater to increase the efficiency of desolvating the ions. The tube lens has two functions, firstly to restrict the expansion of the ion beam from the ion transfer tube and secondly to focus the ions towards the opening of the skimmer. The latter is achieved by applying a voltage to the tube lens. The skimmer prevents large ions from entering the second MS analyser (ion trap) as well as acting as a vacuum baffle between the high pressure capillary skimmer region and the low pressure square quadrupole region. The square quadrupole traps negative ions while leaving positive ions pass through to the octopole. The octopole focuses the ions to then pass to the ion trap through an entrance electrode to which they are attracted due to a DC voltage opposite to the charge of the ions being applied. A large radio frequency traps the ions within the ion trap in a stable eight shaped trajectory. The ions exit the ion trap according to m/z ratio by increasing the voltage applied to the ring electrode and endcap electrode. Ions with increasing m/z ratios come into resonance, which alters their trajectory and exit via the entrance and exit endcaps, however, only ions exiting via the exit endcap are detected. Helium is used in the ion trap to slow ions which prevents them from leaving the ion trap. It also serves as collision gas for second phase fragmentation of the ions.

Due to the fragmentation procedures, many daughter species with varying molecular masses can be detected. The peptide mass fingerprint is a collection of all these daughter ions, which can be used to accurately identify proteins with the use of databases (Thiede, Hohenwarter et al. 2005).

1.7.6.4 Protein identification by database searching

The idea that a specific enzymatic digestion or cleavage of a protein may be used as a unique fingerprint, enabling a database search to reveal the identity of a protein, was first

suggested in 1977 (Cleveland, Fischer et al. 1977). Peptide mass fingerprinting (PMF) has lead to widespread identification of proteins and the construction of many databases from information gained from previous studies (James, Quadroni et al. 1994).

PMF starts with the digestion of a protein by chemicals or protease (in this study trypsin), producing peptides. The mass spectrometer determines the molecular masses of these peptides. These masses are searched against a set of mass profiles produced by theoretical fragmentation by the chemical/enzyme on a database to find a protein, which generates a similar pattern (Cottrell 1994).

Within the mass spectrometer, the peptides are fragmented by cleavage at the peptide bonds. There are three types of bond which can cleave: the NH-CH, CH-CO and CO-NH bonds. When a bond is broken, two species arise: one neutral and one charged species. The charged species is identified by the mass spectrometer. So-called a, b and c ions are produced when the charge is retained on the N-terminal fragment and x, y and z ions are produced when the charge is retained on the C-terminal fragment. Most commonly, the cleavage site is at the CO-NH bond, producing b and or y ions. The ion including only the first residue of the peptide is labelled 1 and the remaining ions are numbered progressively higher along the peptide chain. The mass difference between two adjacent b ions, or y ions indicates a specific amino acid residue (Downard 2004).

As mentioned earlier, there are many databases available to perform a protein search, including Mascot, MOWSE, NCBI and the database used in this study, SEQUEST.

For a selected peptide ion, SEQUEST will identify an amino acid sequence of the same measured mass and predict the expected fragmentation pattern for that sequence. The expected fragmentation pattern is compared to that generated by the MS/MS spectrum and amino acid sequences are reported. Each peptide suggested by the database is assigned a score, including the X correlation score, the charged state of the peptide and the Delta correlation score (amongst others). The X correlation score values the correlation between the observed spectrum and the theoretical spectrum for the sequence. The Delta correlation describes how different the first peptide match is from the second peptide match. For peptide identification each score must be filtered to produce reliable

classification. A single MS/MS run generates many spectra. While SEQUEST can identify a peptide/protein from a single spectrum, having MS/MS spectra with matching peptides from a single protein increases the confidence in the identification (Lundgren, Martinez et al. 2009).

1.8 Aims and objectives

This study aims to identify which *S. coelicolor* secondary metabolite genes are expressed under environmental conditions, in particular in its natural habitat soil and sand. Factors affecting expression of these genes under environmental conditions will also be investigated, notably how nutrient availability and other biologically active organisms may affect secondary metabolite gene expression in soil. Ultimately, the results from the work described in this thesis are hoped to give an insight into how secondary metabolites contribute to *in situ* competitiveness of *S. coelicolor* in soil.

These questions will be addressed by growing *S. coelicolor* in sterile soil and sand microcosms, followed by analysing the expression of selected genes encoding for key enzymes involved in the biosynthesis of the full range of secondary metabolites by applying transcriptomic and proteomic approaches. Due to the low level of RNA extracted from soil, RNA amplification methodology will be optimised. It is hoped that this method will yield quantities of RNA sufficient for microarray analysis. For this study, amplified RNA will be used as an indicator of gene expression, through the use of endpoint RT-PCR and qPCR to reveal gene expression profiles.

This study also aims to elucidate the proteome of *S. coelicolor* in environmental conditions. Due to difficulties in extracting proteins from soil, sand will be used as an alternative media for the microcosms. In particular, the proteins involved in secondary metabolism and their central carbon metabolism precursors will be investigated. This will be achieved by GeLCMS.

Finally, this study aims to compare the transcriptomic and proteomic data for further insights into *S. coelicolor* grown under natural conditions.

Chapter 2 Methods and materials

2.1 Soil

2.1.1 Soil preparation

Soil was collected from Lower Hardingsdown Organic Farm, Gower. Swansea. The soil was spread over large trays after the removal of large stones, large organic matter (e.g. roots and grasses) and organisms e.g. worms. The soil was left for 72h to air-dry, with a rotation after 24h. After air drying, the soil was ground with a pestle and mortar to break down the larger soil aggregates. The soil was then sieved to a diameter of 2mm, autoclaved twice at 121^{0}C for 1h and kept at ambient temperature.

2.1.2 Soil water content

Soil water content was measured gravimetrically by calculating the difference in soil mass before and after oven drying. The measurement procedure started with recording the mass of a small oven proof container, followed by recording the mass after a sample of soil has been added. This was then placed in an oven at 105^{0}C for 24h. The mass was measured again until constant. The water content was calculated using equation 1:

Eq. 1 $W_{content}= ((W_{air\ dry}-W_{oven\ dry})/W_{oven\ dry})*100$

2.1.3 Soil water saturation testing

For water saturation testing, a metal cylinder container of 100cm^3 was fitted with a sheet of filter paper over the bottom and attached using an elastic band. The metal container was then filled to capacity with oven dried soil. The mass was recorded and the container was placed in a tray filled with water and left overnight. During this time, the soil filled with water to saturation point. The mass was measured again before placing the container in an oven at 105^{0}C for 24h. The oven-dried mass was then recorded. The water content at saturation could then be calculated using equation 2:

Eq. 2 $W_{content}= ((W_{saturated}-W_{oven\ dry})/W_{oven\ dry})*100$

2.1.4 Soil pH

Air-dried soil was used to measure soil pH. 25ml of 0.01M $CaCl_2$ was added to 10g of soil (or 1:25 ratio of soil:$CaCl_2$) . This was then mixed well for 10 min. The solution was then left to settle for 1h before being agitated and stirred again. After a further 1h of standing, the pH of the layer above the soil was measured using a pH meter (Hanna) calibrated with pH buffers (Sigma).

2.1.5 WDPT (Doerr 1998)

Onto the surface of approximately 1g of soil (in a Petri dish), 10µl of water was pipetted gently. The penetration time (the time for the droplet to completely absorb into the soil) was recorded.

2.2 Buffers/media

2.2.1 Bradford Reagent

For 100ml of Bradford reagent, 10mg Coomassie Blue G250 was dissolved in 5ml 95% ethanol. 10ml 85% orthophosphoric acid was added and the solution made up to a total volume of 100ml with distilled water.

2.2.2 DNA loading dye

Before loading a DNA sample onto an agarose gel, the sample was mixed with 10x loading dye. This was prepared by adding 20 mg Orange G (Fisher) to 3ml glycerol and 7ml DNase free water.

2.2.3 FA buffer

FA buffer (10x) comprised of 200mM MOPS, 50mM sodium acetate and 10mM EDTA (adjusted to pH 7 with NaOH). This was autoclaved, kept at room temperature and away from light by cover with foil (Sambrook et al 1989).

2.2.4 FA gel denaturation mix

The FA denaturation mix was prepared with a 20:34:100 ratio of 10X FA running buffer:formaldehyde:formamide (Sambrook et al 1989).

2.2.5 FA running buffer

To produce 1 litre of FA running buffer, 100ml 10X FA buffer and 20ml 37% formaldehyde was added to 880ml RNase free (double autoclaved) distilled water (Sambrook et al 1989).

2.2.6 LB agar/broth

LB broth was prepared by dissolving 10g Difco Bacto tryptone, 5g Difco yeast extract, 5g NaCl and 1g glucose in 1 litre distilled water. For LB agar, 2% agar (Melford) was added. The broth/agar was then autoclaved.

2.2.7 Liquefied phenol

Crystallised phenol was melted my mixing with an equal volume of 1M Tris HCl (pH 8) and immersing it in a water bath at 68^0C. The liquefied phenol was recovered and mixed with an equal volume of 1M Tris HCl (pH 8). This was then inverted to mix and left to stand until a clearly defined phenol:aqueous interphase was formed. The pH of a sample of the upper aqueous phase was determined my mixing 2ml sample with 8ml of methanol and 10ml

distilled water. This was mixed and the pH determined using a pH meter. If the pH was higher than 8, the liquefied phenol was again mixed with an equal volume of 1M Tris-HCl (pH8) until a pH of lower than 8 was achieved. Next, an equal volume of 0.1M Tris-HCl was added and mixed and left to stand until a clearly defined phenol:aqueous interphase formed. This aqueous layer was again removed and an equal volume of TE buffer added. This was then stored for up to 3 months at 2-8 ^{O}C, protected from the light.

2.2.8 Ringers solution

To produce Ringer's solution the following were dissolved in 1 litre of distilled water; 6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 1 mole of sodium bicarbonate is dissolved in one litre of distilled water.

2.2.9 SDS PAGE solutions

Solution B (4x separating gel buffer) was prepared using 75ml 2M Tris (adjusted to pH 8.8 with HCl), 4ml 10% SDS and 21ml distilled water. The final concentrations were 1.5M Tris-HCl and 0.4% SDS. The solution was stored at 4^{0}C.

Solution C (4x stacking gel buffer) was prepared using 50ml 1M Tris (adjusted to pH 6.8 with HCl), 4ml 10% SDS and 46ml distilled water. The final concentrations were 0.5M Tris-HCl and 0.4% SDS. The solution was stored at 4^{0}C.

Electrophoresis buffer (1x) was prepared by dissolving 3g Tris, 14.4g glycine and 1g SDS in a small volume of distilled water before being made up to 1 litre. The final concentrations were 25mM Tris, 192mM glycine and 0.1% SDS.

Sample buffer was prepared by combining 0.6ml 1M Tris (adjusted to pH 6.8 with HCl), 5ml 50% glycerol, 2ml 10% SDS, 0.5ml 2-mercaptomethanol, 1ml 1% bromophenol blue and 0.9ml distilled water. The final concentrations were 60mM Tris-HCl, 25% glycerol, 2% SDS, 14.4mM 2-mercaptomethanol and 0.1% bromophenol blue.

2.2.10 Soya flour mannitol agar

SFM was prepared by adding 2g soya flour, 2g mannitol and 2g of Lab M agar to 100ml of tap water in a 250ml conical flask (Kieser et al 2000). The flask was autoclaved at 121^{o}C for 15 min. After cooling, the SFM was poured into 90mm Petri dishes and dried.

2.2.11 Singleton et al protein extraction buffer

The extraction buffer was prepared with final concentrations of 50mM Tris-HCl, 10% sucrose, 2mM DTT, 4mM EDTA and 0.1% Brij. This was adjusted to pH 7.58 with ammonia (Singleton et al 2003).

2.2.12 T10E1 (TE buffer)

T10E1, also known as TE buffer was prepared by the addition of 1ml 1M Tris (adjusted to pH 8 with HCl) to 0.2ml 0.5M EDTA which was then made up to 100ml using distilled water. The final concentrations were 10mM Tris and 1mM EDTA.

2.2.13 Taq extraction buffers

Buffer A was prepared with 50mM Tris-HCl pH 7.9, 50mM glucose and 1mM EDTA.

Buffer B was prepared with 10mM Tris-HCl pH 7.9, 50mM KCl, 1mM EDTA, 1mM PMSF, 0.5% Tween and 0.5% Nonidet P40.

Storage buffer was prepared with 50mM Tris-HCl pH7.9, 50mM KCl, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF and 50% glycerol

2.2.14 TBE

A 10X TBE solution was prepared by dissolving 106g Tris and 55g boric acid in 500ml distilled water. Next, 40ml of 0.5M EDTA (adjusted to pH8) was added before the solution was made up to a final volume of 1L with distilled water.

2.2.15 YEME

YEME was prepared according to 'Practical Streptomyces Genetics' (Kieser et al 2000), combining 3g Difco yeast extract, 5g Difco Bacto-peptone, 3g oxoid malt extract, 10g glucose and 340g sucrose into a final volume of 1L with distilled water. The media was then autoclaved for 15 min at 121^0C.

2.3 *Streptomyces* methods

2.3.1 Spore stock production

To produce spore stocks of *S. coelicolor* M145, 70µl of concentrated spore stocks was spread evenly over SFM plates using sterile glass pipettes. These were placed in an incubator at 30^0C for four days. Sporulation was indicated by the lawn turning grey in colour.

Once the M145 plates had matured and the grey spore colour could be seen, 2ml of autoclaved 20% glycerol was added to each plate. Using a sterile quarter piece of cotton pad and sterile tweezers the spores were gently rubbed into suspension. A sterile 10ml syringe was then used to remove the spore solution through from the cotton pad, ensuring agar was not collected.

2.3.2 Spore concentration glycerol stocks

To determine the concentration of spores, spore stocks were fully defrosted and vortexed extensively to make a homogenous mixture (spores produce chains which would make the

counting inaccurate). From the spore stocks, 100x and 1000x dilutions were prepared in microcentrifuge tubes using distilled water. 70µl of the spore dilutions were added to a bacterial counting chamber (0.02mm depth, Hawksley). Spores were counted per block of 16 squares using a light microscope at 40x magnification. The number of spores per ml was calculated using equation 3:

Eq. 3 Spores per ml= $2x10^7$ x spores per little square x dilution factor

2.4 Microcosms methods

2.4.1 pH adjustments

To adjust the pH of the soil to neutral, soil was mixed with phosphate buffer and sodium hydroxide. To start 10mM, 50mM and 100mM phosphate buffer (K_2HPO_4 + NaH_2PO_4) was mixed with 10g of soil. Volumes ranged from 0.5 to 15 ml. The pH of the soil was then determined as described in Section 2.1.4. The effect of adding sodium hydroxide was also tested using 1 ml of 20-100mM NaOH.

2.4.2 Soil microcosms set up

In a sterile volumetric flask, 10g of air-dried, sieved and autoclaved soil was mixed with 1ml of 100mM NaOH, 630µl of distilled water (up to 40% water saturation) and 10^7 spores g^{-1}. This was mixed thoroughly in an aseptic manner. The mixture was placed in a sterile 12 g plastic container (Nunc, Thermo) pot and a lid created with gas permeable adhesive seals (Breathable film, Starlab). The microcosms were then incubated at 28^0C at 85% relative humidity and harvested daily for five days. Each microcosm pot was split for RNA extraction and growth curve measurements using spore cfu counts.

For nutrient amendments, 1% soluble starch (BDH Laboratory Supplies) and/or 1% crab shell chitin (Sigma) was added to the soil, which was then re-autoclaved before inoculation. For microcosm amendment with nematode extract, a solution of *Steinernema kraussei* (provided by Dr. M. A. Ansari) (>1000/ml) was blended and 100µl added to each microcosm

(in place of 100µl of water). For the fungi amendment, $1x10^7$ spores of the bioactive entomopathogenic fungus *Metarhizium anisopliae* V245 (provided by Dr. F. A. Shah) were added per gram of soil.

The metal content of the microcosms were amended individually:

359.5mg of zinc sulphate (AnalaR) was dissolved in 1 ml distilled water. This was filter sterilised, and 1µl per gram of soil was added to the microcosms resulting in a final Zn^{2+} concentration of 115ppm. 216.5mg of iron (III) oxide (Sigma) was dissolved in 1ml distilled water. This was filter sterilised and 100µl per gram of soil was added to the microcosms resulting in a final Fe^{3+} concentration of 32725ppm. 106.95mg of copper sulphate (Fisher) was dissolved in 1ml distilled water. This was filter sterilised and 1µl per gram of soil was added to the microcosm resulting in a final Cu^{2+} concentration of 38ppm. The volume of metal solution used was taken from the water allocation for each microcosm.

2.4.3 Sand microcosms set up

Sand microcosms were set up in the same manner as the soil microcosms. Sand microcosms, however, were saturated to 60% water saturation (14.2% w/v). Each microcosm was amended with 1% starch and 1% chitin as described above. The 60% saturation level was maintained by daily addition of sterile tap water.

2.4.4 Growth curve measurements of microcosms

The *S. coelicolor* biomass was first extracted from the soil by shaking 1g of microcosm soil/ sand in 9mls of one-forth strength Ringer Solution for 10 min at room temperature (Herron and Wellington 1990). Serial dilutions of these solutions were then plated onto SFM agar and colony-forming units (cfu) were determined after 2-3 days of incubation at 30^0C. For spore counts, the solutions (or appropriate dilutions of the solutions) were analysed on a bacterial counting chamber (see spore counts).

Doubling time was calculated using equation 4, where t = time interval in hours or minutes, B = number of bacteria at the beginning of a time interval and b = number of bacteria at the end of the time interval.

Eq. 4 Doubling time = t/ 3.3(log b/B)

2.4.5 Antibiotic assays

To 1g of microcosm sand 9ml of ¼ strength Ringer's solution was added. For Act determination, 3N KOH was added 1:1 to the microcosm-Ringers sample. This was shaken overnight at 4^0C before being centrifuged at 4000xg for 10 minutes. The absorbance of the supernatant was then measured at 640nm in triplicate. The total Act concentration was determined using equation 5.

Eq. 5 E_{A640}= 25,320 X M^{-1} X cm^{-1} X dilution factor

For Red determination, acidified MeOH (pH 1.5) was added 1:1 to the microcosm-Ringers sample. This was shaken overnight at 4^0C before being centrifuged at 4000xg for 10 minutes. The absorbance of the supernatant was then measured at 530nm in triplicate. The total Act concentration was determined using equation 6.

Eq. 6 E_{A530}= 100,500 X M^{-1} X cm^{-1} X dilution factor

2.5 Molecular Biology methods

2.5.1 Genomic DNA (gDNA) extraction

For extraction of gDNA, 50µl of spore stock was added to 100ml of YEME in a sterile conical flask with a 30cm coiled metal spring with 1.3cm diameter (Spring Co, London). *S. coelicolor* M145 was grown at 30^0C in a shaker set at 250rpm. Before proceeding, the culture was examined microscopically for contamination. The mycelium was harvested by centrifugation. A 50ml tube was filled with half the inoculum and spun for 10 min at 8000xg. The supernatant was removed and the remaining culture added to the tube and spun again

for 10 min at 8000xg. The cell pellet was washed with T10E1. The pellet was then resuspended in 10ml T10E1 + lysozyme (final concentration 1mg/ml). This was incubated for 15 min at 30^{0}C. A lysate was produced by adding SDS and NaCl to final concentrations of 1% SDS and 1M NaCl. The lysate was homogenised completely. 3mg of RNase (10 mg/ml stock) was added to the lysate, which was incubated for 15 min at 55^{0}C. Next, 770µg proteinase K (from 10mg/ml stock) was added and it was again incubated for 15 min at 55^{0}C. Next, 2.7ml of 5M sodium percolate and 1 volume chloroform-isoamylalcohol (v:v 44:1) was added and the mixture was shaken vigorously for 30 min at 4^{0}C. The mixture was then centrifuged for 10 min at 8000xg. Two volumes of cold 96% ethanol were added to the supernatant to precipitate genomic DNA. The gDNA precipitate was transferred to a clean tube with cold 70% ethanol using a sterilised glass pipette. The gDNA was then transferred to a clean microcentrifuge tube and excess ethanol removed by gently pressing the precipitate against the wall of the tube with the sterilised glass pipette. The ethanol-free gDNA was then transferred to a clean microcentrifuge tube where it was dissolved in 1ml T10E1. It was then stored at 4^{0}C.

2.5.2 RNA extraction from SFM-grown *S. coelicolor* using a modified Promega SV Total RNA Isolation Kit (van Keulen et al 2004)

SFM agar was overlaid with sterile cellophane membranes (325P, diameter 80mm, AA Packaging) before inoculation with spores of *S. coelicolor* M145 for efficient harvesting of grown mycelium after incubation. The cellophane membranes were prepared by soaking in distilled water until softened and layering them between wet filter paper in an autoclavable container before autoclaving at 121^{0}C for 15 min. The membranes were then transferred aseptically onto SFM agar using sterilised tweezers. The plates were then inoculated with 70µl of spores from the glycerol stocks (~2X10^{11}spores) and spread using a glass pipette. The plates were incubated at 30^{0}C for 3 days. *S. coelicolor* was harvested using a single edge razor blade (Fisher). A pestle and mortar was washed thoroughly with Pyroneg and water and rinsed with ethanol. After the ethanol had evaporated, liquid nitrogen was used to cool both the pestle and the mortar. The *S. coelicolor* biomass was then grounded to power and transferred into a microcentrifuge tube. 1ml of Trizol was added to the powder, followed by

addition of 0.2ml chloroform. This was then mixed and incubated at room temperature for 2 min before centrifugation at maximum speed for 15 min. The upper phase was transferred to a clean microcentrifuge tube. From this point the Promega kit was used. Firstly, 750µl of the kit's dilution buffer was added and mixed. The tube was then centrifuged for 10 min (expecting a blue pellet). The supernatant was transferred into a clean microcentrifuge tube containing 50µl 96% ethanol, which was mixed by pipetting. The kit's spin column was then set up and the sample pipetted onto the column which was then centrifuged at high speed for 1 min. The column was then washed with 600µl of the kit's RNA wash buffer and centrifuged for another min. The kit's yellow core buffer was then prepared as follows: 5µl 0.09M $MnCl_2$ and 5µl DNase. 50µl of prepared yellow core buffer was added to the column and incubated at room temperature for 15 min. Next, 200µl of the kit's DNase stop solution was added and the column was centrifuged at high speed for 1 min. The column was then washed with 250µl of the kit's RNA wash buffer and centrifuged at high speed for 2 min. The column was then transferred to and clean elution tube before adding 50µl of RNase free water to the column. After incubating at room temperature for 1-2 min the RNA was collected by centrifuging for 1 min. The tube was placed directly into liquid nitrogen and stored at -80^{0}C.

2.5.3 DNA/RNA quantitation: UV spectrophotometer and Nanodrop

DNA and RNA quantities were determined using either a UV spectrophotometer or a Nanodrop spectrophotometer, ND1000 (Nanodrop). Each machine works by calculating a measurement of optical density at 260nm and 280nm. At 260nm, nucleic acids absorption peaks in direct proportion to quantity. An absorption reading of 1 at 260nm indicates 50µg DNA or 40µg RNA is present. The 260:280 ratio gives an indication of purity. A ratio of 1.8 indicates pure DNA whereas a ratio of 2 indicates pure RNA. Lower ratios indicate contamination with protein. A low ratio of 260:230 indicates contamination with solvents and organic molecules such as phenol (Yeates, Gillings et al. 1998).

2.5.4 DNA agarose gel electrophoresis

Agarose gels were used to separate and visualise DNA. For a 1% gel (standard used in this project), 0.5g of agarose was added to 50ml TBE buffer and heated in a microwave until all crystals had dissolved. After cooling, 3µl ethidium bromide stock (5mg/ml) was added and poured into gel trays. The DNA samples were mixed with 10x loading buffer and run at 40mA for 45 min.

2.5.5 RNA formaldehyde (FA) gel electrophoresis

For a 1.2% FA gel, 4.8g of agarose was dissolved in 4ml 10x FA buffer and 35ml of RNase-free water (double autoclaved). After cooling of the gel solution, 0.72ml 37% formaldehyde was added along with 2µl of stock ethidium bromide (5mg/ml). After pouring and setting, the gel was equilibrated for 30 min in 1X FA running buffer. 8µl of denaturation mix was added to 1-2µg RNA. The sample was then incubated at 55^0C for 15 min. The samples were then loaded onto the prepared RNA gel and run for 2h at 40mA.

2.5.6 Endpoint PCR methods

2.5.6.1 Genes and primer design (web primer)

The primers for endpoint PCR were designed using the online software package Web Primer (http://www.yeastgenome.org/cgi-bin/web-primer). Criteria search for included: internal fragment of 500bp preferred, optimum Tm ~ 60^0C, max Tm 72^0C, optimum primer length 18-22 bases, and optimum GC 57%, max GC 72%, min GC 50%.

Once valid primer pairs had been found, a DNA motif search was performed to ensure specificity (http://www.sanger.ac.uk/cgi-bin/yeastpub/strep_dna_motif_search.p).

Primer pair specificity was determined by performing a gradient PCR. The annealing temperature was optimised for each primer set using genomic DNA. Specificity was determined where a single discreet band of the appropriate size was observed on an agarose gel. These specific annealing temperatures for each gene pair were used for all subsequent PCR reactions.

2.5.6.2 Taq polymerase

E. coli DH5α (ATCC-53868) containing plasmid pTaq was used for overexpression of Taq DNA polymerase (provided by Dr. G. Van Keulen). A LB starter culture was grown overnight at 37^0C by picking one colony with a sterile toothpick and placing it in 3ml of LB broth supplemented with 100 μg/ml of ampicillin. 2.4L of LB broth was inoculated with the overnight culture and incubated at 37^0C until it reached an OD600nm of 0.6-0.8. IPTG was added to a final concentration of 0.25mM to induce Taq expression. The culture was then grown for a further 12 h at 37^0C. The cells were collected by centrifugation for 10 min at 7000xg at room temperature. The cells were washed in 400ml of buffer A. They were then centrifuged for 10 min at 7000xg. The pellet was resuspended in 80ml of buffer A containing 4mg/ml lysozyme (slowly added). This was incubated for 15 min at room temperature before adding 80ml of buffer B. This was then incubated for 1 h at 75^0C in a water bath with occasional shaking. This was centrifuged at 4^0C for 15min at 8000rpm. 30g of ammonium sulphate was added per 100ml of supernatant with rapid stirring at room temperature. The protein precipitate was harvested by centrifuging for 15 min (both pellet and surface). This was resuspended in one-fifth of initial volume in buffer A. The sample was then dialysed twice against storage buffer for 12 h (the volume shrunk to 1/3 initial volume). The dialysed protein was then diluted 1:1 with storage buffer, aliquoted and stored at -20^0C. The concentration of units of Taq was estimated at 10 units/μl using end-point PCR titration (Leelayuwat et al 1997).

2.5.6.3 Endpoint PCR set up

For end-point PCR (final volume 10μl), 1μl forward and 1μl reverse primer (10μM each) were added to 1μl template DNA (1ng/μl gDNA). PCR buffer was added (1 μl of 10x buffer) along with 0.5μl of DMSO, 0.2μl of 10mM dNTPs, 4.3μl of DNase-free water and finally 1μl Taq polymerase (10 units).

Initial denaturation was set to 94^0C for 5min. There were forty cycles of 30sec denaturation at 94^0C followed by 40sec annealing at the desired annealing temperature (primer specific)

and 15sec at 72^{0}C extension. After the cycles had finished, a final extension step consisted of a further 5min at 72^{0}C.

2.5.7.1 Reverse transcription

In a 0.2ml microcentrifuge tube 5µg RNA was mixed with 0.5µl of hexamer primers (Invitrogen) and MilliQ water to a final volume of 12ul. This was denatured by incubation for 10 min at 70^{0}C, which was then placed directly on ice. Next, 4µl of 5x first strand buffer, 2µl of 0.1M DTT and 1µl of 10nM dNTPs were added and mixed carefully. This was incubated for 10 min at 25^{0}C. After incubation, 1µl of 10U/µl RNase Inhibitor (Invitrogen) was added with 1µl of Superscript II Reverse Transcriptase (200units/ µl, Invitrogen) and the mixture was incubated for 1 h at 42^{0}C. The reaction was stopped by incubation for 15 min at 70^{0}C.

2.5.7.2 Roche Transcriptor High Fidelity cDNA synthesis kit

All reagents were thawed thoroughly, briefly centrifuged and kept on ice. The template-primer mix was prepared by mixing 1ng-4µg of RNA with 2µl of random hexamer primer (vial 6) and making up to a final volume of 11.4µl with water (vial 8). Denaturation took place by incubating at 65^{0}C for 10 min and immediate cooling on ice. To the reaction 4 µl of reaction buffer (vial 2) was added with 0.5µl of RNase inhibitor (vial 3), 2µl of deoxynucleotide mix (vial 4), 1 µl of DTT (vial 7) and 1.1µl of reverse transcriptase (vial 1). After mixing carefully the reaction was incubated for 30 min at 55^{0}C followed by 5 min at 85^{0}C. The products were then stored short term at 4^{0}C or long term at -20^{0}C. For PCR, 1µl was used in a 10µl reaction volume.

2.5.8 qPCR

qPCR was performed using SensiMix™ SYBR® No-ROX in a RotorGene 6000 (Corbett Research). The qPCR reagents thawed, vortexed gently and kept on ice. For a final reaction volume of 10µl, 5µl 2x SensiMix SYBR was added along with 250nm final concentration of

both forward and reverse primer and 1µl template cDNA from Roche Transcriptor High Fidelity cDNA synthesis Kit. For the 16S primer, the cDNA was diluted 25x with DNase free water.

To start, the samples were completely denatured and the hot start enzyme activated by incubation for 10 minutes at 95°C. The cycling sequence started with a denaturation step of 95°C for 10 seconds. The annealing temperature was determined by the melting temperatures of the primers. It was usually around 5°C lower than the melting temperature. This was held for 30 seconds followed by extension at 72°C for 10 seconds. Data acquisition was after this elongation step. Cycle numbers varied between 35-40 cycles (sufficient for low copy numbers). An additional extension step was carried out after the cycling stage to ensure all DNA was in double stranded form. After the elongation step was complete a melt curve analysis was carried out. Data was acquired every 1°C up to 90°C.

For quantitation it is vital to determine the efficiency of the primers. Ideally, each cycle should result in double the amount of amplified DNA; however, due to a number of variables this is not always true. By performing a standard curve of the primers the efficiency was determined, which was then used for quantitation. A standard curve was plotted as Ct against the log of the standards concentrations (gDNA serial dilution) for each set of primers and for each run. The slope of the graph is related to the efficiency and percentage efficiency was calculated using equation 4:

Eq. 4 $\text{Efficiency} = ((10^{-1/\text{slope}})-1)*100$

A slope of -3.322 equals 100% efficiency i.e. the DNA concentration is doubling per cycle. Efficiency can be as much as 110% if primer-dimers are being formed. Efficiencies should not however be lower than 80%. This indicates problems with primer design, amplicon length or the presence on inhibitory substances.

After determining specific primer efficiencies, the logarithm of starting quantity of each sample was calculated using equation 5:

Eq. 5 Log starting quantity= (cT-intercept)/-slope

The power 10 of this number gave the starting quantity. In order to look at gene expression of a specific gene it was essential to normalise this number against a house keeping gene. As the cDNA was diluted 25x for use with the housekeeping gene primer, the RTA was multiplied 25 times. The samples were normalised by dividing average starting quantity of the gene of interest by the average starting quantity of the 16S gene, which was used for normalisation. Statistical tests were performed to determine significant differences of the qPCR results.

2.5.9 RNA amplification amended from Gao et al 2007

2.5.9.1 First strand synthesis

First stand synthesis followed the protocol for Roche cDNA synthesis (Section 2.5.7.2), except replacing 2µl of random hexamer primer (vial 6) with 1µg of T7 primer and excluding the addition of 0.5µl of RNase inhibitor (vial 3). The amount if RNase-free water was adjusted accordingly.

2.5.9.2 Second strand synthesis

For second strand synthesis a 150µl solution was prepared with all of the reverse transcription products (from first strand synthesis), 40 units of *E. coli* DNA polymerase I (NEB), 2 units of *E. coli* RNase H (NEB), 10 units of *E. coli* DNA ligase (NEB) and 1x second strand buffer (NEB). This mixture was incubated for 2 h at 16^0C. After the addition of 20 units of T4 DNA polymerase (NEB) the mixture was incubated for 5 min at 16^0C. To stop the reaction 10µl of 0.5M EDTA was added followed by addition of 10µl of NaOH. This was incubated for 10 min at 65^0C. To neutralise, 25µl of 1M Tris HCl (pH 7.5) was added. The solution was incubated with 100-200ug/ml proteinase K and 0.5% SDS for 30 min at 50^0C. An equal volume of phenol:chloroform (v/v 1:1) was used to remove contaminants. This was centrifuged for 10 min at 13000xg and the aqueous phase was removed. To prevent carryover of phenol, an equal volume of chloroform was added and centrifuged at high speed for 10 min. 1ug/µl of glycogen was added to the aqueous phase as a co-precipitant

and the dscDNA was precipitated in 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of 96% ethanol prior to incubation overnight at -20^{0}C. The dscDNA was pelleted by centrifugation for 20 min at 13000xg and air-dried for 5 min before being re-suspended in 16µl of DNase free water.

2.5.9.3 *in vitro* transcription

Up to 1µg of the dscDNA was denatured by heating to 95^{0}C for 15 min and placing it directly on ice. The MEGAscript T7 Kit (Ambion) was used to produce the amplified RNA from the dscDNA. All the reagents were thawed and kept on ice (except 10x reaction buffer which was kept at room temperature). A 20µl solution was prepared containing the products of the dscDNA synthesis and (from the MEGAscript kit) 2µl ATP, 2µl UTP, 3µl CTP, 3µl GTP, 2µl enzyme mix, 2µl 10x reaction buffer (added last) and DNase free water. The tube was mixed and microcentrifuged briefly before being incubated at 37^{0}C for 4 h. To denature any remaining DNA at this point, 1µl of the kit's Turbo DNase was added and mixed well into the solution before re-incubating at 37^{0}C for 15 min. To stop the DNase reaction, 115µl of water and 15µl of ammonium acetate stop solution were added. Next, an equal volume of phenol: chloroform (v/v 1:1) was added and vortexed. The phases were separated by centrifugation for 10 min at 13000xg. To the aqueous phase, one volume of chloroform was then added, vortexed and centrifuged for 10 min at 13000xg. The aqueous phase was then recovered and the RNA precipitated by the addition of one volume of isopropanol and glycogen (final concentration 1ug/µl). This was incubated for 15 min at -80^{0}C and centrifuged at 13000xg for 15 min at 4^{0}C. If the pellet was small, the solution was re-incubated at -20^{0}C. The pellet was then air-dried to remove any excess solvent and resuspended in RNase free water.

2.5.10 RNA extractions from soil

2.5.10.1 PowerSoil Total RNA Isolation Kit (Mobio)

Up to 2g of microcosm soil was added to a 15ml bead tube. To this, 2.5ml Bead solution was added and vortexed, followed by the addition of 0.25ml SR1, which was further vortexed.

0.8ml of SR2 was added next and the tube was vortexed for 5 min. Vortexing took place on a platform mounted to a Vortex Genie II to ensure sufficient cell lysis. While the solution was vortexing, a phenol:chloroform:isoamyl alcohol (v/v/v 25:24:1) was prepared and 3.5ml was added to the tube followed by 10 min vortexing. The mixture was then separated by centrifugation at 2500xg for 10 min and the upper phase was then transferred to a 15ml collection tube. 1.5ml of solution SR3 was then added, vortexed and incubated at 4°C for 10 min. Centrifugation at 2500xg for 10 min followed and the supernatant was transferred to a clean collection tube. Next, 5ml of SR4 was added, vortexed and incubated at -20^{0}C for 30 min. After this time the tube was centrifuged at 2500xg for 30 min and the supernatant decanted. The tube was inverted onto a paper towel for 5 min to air-dry the pellet. The pellet was then resuspended in 1ml of SR5. This was aided by heating to 45°C for 10 min and vortexing if the pellet was hard to dissolve. The RNA capture column was then prepared by placement in a 15ml collection tube and the addition of 2ml of SR5 which was left to run through by gravitation. The sample was then placed on the column followed by a wash with 1ml of SR5. The RNA was eluted by adding 1ml of SR6 to the column and allowing it to flow through by gravitation into a clean 15ml tube. To this, 1ml of SR4 was added and mixed through inversion followed by incubation at -20^{0}C for 10 min. This was centrifuge at 13,000xg for 15 min and the supernatant decanted. The tube was then inverted onto a paper towel to air-dry the pellet. The pellet was then resuspended in 50µl of SR7, frozen in liquid nitrogen and stored at -80^{0}C.

2.5.10.2 Nucleospin RNA from Plant Tissue (ABgene)

The Nucleospin kit is designed for RNA extraction from plant tissue, adjustments to the protocol were made to suit a soil sample. To start, 100mg of microcosm soil was ground under liquid nitrogen in a clean mortar and pestle. To the ground soil 350µl of RA1 and 3.5ml β-mercaptoethanol was added and vortexed. The solution was run through the violet filter column, aided by centrifugation for 1 min at 11000xg. The solution was transferred to a clean microcentrifuge tube to which 350µl of 70% ethanol was added and mixed gently by pipetting. The lysate was then loaded onto the blue column and was centrifuged for 30 sec at 11000xg. Next 350µl of the kit's membrane desalting buffer (MDB) was added followed

by centrifugation for 1 min at 11000xg. A DNase solution was then prepared (10µl DNase plus 90µl DNase reaction buffer) and 95µl of this solution applied to the column. This was incubated for 15 min at room temperature. The column was then washed with 200µl of RA2, 600µl of RA3 then 250µl of RA3 respectively centrifuging for 1 min at 11000xg each time. The RNA was then eluted using 60µl of RNase free water, which was incubated on the column for 2 min before centrifuging for 1 min at 2000xg. The RNA was then frozen using liquid nitrogen and stored at -80^{0}C.

2.6 Proteomics methods

2.6.1 Bradford assay

For a Bradford protein determination, a standard curve was produced using a serial dilution from a 1mg/ml stock of bovine serum albumin (BSA). 20µl of each dilution was added to 1ml of Bradford reagent and vortexed. After incubating at room temperature for 2min the OD595 was recorded. Unknowns were calculated by interpolation into the straight line equation produced from the standard curve.

2.6.2 Protein extraction from soil: Benndorf et al. (Benndorf et al 2007)

From the *S. coelicolor* microcosms, 5g of microcosm soil was treated with 10ml of 0.1M NaOH for 30 min. The sample was then centrifuged for 10 min at 16000xg at 20^{0}C. To remove the humic acids, the supernatant was mixed with 19ml liquid phenol and 10ml water. This was shaken at 250 rpm for 1 h at 20^{0}C. The phases were separated by centrifugation for 10 min at 14000xg. The lower phenol phase was collected and washed with 15ml water followed by 5 min of shaking at 20^{0}C and centrifugation for 10 min at 14000xg. The proteins were then precipitated with five fold volume of 0.1M ammonium acetate in methanol. This was incubated overnight at -20^{0}C. The sample was then centrifuged for 10 min at 16000xg at 0^{0}C and the pellet resuspended by ultrasonication in 10ml of 0.1M ammonium acetate in methanol. The pellet was then washed in 2ml of 0.1M ammonium acetate in methanol followed by 2ml of 80% acetone, then 2ml of 70% ethanol,

each wash step included 15 min incubation at -18^{0}C and centrifugation for 10 min at 16000xg at 0^{0}C.

2.6.3 Protein extraction from soil: Singleton et al. (Singleton et al 2003)

One gram of microcosm soil was placed in a microcentrifuge tube with 2nM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). To this, 1ml of extraction buffer was added and the sample was vortexed for 10 sec. The lid of the microcentrifuge tube was then pierced several times before subjecting it to four freeze-thaw cycles (liquid nitrogen to 25^{0}C). This was then centrifuged for 15 min at 20817xg at 4^{0}C. The supernatant was then removed, re-centrifuged and the supernatant removed and stored at -20^{0}C.

2.6.4 Protein extraction from soil: amended from Masciandaro et al 2008

All glassware was soaked overnight in 1% HCl to remove keratin. For total protein extraction, 10g of microcosm soil or sand was suspended in 10ml of distilled water. 100µl of Protease Inhibitor Cocktail (Sigma) was added and incubated in a shaker at 150rpm at RT for 15 min. The suspension was sonicated with a sonication probe approximately 10 times in 2 min. Next, a further 100µl of protease inhibitor cocktail was added and incubated in a shaker at 150rpm at RT for 15 min. 300ml of 0.5M potassium sulphate and 30ml 0.1M EDTA was added to this solution. This was shaken at 15rpm for 1 h at room temperature. The soil mixture was then centrifuged for 15 min at 14300xg at 5^{0}C. The supernatant was then filtered through Whatman number four filter paper and then re-filtered using 0.2µm sterilisation syringe filters. The solution was diluted four times using MilliQ water and dialysed overnight using a 10kD cut-off dialysis membrane against water. The solution was then concentrated down to 10ml using a 10kD cut-off Pall Minimate membrane. After the sample volume had reduced to around 20ml, it was frozen at -80^{0}C before being freeze-dried. The sample was re-suspended in 2ml of distilled water and the protein concentration was determined using Bradford reagent. For further desalting purification, the sample

volume was increased to 5ml before being concentrated in spin columns down to ~1ml. This process was repeated several times to ensure little salt contamination.

2.6.5 SDS PAGE

For preparation of 1D-SDS-PAGE minigels, a back plate and front plate were set up in the stand and sealed along the bottom with 1-2ml 2% agar. The separating gel was prepared, adding TEMED and APS last, according to the Table 2.1. This was mixed thoroughly and pipetted into the glass plate setup up to 1cm away from the top. An overlay of buffer-saturated butanol was placed on the gel and it was left to polymerise for 45 min. The buffer-saturated butanol was then washed off and stacking gel was prepared according to the table below. After mixing the stacking gel thoroughly (Table 2.1), it was poured over the running gel and a comb was gently inserted. It was ensured that no bubbles had been introduced, which was aided by inserting the comb at an angle. The stacking gel was left to polymerise for 30 min, after which the comb was gently removed.

Table 2.1 SDS-PAGE Separating and stacking gel proportions

Reagents	12% Separating Gel	Stacking Gel
5% Acrylamide	4ml	0.67ml
Solution B	2.5ml	-
Solution C	-	1ml
Distilled water	3.57ml	2.3ml
10% Ammonium Persulphate	50µl	30µl
TEMED	5µl	5µl

The soil/sand protein samples were prepared by adding 5x sample buffer and boiling (100^0C) in a heating block for 2 min. The samples were then loaded onto the gel (max 30µl per well) along with a molecular weight marker, 5µL of Protein Ladder (10-250kDa, NEB)

Next, electrophoresis tanks were set up and filled with 1x running buffer. After placing the gels in the cassettes the gel was run at 100V through the stacking gel and turned up to 200V to run through the separation gel.

For larger, 16x18cm gels (Protean II xi gel rig), all solutions were as the minigels. The gels were however run at 16mA through stacking gel and 24mA through separation gel, at 4°C.

2.6.6 Gel staining

The staining technique used was determined by how much protein was loaded per lane. Coomassie staining was used when protein was expected to be between 15 and 20μg per lane. Where there was less protein, silver staining was used. This technique was used where proteins amounts were between 1 to 4μg per lane.

2.6.6.1 Coomassie staining

Coomassie stain was prepared with 1% w/v Coomassie R 250 in 10% acetic acid and 45% methanol. The gels were stained for 30 min in 25ml of the coomassie stain solution. After staining, the gels were rinsed in MilliQ water. Destain solution was then applied (10% acetic acid and 40% methanol) and the gels were left to destain for 30 min. To aid destaining, on occasion the gels were gently heated in a microwave (for a few sec only) in the destain solution. Once destaining was complete the gels were rinsed with MilliQ water to remove excess unbound dye and stored in water at 4^0C.

2.6.6.2 Silver Staining

For silver staining, the gels were fixed in 50% methanol and 10% acetic acid for 1 h to overnight. The gels were rinsed twice in 50% methanol and washed in MilliQ water three times over 20 min. The gels were then sensitised for 90 sec in 0.02% sodium thiosulphate before being rinsed three times for 30 sec in MilliQ water. The gels were then stained in

0.2% silver nitrate at 4^0C for 30 min. The stain solution was replaced with MilliQ water for 30 sec before adding developer solution (6g of sodium carbonate, 2ml of 0.02% sodium thiosulphate, and 50µl of 37% formaldehyde made up to 100ml with MilliQ water). The bands were left to develop for up to 10 min after which stop solution (5mM of Na-) was added. Gels were stored in MilliQ water at 4^0C.

2.6.7 Band excision and treatment

After excision of each protein band using a scalpel, gel pieces were placed in individual microcentrifuge tubes. The gel pieces were then covered in acetonitrile and left to soak for 5 min before being dried in a vacuum concentrator. The samples were than stored at -20^0C until ready for trypsin digest.

2.6.8 Trypsin Digestion

To each tube containing a gel piece, 25µl of 20mM ammonium bicarbonate and 0.5µl of Trypsin Gold (1µg/µl in 50mM acetic acid) (Promega) was added before being centrifuged briefly. The gel bands were incubated with trypsin on ice for 45min. The excess liquid was removed and another 25µl of 20mM ammonium bicarbonate was added to the sample. The tubes were incubated overnight at 37^0C. The digestion reaction was stopped by acidification with a four times dilution in 0.1% trifluoroacetic acid.

The excess liquid was collected before adding 50µl of seven parts 5% acetic acid to three parts acetonitrile (ACN) to the gel piece. The tube was sonicated in a sonication bath for 15min. The excess liquid was again removed and pooled with the previous solution. This extraction step was repeated twice and all the digestion solutions pooled together. The extracted peptide mixture was dried in a vacuum centrifuge at 45^0C.

2.6.9 Zip-Tip protein purification

To purify the samples they were treated with a C18-bonded silica-based column piped "Zip-Tip" (Supel-Tips C18 micropipette tips TPSC18, Sigma-Aldrich). First, the dried samples were resuspended in 0.1% TFA. A Zip-tip column was wetted with 10µl of 70% ACN and 0.1% TFA solution. The column was washed three times with 10µl of 0.1% TFA. The peptides sample was bound to the column by aspirating and dispensing 20 times. The column-bound sample was then washed five times with 10µl of 0.1% TFA. Lastly, the sample was eluted three times in 10µl of 70% ACN and 0.1% formic acid. Then, 30µl of clean sample was dried in a vacuum centrifuge at 45^0C.

2.6.10 LC-ESI MS/MS

Samples were analysed by nano-reverse phase-liquid chromatography (Ultimate Pump, LC-Packing, Dionex, The Netherlands) using an electrospray-ion-trap MS (LCQ Deca XP, ThermoElectron, Hemel Hempstead, UK). LC-ESI MS/MS separations were performed using a 10cm x 75mm I.D. pulled-tip capillary column that was slurry packed in-house with 3µm, 300Å pore size C18 silica bonded stationary phase (PepMap, Dionex, Camberley, UK). The autosampler was fitted with a 5µl injection loop and was refrigerated at 4^0C during analysis.

Samples were injected onto the analytical column for 5min with 98% buffer A (0.1% formic acid in water v/v), peptides were then eluted using a stepwise gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 60min and then to 100% B in 10min with a constant flow rate of 0.2µl/min. The electrospray-MS was operated in a data-dependent mode in which each full MS scan was followed by three MS/MS scans, in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalised collision energy of 35%. The temperature of the heated capillary and electrospray voltage was 160^0C and 1.6kV, respectively.

2.6.11 Data processing and mining

The data produced by the LC-ESI MS/MS in Excalibur was processed using Bioworks Browser 3.2 (ThermoElectron, Hemel Hempstead, UK). Protein data (RAW files) was searched against with the 2011 indexed non-redundant (nr) protein sequence database using SEQUEST, comparing found peptide masses and charges. The resulting SRF (search result file) files were filtered using the peptide/xcorr vs charge state filter. XCorr is the cross-correlation value between the observed peptide fragment mass spectrum and the one theoretically predicted. The filter was set for high stringency cross correlation on charge states (z) 1=1.80 2=2.00 3=2.50. All found *S. coelicolor* A3(2) proteins with two or more different peptide matches or two or more identifications with one peptide were exported to an Excel spreadsheet. Individual proteins were selected for best ID. When more IDs for the same protein were found in different samples, only the best ID was kept. Selection went according to best Xcorr scores, which is the cross-correlation value between observed and predicted peptides thus also representing the number of different peptides matched. In equal Xcorr scores, the ID with lowest P (peptide probability) was kept and the other(s) discarded. P shows best probability from the matched peptides. P is the probability of this peptide being found at random in this search. The lower the P, the better the match is. Subsequently, protein sequences were aligned with the nr (protein) database to confirm annotation, using BLAST search on the NCBI website (Altschul, Madden et al. 1997). Proteins were then clustered according to their (predicted) function. Samples from which only one clear ID could be deduced were treated as pure single protein and their spots analysed for abundance using visual tools.

2.6.12 Visual analysis for protein abundance

Digital images of triplicate stained gels were made using a gel-doc imager (Gel Doc XR, Bio-Rad) and a PC (Quantity One, Bio-Rad). The images were analysed with ImageJ software (v1.43U, NIH, USA). The background was removed as much as possible using ../Process/Subtract Background/.. , a Rolling Ball Radius of 30.0 pixels was set. Band intensity was obtained by selecting the lane horizontally using ../Analyze/Gel/Select First

Lane.. To measure, the lane was plotted using the ../Plot Lanes.. option. ImageJ automatically corrects the height of the selected lanes areas to the height of the first lane. Plotting the lane resulted in an image displaying the intensity of colours (bands) in a Y-signal, plotted against the distance from the top down as X-axes. To obtain individual peaks from this signal, a baseline was set for 20 pixels above y=0 using the "Straight" tool. This tool was also used to slice off any side-peaks due to smearing. Next, using the "Wand" tool, each individual peak was selected and measured with ../Analyze/Measure.. yielding a data list in the Results window. This window listed peak areas for each of the temporal samples for comparison. The Table was exported into Excel (Office Excel 2002, Microsoft). To normalise the data, all peak area values were divided by the total for that repeat (gel) and lane. The normalised intensities were displayed in a histogram chart displaying changes in the relative abundance of the protein. The height for the histogram was set relative to the timepoint of highest abundance, this bar stretching the full height of the chart. Some supporting and annotated images were edited using MS Paint (v. 6.1, Microsoft Windows 7).

2.7 Statistical analysis

2.7.1 qPCR

Mann Whitney tests were used to check for significant differences between the RNA used for cDNA synthesis (negative control) and the cDNA samples. Mann Whitney tests were chosen as an alternative to a t-test as the data is not normally distributed. This non-parametric test uses medians rather than means for analysis and is therefore more suitable for not-normally distributed data. The statistical analyses were performed using Minitab software (Minitab Statistical Software 14). Mann Whitney tests were also used to check for significant differences between starting cDNA quantities for each sample/primer. Because the data was not normally distributed, 95% confidence intervals were used for error bars on all graphs rather than standard deviation as standard deviation relies on normally distributed data.

2.7.2 Doubling time analysis

The standard deviation of the doubling times was calculated, along with the Coefficient of Variation. To test the deviation of the extreme values the upper/lower doubling time values were calculated by dividing the standard deviation by the upper or lower value minus the mean. Both values were checked against the standard value of +/- 1.64.

Chapter 3 Soil characterisation and optimisation of molecular techniques

In this chapter the results from soil characterisation techniques and the optimisation of molecular techniques are described and discussed. Firstly, soil was collected from Lower Hardingsdown Far, Gower Swansea and classified according to texture, organic carbon content, water capacity and pH. The pH of the soil was adjusted for microcosm studies and growth curves of *S. coelicolor* established. Several methods for RNA extraction from soil were tested and the resulting RNA used for the development of a T7 polymerase-based RNA amplification method. This method proved troublesome and so was optimised extensively. PCR primers were designed and optimised for end-point PCR and qPCR. To conclude Chapter 3, the growth of *S. coelicolor* in soil is discussed along with the results for the RNA extraction and amplification techniques.

3.1 Soil Sampling

A sample of soil was taken from several sites around the Gower Peninsula, Swansea (UK), from arable (non-ploughed) and pastures (permanent and rotation). The soil used for the microcosms was sourced from Lower Hardingsdown Farm, in the Gower at latitude 51°35'40.54"N and longitude 4°14'32.85"W in November 2007. Figure 3.1 demonstrates the location in relation to Swansea. The land at this site was arable and non-ploughed. At time of sampling, the field was in use for growing turnips.

Figure 3.1 Google earth image of organic farm sampling site (Google earth).

3.2 Soil characterisation

The organic carbon content of soil is the measure of residual plant and animal material, generally decayed by bacteria. The soil carbon content of the organic farm soil was assessed by colleagues in the Geography department and was measured to be 2.47% TOC.

The ability of the soil to take up and hold water was crucial to the study. If the soil was hydrophobic, i.e. unable to be wetted, it would have been unsuitable for *S. coelicolor* microcosm studies. According to Doerr (Doerr, Shakesby et al. 2006), any soil with a water penetration time (WPT) of less than 60 seconds is hydrophilic. The results of the water penetration droplet test (WDPT) on the farm soil deemed it highly hydrophilic with a WDPT of 10 seconds.

In order to establish soil texture classification, the soil particles are grouped according to their size. These groups are referred to as 'soil separates', namely clay, silt and sand (and for courser soil, pebbles also). Soil texture is important to understand as it has an effect on not only the movement and retention of water in the soil but also aeration. These alter the suitability of a soil as a medium of plants and in this case, microbes to grow.

To identify the soil texture classification, the soil separate fractions were calculated (as a cumulative total). The soil analysis data (Table 3.1 and Fig 3.2) indicated the organic farm soil to be 2.5% clay, 46% silt and 51% sand. The mean diameter of particles was 53.26μm, the median 51.09μm, the mean/median ratio 1.043 and the mode 87.90μm. This data was inputted into the soil textural triangle (Fig 3.2b) finding the soil to be a sandy loam.

Table 3.1 Soil particle analysis results.

Particle Diameter µm	Volume < (cumulative percentage)	% of total mass
2	2.49	2.49
5	4.59	2.1
16	15	10.41
20	19	4
32	30.8	11.8
50	48.9	18.1
125	99.1	50.2
250	100	0.9
500	100	0
1000	100	0
2000	100	0

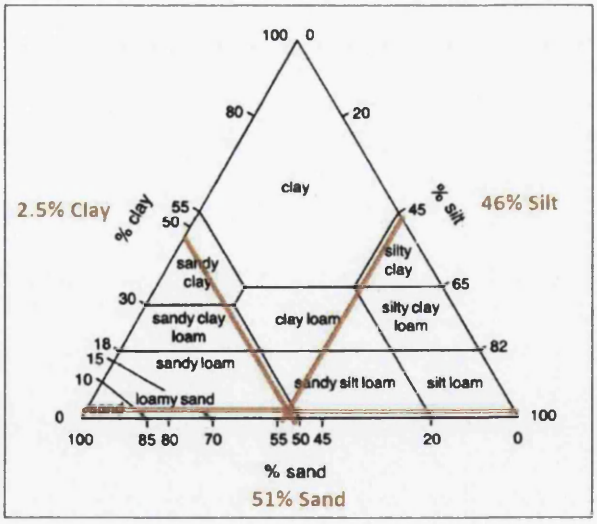


Fig 3.2 Soil textural triangle (DEFRA 2007)

The point of water saturation is reached when all soil pores are filled with water giving a soil water potential of zero. Different soils have different water holding capacity according to texture, organic content and carbonate content. The desired soil saturation for the microcosms was 40% (Herron and Wellington 1990). In order to calculate the volume of water needed to produce the desired 40% water saturation, total water saturation was calculated.

Soil samples were fully saturated with water. Measurements of water content showed average water saturation to be 42.03% (Table 3.2). The residual water content in air-dried soil was also calculated (Table 3.3) and averaged at 3.11%.

Table 3.2 Summary of soil water saturation test results.

	Container (g)	Container+ air- dry soil (g)	Container+ saturated soil (g)	Container+ oven-dry soil (g)	Water saturation %
Sample 1	95.74	223.69	271.00	220.39	40.6
Sample 2	97.42	218.12	264.40	214.84	42.2
Sample 3	96.68	216.71	263.96	213.43	43.3

Table 3.3 *Water content of air-dry soil*

	Pot (g)	Pot + air dry soil (g)	Pot + oven dry soil	Water content %
Sample 1	16.3346	25.9269	25.6440	3.14
Sample 2	15.9876	26.5310	26.2174	3.07
Sample 3	24.7549	34.1416	33.8574	3.12

The pH of the farm soil was determined to be 5.5. For most *Streptomyces* soil microcosms a neutral pH is preferred. In order to adjust the soil pH for a 10g microcosm several methods were tested, including the addition of variable concentrations of phosphate buffer. The maximum volume to be added to each 10g microcosm was 1.63ml as this is the volume where the microcosm would reach the desired 40% saturation. Using the phosphate buffers,

both 10mM and 100mM, the volume needed to change the pH to neutral was too large (Table 3.4a). Sodium hydroxide was therefore tested as an alternative to phosphate buffer. The results revealed that a more or less neutral pH of 6.8 was achieved with addition of 1ml of 100mM NaOH (Table 3.4b). For all 10g soil microcosms, 1ml 100mM NaOH was then used to neutralise the soil.

Table 3.4a Changing soil pH using phosphate buffer (K_2HPO_4+NaH_2PO_4) in 10g soil.

i. Using 10mM phosphate buffer:

Buffer volume (ml)	Soil pH
1	5.08
2	5.24
5	5.28
10	5.58
15	5.88

ii. Using 100mM phosphate buffer:

Buffer volume (ml)	Soil pH
0	5.96
0.5	5.83
1.0	5.91
1.5	5.85
2.0	5.91

Table 3.4b Changing soil pH using NaOH in 10g soil with a final volume of 1ml.

NaOH concentration (mM)	Soil pH
0	5.88
20	5.91
40	6.18
60	6.33
80	6.51
100	6.8

3.3 *S. coelicolor* growth curves

Once microcosm set up was devised it was important to check that the organic farm soil supported *S. coelicolor* growth. Using a total viable count method, growth curves were established and repeated to ensure reproducibility. The average of three growth curves from *S. coelicolor* microcosms amended with 1% starch plus 1% chitin can be seen in fig 3.3. Total viable count dropped between day zero and day one. Exponential growth was seen between days one and three with a doubling time of 365min. The microcosm reached early stationary phase after day three which then continued into stationary phase.

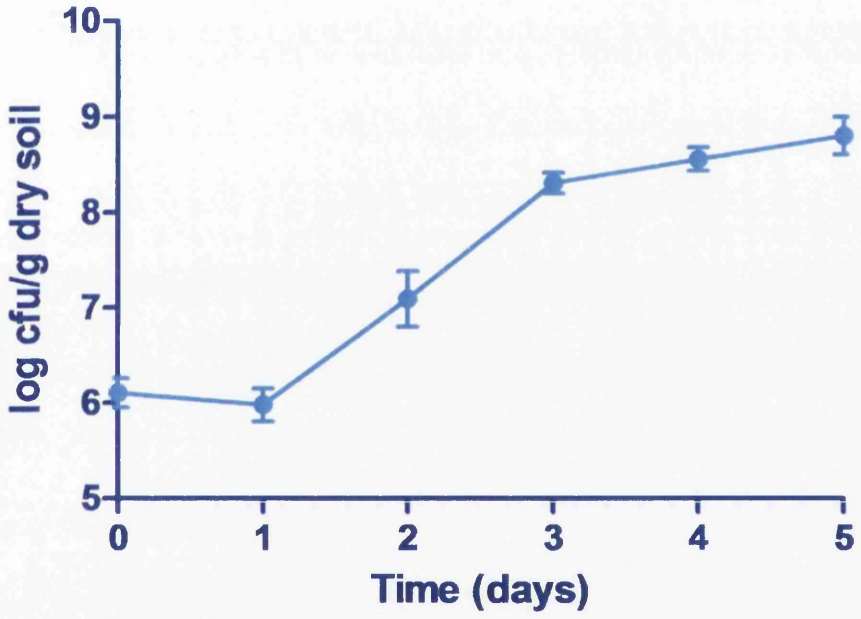


Fig 3.3 Total viable counts of S. coelicolor form soil microcosms. Summary of triplicate samples. Error bars showing standard deviation.

3.4 Comparison of RNA extraction methods from soil

RNA was used as an indicator of gene expression. The presence of humic acid and other inhibitors in soil were anticipated to cause problems in downstream reactions. Therefore, three protocols were tested for RNA extraction from soil. Protocol one was the PowerSoil

Total RNA Isolation Kit from Mobio, protocol two was the SV total isolation kit from Promega and protocol three was the Nucleospin RNA plant kit. Each protocol resulted in the successful extraction RNA from three-day old *S. coelicolor* microcosms. The RNA samples were separated by electrophoresis under denaturing conditions in a FA gel (Fig 3.4, Table 3.5). The total RNA yield was calculated as 1.38µg from protocol one, 0.96µg from protocol two and 0.99µg from protocol three. After RNA electrophoresis of 1.5µg of each sample (fig 3.4b) smearing could be seen in lanes two and three indicating the degradation of the RNA. However, colourless RNA extracted using the Mobio PowerSoil kit showed discreet 16S and 23S bands. Furthermore, the RNA suspensions produced from the Promega and the Nucleospin kits were brown in colour, indicating the presence of humic acids, which interfere with downstream processes. The Mobio PowerSoil Total RNA Isolation was therefore used for all future RNA isolations from soil.

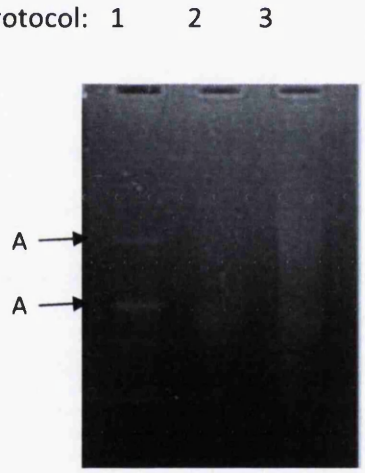


Fig 3.4a Formaldehyde agarose gel showing RNA extracted form soil microcosms, *lane 1: Mobio (RNA PowerSoil); lane 2: Promega (SV Total RNA Isolation System) and lane 3; Macherey Nagel (Nucleospin RNA Plant).*

Table 3.5 Total RNA yield from each method tested.

Protocol	Yield (µg)
RNA PowerSoil	1.38
SV Total RNA Isolation system	0.96
Nucleospin RNA Plant	0.99

3.5.1 RNA amplification procedure optimisation

A RNA amplification protocol was optimised to increase yield of RNA from analysis. A protocol from Gao *et al* (Gao, Yang et al. 2007) was tested and produced amplified RNA from *Streptomyces* microcosms, however, optimisation was required.

Firstly, for first strand synthesis two reverse transcriptases were tested. The first was Superscript III reverse transcriptase (as suggested by Gao *et al*). The result for this was compared to the Roche Transcriptor High Fidelity cDNA Synthesis kit. Spectrophotometric data indicated the production of more second strand DNA with better purity using the Roche kit (216ng compared to 72.9ng) and so the Roche kit was used for further amplifications.

After first strand synthesis, the first strand DNA was used to generate dscDNA. The conversion of this dscDNA to amplified RNA was performed using the MEGAscript T7 kit. The protocol suggested optimisation of the incubation time between four and 16 hours. Equal volumes of dscDNA from one *S. coelicolor* soil microcosm (day three) was incubated for either four or 16 hours. The final yield was 33.6µg (2.1 µg/µl) after four hours and 4.8µg (0.3ug/µl) after 16hours. After 16 hours the initially produced RNA had degraded, producing a lower yield. An incubation time of four hours was subsequently used for RNA amplifications.

3.5.2 Troubleshooting and optimisation

Amplified RNA was repeatedly run on an FA gel with no band/smear seen. Spectrophotometric data showed the presence of RNA for each sample. To check for the presence of inhibitors the second strand DNA was mixed with the control plasmid (pTRI-Xef) provided with the MEGAscript kit. The products were run on a FA gel, seen in fig 3.5. The gel shows no product for the microcosm sample but RNA presence for second strand mixed with plasmid and plasmid by itself. This indicates no inhibitors were present in the second strand sample.

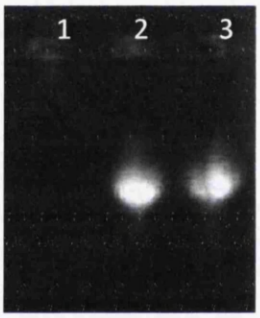


Fig 3.5 Formaldehyde/agarose gel showing products for* hrdB *of RNA amplification. *Lane 1: second strand DNA; lane 2: second strand DNA mixed with plasmid; lane 3: plasmid.*

To check integrity of the amplified RNA shown by the spectrophotometric data, reverse transcription PCR was performed using *hrdB* primers on amplified RNA (fig 3.6) and PCR performed on the second strand DNA (fig 3.7). The gels showed that both the second strand DNA and the amplified RNA underwent PCR/rtPCR with positive results. The number of cycles need for optimum results from rtPCR on the amplified RNA was tested. The best results were achieved with 40 cycles (fig 3.8) which was used for all subsequent PCRs.

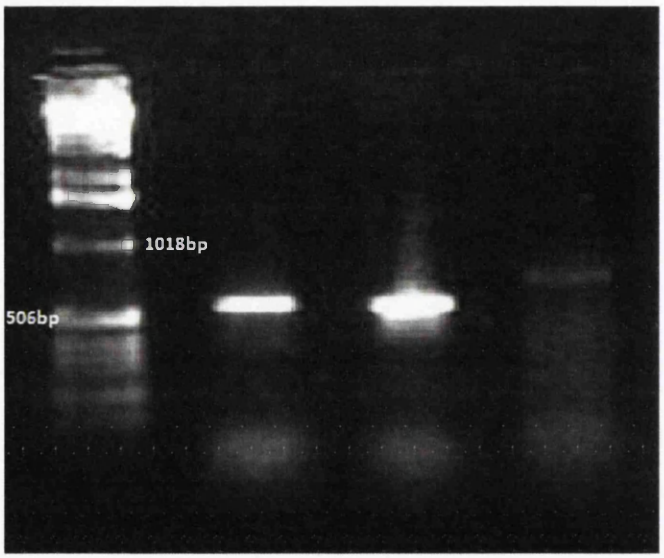


Fig 3.6 Agarose gel showing products of PCR using hrdB primers and different templates. *Lane 1: Ladder (1Kb Plus NEB); lane 2: amplified RNA from day 3 microcosm; lane 3: genomic DNA; lane 4: No template control (NTC).*

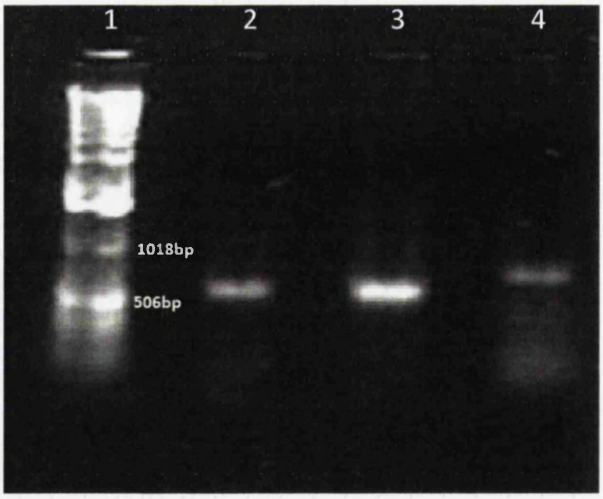


Fig 3.7 Agarose gel showing PCR products using hrdB primers and different templates. *Lane 1: Ladder (1Kb Plus NEB); lane 2: second strand DNA; lane 3: genomic DNA; lane 4: NTC.*

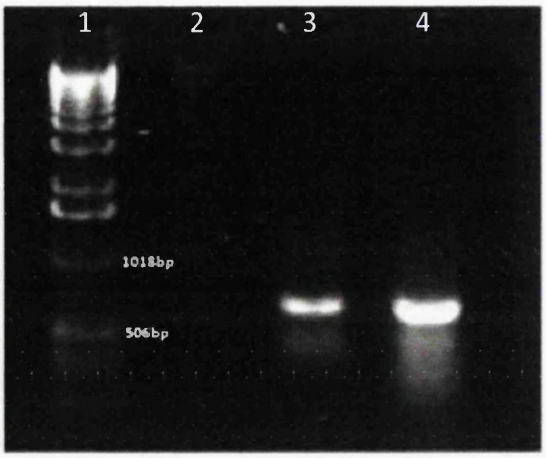


Fig 3.8 Agarose gel showing results of rtPCR products from amplified RNA. *Lane 1: Ladder (1Kb NEB); lane 2: 30 PCR cycles; lane 3: 35 PCR cycles; lane 4: 40 PCR cycles*

Protocol development also included adding a purification step after second strand synthesis. Proteinase K and SDS were used followed by a phenol chloroform extraction to further purify the DNA before inclusion in the RNA synthesis. Before using the MEGAscript kit a denaturation step was added, which resulted in an increase in the yield of amplified RNA (data not shown). To further increase yield, the ratios of ATP and UTP to GTP and CTP were altered to reflect the fact that *S. coelicolor* has a high GC content. These changes increased the yield from ~22µg to ~32µg when starting with 100ng of RNA.

Although the RNA amplification protocol had produced amplified RNA capable of successful rtPCR, there were intermittent problems resulting in no RNA being produced. The T7 primer sequences were compared to the suggested sequences in the MEGAscript kit. They were shown to be not ideal. Therefore new primers were designed (Table 3.6). An agarose gel showed production of the first strand (Fig 3.9) and second strand DNA (Fig 3.10). The four new T7 primers produced consistent positive results with *hrdB* PCR (Fig 3.11).

Table 3.6 New primer sequences N6, N9, shortN6, minimumN6

Primer name	Primer Sequence
N6	GGAATTAATACGACTCACTATAGGGAGANNNNNN
N9	GGAATTAATACGACTCACTATAGGGAGANNNNNNNNN
shortN6	GGAATTAATACGACTCACTATAGGGNNNNNN
minumumN6	TAATACGACTCACTATAGGGNNNNNN

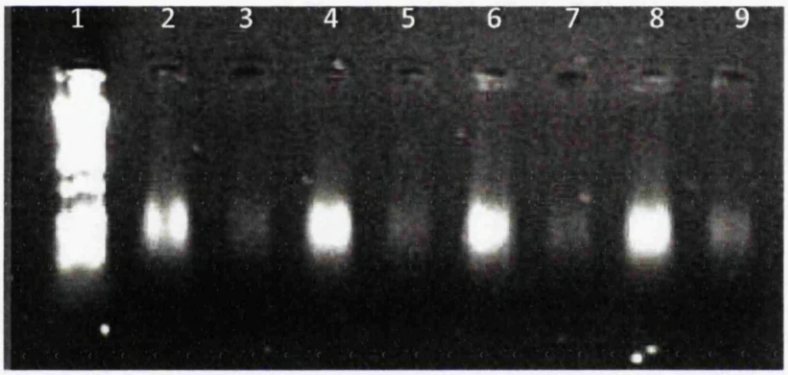


Fig 3.9 First strand DNA on agarose gel. *Lane 1: standard; lane 2: N6 126ng starting RNA; lane 3:N6 63ng RNA; lane 4: N9 126ng starting RNA; lane 5:N9 63ng RNA; lane 6: N6short 126ng starting RNA; lane 7: N6short 63ng RNA; lane 8: N6minimum 126ng starting RNA; lane 9:N6minimum 63ng RNA.*

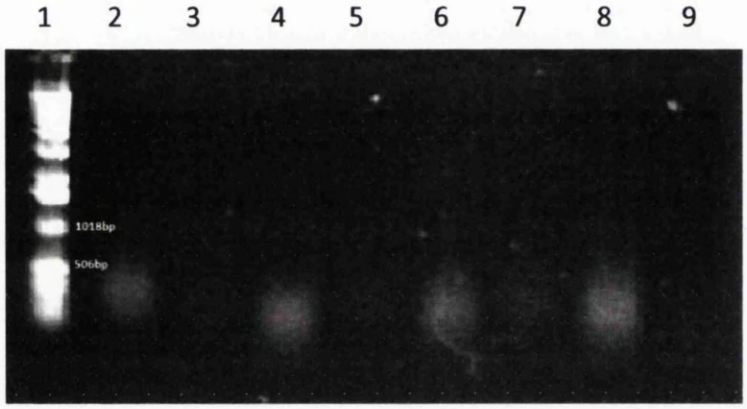


Fig 3.10 Second strand DNA on agarose gel. *Lane 1: standard; lane 2: N6 126ng starting RNA; lane 3:N6 63ng RNA; lane 4: N9 126ng starting RNA; lane 5:N9 63ng RNA; lane 6: N6short 126ng starting RNA; lane 7: N6short 63ng RNA; lane 8: N6minimum 126ng starting RNA; lane 9:N6minimum 63ng RNA.*

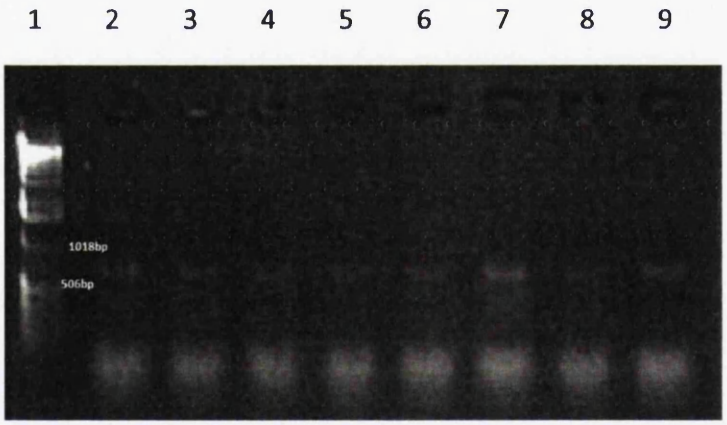


Fig 3.11 hrdB rtPCR results. *Lane 1: standard; lane 2: N6 126ng starting RNA; lane 3:N6 63ng RNA; lane 4: N9 126ng starting RNA; lane 5:N9 63ng RNA; lane 6: N6short 126ng starting RNA; lane 7: N6short 63ng RNA; lane 8: N6minimum 126ng starting RNA; lane 9:N6minimum 63ng RNA.*

The maximum efficiency of the RNA amplification procedure was tested using lower starting quantities of RNA. It was limited to 1ng starting material due to the limitations of the cDNA synthesis kit used for first strand synthesis. Utilising the final optimised protocol for the RNA amplification an amplification factor of 12525 was achieved (Table 3.6).

Table 3.6 Summary of results from RNA amplification with decreasing starting quantities of RNA.

Starting RNA quantity (ng)	2nd strand DNA (ng/µl)	amplified RNA (µg/µl)	Total RNA yield (µg)	RNA amplification factor
500	263	0.303	4.545	45.45
250	610	0.475	7.125	142.5
125	328	0.399	5.985	239.4
10	250	0.271	4.065	2032.5
1	190	0.167	2.505	12525

3.6 PCR

3.6.1 Genes of interest (GOI)

At the beginning of the project, 23 secondary metabolite gene clusters were identified from the genome sequence (Bentley, Chater et al. 2002). For each gene cluster a single gene was chosen which was deemed to be an important gene in the secondary metabolite biosynthesis and not in its regulation.

PKS

For each polyketide cluster, a PKS gene was chosen. For the Cpk cluster, *sco6273* from the cpkC unit was identified. This unit contains extension module 5 and the reductase module

for polyketide synthesis. From the *whi* cluster, *sco5318* was identified as ORFIII, a ketoacyl synthase, part of the minimal PKS (Kelemen, Brian et al. 1998). For the other polyketide clusters (unknown polyketide *sco6826-6827*, flaviolin, germicidin and a putative chalcone), (putative) PKS-encoding genes were also identified.

Fatty acid

In the eicosapentaenoic acid cluster, SCO0126, a putative beta-ketoacyl synthase is involved in fatty acid synthesis forming an acetoacyl ACP. Similarly, in the putative fatty acid secondary metabolite (*sco1265-sco1273*), *sco1271* encodes a putative oxoacyl synthase, also involved in acetoacyl production in fatty acid biosynthesis.

Terpenoids

In the isorenieratene cluster, crtE (encoded by *sco0185*) is a trans-isoprenyl diphosphate synthase that catalyses the successive 1'-4 condensation of the 5-carbon IPP to allylic substrates geranyl-, farnesyl-, or geranylgeranyl-diphosphate (NCBI).

The terpene synthase, SCO5222, catalyses the cyclisation of FPP to epi-isozizaene in albaflavenone biosynthesis.

In geosmin biosynthesis, a germacradienol synthase (SCO6073) catalyses Mg^2+ dependent conversion of FFP to the intermediate germacrene D (Cane and Watt 2003).

In the hopanoid cluster a phyotene synthase gene was chosen (*sco6759*), which is part of a super family, which catalyses the head to tail condensation of polyisoprene pyrophosphates (Altschul, Gish et al. 1990) in hopanoid biosynthesis.

For production of methylisoborneol production, the terpene synthase, SCO7700, works in combination with the *C*-methyl transferase SCO7701 to catalyse the two-step conversion of GPP to 2-methyl-isoborneol with the intermediate 2-methylgeranyl diphosphate (Wang and Cane 2008).

Peptides

For three of the peptide secondary metabolite gene clusters, genes encoding NRPSs were identified as biosynthetically important genes. For the coelichelin cluster, the NRPS gene *sco0492* (cchH) was chosen, along with *sco3220* for CDA and *sco7682* for coelibactin.

For the two lantibiotic gene clusters, LanC cyclases were identified and chosen as the genes of interest. LanC catalyses the addition of cysteine thiols to the amino acid compounds resulting in thioether formation, generating lanthionine or methyl-lanthionine (Patton and van der Donk 2005).

The genome sequence unearthed another putative NRPS secondary metabolite cluster, *sco6429-6438*. The structure of the product of these genes in unknown (Bentley, Chater et al. 2002). *sco6431* is thought to be biosynthetically important as it encodes a putative peptide synthase.

Miscellaneous secondary metabolites

The genome sequence exposed a putative deoxysugar secondary metabolite cluster, *sco0381* encodes a glycosyl transferase.

In the desferrioxamine cluster (*desA-D*), *desD* (encoding a cyclase), catalyses the oligomerisation of hydroxamic acid followed by cyclisation producing a macromolecule of desferrioxamine G and E (Barόna-Gomez, Wong et al. 2004; Barona-Gόmez, Lautru et al. 2006).

For the putative siderophore synthetase secondary metabolite cluster, *sco5800* was chosen as gene of interest due its similarity to a Rhizobium siderophore biosynthesis gene (*rhsC*).

The oligopyrrole undecylprodigiosin (Red) cluster contains 23 genes. *RedL* (*sco5892*) is a polyketide synthase which contains an A, KS, AT and ST domain, important for biosynthesis of the metabolite.

Within the SCB cluster, *sco6266* encodes scbA which along with regulatory properties has homology to fatty acid synthases and is able to synthesise SCB1, although the biosynthetic pathway is not known (Hsiao, Soding et al. 2007).

For production of methylenomycin, mmyC (*SCP1.233B*) catalyses the decarboxylative condensation of malonyl-MmyA with acetyl-CoA to form acetoacetyl-MmyA, the mid stage in methylenomycin production (Corre and Challis 2005).

3.6.2 Primer design

For each of these GOI, PCR primer pairs were designed using webprimer software (Table 5). The annealing temperature was optimised for each primer set using a gradient PCR on genomic DNA. The optimised PCR products can be seen in fig 3.12. Throughout the project, a further three secondary metabolite gene clusters were identified and GOI chosen and primers designed (Table 3.7). All primers pairs resulted in PCR products of predicted length.

Table 3.7 Webprimer PCR primer list

Secondary Metabolite	*GOI*	Ta	Forward Primer	Reverse Primer
Eicosapentaenoic acid	*SCO0126*	50	GACCCCGAGGAGAGACCATGT	CACACAGCCTGCGCCTTG
Isorenieratene	*SCO0185*	50	GAGAGTGAGAAGGGGCTGCG	TACCGAGTGCCGTCTCGGTC
Lantibiotic 1	*SCO0270*	48	TATCTCACGGCGGGAACCC	GCCCAGGTTGAGATGGCCT
Deoxysugar	*SCO0381*	48.5	CGGCAAGGGGGATTGGTC	GGCGATCAGATCGCGCAC
Coelichelin	*SCO0492*	48.5	GGAACCGACCGCTTCTCTCG	ACCATCTCGGCGGAGCGT
Albaflavanone	*SCO1206*	48.5	GGAGGAGACGCTGGAGCTGG	GGGGATCAGGTACGAGCCG
Putative type II FAS	*SCO1271*	48.5	GACCGGCATCGGCGTCTAC	TGTCGTCCCAGGTGAGCCC
Desferrioxamine	*SCO2785*	50	ACCTGACCCCCGAACGCT	CTCGTCCCGGACGAAGGACT
CDA	*SCO3230*	48	CTCGCCCAGCAGCTGGAT	ACACCGGCGATGACCACG
Actinorhodin	*SCO5087*	48.5	TCGGCCTTCGAGCCTCCT	ATCGGGGTGATCGGGGTG
Epi-isozizaene	*SCO5222*	50	GTCTCCCGGTGATCGAGGC	ACTCGGCTCCAGCAGGTCG
TW95a	*SCO5318*	48	GTTCTGGAGGCTGCTGTCCG	ACGTGCACCCGGTGGAGA
Siderophore synthetase	*SCO5800*	48	AGTGGCGGTCGGCCCACC	GGAGGAAGCGGCGGAAGG
Undecylprodigiosin	*SCO5892*	48.5	AACGTGAGCCACGGGCCGA	TGGACTCGCCGGTGTAGAGG
Geosmin	*SCO6073*	50	CACTTCTACCTGCCGCACCCC	GACCTCGCGCTGGTAGGAGA
SCB1	*SCO6266*	48.5	TTGTCCACCGGACCAGGG	TACGTGGTCGTTGGGGCG
cpk	*SCO6273*	50	CCTCAAGTGGGTGACGACCG	GAAGGTGTACGCGATCCGGC
Peptide	*SCO6431*	48	CGGACTCGTCGGCCACCTAC	CCAGGATGCCGGAGAGCAG
Hopanoids	*SCO6759*	50	AAGGCCGCCGACGAGAACT	CGCCTCCTGGACGTGGAA
Polyketide 1	*SCO6827*	50	TGCCTCACGACTCCACCCGC	GCACTCACCGAGCCGGATC
Lantibiotic II	*SCO6929*	48.5	CATACGGGAGCTGCATCACC	CGTTGTTGGCGTGCCCTT
Germicidin	*SCO7221*	48	TCGTCGAGGAGGTACGCGG	CAGTGCCAGGGAACGGGTC
Putative Chalcone	*SCO7671*	50	ACATGCGTGTCCCCGTGC	GAACAGCGGGAGCCGCTT
Coelibactin	*SCO7682*	50	GCTGGAGATCGCACCGGA	GGGTGTGGGTGTTGCGCT
Methylisoborneol	*SCO7700*	50	TACATGGTCGGCTGCCATC	GGGGCGGAAGTTGTTGAACTG
Methylenomycin	*SCP1.233B*	48	ACCAACGAGGAGCTGTCCCG	AGCCGGTACGGTCGAGCA

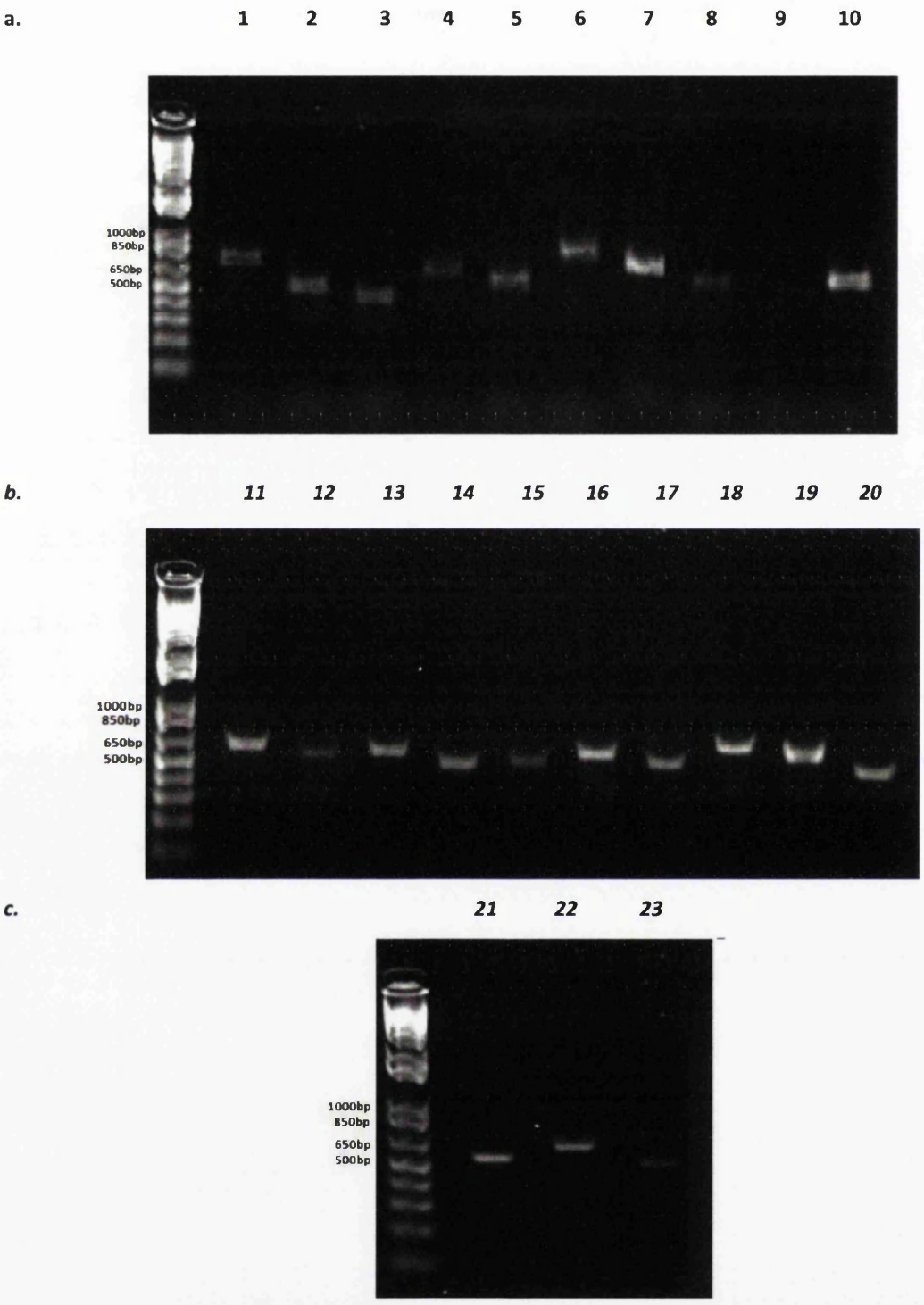


Fig 3.12 PCR products from secondary metabolite primers showing specificity. ***a.*** *Sco numbers: 1: 5087; 2: 7221; 3: 5318; 4:6266; 5:5892; 6: 3230; 7: 6073; 8:5222; 9: scp.1233; 10:0492;* ***b.*** *11: 2785; 12:7682; 13:6759; 14:0126; 15:0185; 16:1206; 17:6273; 18:6431; 19:6827; 20:7671;* ***c.*** *21:5800; 22:1271; 23:0381.*

For reproducibility purposes, for a repeat experiment, new primers pairs were designed for each gene cluster using Beacon software (Table 3.8). The annealing temperature was optimised for each primer set using a gradient PCR. All primers pairs resulted in PCR products of predicted length (not shown).

Table 3.8 Beacon PCR primer list

Secondary Metabolite	GOI	Ta	Forward Primer	Reverse Primer
Eicosapentaenoic acid	*SCO0126*	61	CACGATTCTGCTGTTCTC	TGCTGGAGGACTTCATTG
Isorenieratene	*SCO0185*	64	GACCCGAAGTCCGAGAAG	GATCTTCGTCTCCACCAG
Lantibiotic 1	*SCO0270*	63	CTGAACTGCCGTTCGGACTG	CGTCGAGGTAGCCGGAGAC
Deoxysugar	*SCO0381*	60	CTCACTGTTCATGGCGAC	AAGATCCAGAGTTTGATGTTG
Coelichelin	*SCO0492*	60	GAGTACGGTCCAGTGATC	GAGTGGTCATCGAGGTGA
Albaflavanone	*SCO1206*	58	ATCGACGTGATCATCTAC	GAAACCCATCTCGTTGAT
Putative type II FAS	*SCO1271*	61	CACGAGGACGGCTATATC	CATCTCGACGAAGGTGTC
Desferrioxamine	*SCO2785*	59	CTGGACTTCTTCATCGAG	GATCTCCTCCAGGTAGAC
CDA	*SCO3230*	58	AGAAATCCTCTGCACACT	GAGGTCGAAGAAGTTGTC
Actinorhodin	*SCO5087*	61	CGACGATGACGACGACCA	GCCCGTGATGACGACTCT
Epi-isozizaene	*SCO5222*	58	AGACGAGGTCCACAATCT	ACTTCTCCGATGGCTTCT
TW95a	*SCO5318*	63	CAACGCCTACCACATGAC	GAGTTCCGCCATGTCCAG
Siderophore synthetase	*SCO5800*	62	CGAACACAGCGACACGTT	ATGAGACCGAACACGTTGT
Undecylprodigiosin	*SCO5892*	63	GTCTCACCGACCGCTTCC	GATGATGTCCTGCACCGT
Geosmin	*SCO6073*	59	CTCCCTCATCACCGACTG	TGGCTGCGTTTGTACTTC
SCB1	*SCO6266*	59	GCCCGAAGCAGTAGTTTTGA	CCATCGGAACCGGCAATG
cpk	*SCO6273*	60	ACCATGATCGCCCTCCAA	CGATGGTGAGGTGGTGTT
Peptide	*SCO6431*	60	GTCGGCTGGACCAACTAC	GTCGTACTTGACCTGGAA
Hopanoids	*SCO6759*	63	TACGAGACCTACGACGACC	TGACGGCGAGGACGAGAC
Polyketide 1	*SCO6827*	60	TACCTGGAACTCAGCAAA	GCGTGGAAGGAAAGGTTG
Lantibiotic II	*SCO6929*	61	ATGAATCTGCGTGACAGC	CCGTATGTCCTGTGGTGA
Germicidin	*SCO7221*	62	GTCCTGGTCGTGATCTCC	GACGATCAGGGACTCCAG
Putative Chalcone	*SCO7671*	64	CACGCGCAGTCACCTGTA	GATCACGACACGGAAGCC
Coelibactin	*SCO7682*	62	GAGATCGCACCGGAGGAG	GGACATCAACGCCAGTGA
Methylisoborneol	*SCO7700*	60	GACATGCAACGGGTCATC	TGTTGAGTTCCTTGGTGTAG
Methylenomycin	*SCP1.233B*	62	GGTTTCATCTACGGACTC	ATCGAGGATGGTGGAATA

3.7 Chapter 3 discussion

3.7.1 Growth of *S. coelicolor* in soil

S. coelicolor was inoculated into the soil microcosm at $1x10^7$ cfu/g dry soil which resembles naturally occurring streptomycete cfu's (Saadoun, Mohammad et al. 1998). *S. coelicolor* was able to grow in the sterile soil microcosms without further nutrient amendments (Chapter 4, Fig 4.1), indicating that autoclaved soil contains enough nutrients and other trace elements for normal growth. As spores were suspended in 20% glycerol solution, each microcosm contained between 3.25 and 6.5nM of glycerol which correlates to $5.1x10^{-6}$% glycerol. Typically, studies investigating glycerol as a carbon source for *Streptomyces* use up to 5.9% glycerol (Winkelmann 2002; Chen, Tang et al. 2011), so the effect of such a small amount used in this study would be minimal. Growth curves displayed typical *Streptomyces* growth patterns but over a longer timescale, reaching stationary phase in four days, compared to 30h in R5 media (Huang, Lih et al. 2001). A common feature for all growth curves in soil was a drop in total viable count between inoculation and day one. This is the usual growth pattern of *Streptomyces* in soil and may be attributed to the death phase of young germlings (Burroughs, Marsh et al. 2000), or the number of spores that germinated and became undetectable by the extraction technique (Herron and Wellington 1990). After day one, *S. coelicolor* entered exponential growth as the spores underwent germination and substrate mycelia were formed. By day three, after apparent nutrient depletion, *S. coelicolor* growth slowed and the bacteria entered stationary phase.

3.7.2 RNA extraction and amplification

RNA quality is a critical determinant in the analysis of gene expression. Preparation of high quality RNA from soil has proved challenging (Burgmann, Widmer et al. 2003; Han and Semrau 2004; Luis, Kellner et al. 2005). Published methods process small amounts of soils, yielding low amounts of RNA, and often result in the co-extraction of humic and other inhibitory substances. Humic substances can interfere with downstream processes, especially those involving enzymic reactions (Tebbe and Vahjen 1993). Of the tested

extraction methods, the RNA PowerSoil kit from Mobio yielded high-quality total RNA, as indicated by sharp 16S and 23S bands (Fig 3.4a). Smears on a denaturing RNA gel indicated RNA degradation in the samples from the two other extraction methods. Also, where the RNA extract solution was colourless for the RNA PowerSoil kit, the other kits resulted in a brown-tinted solution, indicating the presence of inhibiting humic acids. Using RNA PowerSoil kit the humic acids were removed in a solvent extraction step, which limited the carryover of inhibitors.

The RNA amplification method used was adapted from Gao (Gao, Yang et al. 2007). Due to the high-GC content of *S. coelicolor* (72.1%) the ratios of ATP and UTP to GTP and CTP were altered for the amplification step (MEGAscript kit). Proteinase K and SDS were used followed by a phenol chloroform extraction to further purify the DNA before inclusion in the RNA synthesis. Before using the MEGAscript kit a denaturation step was added, increasing the yield of amplified RNA. These changes increased the yield from ~22ug to ~32ug when starting with 100ng of RNA.

3.8 Summary

To summarise, soil was collected from an organic farm in the Gower, Swansea. The soil was classified as a sandy loam, with 2.47% TOC, pH 5.9 and was deemed highly hydrophilic. The air dried water content was 3.11% and water saturation was 42.03%. /after inoculation with spores, *S. coelicolor* growth curves were established indicating that *S. coelicolor* had entered the stationary growth phase after four days incubation. Comparisons of protocols for RNA extraction from soil were undertaken, with Mobio PowerSoil Total RNA extraction kit yielding the highest amount and highest quality RNA. An RNA amplification protocol was optimised, by the design of new primers, changing the rations of ATP, UTP, GTP and CTP and the addition of a purification step. For each secondary metabolite gene cluster, a single gene was chosen which was deemed to be an important gene in the secondary metabolite biosynthesis and not in its regulation, for which PCR primers were designed and annealing temperatures optimised.

Chapter 4: Secondary metabolite gene expression profiles in *S. coelicolor* grown in soil microcosms

In this chapter the results from transcriptomic studies on *S. coelicolor* grown in sterile soil microcosms are described and discussed. This chapter describes and discusses the results. Firstly, growth of *S. coelicolor* in soil was analysed. Microcosms were subjected to nutrient, biotic and metal amendments. RNA was extracted from these microcosms over a time-course of five days, which was then amplified using an optimised T7 RNA polymerase RNA amplification protocol (Chapter 3). The transcripts were analysed using reverse transcription-PCR firstly. Four genes were then selected for in-depth analysis using quantitative PCR to measure relative transcript abundance. The nutrient amended microcosm expression profiles are discussed firstly in secondary metabolite class order, followed by the biotic-amended expression profiles. The siderophore clusters are the last to be discussed, with gene expression profiles from nutrient-amended microcosms, biotically-amended microcosms and metal amended microcosms, with data from endpoint rtPCR and qPCR.

4.1 Nutrient amendments

The effect of changing nutrient availability on the growth of *S. coelicolor* in soil microcosms was investigated. Amendments to the soil included the addition of 1% starch and 1% chitin individually and combined. Chitin and starch were chosen as they represent environmentally available carbon and nitrogen nutrients, which have been shown to benefit selectivity, growth and cell number of actinomycetes (Küster and Williams 1964; Hsu and Lockwood 1975; Abdel-Fattah and Mohamedin 2000). In addition, both chitin and starch are used routinely in *Streptomyces* soil microcosm studies as sources of carbon and nitrogen (Herron and Wellington 1990; Ravel, Wellington et al. 2000; Anukool, Gaze et al. 2004). The results were compared to growth in sterile soil (2.47% TOC), without nutrient amendments. Fig 4.1 shows the growth curves for the nutrient-amended soil microcosms. Each of the four growth curves shows very similar growth patterns. The cfu dropped between a 0.5 and 1.5 log from day zero to day one, which is linked with spore germination. The microcosms were

growing exponentially between days one and four. Between days four and five the growth rate decreased in the nutrient-amended microcosms, indicating the transition to the stationary growth phase. In the sterile microcosm, however, the growth did not slow by day five, which indicated the culture was still undergoing exponential growth.

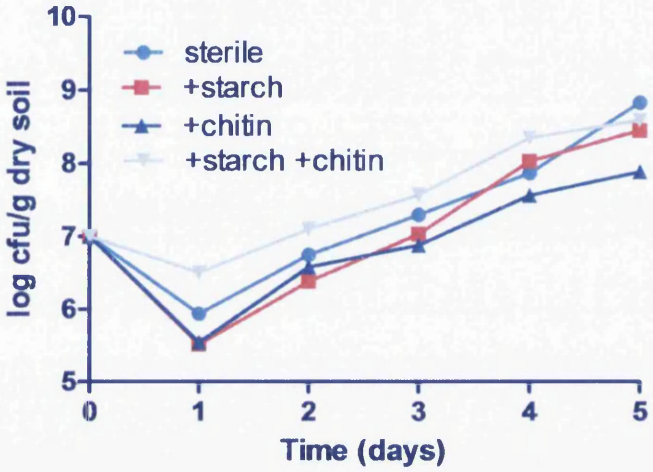


Fig *4.1 Nutrient-amended growth curves:* *S. coelicolor growth (log_{10} cfu/g dry soil) over five days in soil nutrient amended microcosms.*

The doubling time of *S. coelicolor* in sterile unamended soil was 674 min (11.2h). The doubling time was reduced for the microcosm with 1% starch by 156 min to 518 min but only by 31 min in the microcosm with 1% chitin to 643 min. However, microcosms with double amendment of starch plus chitin showed an increase in doubling time by 31 min to 705 min. The standard deviation of the doubling times was 82 min, with a Coefficient of Variation of 12.9%. Deviation of the extreme values was calculated at 0.85 for the upper value and -1.43 for the lower value. Both values were within the standard value of +/- 1.64. Variation between the doubling times of unamended and amended microcosms was therefore limited and the growth curves were therefore not significantly different. This indicates that putative differences in phenotype are not due to growth rate.

RNA was extracted from the microcosms as an indicator of gene expression. RNA was extracted every 24h for four days. The yield of RNA extracted is shown in Table 4.1. Extractions yielded between three and four µg of total RNA per gram of soil. Due to the low yield of RNA a T7 RNA polymerase-based amplification procedure (Chapter 3) was applied and was yielding up to 52µg of amplified RNA from 500ng starting material (Table 4.1). The sample RNA was amplified to provide sufficient material for gene expression analyses. Random PCR-based amplification may not have been appropriate due to amplification bias and therefore the loss of reliable information (Wang 2005; Nygaard and Hovig 2006). The yields along with final RNA amplification factor are shown in Table 4.1.

Table 4.1 RNA extraction from microcosm-grown* S. coelicolor *and amplification. *The total yield of RNA extracted from each microcosm is given with total yields after T7 RNA polymerase amplification (500ng starting material).*

Microcosm/Amendment	Day	Amount of RNA Extracted (µg)	Total RNA yield after amplification (µg)	RNA amplification factor
Sterile soil	**1**	**3.5**	**30.1**	**301**
	2	2.2	52.8	566
	3	**5.3**	**12.0**	**121**
	4	2.3	33.1	331
+Starch	**1**	**1.5**	**18.5**	**185**
	2	2.5	31.6	316
	3	**3.9**	**30.1**	**300.7**
	4	4.4	27.3	273.5
+Chitin	**1**	**3.5**	**31.8**	**318.5**
	2	1.7	30.9	416.8
	3	**2.5**	**37.1**	**371.3**
	4	2.0	33.9	404.6
+Starch +Chitin	**1**	**1.9**	**19.9**	**245**
	2	2.5	30.5	305
	3	**3.3**	**22.3**	**223**
	4	2.3	33.8	348

Expression of genes was shown by the presence of product resulting from reverse transcription-PCR performed on the amplified RNA. One primer pair for one of the core biosynthetic genes in each secondary metabolite gene cluster (as described in Chapter 3) was used, totalling 26 genes studied. *hrdB* was used as internal control, which is a commonly used housekeeping gene as it encodes for the constitutively expressed sigma factor that promotes the attachment of RNA polymerase to specific initiation sites before being released (Tetsuo, Kan et al. 1991). To ensure the RTA of each treatment was not obscured by differences in growth rate, each RTA was normalised to the housekeeping gene RTA. This ensured comparable results by taking into account the amount of growth under each condition. In parallel, control PCRs were performed using RNA before the reverse transcription step to confirm that amplified products were not derived from (contaminating) chromosomal DNA. Fig 4.2 shows a summary of the 26 reverse transcription-PCR experiments performed. Overall, 19 of the 26 secondary metabolite biosynthetic genes studied were expressed in microcosms with at least one of the amendments.

		sterile				+S				+C				+S+C			
(Predicted) compound, enzyme name	**SCO #**	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Eicosapentaenoic acid, ketoacyl synthase	*SCO0126*																
Isorenieratene(carotenoid), geranylgeranyl	*SCO0185*																
Lantibiotic 1, cyclase	*SCO0270*																
Deoxysugar, glycosyl transferase	*SCO0381*																
Coelichelin, NRPS	*SCO0492*																
Tetrahydroxynaphthalene/flaviolin, polyketide synthase	*SCO1206*																
Putative type II FAS, oxoacyl synthase	*SCO1271*																
Desferrioxamine, putative siderophore synthetase	*SCO2785*																
CDA, NRPS	*SCO3230*																
Actinorhodin, polyketide synthase	*SCO5087*																
Epi-isozizaene, terpene synthase	*SCO5222*																
TW95a, polyketide synthase	*SCO5318*																
Putative siderophore synthetase, siderophore synthetase	*SCO5800*																
Undecylprodigiosin, polyketide synthase	*SCO5892*																
Geosmin, sesquiterpine cyclase	*SCO6073*																
SCB1, scbA protein with homology to a fatty acid synthase	*SCO6266*																
cpk, type I polyketide synthase	*SCO6273*																
Peptide, polyketide synthase	*SCO6431*																
Hopanoids, phyotene synthase	*SCO6759*																
Polyketide 1, polyketide synthase	*SCO6827*																
Lantibiotic II, cyclase	*SCO6929*																
Germicidin, type III polyketide synthase	*SCO7221*																
Putative Chalcone, putative chalcone sythase	*SCO7671*																
Coelibactin, NRPS	*SCO7682*																
Methylisoborneol, terpene synthase/ putative cyclase	*SCO7700*																
Methylenomycin, ketoacyl synthase	*SCP1.233B*																

Fig 4.2 Reverse transcription-PCR results for each secondary metabolite biosynthetic gene within sterile nutrient-amended* S. coelicolor *microcosms. *Red indicates no expression, green indicates expression. Microcosms amended with 1% starch are indicated by +S, 1% chitin-amended microcosms are indicated by +C, microcosms amended with both 1% starch and 1% chitin are indicated by +S+C.*

The expression profiles for each growth condition/gene have been grouped according to their expression pattern: no expression, constitutive expression, nutrient-dependent expression and other expression pattern.

Seven genes were not expressed under any nutrient-related conditions tested. These included *sco0126*, which codes for a ketoacyl synthase in the eicosapentaenoic acid cluster, *sco2785 (desD)*, a gene involved in desferrioxamine biosynthesis, *sco3230* (*cdaPSI*), an NRPS gene in the CDA gene cluster, *sco5892* (*redL*), a polyketide synthase gene in the Red gene cluster, *sco6073* (*geoA*), a sesquiterpene cyclase/synthase in the geosmin gene cluster, *sco7671*, a putative transferase gene in a putative chalcone synthase cluster and finally *SCP1.233B (mmyC)*, a ketoacyl synthase in the methylenomycin gene cluster. The latter was used as an internal negative control as M145 (the strain used in this study) does not contain SCP1, the linear 365kb plasmid, which encodes for the *mmy* cluster (Kieser, Kieser et al. 1992).

Only one gene showed constitutive expression in each nutrient-amended microcosm. This was *sco7682*, encoding the first NRPS gene in the coelibactin gene cluster (Kallifidas, Pascoe et al. 2010).

Many of the genes studied showed nutrient-dependent and/or temporal expression patterns. In the microcosms containing starch only both *sco0492* (*cchH*), a NRPS gene in the coelichelin gene cluster and *sco6266 (scbA)*, encoding the ScbA protein in the *SCB1* gene cluster were expressed on days two, three and four exclusively. Two genes showed constitutive expression in the microcosms amended with 1% starch. These were *sco5800* a putative siderophore synthase gene, which was cryptic up to now, and also *sco7700*, a putative terpene synthase/cyclase gene in the methylisoborneol gene cluster. Two genes were expressed only in microcosms in the presence of 1% chitin. These were *sco6273*, a type I PKS gene in the *cpk* gene cluster, which was expressed only on day two, and *sco6929*, a cyclase gene from the lantibiotic II gene cluster expressed on days one and two. A pattern emerged from the microcosms where genes were expressed in the sterile microcosms and also in the microcosms containing chitin and starch plus chitin; however, no expression was seen in the starch only microcosms. This indicates that they are not induced by starch in the absence of chitin. Both *sco0185* (*crtE*), a geranylgeranyl pyrophosphate synthase in the

isorenieratene gene cluster, and *SCO5087* (*actI-ORF1*), a polyketide synthase gene from the actinorhodin gene cluster, showed expression on days two to four in the sterile microcosms. This was replicated in the chitin and starch plus chitin microcosms, however, there was also expression on day one in these microcosms. *SCO5318 (whiE-ORFIII)*, a polyketide synthase gene in the TW95a (whiE) gene cluster was expressed on days two to four in the sterile microcosm. In the presence of 1% chitin, *sco5318* expression was constitutive. In the 1% starch plus 1% chitin microcosms, *sco5318* expression was detected from day one to day three, i.e. expression on the fourth day was not observed. Growth in the presence of starch resulted in silencing of *sco5318 (whiE-ORFIII).* A mostly similar pattern was found for *sco7221.* Constitutive expression of *sco7221,* a type II polyketide synthase gene in the germicidin gene cluster, was shown in sterile microcosms and in the chitin and starch plus chitin amended microcosms, whereas its expression was not found in starch-amended microcosms.

Other nutrient-dependent patterns included that of *sco0270,* a cyclase gene from the lantibiotic I gene cluster, and *sco0381,* a glycosyl transferase gene in the deoxysugar gene cluster. Both these genes were not expressed in either the sterile microcosms or the microcosms containing both starch and chitin. However, in the starch microcosm, *sco0270* and *sco0381* were expressed constitutively, whereas in the chitin microcosms their expression was induced only on day two. Expression of *sco5222* (*eizA*), a terpene synthase gene from the albaflavenone gene cluster, was found in both the starch and the starch plus chitin microcosms on day four only. *sco5222* expression was not observed under any other condition on any other day. Expression of *sco6759,* a phytoene synthase gene in the hopanoid gene cluster, was induced by the addition of starch on days one to three. It was also expressed on day three only in the chitin microcosm, whereas there was no expression in either the sterile soil microcosms or the starch plus chitin microcosms.

Other expression patterns included that of *sco1206 (rppA),* a PKS gene from the tetrahydroxynaphthalene/flaviolin gene cluster. In sterile soil microcosms, this gene was expressed on days three and four. With the addition of starch, gene expression was delayed to day four, whereas with the addition of chitin *sco1206* was expressed on days one and two. S*CO1206* was not expressed at all in the starch plus chitin microcosms. *sco1271* (*fabH3*), an oxoacyl synthase gene in the putative type II FAS gene cluster, was also

expressed on days three and four in the sterile soil microcosms. However, the only nutrient-supplemented microcosm where this gene was expressed was the starch plus chitin microcosm on day two. *sco6431*, a cryptic peptide synthase gene from a putative NRPS gene cluster was expressed on days three and four in the sterile microcosms. The addition of chitin and/or starch did not result in induced expression on days three and four. However, when chitin was added, both singularly and with starch, expression was induced temporally on day two only. A cryptic type I PKS gene (*sco6827*) from a putative polyketide gene cluster was expressed on day four in the sterile soil microcosms. With the addition of starch, expression was found but somewhat earlier on day three only. Gene *sco6827* was found to be induced temporally on days one and two in the presence of chitin, but in contrast was not at all expressed in the 1% starch and 1% chitin microcosms.

4.2 Biotic amendments

The effect of the addition of entomopathogenic microorganisms, which have been shown to environmentally produce bioactive secondary metabolites, on the growth of *S. coelicolor* in soil was also tested. This included addition of an extract of the nematode *Steinernema kraussei* or of spores of the fungus *Metarhizium anisopliae* to *S. coelicolor* microcosms containing starch and chitin. The family to which this species of nematode belongs, Steinernematidae, plays an important role in regulating soil food webs (Strong, Kaya et al. 1996; Jaffee and Strong 2005), acting as biological insecticides (Shapiro-Ilan and Gaugler 2002; Grewal, Ehlers et al. 2005). They are parasitic due to the release of bacterial symbionts from the gut, which feed of insects (Han and Ehlers 2000). Species from the *Metarhizium* genus are also used as biocontrol agents, preventing infestations of a wide variety of insects (Lomer, Prior et al. 1997; Milner 1997; Milner and Pereira 2007). For instance, *M. anisopliae* has been shown to be effective in the control of malaria-vectoring mosquitoes (Bischoff, Rehner et al. 2009).

Chitin and starch amended microcosms were chosen over sterile and singular nutrient-amended microcosms as the least number (eight) of secondary metabolite genes were expressed under these conditions, whereas the other conditions expressed nine to thirteen

genes. The expression profiles of the biotically-amended microcosms were compared with the starch plus chitin microcosms. Fig 4.3 shows the growth curves for *S. coelicolor* in microcosms with biotic amendments. The microcosms amended with 1% starch and 1% chitin showed exponential growth of *S. coelicolor* between day one and four. Growth slows by day five, indicating the beginning stage of the stationary phase was reached. However, *S. coelicolor* growing in the fungi-amended microcosm displayed a longer lag phase and reached the stage of exponential growth after day two with exponential growth continuing to day five. *S. coelicolor* growing in the nematode extract-amended microcosms showed exponential growth from day one, which continues to day five with a slight dip in growth rate on day three possibly indicating biphasic growth. During determination of cfu on SFM agar using microcosm dilutions, growth of *M. anisopliae* was not observed in dilutions of the fungi-amended microcosms during the period of incubation used. *M. anisopliae* usually grow to visual colonies after four days on SFM agar at 28^0C, the culturing conditions used in this study (Schoenian et al 2011). Slimy, spreading colonies and sections appeared on the SFM plates inoculated with the less diluted fractions from nematode extract-amended microcosms, although this did not interfere with determining the cfu of *S. coelicolor*.

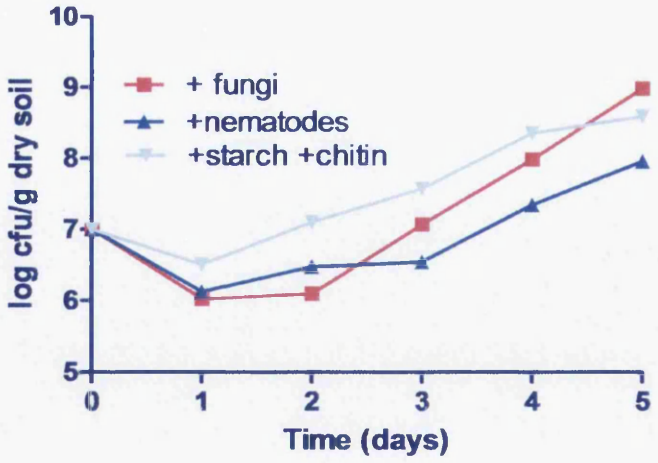


Fig 4.3 Growth curves of S. coelicolor grown in microcosms amended with nematode extract, fungi and starch plus chitin.

The doubling time of *S. coelicolor* in the microcosm amended with starch and chitin was 705 min. This was reduced considerably (by 36%) with the addition of fungi to 451 min. However, the addition of nematodes increased the doubling time of *S. coelicolor* by 175 min to 880 min.

The standard deviation of the doubling times for both biotic amendments and the microcosms amended with starch and chitin was 215.7, with a Coefficient of Variation of 31.8%. Deviation of the extreme values calculated at 0.93 for the upper value and -1.2 for the lower value. Both values were within the standard value of +/- 1.64. Variation between the doubling times was therefore limited and the growth curves are consequently not significantly different. Therefore differences in phenotype are not due to a growth rate phenotype.

RNA was also extracted from the microcosms every 24h for four days as an indicator of gene expression. The yield of RNA extracted from each microcosm is shown in Table 4.2. RNA amplification was successful producing yields of up to 37μg from 500ng starting material. The yields along with final RNA amplification factor are shown in Table 4.2.

Table 4.2 RNA extraction from biotic amended S. coelicolor microcosms and RNA amplification yields. *The total yield of RNA from each microcosm is given with yields after T7 RNA polymerase amplification (from 500ng starting material).*

Microcosm	Day	Yield of extracted	Total amplified RNA	RNA amplification
Nematodes	**1**	**3.6**	**37.5**	**375**
	2	5.9	32.8	328
	3	**4.8**	**33.3**	**334**
	4	4.8	26.6	266
Fungi	**1**	**3.7**	**26.1**	**261**
	2	4.0	24.3	243
	3	**4.3**	**28.6**	**286**
	4	5.8	21.4	214

Expression profiles for each secondary metabolite biosynthetic gene were established for each amendment over the time course with *hrdB* used a as an internal control for all PCRs. RNA before the reverse transcription step was used as a negative control to confirm that amplified products were not derived from carried over chromosomal DNA. The results of the duplicate reverse transcription PCRs are show in Fig 4.4.

		+S+C				+S+C+F				+S+C+N			
Predicted compound, enzyme name	SCO number	1	2	3	4	1	2	3	4	1	2	3	4
Eicosapentaenoic acid, ketoacyl synthase	*SCO0126*												
Isorenieratene(carotenoid), geranylgeranyl pyrophosphate	*SCO0185*												
Lantibiotic 1, cyclase	*SCO0270*												
Deoxysugar, glycosyl transferase	*SCO0381*												
Coelichelin, NRPS	*SCO0492*												
Tetrahydroxynaphthalene/flaviolin, polyketide synthase	*SCO1206*												
Putative type II FAS, oxoacyl synthase	*SCO1271*												
Desferrioxamine, putative siderophore synthetase	*SCO2785*												
CDA, NRPS	*SCO3230*												
Actinorhodin, polyketide synthase	*SCO5087*												
Epi-isozizaene, terpene synthase	*SCO5222*												
TW95a, polyketide synthase	*SCO5318*												
Putative siderophore synthetase, siderophore synthetase	*SCO5800*												
Undecylprodigiosin, polyketide synthase	*SCO5892*												
Geosmin, sesquiterpene cyclase	*SCO6073*												
SCB1, scbA protein with homology to a fatty acid synthase	*SCO6266*												
cpk, type I polyketide synthase	*SCO6273*												
Peptide, polyketide synthase	*SCO6431*												
Hopanoids, phytoene synthase	*SCO6759*												
Polyketide 1, polyketide synthase	*SCO6827*												
Lantibiotic II, cyclase	*SCO6929*												
Germicidin, type III polyketide synthase	*SCO7221*												
Putative Chalcone, putative chalcone sythase	*SCO7671*												
Coelibactin, NRPS	*SCO7682*												
Methylisoborneol, terpene synthase/ putative cyclase	*SCO7700*												
Methylemomycin, ketoacyl synthase	*SCP1.233B*												

Fig 4.4 Reverse transcription-PCR results for each secondary metabolite gene with in* S. coelicolor *microcosms with biotic amendments. *Red indicates no expression, green indicates expression. Microcosms amended with 1% starch and 1% chitin are indicated by +S+C, microcosms amended with 1% starch and 1% chitin plus fungi are indicated by +S+C+F, microcosms amended with 1% starch and 1% chitin plus nematode extract are indicated by +S+C+N.*

Introduction of biological organisms producing bio-active compounds themselves did not result in a significant reorganisation of secondary metabolite gene expression in *S. coelicolor*. Compared to the eight secondary metabolite genes expressed in the starch and chitin-amended microcosms, upon the addition of fungal spores this number fell to seven, and six with the addition of nematode extract. There were only five instances where a gene was expressed in either the fungi-amended microcosm or the nematode-amended microcosm but not in the presence of starch and chitin. Firstly, *sco0381* from the deoxysugar gene cluster was expressed on day four in the fungi-amended microcosm and on days one and two in the nematode-amended microcosm. *sco6273* from the *cpk* gene cluster was expressed on day two in the fungi-amended microcosm. The same expression pattern was also shown in chitin-amended microcosms. *sco6929* from the lantibiotic II gene cluster was expressed on day one in the nematode-amended microcosms. This however is not novel as this gene was expressed on days one and two in the 1% chitin-amended microcosm (see Section 4.1).

The global differences in gene expression profiles with biotic amendments were when genes were silenced upon addition of fungal spores or nematode extract. Of the 26 genes studied, four were constitutively expressed under starch plus chitin conditions. However, when either fungi or nematode extract were added these genes were no longer constitutively expressed. *sco0185* from the isorenieratene/carotenoid gene cluster was not expressed on days three and four in the fungi-amended microcosm and showed no expression on day two in the nematode-amended microcosm. *sco5087* from the actinorhodin gene cluster was not expressed on days one and three in the fungi-amended microcosm and on days two and four in the nematode-amended microcosm. *sco7221* from the germicidin gene cluster was not expressed on day three in the fungi microcosms and days one and four in the nematode microcosms. *sco7682* from the coelibactin cluster was not expressed on day one in the fungi microcosms and day four nematode microcosms. This gene, however, was expressed constitutively in sterile soil and all nutrient-amended microcosms.

Other expression profiles for the biotic-amended microcosms include that of *sco1271* from the putative type II FAS gene cluster and *sco6431* from a NRPS gene cluster. Both genes were not expressed in either the fungi or nematode microcosms but were expressed on day two in the starch plus chitin amended microcosms. Similarly, sco*5222* from the epi-

isozizaene gene cluster was not expressed in the fungi- or nematode-amended microcosms but was expressed on day four in the starch plus chitin microcosm. *sco5318* from the TW95a gene cluster was expressed on days one to three in both the starch and chitin amended microcosms and also in the fungi-amended microcosms but showed no expression with the addition of nematode extract.

4.3 Siderophore gene cluster expression

After analysis of the secondary metabolite gene cluster expression profiles an interesting pattern emerged from the siderophore gene clusters. *sco0492* expression was induced by the addition of 1% starch on days two, three and four, but was silent under all other conditions tested. *sco5800* expression was constitutive in the presence of starch, but silent under the three other conditions. Sco2785 was not expressed under any condition. Surprisingly, *sco7682* was constitutively expressed in sterile soil and across all nutrient amendments tested. qPCR was used to quantify the changes in expression of the siderophore gene clusters indicated by endpoint PCR.

The negative controls used were non-reverse transcribed RNA from each sample. The negative control results were scored for significant differences to the samples (Table 4.3) using the Mann Whitney test. When the critical threshold value (cT) of the sample was significantly lower than the negative controls, the sample gene is expressed. The 16S rRNA gene was used as positive control and for normalisation. This gene encodes for 16S rRNA, a component of the 30S subunit of a ribosome. During each qPCR cycle, the integrity of the sample cDNA was tested using the 16SrRNA gene as positive control. Mann Whitney tests indicated that the cT values were significantly lower than the no template control (NTC) for all cDNA samples (p values in Table 4.3). The cT values were then used for normalisation of relative transcript abundance (RTA).

Table 4.3 P values from comparison of the cT values from the negative control and the sample cDNA RTA in sterile and nutrient amended microcosms. *Highlighted in red are p values above 0.005 showing no significant difference. Highlighted in blue are p values below 0.005 showing a significant difference. Where there is no p value, the cT value from the cDNA was either the same or higher than the negative control. RTA is the relative transcript abundance (normalised to 16S expression).*

Gene	DAY	Sterile		+S		+C		+S+C	
		P value	RTA	P value	RTA	P value	RTA	P value	RTA
16S	1	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
	2	0.0046	1.000	0.0046	1.000	0.0041	1.000	0.0046	1.000
	3	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
	4	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
sco0492	1			0.4486		0.0128	0.0047	0.8082	
	2	0.8082				0.0046	0.0006	0.0046	0.0713
	3			0.0128	0.0032	0.0046	0.0010	0.0046	0.0117
	4			0.0046	0.0005	0.0128	0.0011	0.0046	0.0467
sco2785	1							0.3734	
	2			0.0046	0.0027			0.0046	0.3914
	3			0.3734		0.0046	0.0011	0.0046	0.0550
	4					0.3734		0.0046	0.1652
sco5800	1			0.0046	0.0056	0.1242		0.0809	
	2			0.0046	0.0025	0.1242		0.0809	
	3	0.0046	0.0110	0.0046	0.0028	0.0046	0.0018	0.0809	
	4			0.0046	0.0003	0.0289	0.0073	0.1904	
sco7682	1			0.0046	0.0053	0.0046	0.0008		
	2			0.0128	0.0025	0.0046	0.1158	0.0046	0.0026
	3	0.0046	0.0010	0.0046	0.0016	0.0041	0.0068	0.0289	0.0007
	4	0.0046	0.0009	0.0046	0.0004	0.0046	0.1079	0.0046	0.0022

The RTA for each of the four siderophore genes was compared for each microcosm amendment in order to determine the effect of each amendment. The qPCR data for *S. coelicolor* grown in sterile microcosms (Fig 4.5) indicated no expression of *sco0492* or

sco2785. There was comparatively high expression of *sco5800* on day three only, although with a large standard error (discussed with Fig 4.11). Expression of *sco7682* was reduced by 90% compared to *sco5800* on day three and this was continued on day four.

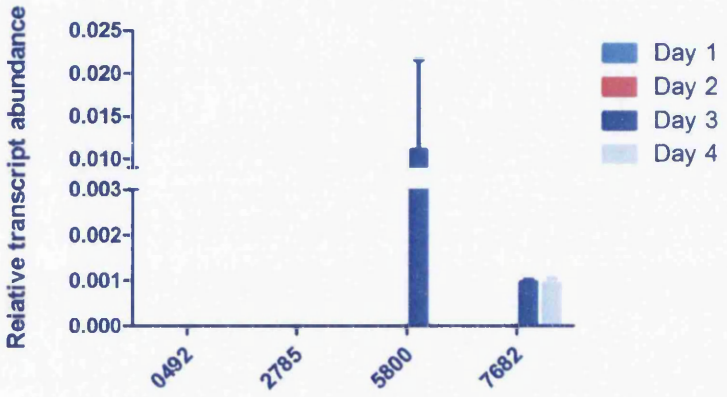


Figure 4.5 Relative transcript abundance of siderophore genes from sterile soil microcosms. *Normalised temporal RTA from* S. coelicolor *grown in sterile soil microcosms. The numbers on the X axis represents sco gene numbers. Error bars indicate the standard error of the mean (SEM).*

In the starch-amended microcosms (Fig 4.6) *sco0492* was not expressed on days one or two, but expression was induced on day three which was then reduced significantly on day four to 20%. *sco2785* was expressed on day two only. The RTA of *sco5800* varied across days one to four. There was a significant drop in expression from day one to day two. A slight increase could be seen on day three, although not significant at p=0.3734. This was followed by a significant drop of 90% in RTA on day four. *sco7682* was expressed highly on day one. The RTA decreased significantly from day two with a final reduction of 68% by day four.

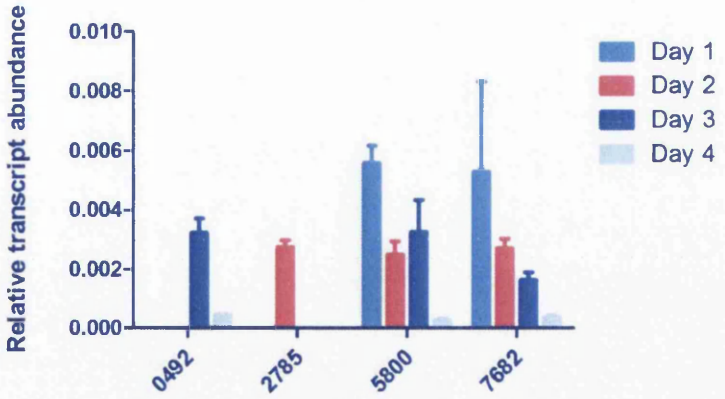


Figure 4.6 Relative transcript abundance of siderophore genes from 1% starch amended soil microcosms. *Normalised temporal RTA from* S. coelicolor *grown in soil microcosms with 1% starch added. The numbers on the X axis represents sco gene numbers. Error bars indicate the SEM.*

sco0492 was expressed across all days in the 1% chitin amended microcosms (Fig 4.7). There was a significant increase (two-fold) in expression from days two to three. There was no expression of *sco2785* on days one, two and four, with low expression on day three. *sco5800* was expressed only on days three and four with a four-fold increase on day four. *sco7682* had limited expression on day one with a significant increase of over 100-fold in RTA by day two. Expression then significantly decreased to 6% by day three and rose significantly again on day four. The levels of expression on day two and day four were not significantly different.

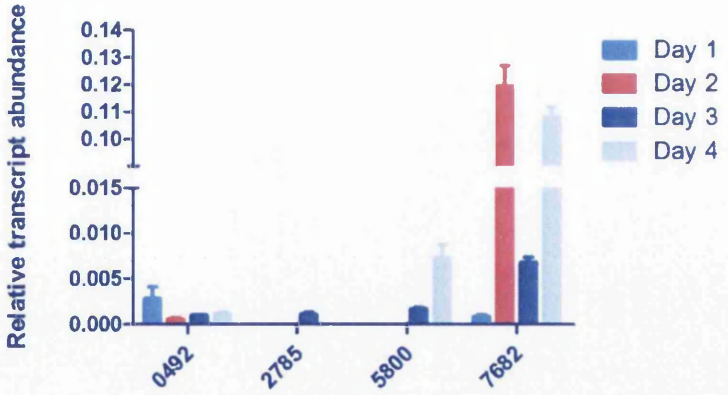
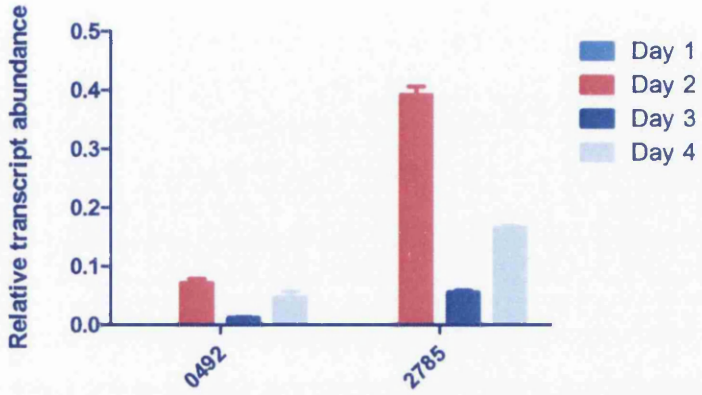


Figure 4.7 Relative transcript abundance of siderophore genes from 1% chitin amended soil microcosms. *Normalised temporal RTA from* S. coelicolor *grown in soil microcosms with 1% chitin added. The numbers on the X axis represents sco gene numbers. Error bars indicate the SEM.*

For the double amended microcosms, with starch plus chitin (Fig 4.8), there was no expression of any of the siderophore genes on day one. On day two *sco0492* was expressed highly with a significant drop in RTA by day three (Fig 4.8a). The RTA increased significantly again on day four to the same level as day two. *sco2785* followed the same pattern as sco0492, but with higher RTA, although there was a significant difference between expression on days two and four (Fig 4.8a). *sco5800* was not expressed on any day (Fig 4.8b). Expression of *sco7682* (Fig 4.8b) was very limited compared to other expressed siderophore genes. However, the same expression pattern as sco0493 and *sco2785* emerged. The RTA dropped significantly to 25% from day two to three and rose significantly from day three to four. Expression on days two and four were comparable with no significant difference between expression levels.

A



B

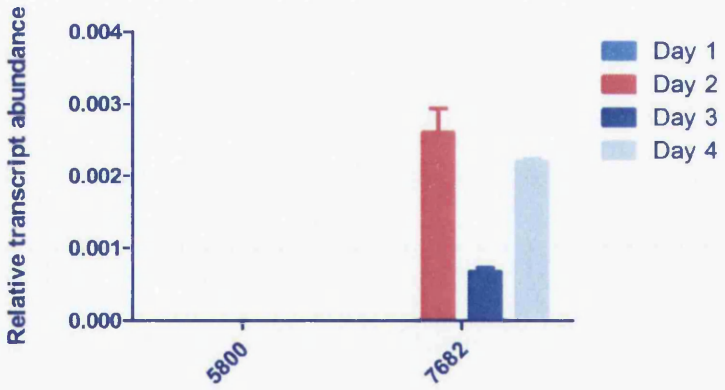


Figure 4.8 Relative transcript abundance of siderophore genes from 1% starch plus 1% chitin amended soil microcosms. *Normalised RTA from* S. coelicolor *grown in soil microcosms with 1% starch plus 1% chitin added. The numbers on the X axis represents sco gene numbers. Error bars indicate the SEM.*

After analysis of each microcosm, comparisons were also performed per siderophore gene in order to identify changes in siderophore gene expression in relation to microcosm

amendment. The coelichelin gene (*sco0492*-Fig 4.9) was not expressed in unamended sterile soil. Chitin induced constitutive expression of *sco0492*, whereas, gene expression was induced on days three and four only in the 1% starch-amended microcosm. The double amendment of both starch and chitin resulted in significantly increased RTA on day two by over 120-fold, day three by over 12-fold and day four by over 40-fold. However, the induction of expression from chitin in day one is not seen when added together with starch.

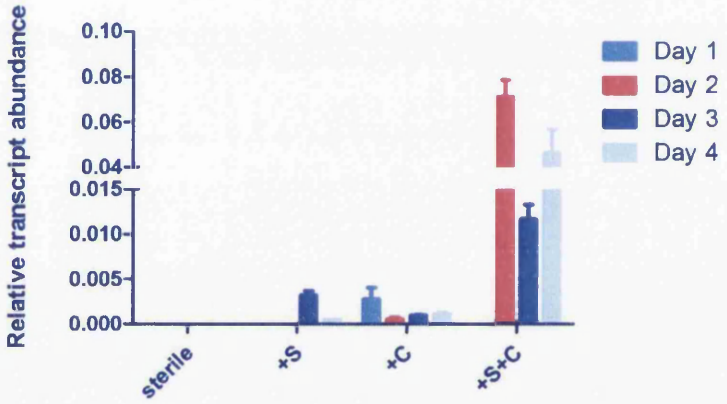
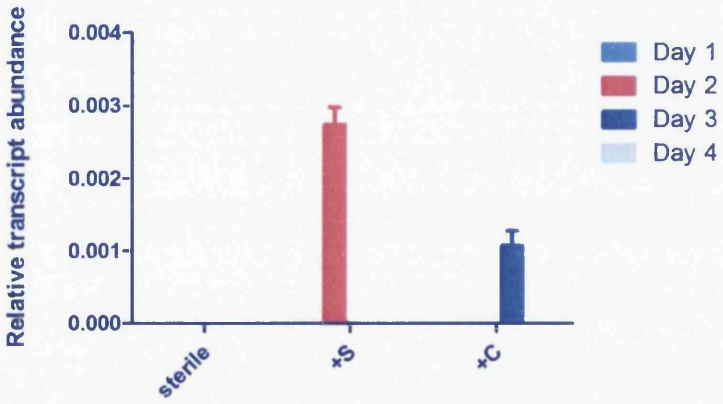


***Figure 4.9 Normalised temporal relative transcript abundance of SCO0492 in nutrient amended soil microcosms of* S. coelicolor.** *+S: 1% starch-amended microcosms; +C: 1% chitin-amended microcosms and +S+C: 1% starch plus 1% chitin-amended microcosms. Error bars indicate the SEM.*

The *sco2785* desferrioxamine gene (Fig 4.10) was not expressed on any day in the sterile microcosms. The expression of this gene was induced under certain nutrient amendments. When compared to double addition of starch plus chitin there was very limited expression of *sco2785* in the starch or chitin amended microcosms. The expression of the *sco2785* gene was significantly increased in the 1% starch and 1% chitin-amended microcosm on day three by 50-fold compared to the 1% chitin-amended microcosm and on day two by 140-fold

compared to the 1% starch-amended microcosm (Fig 4.10a). *sco2785* was expressed on day four only in the starch plus chitin amended microcosms (Fig 4.10b).

A



B

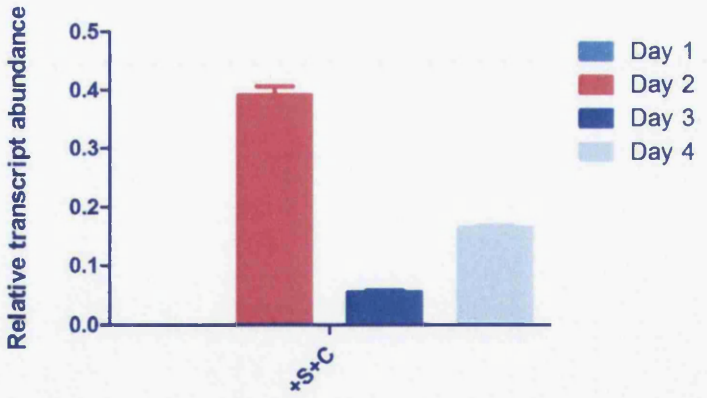


***Figure 4.10 Normalised temporal relative transcript abundance of sco2785 in nutrient amended soil microcosms of* S. coelicolor.** *+S: 1% starch amended microcosms; +C: 1% chitin amended microcosms and +S+C: 1% starch plus 1% chitin amended microcosms. Error bars indicate the SEM.*

In the sterile soil microcosm the putative siderophore synthetase gene sco*5800* (Fig 4.11) was expressed on day three only. Although the large standard error may question the reliability of the amplification, expression of the gene was observed on day three in the starch and chitin-amended microcosms also. The addition of 1% starch induced expression on days one, two and four whilst it had no significant effect on RTA on day three (Mann Whitney p value 0.3734). As explained, chitin also induced expression on day three yet significantly increased the RTA on day four by 25-fold compared to the starch microcosm. However, when starch and chitin were added together no induction occurred.

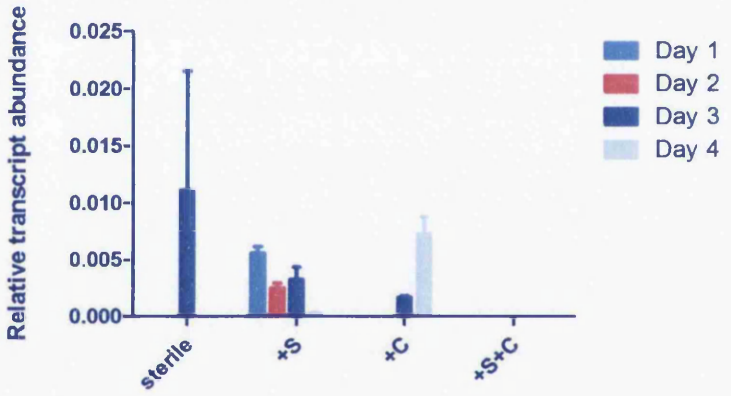


***Figure 4.11 Normalised temporal relative transcript abundance of sco5800 in nutrient amended soil microcosms of* S. coelicolor.** *+S: 1% starch amended microcosms; +C: 1% chitin amended microcosm and +S+C: 1% starch plus 1% chitin amended microcosms. Error bars indicate the SEM.*

Finally, expression of the coelibactin gene *sco7682* (Fig 4.12) was very limited in sterile soil, with low RTA on day three and four. Expression was induced on days one to three after addition of starch, although expression levels decreased as the days progressed. Expression was also induced on all days in the chitin-amended microcosms. The RTA on day four was significantly increased from day three by 16-fold. For the double amended microcosms, the induction of expression on day one did not occur. Expression on day two was equal to that in the starch-amended microcosms, but significantly lower than in the chitin-amended

microcosm. Expression on day three in the double-amended microcosm was significantly lower than that in the singularly amended microcosms. The double amendment significantly increased the expression of *sco7682* on day four compared to the sterile microcosm and the 1% starch-amended microcosm, but was still significantly less than in the 1% chitin-amended microcosm.

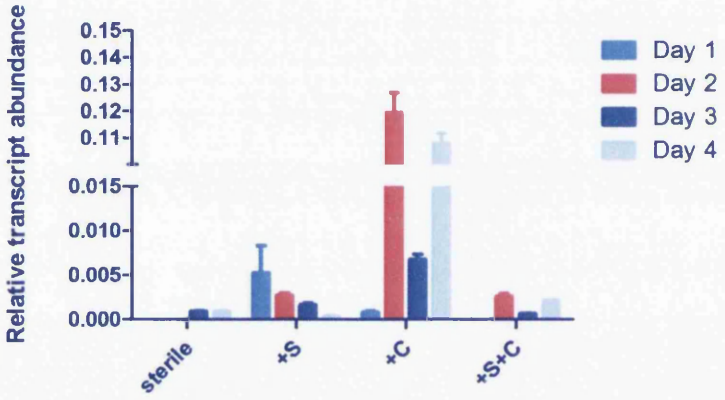


***Figure 4.12 Normalised temporal relative transcript abundance of* sco7682 *in nutrient amended soil microcosms of* S. coelicolor.** *+S: 1% starch amended microcosms; +C: 1% chitin amended microcosms and +S+C: 1% starch plus 1% chitin amended microcosms. Error bars indicate the SEM.*

4.4 Endpoint PCR vs qPCR

There were some discrepancies between data obtained from the endpoint PCR analysis and qPCR analysis of the siderophore genes. In sterile soil, endpoint PCR data indicated constitutive expression of *sco7682*, whereas qPCR indicated expression only on days three and four. Both methods concluded that sco0492, *sco2785* and *sco5800* were not expressed.

In the microcosms amended with both 1% starch and 1% chitin qPCR and endpoint data indicated expression of *sco7682* on days two to four, whilst end point PCR also indicated expression on day one. Also in the double amended microcosms, qPCR data indicated expression of *sco0492* on days two and three, *sco2785* on days one to four and *sco5800* on

day three, whereas the endpoint PCR data indicated no expression of these genes. Both techniques concluded no expression on the other time points.

In the starch-amended microcosms, data for *sco5800* and *sco7682* corresponded largely for both PCR techniques. For *sco0492*, the data corresponded for days one, three and four whilst only endpoint PCR indicated expression on day two. Endpoint PCR indicated no expression of *sco2785* in the starch-amended microcosms, yet qPCR indicated expression on day two.

In the chitin-amended microcosms both methods concluded expression of *sco7682* on days one to four. Endpoint PCR indicated no expression of *sco0492*, *sco2785* or *sco7682*. However, qPCR indicated expression of *sco0492* on days one to four, *sco2785* on day three and *sco5800* on days three and four.

4.5 Soil metal analysis

Siderophore gene expression normally indicates iron limitation for growth (Buyer, Sikora et al. 1989; Winkelmann 2001; Winkelmann 2002). The metal content in the organic farm soil used for the microcosm studies was therefore analysed. A summary of the pseudototal contents of metals is shown in Table 4.4 together with a comparison to the metal content in a baseline soil (Morley and Ferguson 2001). In 2001, the British Geological Survey showed that the Swansea-Neath-Port Talbot in general has elevated levels of heavy metals compared to those of a baseline soil (Morley and Ferguson 2001). However, metal analysis on the organic farm soil used for the studies described here showed lower levels of metals compared to both Swansea and baseline. In particular, copper levels were 3.5-fold lower, iron was 2.7-fold lower, nickel was 3.8-fold lower, lead 5.8-fold lower and zinc 3.5-fold lower in the organic farm soil than in the baseline soil.

Table 4.4 Total metal content of organic farm soil and baseline level. (1) (Morley and Ferguson 2001)

Analyte	Baseline ppm[1]	Organic Farm ppm
As	15	8.2
Ba	504	27.9
Cd	2	0.193
Co	24	6.5
Cu	38	10.8
Fe_2O_3/Fe	46800	17595
Ni	25	6.5
Pb	104	17.8
Sn	8	0.28
U	1.7	5.739
V	99	29.5
Zn	115	33.1

4.6 Metal-amended microcosms

Expression of siderophore genes in *S. coelicolor* in our microcosms was therefore not surprising considering the lower levels of metals and the requirement of certain metals for growth. Microcosms were prepared with adjusted baseline levels of iron, zinc and copper using ferric oxide, zinc sulphate, and cupric sulphate to test whether siderophore gene expression was reduced or totally repressed, with the latter indicating metal was not limiting. Iron, zinc and copper were chosen as amendments for three reasons; firstly, they are all essential for microbial growth, secondly they are all deficient in the organic farm soil compared to base line soil and finally, these three metals have been shown to increase secondary metabolite production in *Streptomyces* (Paul and Banerjee 1983; Raytapadar, Datta et al. 1995). The metal-amended microcosms were prepared with 1% starch plus 1% chitin, therefore comparisons for expression profiles are made with the data obtained from starch and chitin microcosms.

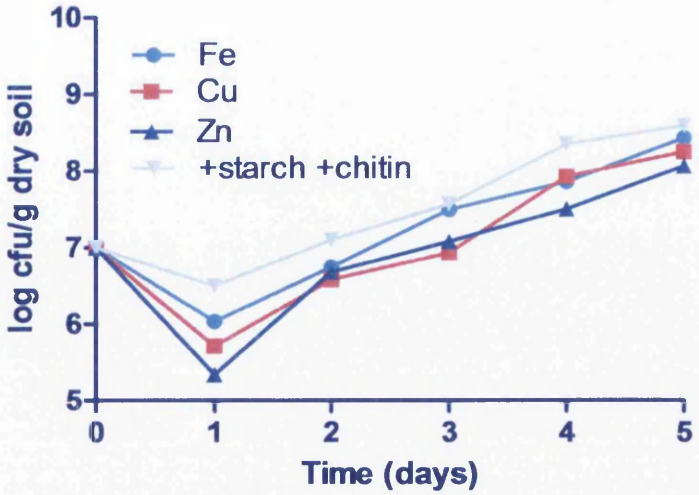


Fig 4.13 Growth curves of S. coelicolor grown in microcosms amended with ferric (Fe), zinc (Zn) or cupric (Cu) ions.

The doubling time of *S. coelicolor* in the microcosm amended with starch and chitin was 705 min. This was reduced with the addition of each metal amendment to 674min in ferric ion amended microcosm, 629min for cupric ion amended microcosms and 621min for zinc ion amended microcosms.

The standard deviation of the doubling times for all metal amendments and the microcosms amended with starch and chitin was 39.4, with a Coefficient of Variation of 10.6%. Deviation of the extreme values calculated at 0.83 for the upper value and -1.09 for the lower value. Both values were within the standard value of +/- 1.64. Variation between the doubling times was therefore limited and the growth curves are consequently not significantly different. Therefore differences in phenotype are not due to a growth rate phenotype.

RNA was extracted every 24h for four days as an indicator of gene expression. The yield of RNA extracted can be seen in Table 4.5. Due to the low yield of RNA from the extraction procedures, averaging between three and four µg per gram of soil, the T7 RNA polymerase-based amplification procedure was applied. This was successful, producing yields of up to

67µg from 500ng starting material. The yields along with final RNA amplification factor are shown in Table 4.5.

Table 4.5 RNA extraction and amplification. *The total yield of RNA from each microcosm is given with yields after T7 RNA polymerase amplification (from 500ng starting material).*

Microcosm/ Amendment	Day	Amount of RNA Extracted (µg)	Total RNA yield after amplification (µg)	RNA amplification factor
Zn II	**1**	**2.60**	**57.8**	**578**
	2	3.51	67.0	671
	3	**5.32**	**61.0**	**611**
	4	3.82	52.7	527
Fe III	**1**	**2.22**	**65.1**	**705**
	2	1.94	61.8	766
	3	**3.14**	**67.9**	**679**
	4	2.63	59.0	591
Cu II	**1**	**3.13**	**53.4**	**534**
	2	4.53	66.8	668
	3	**5.32**	**65.8**	**658**
	4	1.20	53.3	1069

The expression of siderophore genes were quantified using qPCR as described above. Mann Whitney tests indicated that the cT values were significantly lower than the NTC for all cDNA samples (p values in Table 4.9). The cT values were then used for normalisation of relative transcript abundance (RTA) shown in Table 4.6.

Table 4.6 p values from comparison of the cT values from the negative control and the sample cDNA RTA for metal-amended microcosms and 1%starch plus 1% chitin-amended microcosms. *Highlighted in red are p values above 0.005 showing no significant difference. Highlighted in blue are p values below 0.005 showing significant differences. Where there is no p value, the cT value from the cDNA was either the same or higher than the negative control. RTA is the relative transcript abundance (normalised to 16S expression). +S+C: 1% starch plus 1% chitin amended microcosms, Fe: ferric ion amended microcosm, Zn: zinc ion amended microcosms and Cu: cupric ion amended microcosms.*

Gene	DAY	+S+C		Fe		Zn		Cu	
		P value	RTA	P	value	P value	RTA	P value	RTA
16S	1	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0041	1.000
	2	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
	3	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
	4	0.0046	1.000	0.0046	1.000	0.0046	1.000	0.0046	1.000
SCO0492	1	0.8082						0.0289	
	2	0.0046	0.0713			0.0046	0.0007	0.3734	
	3	0.0046	0.0117			0.0289	0.0010	0.0046	0.0001
	4	0.0046	0.0467						
SCO2785	1	0.3734				0.8082		0.3734	
	2	0.0046	0.3914			0.0046	0.0001	0.01188	<0.0001
	3	0.0046	0.0550	0.3005		0.0046	0.0005	0.0046	<0.0001
	4	0.0046	0.1652	0.4486		0.0046	<0.0001	0.0046	<0.0001
SCO5800	1	0.0809				0.1044			
	2	0.0809							
	3	0.0809				0.0046	0.0201	0.8082	
	4	0.1904							
SCO7682	1					0.0046	0.0002	0.1242	
	2	0.0046	0.0026			0.0046	0.0001	0.0041	0.0001
	3	0.0289	0.0007			0.0046	0.0003	0.0046	0.0001
	4	0.0046	0.0022			0.1242			

The RTA of each of the four siderophore genes studied was compared for each soil microcosm amendment. The qPCR data for the microcosm adjusted with ferric oxide indicated that none of the four genes studied were expressed at any of the time points

studied. Although the cT value for *sco2785* on days three and four were lower than that of the negative control, Mann Whitney tests revealed that there was no significant difference (p values shown in Table 4.9) and therefore no expression.

For the zinc-amended microcosm (Fig 4.14), *sco0492* was not expressed on days one or four. The RTA increased significantly (three-fold) between days two and three. Similarly, *sco2785* was not expressed on day one in the zinc-amended microcosm with the RTA increasing four-fold between days two and three. The RTA was only 10% of that on day three. *sco5800* was not expressed on days one, two or four. However, the high level of RTA on day three was over ten times that of the next highest in the zinc-amended microcosms (*sco0492*, day two). *sco7682* was expressed on days one, two and three. There was no significant difference between the RTA on days one and two and between days one and three, whereas the RTA of *sco7682* increased significantly (4-fold) from day two to three.

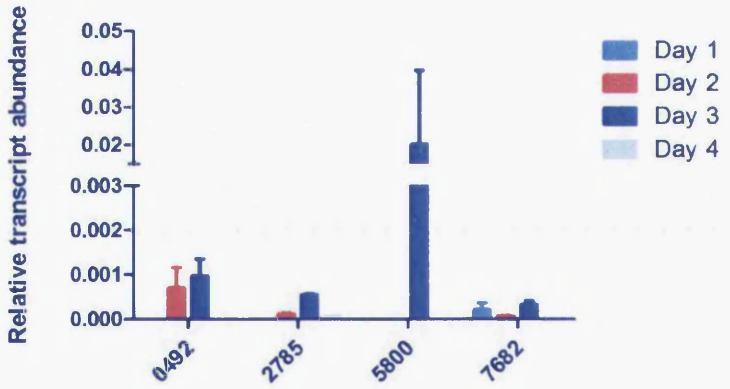


Figure 4.14 Normalised temporal relative abundance of siderophore genes from a soil microcosm adjusted with zinc ion. *Normalised temporal RTA from* S. coelicolor *grown in soil microcosm adjusted to baseline levels of zinc. The numbers on the x-axis represent sco gene numbers. Error bars indicate the standard error of the mean (SEM).*

For the cupric-amended microcosms (Fig 4.15), *sco0492* was expressed only on day three. *sco2785* was not expressed on day one, but its RTA was steady over days two to four with no significant difference in RTA between any of the days. *sco5800* was not expressed on any

day. *sco7682* was expressed on days two and three only. There was no significant difference between RTA on these days.

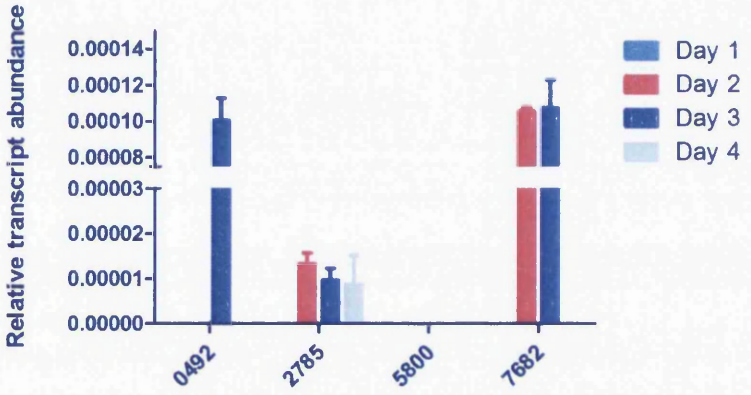


Figure 4.15 Normalised temporal relative abundance of siderophore genes from a soil microcosm adjusted with cupric ion. *Normalised temporal RTA from* S. coelicolor *grown in soil microcosms adjusted to baseline levels of copper. The numbers on the x-axis represent sco gene numbers. Error bars indicate the standard error of the mean (SEM).*

After expression analysis of each of the metal-amended microcosms, comparisons were made for each gene's expression in relation to metal added.

In the microcosm adjusted with 1% starch and 1% chitin, the coelichelin cluster gene *sco0492* (Fig 4.16) was expressed from day two up to day four. With the addition of ferric oxide, the gene was switched off on all days. *sco0492* was expressed, however, on days two and three in the zinc-adjusted microcosms and on day three in the cupric-adjusted microcosm, though the RTA was significantly lower by at least nine-fold when compared to those in the starch and chitin amended microcosm.

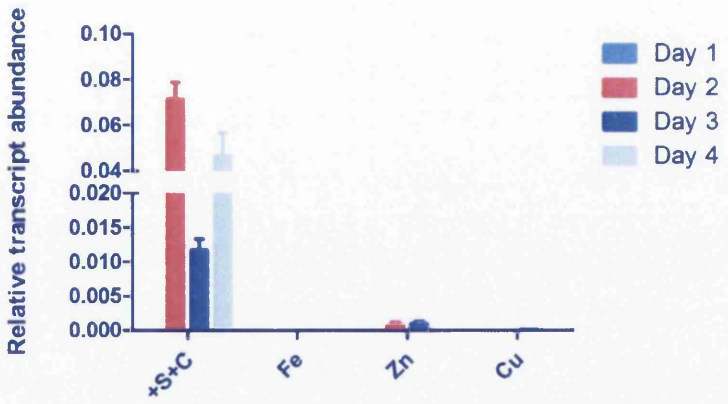
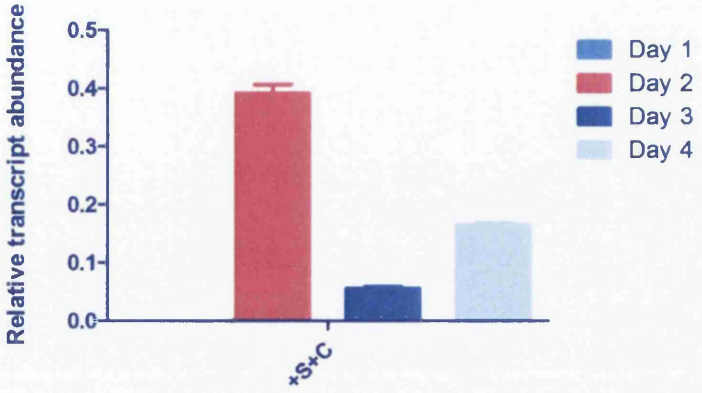


Figure 4.16 Normalised temporal relative transcript abundance of SCO0492 in* S. coelicolor *soil microcosms amended with 1% starch and 1% chitin (+S+C) and also adjusted with ferric (Fe), zinc (Zn) or cupric (Cu) ion. *Error bars indicate the SEM.*

sco2785, a gene involved in desferrioxamine biosynthesis, was expressed on days two to four in the 1% starch and 1% chitin microcosm (Fig 4.17a). The expression of this gene was completely repressed when ferric ion was added and partially repressed in the zinc and cupric ion adjusted microcosms (Fig 4.17b). *sco2785* was expressed on days two to four in both the zinc and cupric ion adjusted microcosm although the RTA was on average over 21,000-fold less in the copper microcosms and over 800-fold less in the zinc microcosms.

A



B

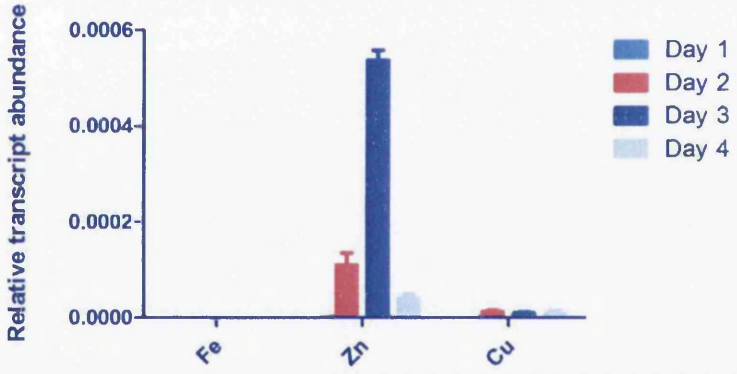


Figure 4.17 Normalised temporal relative transcript abundance of* sco2785 *in* S. coelicolor *soil microcosms amended with 1% starch and 1% chitin (+S+C) and also adjusted with ferric (Fe), zinc (Zn) or cupric (Cu) ion. *Error bars indicate the SEM.*

There was no expression of the siderophore synthetase gene *sco5800* (Fig 4.18) in the 1% starch and 1% chitin microcosms along with those adjusted with ferric ion and copper ion. However, the introduction of zinc ion induced expression on day three.

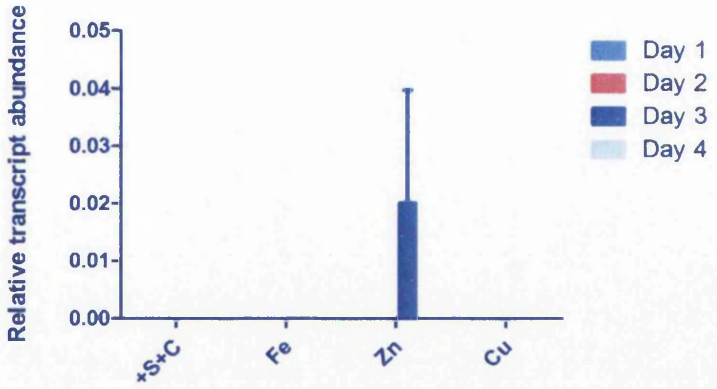


Figure 4.18 Normalised temporal relative transcript abundance of* sco5800 *in* S. coelicolor *soil microcosms amended with 1% starch and 1% chitin (+S+C) and also adjusted with ferric (Fe), zinc (Zn) or cupric (Cu) ion. *Error bars indicate the SEM.*

The coelibactin NRPS gene *sco7682* was expressed on days two to four in the starch and chitin-amended microcosms (Fig. 4.19). The addition on Fe to the microcosm turned off gene expression on each day. With the addition of zinc, expression was found on days one, two and three. Expression on days two and three was significantly less in the zinc-amended microcosms compared to the 1% starch and 1% chitin microcosm. The addition of copper to the microcosms turned off *sco7682* gene expression on day four and significantly lowered the RTA on days two and three.

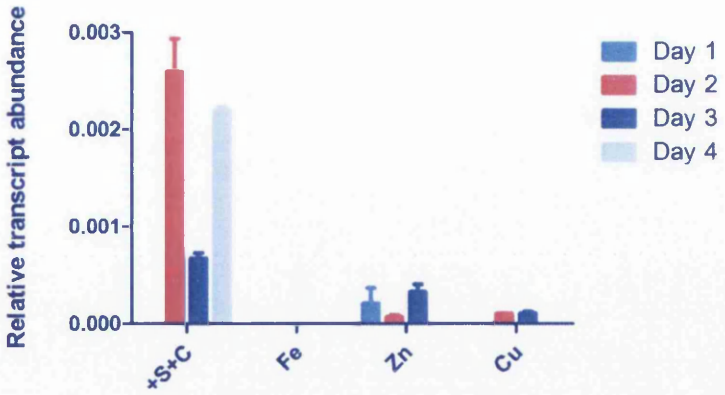


Figure 4.19 Normalised temporal relative transcript abundance of* sco7682 *in* S. coelicolor *soil microcosms amended with 1% starch and 1% chitin (+S+C) and also adjusted with ferric (Fe), zinc (Zn) or cupric (Cu) ion. *Error bars indicate the SEM.*

4.7 Discussion of gene expression/secondary metabolite production

There is a general consensus in secondary metabolism studies, including *Streptomyces* biology, that antibiotic gene clusters are generally only expressed after exponential growth has ceased (Wang, Tian et al. 2009). However, most studies looking at secondary metabolite production are undertaken in liquid or on solid media. There is little gene expression data available for *in situ* studies to test this hypothesis. This study looked at expression in a totally different growth medium and uncovered many new/different expression profiles of secondary metabolite biosynthetic gene clusters in *S. coelicolor*.

There is evidence that gene expression in soil correlates to biosynthesis of secondary metabolites. Anukool et al, in 2004, compared the production of streptothricin in *S. rochei* microcosms to liquid culture. Even though streptothricin itself could not be extracted from the soil microcosms, there was correlation of gene expression in bulk soil and liquid culture proving that streptothricin was produced in situ (Anukool, Gaze et al. 2004).

4.7.1 Nutrient-amended gene expression profiles

The growth curves of *S. coelicolor* in nutrient-amended soil followed the same pattern over the same timescale as in sterile soil. The standard deviation of *S. coelicolor* doubling times in the microcosms indicated no significant difference between them. The addition of starch and chitin as nutrient sources therefore did not increase the growth rate. In contrast, Vionis reported increased viable count of *S. lividans* in soil microcosms amended with 1% fungal chitin (Vionis, Niemeyer et al. 1996).

The production of secondary metabolites is influenced greatly by changing the type and concentration of nutrients in the culture media. For example, the effect of carbon source has been investigated for novobiocin production in *S. niveus* where citrate was the preferred carbon source over glucose (Kominek 1972). The kanamycin-producing *S. kanamyceticus* preferably utilized dextrose over maltose, sucrose, and soluble starch (Pandey, Shukla et al. 2011). Furthermore, cephalosporin production in *S. clavuligerus* is regulated by carbon catabolite control, with increasing concentrations of glycerol and maltose decreasing antibiotic production (Aharonowitz and Demain 1978). Nitrogen sources have also been identified as influencers of secondary metabolite production (Aharonowitz 1980; Barbhaiya and Rao 1985). Chitin, a source of both carbon and nitrogen, has been shown to be important in Act and Red production in *S. coelicolor*. GlcNAc, the monomer of chitin, blocks development and antibiotic production under rich growth conditions (R2YE agar >10mM GlcNAc), whereas it triggers Act and Red production and sporulation under poor nutritional conditions (MM agar > 5mM GlcNAc) (Rigali, Titgemeyer et al. 2008).

ActI-ORFI was expressed from mid-exponential growth to stationary phase in the sterile microcosms (Fig 4.2, Chapter 4). With the addition of chitin, expression was induced further from early exponential growth and continued to stationary phase as in the sterile microcosm. With the addition of starch only, expression was switched off. The expression of Act has been widely studied as a pigmented model antibiotic. Act shows the archetypal expression pattern of secondary metabolites in liquid culture and on solid media at the end of exponential growth (Bystrykh, Fernandez-Moreno et al. 1996; Chakraburtty and Bibb 1997). One group of Act regulators, which control the archetypal expression pattern are SARPs (Streptomyces antibiotic regulatory proteins), which regulate both Act and Red

biosynthetic genes during the transition between exponential growth and stationary phase (Takano, Gramajo et al. 1992; Gramajo, Takano et al. 1993). Production of Act is activated by the transcription factor, ActII-ORF4 (Gramajo, Takano et al. 1993). Act biosynthesis is therefore regulated by factors that control production of ActII-ORF4 (van Wezel and McDowall 2011). Regulation of ActII-ORF4 is extremely complex. Its promoter is target for many other genes including AbsA (McKenzie and Nodwell 2007), DasR (Rigali, Titgemeyer et al. 2008) and AtrA (Uguru, Stephens et al. 2005).

The response regulator AbsA2 is a protein encoded by *absA*, which is embedded in the CDA cluster (Sheeler, MacMillan et al. 2005). Disruption of AbsA results in enhanced production of Act (Brian, Riggle et al. 1996; Anderson, Brian et al. 2001). AbsA also negatively regulates Red and CDA as well as the plasmid encoded gene cluster of methylenomycin. Red and CDA were not expressed in any of microcosms, (methylenomycin was an internal negative control), and therefore, it may be assumed that the AbsA system contributed greatly to the regulation of Red and CDA in soil. Yet another regulation system overrode the AbsA system, resulting in expression of Act.

The regulator DasR was first identified as a repressor of the phosphotransferase system (PTS) for GlcNAc (Rigali, Nothaft et al. 2006). It has since been discovered that DasR can bind directly to the promoter of ActII-ORF4 affecting Act production (Rigali, Titgemeyer et al. 2008). DNA binding by DasR is inhibited by glucosamine-6-phosphate, a GlcNAc metabolism intermediate (Rigali, Nothaft et al. 2006). In the soil microcosms, the addition of chitin induced earlier expression of Act, from mid exponential growth in sterile microcosms to early exponential growth in chitin-amended microcosms (Fig 4.2, Chapter 4).

AtrA is transcriptional regulator, which when disrupted decreases the production of Act under certain growth conditions, yet had no detectable effect on the production of Red or CDA (Uguru, Stephens et al. 2005). Overexpression of AtrA causes an increase in Act production under all tested growth conditions (Towle 2007). Along with increasing production of Act, AtrA is thought to adjust acetyl-CoA metabolism, which supplies precursors for polyketides (van Wezel and McDowall 2011).

Several more two component systems have been attributed to be involved in the regulation of Act biosynthesis including AfsQ1/Q2 and CutR/S (Ishizuka, Horinouchi et al. 1992; Chang, Chen et al. 1996), PhoP/R, which regulates the biosynthesis of both Act and Red (Sola-Landa, Moura et al. 2003), and RapA1/A2, which regulates both Act and the type I polyketide cpk cluster (Lu, Wang et al. 2007).

BldA also exerts developmental control of Act production, via the initiation of *actII-ORF4* transcription and a role in Act export (Fernández-Moreno, Caballero et al. 1991). *bldA* encodes the only tRNA in *S. coelicolor* that can efficiently translate the rare leucine codon UUA (Fernández-Moreno, Caballero et al. 1991). AdpA protein transcriptionally activates a large regulon of genes involved in sporulation or antibiotic production. At the translational level, *adpA* depends on the *bldA* tRNA (McCormick and Flärdh 2011).

Interestingly, there was no evidence of production of Red in any of the tested microcosms. This is surprising as Red is repeatedly expressed under normal laboratory conditions after 12h incubation (Chakraburtty and Bibb 1997), although to date no studies have indicated production of Red from *Streptomyces* in soil. As discussed previously, the AbsA system may have contributed to the regulation of Red. The production of prodigiosin is medium dependent. Red production is sensitive to ammonium and phosphate levels in the growth media (Hobbs, Frazer et al. 1990). Production prodigiosin by *Serratia marcescens* is inhibited by the addition of different carbon sources to the tryptic soy agar growth media (Solé, Francia et al. 1997). Prodigiosin production decreased upon increasing concentrations of glucose. This effect, however, was attributed to pH changes caused by the addition of glucose. Whilst the pH of the incubation media increased from 6.70 to 7.03 over 24h in the media with no addition of glucose, with the addition of glucose the pH of the media declined to pH 4.27 after 24h with $5.5x10^{-2}$ mol l^{-1} glucose (Solé, Francia et al. 1997). Wildtype *S. coelicolor* has been shown to change the pH of R2YE media, reducing the pH from 7.2 to 6.0 during substrate mycelial growth followed by an increase in pH up to 7.0 in later stages of growth (Viollier, Nguyen et al. 2001). However, this study showed that a significant amount of alkali was needed to increase the pH of the soil in order to produce neutral pH microcosms (Chapter 3). The soil demonstrated the ability to buffer minor changes, limiting the possibility that *S. coelicolor* was able to change the pH of the soil enough to reduce Red production.

CDA is an acidic lipoprotein antibiotic which limits growth of Gram positive bacteria and forms trans-membrane channels which conduct monovalent cations in the presence of calcium ions (Lakey, Lea et al. 1983). This study revealed that CDA was not being produced in any soil microcosm, as the CDA NRPS biosynthetic gene *sco3230* was not expressed under any condition. This may be attributed to regulation by AbsA2, which is located in the CDA cluster (as discussed above). CDA has variable amino acid residues at four different positions. The amino acids required for CDA biosynthesis are tyrosine, aspartate, asparagine, tryptophan, threonine, glycine, serine, glutamate and oxoglutarate (Kim, Smith et al. 2004). A deficiency in any of these amino acids would stall the production of CDA. For the precursor tryptophan there are four genes (*sco3211-3214*) dedicated to the biosynthesis of tryptophan for CDA biosynthesis and not for general primary metabolism (Hojati, Milne et al. 2002). It is unlikely that tryptophan deficiency would be the cause of no expression of the CDA gene. CDA is also derived from acetyl-CoA and malonyl-CoA, as well as d-4-hydroxyphenylglycine (HPG), which is produced in the pentose phosphate pathway (Kim, Smith et al. 2004).

4.7.1.1 Polyketides

PpGpp is produced in *S. coelicolor* under nutrient stress including glucose and ammonium starvation (Kang, Jin et al. 1998). As discussed in Chapter 1, accumulation of ppGpp signals the beginning of the stringent response and therefore has been linked to the shift from primary to secondary metabolism at stationary phase of growth. It has been suggested that the ACPs of PKSs may influence the switch to secondary metabolism by their contribution the regulation of the *Streptomyces* stringent response (van Wezel and McDowall 2011). However, in this study expression of many genes within PKS gene clusters were expressed from the first day of incubation including *sco5318* and *sco7221*.

The type I polyketide synthase gene (*sco6273*) was expressed temporally during early exponential growth in chitin-amended microcosms (day two). The chitin dependency of expression in the soil microcosms is supported by the work of Rigali et al. Rigali et al suggested a putative positive effect of GlcNAc on the expression of the cpk genes. This is

mediated via the transcriptional regulator DasR and the pathway-specific transcriptional activator gene of the cpk cluster, *cpkO* (Rigali, Titgemeyer et al. 2008).

Expression studies show *cpk* to be expressed in a very limited timescale of between 16 and 22h of incubation in supplemented minimal media (Pawlik, Kotowska et al. 2007), which correlates to mid-transition stage. However, in the chitin-amended microcosms *sco6273* was expressed on day two which was at the exponential growth stage. The fact that the *cpk* cluster was shown in liquid culture to be expressed over a very limited amount of time may indicate that the cluster may have been expressed in the other nutrient amended microcosms, yet may have been missed due to the less frequent sampling interval. CpkO is also regulated by ScbR, a butyrolactone receptor protein, by direct repression through two binding sites located in the *cpkO* promoter region (Takano 2006).

Expression of an uncharacterised and unidentified secondary metabolite biosynthesis gene (*sco6827*) from an unknown polyketide gene cluster was observed. This gene was expressed on day four in sterile soil, day three in starch-amended soil and days one and two in chitin-amended soil. This expression pattern was not similar to any other.

WhiE is a complex locus of the polyketide spore pigment (Kelemen, Brian et al. 1998). It forms part a group of (*whi*) genes which are essential for sporulation. Up until recently it has been thought that the main function of the *whi* genes is to activate genes and processes relating to the various stages of aerial development, from early aerial growth to the onset of sporulation (Chater 2001; Traag, Kelemen et al. 2004). However a recent study revealed the importance of the *whi* genes in the correct timing of developmental *ftsZ* transcription (Willemse, Borst et al. 2011). FtsZ is responsible for the formation of Z-rings for cell division and spore formation (Schwedock, McCormick et al. 1997; Grantcharova, Lustig et al. 2005; Willemse and van Wezel 2009). *sco5318* (*whiEORFIII*), a polyketide synthase gene in the TW95a (*whiE*) gene cluster was expressed from mid-exponential growth to stationary phase in the sterile microcosm. In the presence of 1% chitin, *sco5318* expression was constitutive. In the 1% starch plus 1% chitin microcosms, *sco5318* expression was detected from the start of exponential growth to the transition phase, i.e. expression on the fourth day was not observed. Growth in the presence of starch resulted in silencing of *SCO5318*. The *whiE* genes encode proteins that closely resemble components of Act type II polyketide synthases

(Fernandez-Moreno, Martínez et al. 1992). It is interesting therefore that the *whiE* cluster was expressed in a pattern very similar to the Act cluster, the only difference being no expression of *sco5318* on day four in the starch plus chitin microcosm. On solid media, WhiE is expressed shortly before sporulation in the aerial mycelium, coinciding with Act production (Yu and Hopwood 1995). This study shows that production of these secondary metabolites also coincides in sterile soil from day two and in chitin-amended soil from day one.

The type III PKS gene (*rppA*) from the flaviolin cluster was expressed in sterile soil from late exponential growth to the transition stage. *rppA* has not been shown to be expressed under laboratory growth conditions (on LB plates and LB/TB liquid medium) and was thought to be silent (Izumikawa, Shipley et al. 2003). This is in contrast with the findings of this study. However, flaviolin is expressed in *S. venezuelae* (when used as a heterologous host for the PKS genes) after four days of incubation in Semiselective Media containing 1.5% soluble starch (Maharjan, Park et al. 2010). In this study, *rppA* was expressed also after four days of incubation in the starch amended microcosm.

Production of a PKS, such as flaviolin is dependent on malonyl-CoA. Malonyl-CoA is synthesised from acetyl-CoA by acetyl-CoA carboxylase (ACCase). The production of this type III PKS is extremely substrate specific, utilising only malonyl-CoA (as opposed to other PKS or FAS which can use acetyl-CoA) (Funa, Ohnishi et al. 2002). Production of flaviolin in soil may therefore be limited by the availability of this precursor substrate.

The germicidin biosynthetic gene (*sco7221*) showed a similar pattern of gene expression when compared to Act and the other polyketide gene WhiE, by showing gene expression with constitutive expression in sterile soil, chitin-amended and chitin plus starch-amended soil. This suggests that starch inhibits germicidin expression without the presence of chitin. Expression of the germicidin biosynthetic gene cluster had been observed in *S. coelicolor* M145 where it had reached the stationary growth phase grown in minimal glucose liquid media (Song, Barona-Gomez et al. 2006) and liquid yeast-starch media (Aoki, Matsumoto et al. 2011). In a *bldA* mutant, SCO7221 was greatly over-expressed (Song, Barona-Gomez et al. 2006). Defective production of some secondary metabolites in *bldA* mutants is partially attributed to the presence of the TTA codon in regulatory genes for secondary metabolism

(Chater and Chandra 2008), due to presence of a UUA codon in the mRNA for one or more of the biosynthetic genes (White and Bibb 1997). The TTA codons in *sco7221,* as in many genes in both aerial development and secondary metabolism, rely on BldA for translation. Therefore, whilst *sco7221* may be expressed at the beginning of exponential growth its translation at this stage would be unusual as BldA is associated with bld cascade, which regulates initiation of aerial growth (Kelemen and Buttner 1998; Willey, Willems et al. 2006).

The putative chalcone synthase, *sco7671*, from the putative chalcone biosynthesis cluster *sco7669-sco7671* was not expressed under any condition. This is a cryptic cluster unearthed by the complete genome sequence of *S. coelicolor* (Bentley, Chater et al. 2002). No expression of this cluster has been identified in the literature.

4.7.1.2 Fatty acid

In this study the eicosapentaenoic acid fatty acid synthase biosynthetic gene (*sco0126*) was not found to be expressed under any condition. Production of eicosapentaenoic acid or expression of the biosynthetic gene in *S. coelicolor* has not been published in any literature.

The FAS (FabH), encoded by *sco1271*, was expressed from late exponential growth to the transition stage in sterile soil. In previous studies, FabH was expressed at 20h incubation in liquid YEME in stationary phase (Revill, Bibb et al. 2001). FabH is an essential enzyme in fatty acid biosynthesis (Lai and Cronan 2003). Due to its importance in fatty acid biosynthesis, *S. coelicolor* has a second copy of this gene, so when *sco1271* is not expressed the second FabH catalyses fatty acid biosynthesis (Li, Florova et al. 2005). Therefore, it may be assumed that before the transition stage in the microcosms, *S. coelicolor* expresses the other FabH for fatty acid biosynthesis.

4.7.1.3 Terpenoid

crtE, the geranylgeranyl pyrophosphate synthase gene (*sco0185*), of the carotenoid cluster was expressed from mid-exponential growth to stationary phase in the sterile microcosms.

With the addition of chitin, expression was induced on day one and continued, as in the sterile microcosm, on days two to four. With the addition of starch only, expression was switched off. Carotenoid production in *Streptomyces* does not occur naturally in any known culture condition, but was induced when a stress-response sigma factor (CrtS), was introduced into the cells (Kato, Hino et al. 1995; Lee, Ohnishi et al. 2001) or in a light-induced manner (Takano, Kinoshita et al. 2005).

Carotenoid has been shown to be produced in stationary phase of *S. coelicolor* growth on Bennett's glucose solid media under blue light induction (Takano, Kinoshita et al. 2005). The biosynthesis of the carotenoid gene cluster was specified by LitS, a photo-inducible extracytoplasmic function sigma factor (Takano 2006). The results in this study contradict those results in that microcosms were incubated under dark conditions, without the presence of blue light; therefore expression may have been achieved without LitS, suggesting that the sigma factor may have other forms for transcription control, not only photo-inducible.

The albaflavenone terpene synthase gene (*sco5222*) is part of a two-gene cluster with *sco5223* encoding a cytochrome P450. In this study *sco5222* was expressed solely on day four (stationary phase) in the presence of starch. In the wildtype strain of *S. avermitilis,* albaflavenone is not produced under any growth condition. However, it was produced in a mutant strain culture, which had reached the stationary phase of growth (Takamatsu, Lin et al. 2011). In *S. coelicolor,* albaflavenone has been isolated from cells (co-transformed with the plasmids CYP170A1pET17b and pGro12) in the exponential growth phase in LB broth (personal communication S. Moody and D. Lamb).

Surprisingly, there was no evidence of the expression of the geosmin biosynthetic gene (*cyc2*) under any nutrient amendment. The earthy odorant geosmin is biosynthesised by *sco6073,* a germacradienol synthase that catalyses Mg^{2+}-dependent conversion of FFP to the intermediate germacrene D (Cane and Watt 2003). Volatiles released from *S. coelicolor* during two weeks growth on SFM plates at 30°C included amounts of geosmin easily detectable by GC-MS (Gust, Challis et al. 2003). This is in contradiction to this study where no expression of the biosynthetic gene was observed. In *S. avermitilis* the strong odour is produced in several different media (Cane, He et al. 2006). Instead, in the soil microcosms

the alternative earthy odorant methylisorborneol terpene synthase was expressed. *sco7700*, the terpene synthase gene from the methylisoborneol cluster showed starch-dependent expression as it was solely expressed in the starch-amended microcosms. Terpenoid precursors are produced from acetyl-CoA (with the intermediate mevalonic acid) or are derived from C5 sugars via the methylerythritol phosphate (MEP) pathway (Daum, Herrmann et al. 2009). Both precursors, acetyl-CoA and pyruvate, are products of glucose metabolism, which may explain production of this terpenoid with the addition of starch. However, this is true for many of the secondary metabolites, so there must be other regulators preventing the expression of the geosmin sesquiterpine cyclase gene.

In this study, the hopanoid phytoene synthase gene (*sco6759*) was produced throughout exponential growth in starch-amended microcosms and at mid-late exponential growth only in the chitin amended microcosm. Studies to date reveal that wildtype *S. coelicolor* produced hopanoids on solid media, but not in liquid media. Production of hopanoid has been shown to coincide with sporulation on solid media, with a speculative function of alleviating stress in aerial mycelium by diminishing water permeability across the membrane (Poralla, Muth et al. 2000). In soil however, hopanoids were produced throughout the exponential growth phase in starch microcosms and at mid-late exponential growth in the chitin-amended microcosms. This may be indicative that *S. coelicolor* was experiencing stress due to water limitation and underwent early sporulation in the soil microcosms.

4.7.1.4 Peptides

The lantibiotic II cyclase (SCO6929) was expressed during early exponential growth, solely in singularly amended chitin microcosms. Lantibiotics contain the unusual amino acids 2,3-dihydroalanine and 2,3-dihydrobutyrine, which are derived from serine and threonine (Patton and van der Donk 2005). Serine is produced from 3-phosphoglycerate, an intermediate in glycolysis, and threonine is produced from aspartic acid, which in turn is produced from oxaloacetic acid in the TCA cycle (Yamasaki, Yamada et al. 2001; Lee, Park et al. 2007). The end product of chitin metabolism is fructose-6-phosphate. This feeds directly into glycolysis, and from there indirectly into the TCA cycle, which may feed into production

of components of lantibiotics. This may explain why the addition of chitin may induce expression of this gene. However, the lantibiotic I cyclase was expressed on day two in the chitin-amended microcosm and constitutively expressed in the starch-amended microcosms. This secondary metabolite therefore must rely on precursors from starch metabolisms, yet when grown in soil amended with both starch and chitin, the gene expression induction was lost. Expression of these two lantibiotic genes in *S. coelicolor* has not been indicated in any literature.

Expression of other lantibiotics has been noted during mid exponential growth, namely the lantibiotic microbisporicin in the actinomycete *Microbispora corallina*. Microbisporicin was detected (by antimicrobial activity against *Micrococcus lueus*) after the culture had reached mid-exponential growth (46-50h) in medium V supplemented with sucrose and proline (VSP media). Its presence was then confirmed by MALDI-TOF analysis (Foulston and Bibb 2010).

A lantibiotic-like molecule derived from RamS, SapB, has a primary role as a biosurfactant (Kodani, Hudson et al. 2004). SapB self-assembles at the air-colony interface through hydrophobic interactions where it reduces the surface tension and enables the upward growth of aerial hyphae (Talbot 2003). The *ram* gene cluster in *S. coelicolor* is orthologous to the *S. griseus amf* gene cluster, and was found to be highly conserved in several *Streptomyces* species (San Paolo 2007). SapB production commences during aerial hyphae formation (Kodani, Hudson et al. 2004).

sco6431, a cryptic peptide synthase gene from a putative NRPS gene cluster, was expressed from late exponential growth to the transition stage in the sterile microcosms. This expression was silenced by the addition of starch or chitin. However, the presence of chitin induced expression in early exponential phase in both chitin containing microcosms (i.e. the single amended microcosm, and the microcosm amended with starch and chitin). This is the first evidence of expression of this cluster.

4.7.1.5 Miscellaneous

sco0381, a glycosyl transferase gene in the deoxysugar gene cluster, was not expressed in either the sterile microcosms or the microcosms containing both starch and chitin. However, in the starch microcosm it was expressed constitutively, whereas in the chitin microcosms its expression was induced only during early exponential growth. In a proteomic study, in a series of liquid cultures the abundance of SCO0381 was reduced in a *bldA* mutant along with four other proteins expressed by the deoxysugar cluster (Hesketh, Bucca et al. 2007). The protein SCO0381 was observed from M145 in SMM (glucose) media in exponential and stationary phase (Hesketh, Chandra et al. 2002). The production of SCO0381 in the presence of glucose during these growth phases correlates to the expression of the gene throughout exponential phase and during stationary growth in the starch amended microcosms.

The *scbA* gene (*sco6266*) from the butanolide SCB1 cluster was found expressed solely in starch-amended microcosms from mid-exponential growth to stationary phase. ScbA expression is repressed by ScbR, which also controls cpk, Act and Red production (Takano, Kinoshita et al. 2005), although there was no correlation between the expression profiles of *scbA* and the genes from the Act, Red and Cpk clusters. However, there is evidence of transcriptional activation of its own gene by ScbA, thus ScbA has a possible regulatory function. Production of SCBs is detected at transition to stationary phase, and this also coincides with the transcription of *scbA*. Transcription of *scbA* was undetectable during exponential growth, increased in late transition phase and decreased as the culture entered stationary phase in liquid SMM media (Takano, Chakraburtty et al. 2001). This media used glucose as a carbon source, and so showed the same expression pattern as starch-amended microcosms.

4.7.2 Biotic amendments

The relationship between *Streptomyces* and fungi has been noted for some time. *Streptomyces* are known to produce glucanases, proteases and other exoenzymes, which play a major part in the decomposition of fungi in soil (Williams and Robinson 1981).

Streptomyces produce a variety of antifungal compounds including candicidin (*S. griseus*), natamycin (*S. nataensis*), nikkomycin (*S. tendae*) and nystatin (*S. noursei*) (Kieser, Bibb et al. 2000). It has been suggested that *Streptomyces* are able to sense the presence of fungi in soil, which may lead to a competition stress response (Martín, Sola-Landa et al. 2011). The addition of spores of the entomopathogenic fungus *M. anisopliae* to the soil microcosms reduced the doubling time of *S. coelicolor*, although not significantly. The addition of 1% crab chitin to microcosms in other studies indicated an increase in total viable count of *S. lividans* and *S. griseus* where chitin was added as the only nitrogen/carbon source (compared to sterile soil). The same results were found upon addition of *Aspergillus proliferans* mycelia (Vionis, Niemeyer et al. 1996).

Introduction of *M. anisopliae* spores did not result in a significant reorganisation of secondary metabolite gene expression in *S. coelicolor*. There were only two instances where gene expression was induced in the fungi-amended microcosm, during stationary phase in the deoxysugar cluster (*sco0381*) and early exponential phase in the cpk cluster (*sco6273*). The expression pattern from *sco6273* from the *cpk* cluster was replicated in the chitin-amended microcosm described above. This may indicate that *S. coelicolor* produces cpk in response to chitin in both pure form (from crab shells in the nutrient amended microcosm) and the chitin of fungi cell walls at early exponential phase of growth. *sco0381* was also expressed during stationary phase in the starch-amended microcosm. It is hypothesised that *S. coelicolor* may respond to the presence of fungi by producing secondary metabolism in response to competition. However, although conditions were correct for fungal growth (Schoenian, Spiteller et al. 2011), none was visualised on the SFM plates. It may be that the fungi did not germinate and so the *Streptomyces* hyphae did not react by producing secondary metabolites.

The effect of *Streptomyces* on the growth of nematodes has been noted many times. *Streptomyces* reduced the population density of *Pratylenchus penetrans* (a flowering plant bulb root-lesion nematode) in oatmeal agar plate culture studies (Samac and Kinkel 2001). Dicklow et al reported the reduction of multiplication of several nematode species (*Caenorhabditis elegans, Meloidogyne incognita, Rotylenchulus reniformis* and *Pratylenchus penetrons)* by a novel species of *Streptomyces, Streptomyces CR-43* (Dicklow, Acosta et al. 1993). Numbers of the potworm *Enchytraeus crypticus* fed on *S. lividans* mycelia increased

by 200-fold (Krištůfek, Fischer et al. 1999), although this was dead mycelia whilst the other studies showed competition from live *Streptomyces*. A nematicidal compound has been described with effect against *Belanolaimus iongicaudatus* produced by *Streptomyces* (Nair, Chandra et al. 1995). Although there is evidence for the reduction of nematode growth by *Streptomyces*, there is little information available on the effect of nematodes on *Streptomyces* growth. In this study, nematode extract slowed growth of *S. coelicolor* and delayed the stationary growth phase, although not significantly. The viable count plates were visibly smeared with slimy spreading colonies after a few days incubation, which may have had effect on *S. coelicolor* growth by competition with the nematode gut bacteria. Bacteria have been shown to survive intact in nematode gut (Rae, Riebesell et al. 2008).

Again, introduction of the extract of the nematode *St. kraussei* did not result in an extensive changes in secondary metabolite gene expression profiles in *S. coelicolor*. Two genes were expressed in the nematode-amended microcosms, which were not expressed in the starch- and chitin-amended microcosms. Firstly, expression the deoxysugar gene (*sco0381*) was induced at the beginning of exponential growth and the lantibiotic II gene (*sco6929*) was also induced at the beginning of exponential growth. *St. kraussei* are symbiotic organisms, whose symbiont bacteria, *Xenorhabdus*, are lethal parasites of insects (Burnell and Stock 2000). These symbiotic bacteria were witnessed growing on the total viable count SFM plates. Lantibiotics are a class of peptide antibiotics, and therefore may be produced in reaction to competition with other competitive bacteria found in the gut of the nematode.

4.7.3 Siderophore expression

A common strategy used by many pathogenic and saprophytic microorganisms in conditions of low iron bioavailability is the production and excretion of high-affinity iron chelators known as siderophores (Wandersman and Delepelaire, 2004). The biosynthesis and secretion of siderophores has been attributed to, and is strictly regulated by environmental factors (Duffy and Defago 1999).

In this study, all microcosms were incubated at 28^0C. Siderophore production peaked at 28^0C in *S. filivissimus* (Bendale, Chaudhari et al. 2009). Bendale also identified the best

carbon source for siderophore production in liquid cultures of *S. filivissimus*. After 120h incubation in Chemically Defined Low Iron Medium, sucrose followed by glucose achieved the highest yield of siderophore whilst sodium acetate and lactose did not support siderophore production (Bendale, Chaudhari et al. 2009). This may suggest that, out of all the microcosms, the starch-amended microcosms should produce the most siderophores in soil-grown *S. coelicolor*. The siderophore synthase gene (*sco5800*), was expressed constitutively in the starch-amended microcosms, but was only expressed from late exponential phase to stationary phase in the chitin-amended microcosms and at late exponential phase in the sterile soil microcosms. This is the first time that this gene has been shown to be expressed. The addition of ferric or cupric silenced expression, although with the addition of zinc *sco5800* was expressed during late exponential growth.

The endpoint PCR data indicated starch-dependent expression of *sco0492 (cchH)* from the coelichelin non-ribosomal siderophore peptide cluster as it was expressed constitutively in the starch amended microcosms, but not in any other. However, the qPCR data revealed constitutive expression in the chitin-amended microcosm and expression from mid-exponential growth phase onwards in the starch- and chitin-amended microcosms. A study by Lautru *et al* indicated that coelichelin is produced only under iron-deficient growth conditions (Lautru, Deeth et al. 2005). They demonstrated coelichelin production in *S. coelicolor* M145 in iron-deficient media, but not when ferric iron was added (Lautru, Deeth et al. 2005). This is in agreement with the findings of this study where the addition of ferric to the microcosms repressed the expression of *cchH*. The presence of regulatory DNA sequence motifs similar to iron-dependent repressor binding sites upstream of several genes in this cluster suggests responsiveness to the intracellular iron concentration (Studholme, Bentley et al. 2004).

The results of the endpoint rtPCR indicated that the siderophore cyclase gene, *desD*, involved in desferrioxamine synthesis, was not expressed under any condition. However, qPCR revealed expression at mid-exponential phase in the presence of starch, late exponential phase in the presence of chitin and expression from mid-exponential growth through to stationary in the presence of starch and chitin. In the latter microcosm, expression peaked at mid-exponential growth before the RTA dropped at late exponential and increased again during stationary phase. DesD catalyses the oligomerisation of

hydroxamic acid followed by cyclisation producing a macromolecule of desferrioxamine G and E (Baróna-Gomez, Wong et al. 2004; Barona-Gómez, Lautru et al. 2006). Imert et al found desferrioxamine G and E to be produced when grown in iron-deficient conditions (Imbert, Bechet et al. 1995). Although the endpoint PCR results suggested that there was enough iron for normal growth of *S. coelicolor* and so the siderophore desferrioxamine was not needed, the more specific qPCR data revealed expression. Desferrioxamine B is produced in a variety of conditions in the literature, for example after five days of incubation (to stationary phase) in Bennett's/glucose liquid medium desferrioxamine was extracted from *S. griseus* mycelia (Yamanaka, Oikawa et al. 2005). Desferrioxamine B is important in for growth, morphological development and pigment production on Bennett's/glucose media. The growth impairment of a *des* operon null mutant strain indicated the importance of desferrioxamine for normal growth (Yamanaka, Oikawa et al. 2005). The impairment was attributed to probable iron deficiency. When grown on Bennett's media containing maltose, growth of the mutant returned to normal, linking siderophore production with carbohydrate metabolism (Yamanaka, Oikawa et al. 2005), which could explain the induction of expression in starch-amended microcosms. Two proteins from this *des* cluster were detected in proteomics data from Hesketh et al 2007. SCO2784 protein was more abundant in the *bldA* mutant strain during some of the early timepoints when compared to wildtype *S. coelicolor*, and SCO2785 shows a slightly altered pattern of expression in the *bldA* mutant (Hesketh, Bucca et al. 2007), suggesting regulation by *bldA* (and therefore possibly AdpA as explained above). The addition of ferric ions to the starch- and chitin-amended microcosms completely silenced the *desD* gene. Key intracellular regulatory components may be members of the DmdR (divalent metal-dependent) family. *S. coelicolor* has two such regulators, DmdR1 and DmdR2 (Tunca, Barreiro et al. 2007; Tunca, Barreiro et al. 2009). Disruption of either gene blocks production of desferrioxamine (Tunca, Barreiro et al. 2009). The addition of zinc or cupric down-regulated expression of desferrioxamine by between 1000 and over 30,000 fold. The desferrioxamine B ligand very strongly chelates other metal ions too, including copper(II), nickel(II), and zinc(II) (O'Sullivan, Glennon et al. 1996).

Coelichelin and desferrioxamine are thought to be co-regulated. Intergenic regions in the clusters of both coelichelin and desferrioxamine in *S. coelicolor* contain very similar inverted repeat sequences for the iron-repressor, IdeR (Challis and Hopwood 2003). This repressor regulates the transcription of siderophore biosynthesis in other Gram-positive bacteria, for example *Mycobacteria* (Schmitt, Predich et al. 1995). Many organisms, even those without the necessary biosynthetic pathways to biosynthesis the molecules, have the ability to uptake ferrioxamine complexes (Challis and Hopwood 2003). There is, therefore, a competitive advantage of co-regulation of the two distinct sets of siderophore with separate uptake systems as *S. coelicolor* could survive in habitats with competitors as the ferric coelichelin complex could be selectively absorbed (Challis and Hopwood 2003). Coelichelin and desferrioxamine are an example of contingently acting metabolites, which possess similar biological activity but are independently recognised and used by *S. coelicolor*, giving a competitive advantage (Challis and Hopwood 2003). In this study, coelichelin and desferrioxamine had the same expression profile in both the sterile and starch plus chitin amended microcosms, however, in the singularly amended starch and chitin microcosms expression patterns differed.

In sterile soil and the nutrient-amended microcosms *sco7682* (the first NRPS gene in the coelibactin gene cluster) showed constitutive expression in the endpoint PCR dataset, whereas the qPCR dataset indicated no expression early to mid-exponential growth in the sterile microcosm and no expression at early exponential growth in the microcosm amended with both starch and chitin. Elevated levels of transcription have been shown to down-regulate the activator proteins responsible for switching on Act and Red pathways (Hesketh, Kock et al. 2009). This may explain the non production of Red in the microcosms as coelibactin may be inhibiting its production. This, however, does not explain why Act expression was still observed. Coelibactin is thought to be a zinc-chelating compound (Kallifidas, Pascoe et al. 2010). The zinc-responsive transcriptional repressor, Znr, directly represses a promoter within the coelibactin cluster and also expression of the genes encoding the high-affinity uptake system for zinc (Panina, Mironov et al. 2003). It was therefore surprising that in this study, expression of sco7682 was repressed only during stationary phase in the zinc-amended microcosms. Under low zinc conditions, the AbsC regulator (required for Act and Red biosynthesis) also represses coelibactin production

(Hesketh, Kock et al. 2009). The AbsC binding site overlaps another regulator's binding site, the zinc-responsive transcriptional repressor Zur (Hesketh, Kock et al. 2009). Zur mutant strains are defective in sporulation. This phenotype has been attributed to the overexpression of the coelibactin gene cluster. Each gene in the *S. coelicolor* coelibactin cluster is negatively regulated by zinc when grown in NMMP medium lacking the addition of trace elements solution to mid-exponential phase and then treated with 25μM $ZnSO_4$ (Kallifidas, Pascoe et al. 2010). Again, it is therefore surprising that in this study, expression of sco7682 not repressed completely in zinc-amended microcosms.

In general, this study has demonstrated that the addition of metals silences or down regulates the expression of siderophore related genes, however, other studies have revealed the induction and overexpression of secondary metabolite genes upon addition of metals. For example, scandium causes antibiotic overproduction by 2–25 fold when added at a low concentration (10–100 mM) to *S. coelicolor* cultures (Kawai, Wang et al. 2007). The overproduction was attributed the modulation of ribosomal function, because addition of scandium immediately reduced the level of ppGpp, which is synthesized on the ribosome (Kawai, Wang et al. 2007). Furthermore, scandium along with lanthanum activated the expression of nine genes belonging to nine secondary metabolite biosynthetic gene clusters of *S. coelicolor* when added to the growth medium at low concentrations (Tanaka, Hosaka et al. 2010).

4.7.4 Endpoint PCR and qPCR

As described above, there were some instances where the endpoint PCR data did not match the qPCR data. The qPCR data revealed expression on 15 occasions where endpoint PCR showed a negative result (Table 4.7). This could be explained by the fact that qPCR tends to be more specific and reliable than endpoint PCR. Also, as the primer amplicon length is much shorter (~100bp compared to 500bp), extension may be more efficient. Although qPCR was the preferred method for expression profiling, due to the higher cost in performing the reaction it was only feasible to select genes of particular interest to undergo qPCR analysis.

There were four occasions where endpoint PCR indicated expression of the secondary metabolites genes (Table 4.7). It was noted that each of these occasions were at an early time point preceding established growth and expression. It may also be noted, that as a different set of primers were used for endpoint and qPCR, each primer would amplify a different region of the gene (Fig 4.20). Furthermore, different polymerases were used for endpoint PCR and qPCR. The endpoint PCR used Taq polymerase extracted from an over producing strain of *E. coli* DH5α (ATCC-53868) whereas the qPCR master mix (SensiMix) contained a hot-start polymerase which is activated after 10 minutes at 95°C. The reaction buffers also varied which may cause inconsistencies between the two methods.

Table 4.7 Difference in expression profiles between endpoint PCR and qPCR. *Green indicates expression with at least one method. Red indicates no expression with either method.*

		sterile				**+S**				**+C**				**+S+C**			
(Predicted) compound, enzyme name	**SCO #**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Coelicheliin, NRPS	*SCO0492*	-	-	-	-	-	E	/	/	Q	Q	Q	Q	-	Q	Q	Q
Desferrioxamine, putative siderophore synthetase	*SCO2785*	-	-	-	-	-	Q	-	-	-	-	Q	-	-	Q	Q	Q
Putative siderophore synthetase, siderophore synthetase	*SCO5800*	-	-	Q	-	/	/	/	/	-	-	Q	Q	-	-	-	-
Coelibactin, NRPS	*SCO7682*	E	E	/	/	/	/	/	/	/	/	/	/	E	/	/	/

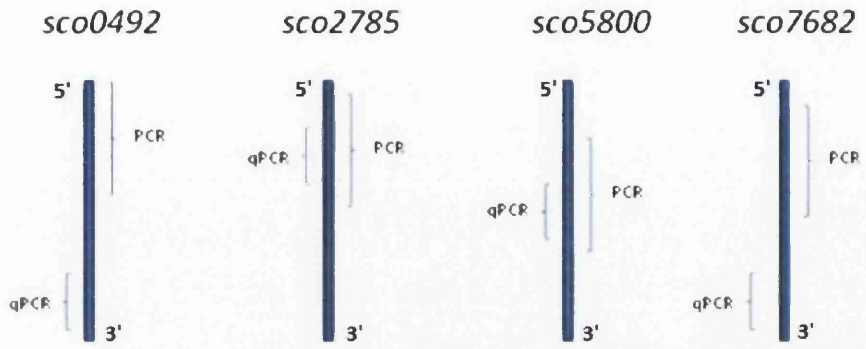


Fig 4.20 Diagrammatic (not-to-scale) representation of siderophore genes and their primer amplification regions.

4.8 Summary

In summary, nutrient, biotic or metal amendments had very little effect on growth of S. coelicolor in soil microcosms. In the nutrient amended microcosms, Act was expressed from the start of exponential growth. The technique of using soil as a growth media proved successful as it uncovered many new and different expression profiles of secondary metabolite biosynthetic gene clusters in *S. coelicolor*. However, it may be noted that findings showed many similarities with data already known from solid/liquid media. Expression of cryptic genes was observed e.g. biosynthetic genes in both lantibiotic gene clusters, the deoxysugar gene cluster and putative NRPS gene cluster. Introduction of biological organisms producing bio-active compounds themselves did not result in a extensive reorganisation of secondary metabolite gene expression in *S. coelicolor*. qPCR indentified an almost constitutive expression profile for the coelibactin biosynthetic gene. The qPCR expression profiles also reveal expression of a cryptic siderophore synthase gene. The addition of ferric ions silenced expression of all siderophore related genes and, in general, zinc and copper down-regulated gene expression.

Chapter 5 Proteomics of *S. coelicolor* grown in Sand Microcosms

In this chapter the results from proteomic studies of *S. coelicolor* grown in sand microcosms are described and discussed. It is acknowledged that sand is a very different medium compared to soil that was used for transcriptomics. The aim of the study was to look at the production of secondary metabolite production in *Streptomyces* under more natural conditions (compared to agar based solid media or liquid broth). Although transcriptomic work was successful on soil, proteomic work proved more difficult. Protein extraction from soil resulted in the carryover of many contaminants and therefore it was decided to use acid washed sand as an alternative. Firstly, growth of *S. coelicolor* in sand was analysed and antibiotic assays executed. Proteins were extracted from sand microcosms and analysed using GelC-MS/MS. All identified proteins were then grouped according to function. Lastly, changes in temporal abundance of selected proteins were quantified. The chapter discussion then focuses to protein expression profiles from the sand microcosms,

5.1 Growth and development

S. coelicolor was found to grow in a highly similar way in sand microcosms amended with starch and chitin when compared to the soil microcosms used for transcriptomic studies (Chapter 4). However, major changes could be observed in the sand microcosms at macroscopic, microscopic and proteomic level over the five day time course itself. Fig. 5.1 displays the growth curve of *S. coelicolor* grown in sand. In the first 48h there was relatively little change with only a small increase in cfu of $6.5x10^5$ cfu per gram of soil. The organism appears to be in the lag phase during this time. From day two to four, cfu increases rapidly (ten-fold in 48h) indicating exponential growth with a doubling time of 766min. Growth stalled between days four and five thus indicating the reaching of early stationary phase.

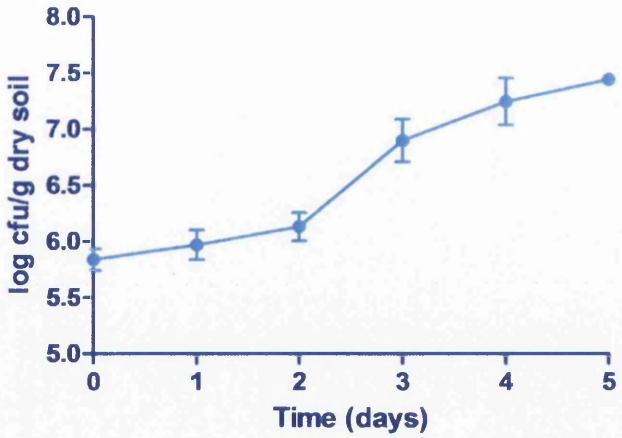


Figure 5.1 : Growth curve of* S. coelicolor *in a sand microcosm. *The graph displays log cfu per gram of microcosm. Error bars show standard error.*

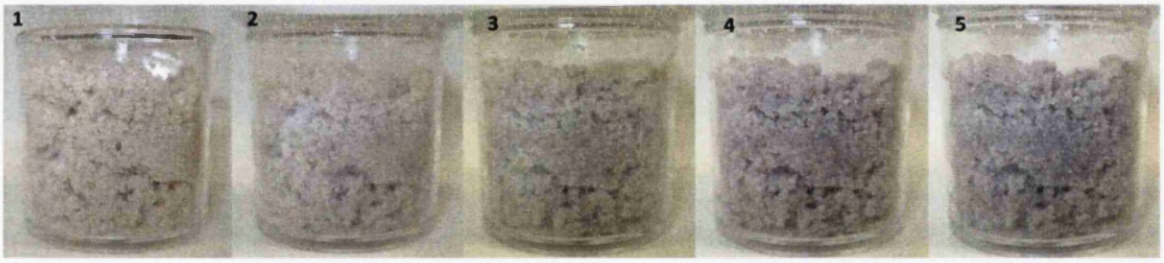


Figure 5.2: Sand microcosm development. *The image shows* S. coelicolor *growing in sand microcosms from day one to day five of incubation.*

The sand microcosms were monitored for phenotypic changes over the period studied. The colour of the sand started to change from a standard beige sand colour to specks of a grey/blue colour after 24h before almost a complete colour change by day five (Fig 5.2), indicating the production of *S. coelicolor* antibiotic pigments. These pigments were observed more closely using a stereomicroscope where they appeared between sand grains (Fig 5.3) after five days of incubation. Light microscopy of a single granule from a three day old microcosm showed mycelium clearly growing on and around the granule (Fig 5.4).

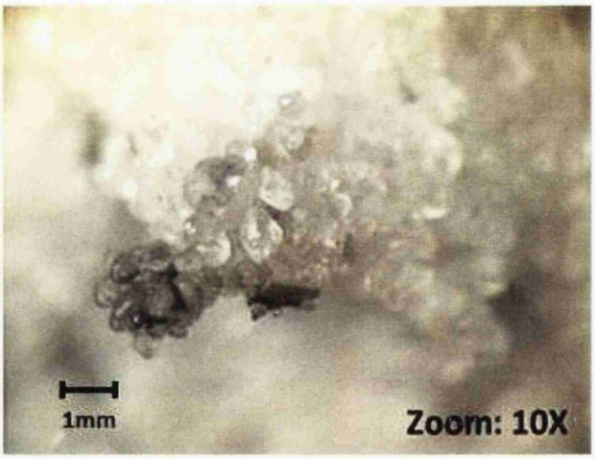


Figure 5.3: Stereomicroscope image of* S. coelicolor *growing in a sand microcosm. *Shown is the tip of a sand microcosm on day 5 of incubation as seen through a stereomicroscope at 10x magnification.*

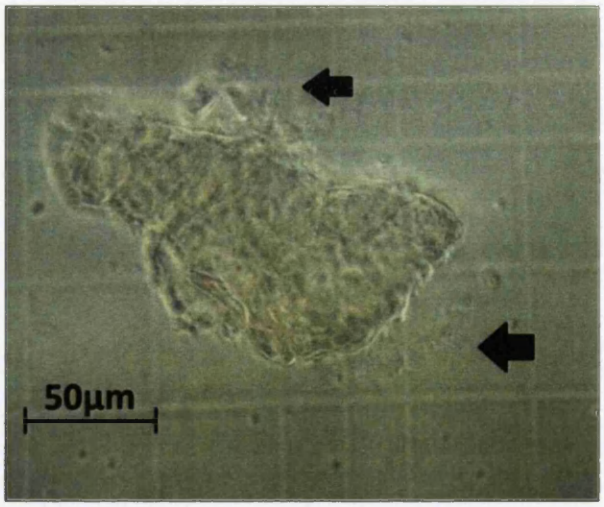


***Figure 5.4: Sand grain with growing mycelium of* S. coelicolor.** *The arrows indicate locations of mycelium apparently attached or growing around the sand particle.*

5.2 Antibiotic production

S. coelicolor is known to produce the pigmented antibiotics Act and Red. A sand microcosm culture was assayed for these two most commonly studied antibiotics. As a dark pigment had already been observed, detection of Act was expected. Fig. 5.5 displays bacterial growth and the measured levels of both antibiotics.

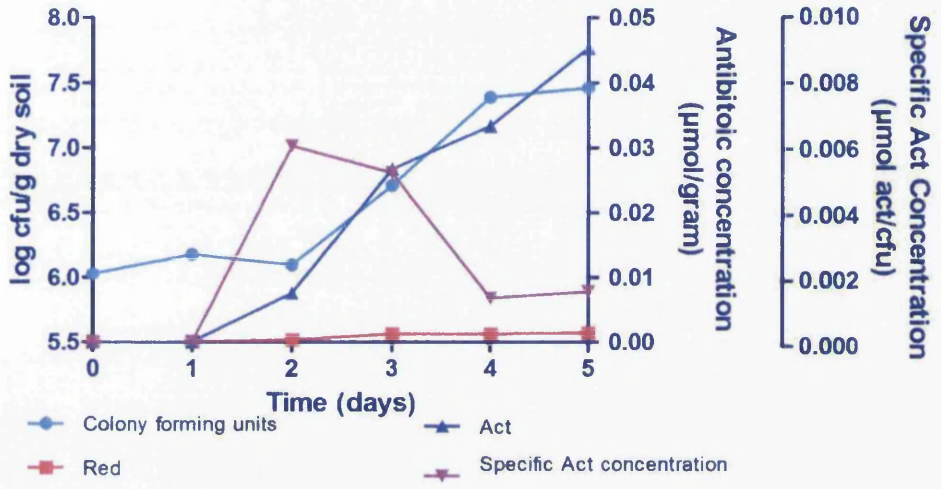


Figure 5.5: Growth and antibiotic production in* S. coelicolor *sand microcosms. *The graph shows a growth curve as measured in the microcosm, together with the absolute and specific Act and Red antibiotic levels.*

The production of Act started at the beginning of exponential growth on day two, with the concentration steadily increasing to over 40µmol per gram of microcosm sand at early stationary phase. However, the specific act concentration varies over the time course. The specific act concentration reached a maximum of 0.030 µmol/cfu at early exponential growth (day two), which decreased to a steady lower specific act concentration of 0.007 µ mol/cfu at the transitional to early stationary phase (day four to five). Very limited production of Red was observed.

5.3 Protein extraction

Proteins were extracted from the *S. coelicolor* sand microcosms to identify proteins and their corresponding expression patterns. Bradford assays were used to determine protein yields after extraction. The standard curve produced a straight line equation of y = 0.0448x + 0.0475. The values from the protein extracted from sand were then interpolated into this equation (Table 5.1). The equation R-squared (R^2) of 0.993 showed a good correlation between concentration standards and absorption.

Extraction from the one day old microcosm yielded little protein (1.5μg), which corresponds well with the culture still being in lag phase (Fig 5.1). Extractions from days two to five showed consistently greater yields (of around 3μg) but this was not proportional to the growth phase.

Table 5.1 Protein determination from* S. coelicolor *sand microcosm time-course. *Measured values of A_{595} for the protein extracted from sand and their calculated concentration and total yield.*

	A_{595} mean	Protein concentration (μg/ml)	Total yield (μg/sample)
Day 1	0.118	76	1.5
Day 2	0.185	155	3.1
Day 3	0.182	139	2.8
Day 4	0.196	160	3.2
Day 5	0.178	142	2.8

5.4 Protein analysis

Gel separations and MS analyses (GelC-MS/MS) were performed to obtain a protein expression profile and protein identifications. The most relevant gels and their resulting protein identifications are shown in this chapter.

From a silver-stained gel, a series of highly abundant protein bands were analysed (fig. 5.6). This gel was run in triplicate, also to serve visual analysis of protein abundance and quantitation. A second run with a higher load of protein was used for more extensive MS analysis. Thus, some pure protein bands could be quantified temporally.

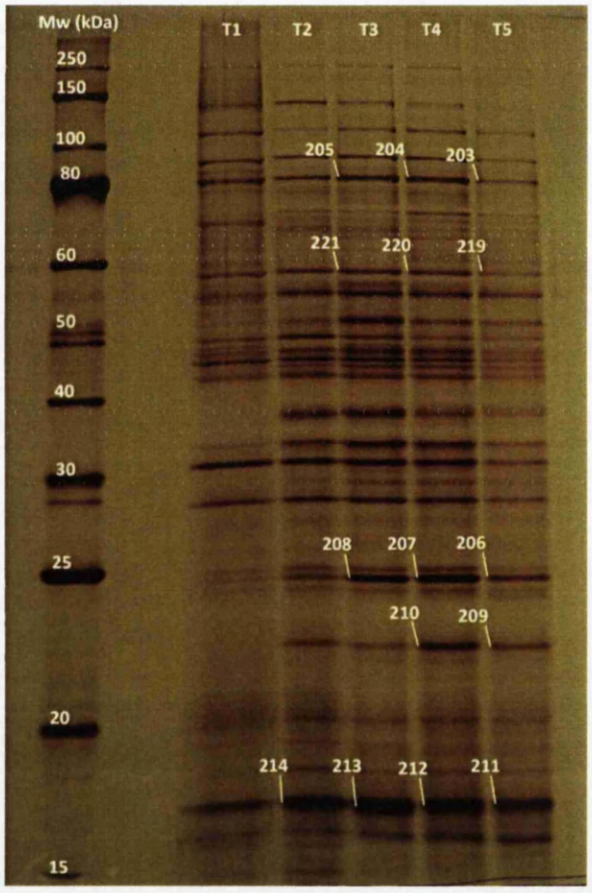


Figure 5.6: Temporal protein expression of* Streptomyces coelicolor *grown in sand microcosms. *The image shows a 1D 10% SDS-PAGE silver stained gel with T1-T5 representing the extracted protein samples from each day of incubation. The marked bands were analysed with protein identifications shown in Table 5.2.*

The clearest bands with highest protein abundance from the gel in Fig 5.6 were used for MS analysis. Samples were selected where a protein of similar molecular weight was being expressed over several days. These bands were selected and analysed using MS. Figure 5.6 also displays a change in protein expression pattern over days one to five. This was demonstrated with bands 210 and 209. This band of the same apparent molecular weight of 23kDa was not present on day one, but was present on days two to five but with different intensities indicating a change in expression over time. This was one of many proteins showing differences in abundance across the bacterial proteome.

For better separation and further analysis, higher amounts of protein from day two and three samples were separated on a long (16x18cm) 10% acrylamide gel (Fig 5.7). Coomassie stain was used as silver stain was deemed too sensitive for the higher amounts of protein. A similar pattern emerged as seen with the silver stained gel (Fig 5.6) from which many distinct bands were sliced out and analysed. TPIS (triose phosphate isomerase) from an unstained polypeptide standard was used as an internal positive control and known protein standard. After MS each identified protein (Table 5.2) was grouped according to function.

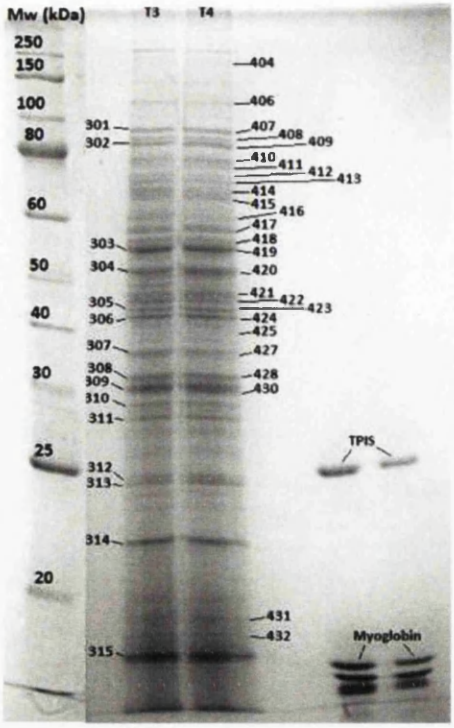


Figure 5.7: Sand microcosm protein extracts separated on Coomassie stained 10% SDS-Page gel. *This image is a combined photograph of two photographs taken of the same gel at different exposure times which were then joined together for a higher resolution. All the marked bands were analysed and most of them led to protein identifications which are shown in table 5.2.*

A total of 90 individual *S. coelicolor* proteins were identified. Of those 90, 67 were validated by either the identification of two or more different peptides from the protein sequence or identification in at least two individual samples taken from the same height in a replicate gel. This identified around 0.85% of the predicted proteome (Bentley, Chater et al. 2002).

At least 46 of the identified proteins belong to primary metabolism and at least six proteins are involved in secondary metabolism. Overall, 10 proteins were unknown or hypothetical proteins with some homology-derived predicted function. Fig 5.8 and Table 5.2 show the clustering of the proteins according to most apparent function deduced from annotations and searches in databases. However, when a protein may have important functions in several pathways, the pathway in which it was clustered to in Table 5.2 is considered the

most important one. For example, some proteins of the central carbohydrate metabolism may also play a role in amino acid metabolism or the production of secondary metabolism precursors. For example, TpiA (SCO1945) and also Pgi (SCO6659) have roles in glycolysis as well as the biosynthesis of secondary metabolites, whilst PdhL functions in glycolysis, TCA and the metabolism of glycine, serine and threonine.

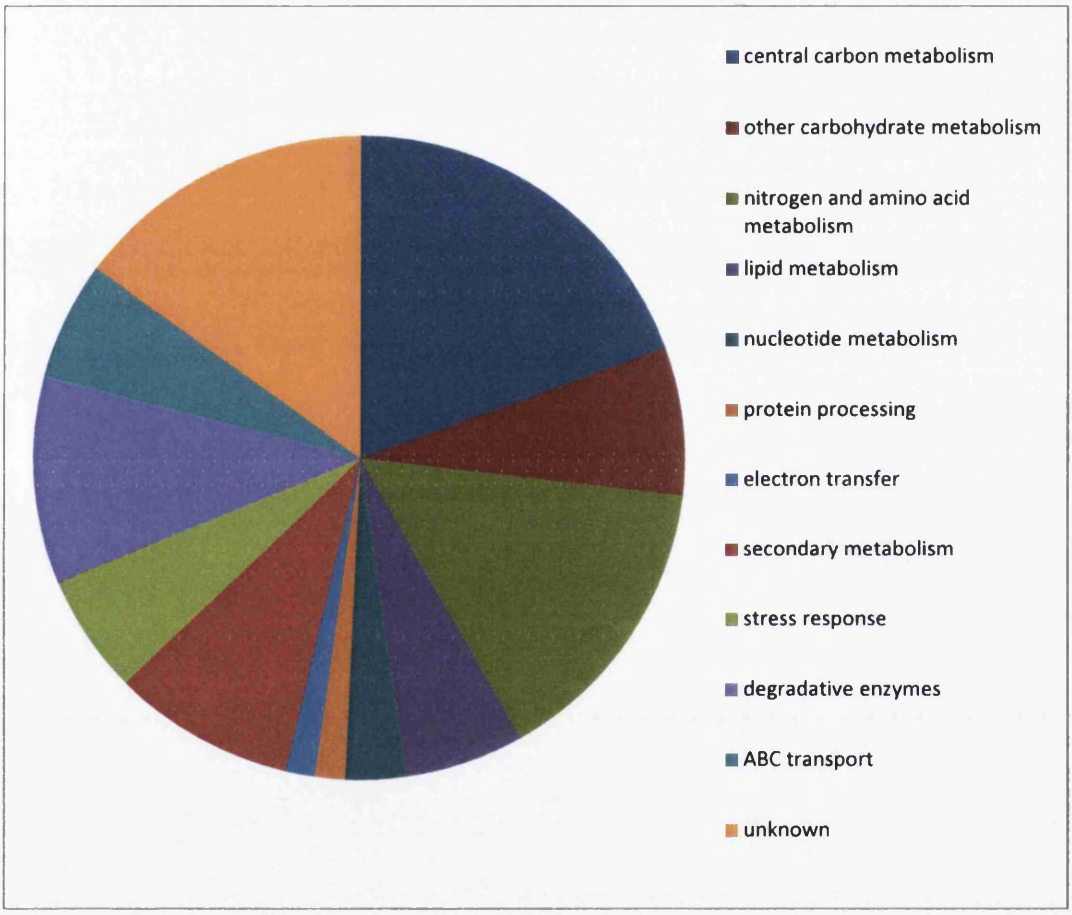


Fig 5.8 Graphical representation of the functions of the identified proteins

Table 5.2: All identified proteins by function.

Function[1]	SCO N°[2]	Description[3]	Gene name[4]	Molecular Weight[5]	Sample[6]
Glycolysis	SCO1945	triosephosphate isomerase	*tpiA*	27736	311
	SCO6659	glucose-6-phosphate isomerase	*pgi*	60424	417
	SCO1942	glucose-6-phosphate isomerase	*pgi2*	60569	416
Pentose phosphate pathway	SCO1936	transaldolase	*tal2*	40587	421
	SCO6662	transaldolase	*tal1*	40407	421
	SCO1937	glucose-6-phosphate 1-dehydrogenase	*zwf2*	56606	418
Citric acid cycle	SCO4827	malate dehydrogenase	*mdh*	34643	428
	SCO2736	type II citrate synthase	*citA*	47744	423
	SCO2180	dihydrolipoamide dehydrogenase	*pdhL*	51396	420
	SCO4809	succinyl-CoA synthetase subunit alpha	sucD	30238	430
	SCO4808	succinyl-CoA synthetase subunit beta	*sucC*	41602	422
	SCO7000	isocitrate dehydrogenase	*idh*	79519	409
	SCO5999	aconitate hydratase	*sacA*	97380	406
Other carbohydrate metabolism	SCO6078	alpha amylase	*treZ*	63952	423
	SCO2786	beta-*N*-acetylhexosaminidase	*hexA*	58568	416
	SCO0948	alpha-mannosidase	*ams1*	110827	406
	SCO1174	aldehyde dehydrogenase	*thcA*	55673	419
	SCO3420	aldehyde dehydrogenase	*SCE9.27c*	51967	419
Nitrogen and amino acid metabolism and protein synthesis	SCO1223	ornithine aminotransferase	*rocD*	42906	425
	SCO2770	agmatinase	*speB*	34302	425
	SCO1773	L-alanine dehydrogenase	*SCI51.13c*	39571	423
	SCO4366	phosphoserine aminotransferase	*SCD19.21c*	38828	423
	SCO4837	serine hydroxymethyltransferase	*glyA1*	51434	420
	SCO2726	methylmalonic acid semialdehyde dehydrogenase	*msdA*	52573	419
	SCO6102	nitrite/sulphite reductase	*SCBAC1A6.26c*	62924	419
	SCO3023	S-adenosyl-L-homocysteine hydrolase	*sahH*	53009	418
	SCO5547	glutamyl-tRNA synthetase	*gltX*	54917	418
	SCO3792	methionyl-tRNA synthetase	*SCH63.39*	59741	416
Lipid metabolism	SCO1565	glycerophosphoryl diester phosphodiesterase	*SCL24.01c,SCL11.21c*	42675	425
	SCO1968	secreted hydrolase	*SC3C9.03*	31419	311
	SCO3079	acetyl-CoA acetyltransferase	*SCE25.20*	42623	421
	SCO5399	acetyl-CoA acetyltransferase	*SC8F4.03*	40929	423
Nucleotide metabolism	SCO2612	nucleoside diphosphate kinase	*ndk*	15110	214
	SCO4087	phosphoribosylaminoimidazole synthetase	*purM*	36834	307
Protein Processing	SCO7510	peptidyl-proplyl cis-trans isomerase	*cypH*	17889	432
Electron transfer	SCO5135	ferredoxin	*fdxA1*	11879	206
Secondary metabolism	SCO5078	hypothetical protein SCO5078	*actVA-ORF3*	30028	310
	SCO0395	epimerase/dehydratase	*SCF62.21*	36410	307
	SCO5077	hypothetical protein SCO5077	*actVA-ORF2*	14422	211
	SCO6433	hypothetical protein SCO6433	*SC1A6.22*	15026	211
	SCO5074	dehydratase	*actVI-ORF3*	23134	209
Stress response	SCO0379	catalase	*katA*	55116	418
	SCO2633	superoxide dismutase	*sodF*	23527	313
	SCO5254	superoxide dismutase	*sodN*	14703	211
	SCO0560	catalase/peroxidase	*cpeB*	80815	410

Table 5.2: All identified proteins by function (continued)

Function[1]	SCO N°[2]	Description[3]	Gene name[4]	Molecular Weight[5]	Sample[6]
Degradative enzymes: nucleases, proteases	SCO2529	metalloprotease	SCC117.02	38245	430
	SCO1230	secreted tripeptidylaminopeptidase	2SCG1.05c	58535	418
	SCO6199	secreted esterase	SC2G5.20	74400	309
	SCO3610	peptide hydrolase	SC66T3.21c	65725	412
	SCO3970	Xaa-Pro aminopeptidase	SCBAC25E3.07c	54048	416
	SCO4589	aminopeptidase	SCD20.07c	33594	308
	SCO2643	aminopeptidase N	pepN	94423	301
ABC transporters	SCO4142	phosphate-binding protein precursor	pstS	38040	427
	SCO2008	branched-chain amino acid-binding protein	SC7H2.22	43503	423
	SCO5113	BldKB, ABC transporter lipoprotein	bldKB	65533	414
	SCO2231	maltose-binding protein	malE	44624	305
Unknown	SCO2847	oxidoreductase	SCE20.21	38104	425
	SCO6418	hypothetical protein SCO6418	SC1A6.07c	54004	416
	SCO6594	secreted protein	SC8A6.15c	63837	416
	SCO6711	hypothetical protein SCO6711	SC4C6.21c	84637	408
	SCO6220	hypothetical protein SCO6220	SC2H4.02	233387	404
	SCO4985	hypothetical protein SCO4985	2SCK36.08	29283	310
	SCO0505	large, multifunctional secreted protein	SCF6.01,SCF34.24	108601	406
	SCO6571	protein function unknown	SC3F9.06	37615	309
	SCO1908	large secreted protein	SCI7.26	64697	412
	SCO6569	secreted solute-binding protein	SC3F9.04	36806	430

[1]Function in pathway considered most important role as on StrepDB (The Streptomyces Annotation Server)

[2]SCO number according to NCBI website

[3]Description as returned by Bioworks Browser

[4]Alternative gene nr or name belonging to SCO number as on StrepDB

[5]Predicted molecular weight as returned by Bioworks Browser

[6]Sample and Band number as found in gel images fig. 5.6 for sample N° 2XX and fig.5.7 for sample N° 3XX, 4XX.

5.5 Primary metabolism

The following proteins were identified with a (putative) role in extracellular hydrolysis and the uptake of nutrients: an alpha-amylase (SCO6078, TreZ), which breaks down starch polymers into maltose units that can be taken up into the cell. A second protein expected to be involved in the ABC transport-dependent uptake of maltose is MalE, maltose-binding protein (SCO2231). Subsequent hydrolysis would yield glucose which can be fed into glycolysis. Enzymes found associated with glycolysis are two glucose-6-phosphate

isomerases (SCO6659 Pgi, SCO1942 Pgi2) and triose phosphate isomerase (SCO1945 TpiA, temporal expression Fig. 5.8). Three enzymes with functions in the pentose phosphate pathway were also identified; glucose-6-phosphate 1-dehydrogenase (SCO1937 zwf2) and two transaldolases (SCO1936 Tal2, SCO6662 Tal1), involved, respectively, in 6-carbon sugar oxidation and decarboxylation and modifications between 3, 4, 5, 6 and 7-carbon phosphorylated sugars. For chitin metabolism, β-*N*-acetylhexosaminidase (SCO2786, HexA) was found, which helps to break down the GlcNAc polymer into smaller subunits. Seven proteins were found associated with the TCA cycle: isocitrate dehydrogenase (SCO7000 Idh, temporal expression (Fig 5.8), which was highly and constitutively expressed throughout growth in sand. Other carbohydrate metabolism enzymes were alpha-mannosidase (SCO0948, AMS1), aconitate hydratase (SCO5999, SacA), succinyl-CoA synthetase subunit alpha and beta (SCO4809 SucD, SCO4808 SucC), type II citrate synthase (SCO2736, CitA), malate dehydrogenase (SCO4827, Mdh) and dihydrolipoamide dehydrogenase (SCO2180, PdhL). The dihydrolipoamide dehydrogenase (SCO2180) is part of a multi-component enzyme system. It is also a component of pyruvate dehydrogenase, involved in pyruvate oxidation to acetyl CoA.

Many proteins from nitrogen and amino acid metabolism were identified including two aminotranferases (SCO1223, RocD and SCO4366). Two tRNA synthetases were also found (SCO5547 GltX, SCO3792 MetG), both having roles in translation and metabolism. SCO5547 (GltX) is involved in the metabolism of porphyrin and chlorophyll, whilst SCO3792 (MetG) metabolises selenocompounds.

A whole range of degradative enzymes (proteases, nucleases) were found including SCO2529, a metalloprotease, which degraded extracellular proteins and peptides for bacterial nutrition prior to sporulation, expressed in the sand microcosm at late exponential- early stationary phase. A total of five aminopeptidases were expressed in the sand microcosms, SCO1230, SCO3970, SCO4589 and SCO2643 (PepN). Aminopeptidases selectively release N-terminal amino acid residues from polypeptides and proteins. They are known to play a role in several significant physiological processes, including the catabolism of exogenously supplied peptides (Gonzales and Robert-Baudouy 1996). A secreted esterase (SCO6199) along with a hydrolase (SCO3610) were also identified amongst the other degradative proteins.

Furthermore, proteins were found involved in each of the following processes: lipid metabolism, nucleotide metabolism, protein processing and ABC transport and finally ten unknown proteins with some conserved domains and predicted functions (see Section 5.8 Other unknown proteins). One of the ABC transporter proteins was the oligopeptide transporter BldKB (SCO5113), which was expressed late exponential/ early stationary phase.

5.6 Secondary metabolism

Four proteins expressed from the Act gene cluster were identified in mid-exponential to stationary phase sand microcosms: SCO5074, SCO5077, SCO5078 and SCO5079. These are respectively, a putative dehydratase (SCO5074, ActVI-ORF3) a probable tailoring enzyme for the antibiotic, a hypothetical protein (SCO5077, ActV-ORF2), a hypothetical protein (SCO5078, ActV-ORF3), a protein with unknown function, and hypothetical protein (SCO5079, ActV-ORF4) a putative (transcriptional) regulatory protein. Another protein considered to be involved with aerial development and morphological differentiation is BldKB (SCO5113). One branched chain amino acid-binding protein (SCO2008) and many amino acid metabolism proteins.

Two more proteins were found which are predicted to be involved in secondary metabolite production. Both belong to 'cryptic' gene clusters with no natural expression reported so far. These are an epimerase/dehydratase (SCO0395), a 36kDa protein expressed by the deoxysugar synthases/glycosyl transferases cluster (*sco0381-0401*) with 68.4% identity in 342 amino acid overlap to a streptomycin biosynthetic enzyme, StrP from S. *glaucescens* (StrepDB), and a hypothetical protein of the cupin superfamily (SCO6433), a 15kDa protein expressed by a cryptic NRPS gene cluster (sco6429-6288). In the proteomic experiments, hypothetical protein SCO6433 was found during mid- to late exponential growth.

5.7 Oxidative Stress

Several proteins associated with the oxidative stress response were identified throughout the culturing period in sand. A total of four different enzymes with similar functions were

found, all protecting the organism against reactive oxygen species. Two enzymes utilise the metal ions of nickel and iron to act as catalyst, these are superoxide dismutases SodN (SCO5254) and SodF (SCO2633). Another identified enzyme that neutralises hydrogen peroxide is the highly expressed catalase (SCO0379, KatA), and also a catalase/peroxidase (SCO0560, CpeB), which catalyses the conversion of hydrogen peroxide to water and oxygen. In addition, SCO0560 (CpeB) may also have further metabolic functions, e.g., in phenylalanine metabolism.

5.8 Other unknown proteins

Ten more proteins were found of which the functions are much less clear (Table 5.2). Four are suggested to be secretion related: SCO6594, SCO1908, SCO6569, and SCO0505. SCO0505 is a large multi-domain protein of which one or more domains may have possible 6-carbon binding capabilities. SCO2847 has possible oxidoreductase activity. SCO6571 was identified but although its crystal structure has been elucidated (Begum, Sakai et al. 2008), the function of this protein is still unknown. The other four unknown proteins are hypothetical proteins with little conserved domains and thus no known function.

5.9 Temporal expression

A time-course abundance plot was constructed for seven selected proteins (Fig 5.9 and Table 5.3). These could be analysed quantitatively (Fig 5.10) as MS analysis showed a pure sample, with only one protein identified per band.

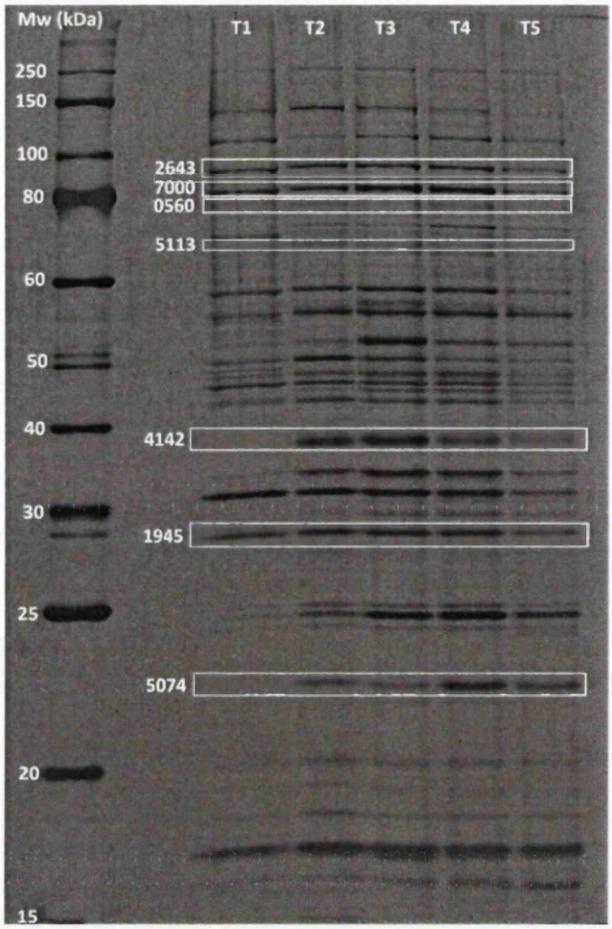


Figure 5.9: Selected pure protein bands used for temporal abundance analysis. *The highlighted horizontal lanes contain the 7 proteins (labelled with SCO numbers) of which MS analysis showed contained only one protein.*

Table 5.3: Proteins selected for temporal abundance analysis.

SCO N°	Function	Gene Name	Molecular Weight (kDa)	Band N° (Fig 5.7)
SCO0560	catalase/peroxidase	CpeB	80815	410
SCO1945	triosephosphate isomerase	TpiA	27736	311
SCO2643	aminopeptidase N	PepN	94423	301
SCO4142	phosphate-binding protein precursor	PstS	38040	427
SCO5074	dehydratase	ActVI-ORF3	23134	314
SCO5113	BldKB, ABC transporter lipoprotein	BldKB	65533	414
SCO7000	isocitrate dehydrogenase	Idh	79519	409

An important TCA cycle enzyme, Idh, and a glycolysis enzyme, TpiA were quantified together with two ABC transporters, a phosphate-transport associated protein, PstS, and the BldKB mycelial differentiation protein. Two more enzymes of primary metabolism, PepN and CpeB, and one from secondary metabolism, the Act-associated protein ActVI-ORF3 were also quantified.

The TCA enzyme Idh and the glycolysis enzyme TpiA were expressed constitutively, with only slight fluctuations over the five days (Fig 5.10). This was also true for PepN, the aminopeptidase enzyme. The phosphate-transport associated enzyme PstS was not expressed on day one but peaked during early to mid exponential growth, which then dropped by 36% during the last stages of the growth curve. Catalase/peroxidase CpeB had varying expression levels. Levels were consistent on days one, three and five, but increased on days two and four (highest on day two), which represent major transitions in the growth curve, respectively, early exponential growth and transition to stationary phase. The mycelial differentiation protein BldKB was not expressed on day one. By day two its expression had peaked and decreased steadily to day five. Finally, the secondary metabolism associated actVI-ORF3 was expressed from day two, with a slight dip on day three followed by increased expression levels on days four and five.

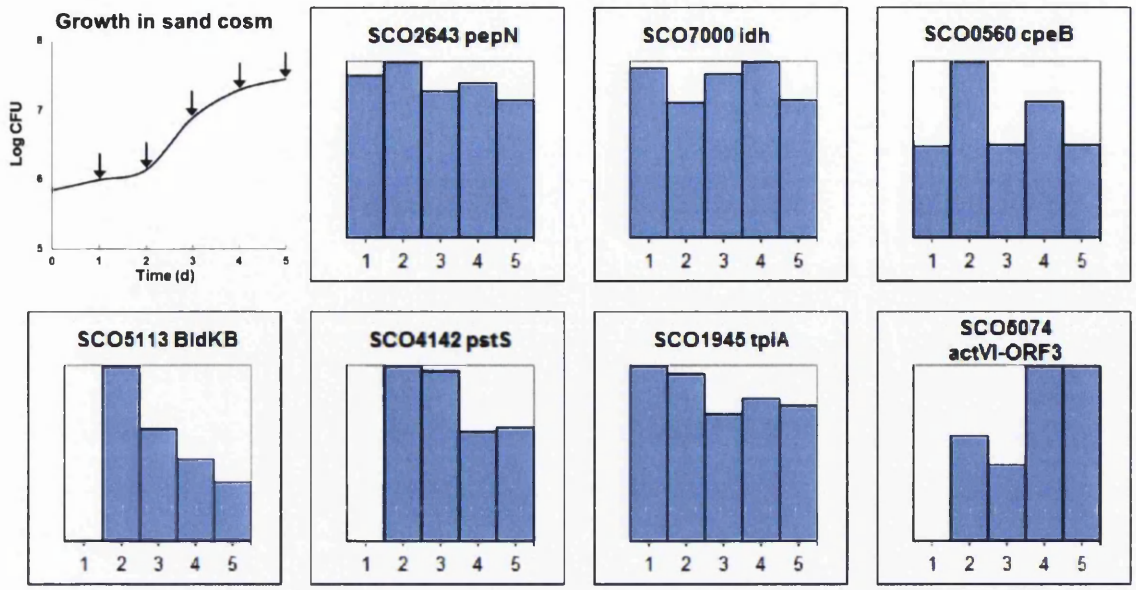


Figure 5.10: Time course abundance of seven proteins expressed in* S. coelicolor *grown in sand microcosms. *The first graph shows the growth curve of* S. coelicolor *in the sand microcosms, with the arrows indicating time of sampling. The histogram bars are normalised (relative to maximum amount) band intensity means taken from three replicate gels. The bands are shown in Fig. 5.9 and proteins listed in Table 5.3.*

5.10 Discussion

5.10.1 *S. coelicolor* growth in sand microcosms

S. coelicolor was able to grow in sand microcosms with the addition of 1% starch and 1% chitin (Chapter 5, Fig 5.2). Growth curves displayed the same growth pattern as the sterile soil/nutrient amended soil microcosms. However, in the sand microcosms the regular pattern of a decline in total viable count due to the death phase of young germlings after one day was not observed.

5.10.2 *S. coelicolor* proteins in sand microcosms

A total of 0.85% of the predicted *S. coelicolor* proteome (Bentley, Chater et al. 2002) was validated by either the identification of two or more different peptides from the protein sequence or identification in at least two individual samples of the same molecular weight in a replicate gel. Proteins were identified from a wide variety of processes including carbon metabolism, degradation, ABC transport, stress response and secondary metabolism.

Of the *S. coelicolor* proteins identified, seven were suitable to be quantified. Even though discrete bands were observed on the long SDS-PAGE gels, resolution was often not high enough to distinguish between proteins of similar sizes. Some of the protein bands, which appeared to be singular, upon MS analysis contained five or more different proteins. Mass spectrometry, with the help of liquid chromatography, is specific and sensitive enough to identify each protein in each sample, although where there is more than one, absolute quantification is impossible. The quantified proteins represented overall a good representative fraction of the total find. They included two central carbon metabolism proteins (SCO1945 and SCO7000), a protease (SCO2643), a Bld protein (SCO5113), an Act protein (SCO5074) along with an ABC transporter (SCO4142) and finally a stress response catalase/peroxidase (SCO0560).

5.10.2.1 Carbohydrate metabolism

As expected, many of the identified proteins were linked to nutrient metabolism. Central carbon metabolism enzymes included glucose-6-phosphate isomerase (SCO6659 and SCO1942), succinyl-CoA synthetase (subunit alpha SCO4809, subunit beta SCO4080), and isocitrate dehydrogenase (SCO7000). Other carbohydrate metabolism-linked proteins included an alpha-amylase, TreZ (SCO6078), for trehalose biosynthesis, an alpha-mannosidase, AMS1 (SCO0948), and beta-*N*-acetylhexosaminidase, hexA (SC02786).

TreZ is a protein of the TreYZ pathway which biosynthesises trehalose. Trehalose is a disaccharide found in the spores of a variety of organisms where it is degrades slowly during dormancy but is rapidly metabolised during vegetative growth (Thevelein 1984). Trehalose

is located in the spore cytoplasm and is not hydrolysed until *S. griseus* spores are germinated (McBride and Ensign 1990). Rapid hydrolysation of trehalose during germination (and the inactivity of trehalase during this time) is attributed to hydration of the cytoplasm (McBride and Ensign 1990). Trehalose is also a precursor to the GlgE pathway for alpha-glucan synthesis (Chandra, Chater et al. 2011). Glycogen, which is a large alpha-glucan, is biosynthesised by the GlgC-GlgA pathway (Chandra, Chater et al. 2011). Recently, a second pathway has been discovered in mycobacteria, the GlgE pathway (Chandra, Chater et al. 2011). This pathway used trehalose as a precursor, and converts it to maltose, linear glucan and finally branched glucan for use, possibly, in capsular alpha-glucan biosynthesis (Chandra, Chater et al. 2011). Trehalose also has been shown to interplay with glycogen during sequential stages of aerial hyphae development in *S. coelicolor* (Schneider, Bruton et al. 2000). The GlgE pathway genes are associated with deposition of glycogen at the initiation of aerial growth and during the first stages of sporulation (Plaskitt and Chater 1995; Schneider, Bruton et al. 2000; Yeo and Chater 2005). Trehalose is also implicated in anhydrobiosis, to help withstand periods of dessication due its water retention capabilities (Leslie, Israeli et al. 1995). The presence of TreZ therefore may suggest that *S. coelicolor* growing in sand is experiencing desiccation. This corroborates the transcriptomic data where expression of phytoene synthase gene (*sco6759*) from the hopanoid biosynthetic cluster also indicated that *S. coelicolor* was experiencing stress due to water limitation.

The enzymatic hydrolysis of alpha-mannosides is catalysed by alpha-mannosidases (glycoside hydrolases). *Streptomyces* mannosidase is bound to the mycelium until lysis occurs (Demain and Inamine 1970). AMS1 is involved in the cleavage of the alpha form of mannose. In this study AMS1 (SCO0948) was detected during stationary phase. This is the first evidence of expression of this protein in the literature. Another glycoside hydrolase was found during stationary phase, HexA (SCO2786). HexA catalyses the conversion of chitobiose to GlcNAc in chitin metabolism by hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues (Kanehisa, Goto et al. 2004). As chitin was added to the sand microcosms, it was expected to find chitin metabolism proteins.

Two proteins were quantified from central carbon metabolism. Firstly, SCO1945 (TpiA) is a triosephosphate isomerase responsible for the isomerisation of the three-carbon phosphorylated sugars dihydroxyacetone phosphate and glyceraldehydes 3-phosphate in

the second stage of glycolysis. Secondly, SCO7000 (Idh) is a isocitrate dehydrogenase from the TCA cycle responsible for the oxidative decarboxylation of isocitrate to α-ketoglutarate. It would be assumed that as the *S. coelicolor* undergo morphological differentiation at the transition to stationary growth that there would be a decrease in central carbon metabolism enzyme expression. However, the normalised quantities of the central carbon metabolism proteins showed this not to be the case completely (Fig 5.10). TpiA showed a decrease in expression from the late exponential stage onwards whilst Idh had fluctuating expression levels which peaked at the transition stage.

5.10.2.2 Degradative Proteins

A range of proteolytic enzymes were identified, which suggest active recycling of biomass. These include four aminopeptidases PepN, SCO1230, SCO3970 and SCO4589. Also identified were an esterase (SCO6199) and a peptide hydrolase (SCO3610). SCO1230 is a tripeptidyl aminopeptidase. Aminopeptidases degrade oligopeptides (Vitale 1999). These enzymes are responsible for the major amino-terminal degradative activity in *S. lividans* (Butler, Binnie et al. 1995). Extracellular proteases are involved in assimilating extracellular proteinaceous nitrogen sources, yet some extracellular proteases are implicated in *Streptomyces* development (Chater, Biro et al. 2010). In *Streptomyces* development, the role of proteases involves elaborate cascades mediated by protease inhibitors and specific proteolytic processing (Chater, Biro et al. 2010). The aminopeptidase PepN (SCO2643) was expressed constitutively with little fluctuation in expression level. PepN is a metalloexopeptidase, which cleaves next to leucine, arginine and lysine in peptide-bond-containing substrates in *S. lividans* (Butler, Aphale et al. 1994). Metalloproteases are involved with other peptidases in the degradation of peptides during extracellular breakdown (Yen et al 1980). In *Streptomyces*, they help to break down substrate mycelium for nutrients to support spore formation (Kang and Lee 1997). PepN participates in the controlled hydrolysis of peptides in the proteolytic pathway (Addlagatta, Gay et al. 2006). In *E. coli*, PepN is the responsible for the majority of aminopeptidase activity (Chandu and Nandi 2003). Expression of PepN in *E. coli* is increased 4-fold upon phosphate starvation and is also regulated by anaerobiosis and carbon source (Gharbi, Belaich et al. 1985). Therefore, in the sand microcosms *S. coelicolor*

may have been experiencing phosphate limitation or oxygen limitation if regulated in a similar manner.

5.10.2.3 ABC Transport

Four enzymes involved with ABC transport were identified: PstS and BldKB along with a maltose-binding protein MalE, SCO2231 and a branched-chain amino acid-binding protein, SCO2008. SCO2008 may have an extra role in providing the precursor amino acids for secondary metabolite biosynthesis. MalE is a maltose-binding protein, responsible for the import of maltose across the membrane. MalE expression is induced by maltose and repressed by glucose (Van Wezel, White et al. 1997). MalE was expressed on day three in the sand microcosm, at mid-exponential stage, indicating the possible depletion of glucose as a carbon source but the presence of maltose at this time point (although the source of maltose in clear). Also found was an alpha-amylase, the enzyme responsible for the breakdown of starch, the polymeric carbon source (SCO6078).

The high-affinity periplasmic phosphate-binding protein PstS (SCO4142) was expressed from the start of exponential growth through to stationary phase. This protein, which is part of the phosphate transport system, is encoded by the first gene of the *pst* operon (*pstSCAB*) (Rodríguez-García, Barreiro et al. 2007). The phosphate transport system is one of the mechanisms that many organisms have developed to help survival under stress conditions, indicating here that *S. coelicolor* is under stress in sand microcosms. Under nutrient limitation conditions (low phosphate) a broad variety of metabolites are produced, mediated by the two-component PhoR-PhoP system (Sola-Landa, Moura et al. 2003), which controls the *pst* operon. In *Streptomyces* depletion of phosphate is known to switch on antibiotic biosynthesis (Martin 2004). Phosphate is an important nutrient and PstS is central to its utilization in *Streptomyces* (Wehmeier, Varghese et al. 2009). In *E. coli* and *B. subtilis* the expression of Pst proteins is linked to phosphate levels in the media, where expression is induced by phosphate starvation and repressed at high phosphate concentrations (Antelmann, Scharf et al. 2000; Aguena, Yagil et al. 2002). In *S. lividans*, no accumulation of PstS was observed at low phosphate concentrations, even after ten days of incubation.

However, upon addition of 5% fructose, PstS accumulated at low phosphate concentrations (Díaz, Esteban et al. 2005). On solid R2YE media, the deletion of *pstS* resulted in the overproduction of actinorhodin. This was attributed to impaired phosphate transport in the mutant strain mimicking a low phosphate concentration (Díaz, Esteban et al. 2005). It is interesting that expression of PstS in the sand microcosm was relatively high during exponential growth and decreased during transition/stationary growth phase, whilst the opposite was true for ActVI-ORF3 (Fig 5.10). These findings are in agreement with Diaz et al, where upon absence of PstS, Act is overproduced.

5.10.2.4 Stress

SCO0560 (CpeB/CatC), is a homologue of the *S. reticuli* CpeB protein, which is a mycelium-associated enzyme with haem-dependent catalase/peroxidase activity and haem-independent manganese-peroxidase activity (Zou and Schrempf 2000). A catalase functions to remove hydrogen peroxide, which is generated as a by-product of aerobic respiration, by decomposing H_2O_2 to O_2 and H_2O. Catalases are either monofuctional or combine catalase-peroxidase activity, where they also reduce H_2O_2 to H_20 using an intracellular reductant (Chelikani, Fita et al. 2004). Expression of CpeB in *S. coelicolor* has been studied in both liquid YEME cultures and on solid R2YE plates, showing different expression patterns (Hahn, Oh et al. 2000). In liquid cultures, CpeB production increased on the procession from early to late exponential growth and decreased slowly until stationary phase. On solid media, CpeB production increased upon production of aerial hyphae and decreased after sporulation (Hahn, Oh et al. 2000). CpeB was present in over two-fold higher concentration in surface-grown cells compared to liquid-grown cells, indicating an aerobic cue for production (Hahn, Oh et al. 2000). In the sand microcosm, expression peaked at the start of exponential stage, reducing at mid-exponential growth and increasing again at the exponential to transition phase. Expression was reduced at stationary phase, which is similar to the expression levels in liquid and in solid-grown cultures. This may suggest that *S. coelicolor* is experiencing oxygen limitation during the stationary growth phase.

5.10.2.5 Secondary metabolism

Detection of Act, the metabolite, and its biosynthetic proteins indicate normally the onset of secondary metabolism, usually after the transition from exponential to stationary growth in liquid and solid cultures (Huang, Lih et al. 2001). However, ActVI-ORF3 protein SCO5074 was found already on the second day of incubation at the very start of exponential growth. The function of this protein is a putative dehydratase (Hesketh, Chandra et al. 2002), for the conversion of (S)-hemiketal to 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-H-naphtho[2,3-c]pyran-3-acetic acid (Kanehisa, Goto et al. 2004). The relative abundance of the protein increased throughout exponential growth through to transition/stationary phase, with a slight dip on day three, which correlated to the absolute Act concentration in sand (Fig 5.5). The presence of Act was observed easily in the sand microcosms as a colour change was obvious (Fig. 5.2). On solid media, the transcription of *act* cluster genes is tightly coordinated temporally, and was up-regulated 2- to 17-fold at later stages of cell growth, as had been observed also in liquid media (Huang, Lih et al. 2001). The transcriptomic data in this study revealed expression of the Act polyketide synthase (*sco5087*) from mid-exponential phase incubation in sterile soil. In the starch plus chitin soil microcosms, as well as just chitin-amended, expression was induced on from the beginning of exponential phase. This shows correlation between the transcriptomics and proteomic data obtained in this study.

As explained previously, secondary metabolism has been thought to begin just before the transition stage, coinciding with or slightly preceding morphological differentiation. This study has revealed expression of secondary metabolite genes much earlier in growth. This was also observed for the production of BldKB (SCO5113), a protein considered to be involved with secondary growth and development, in very early exponential growth indicating that morphological differentiation is ongoing. Bld mutants produce an aerial hyphae-deficient phenotype and have therefore been studied to gain insight into aerial hyphae formation. BldKB is a lipoprotein ABC-type permease involved in mycelial differentiation (Nodwell and Losick 1998). Deletion of the *bldKB* gene results in the Bld phenotype (Claessen, De Jong et al. 2006). BldKB allows the transport of Bld261, a morphogenic oligopeptide (Nodwell and Losick 1998). Bld261 is responsible for the

promotion of early morphological differentiation in rich, but not minimal media (Claessen, De Jong et al. 2006; Chávez, Forero et al. 2011).

BldKB and SCO2582 (a putative membrane metalloendopeptidase), bind SCO2127, a protein produced in stationary growth phase in media supplemented with 100mM glucose (Chávez, Forero et al. 2011), and during the exponential growth phase in minimal media (Chávez, García-Huante et al. 2009). The *sco2127* gene is located upstream from a glucose kinase gene, in a region which restores sensitivity to carbon catabolite repression (CCR) (Chávez, Forero et al. 2011). SCO2127 has been linked in CCR of mycelium differentiation by interaction with BldKB in *S. coelicolor* (Chávez, Forero et al. 2011). In *Streptomyces*, glucose is the carbon catabolite with the most repressive effect (Hodgson 2000). The absence of SCO2127 affects the binding with BldKB which deregulates aerial hyphae formation in the presence of glucose (Chávez, Forero et al. 2011). The repression of mycelium differentiation in the presence of glucose has been reported in *S. griseus*. *S. coelicolor* has a homologous regulatory system, indicating that glucose may also repress mycelium differentiation in this bacteria (Ueda, Endo et al. 2000).

In the sand microcosm, BldKB was produced from early exponential through to early stationary phase in decreasing amounts. This is surprising, as it would be expected that the differentiation protein to be produced during differentiation at the transition stage, where aerial hyphae are typically formed. The presence of BldKB so early in growth therefore may be indicative of early differentiation. This may be due to Bld261 promoting early differentiation in the presence of glucose (from the breakdown of the starch carbon source). After the glucose is expended, there would no longer be CCR of mycelial differentiation by interaction of SCO2127, also indicating the possibility of early differentiation.

The transcriptomic data set revealed expression of cryptic secondary metabolite biosynthetic genes in soil microcosms (see Chapter 4). Protein identification also revealed expression of cryptic clusters (Chapter 5). From a putative NRPS cluster (Fig 5.11), a protein of unknown function, SCO6433, was identified in the late exponential and early stationary phase in sand microcosm. This is correlated with the data from the transcript analysis as a different gene from the cluster, *sco6431*, a peptide synthase, which was expressed in soil

microcosms. This supports the hypothesis that an organism would produce more bioactive compounds when grown in a more natural environment.

The second secondary metabolite cryptic cluster protein found was an epimerase/dehydratase (SCO0395) from the deoxysugar cluster (*sco0381-0401*). This again supports the findings of the transcriptomic data where another gene, *sco0381*, was expressed in the soil microcosm. This is the first evidence of direct expression of genes/proteins from this cluster, however, extracellular carbohydrates have been isolated, indentified and the structures elucidated, although it is unclear whether these extracellular sugars are biosynthesised from the cluster studied (Hesketh, Bucca et al. 2007).

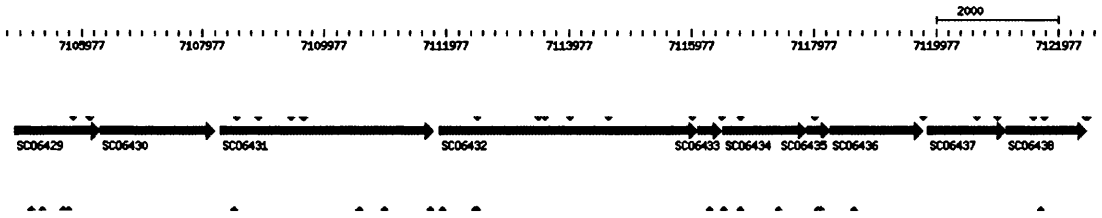


Fig 5.11 Schematic representation of NRPS cluster;* sco6429-sco6438 *adapted from StrepDB.

The biosynthesis of many secondary metabolites is negatively regulated by phosphate (Masuma, Tanaka et al. 1986; Liras, Asturias et al. 1990). Phosphate control has been suggested to be used as mechanism to trigger secondary metabolism (Martín and Demain 1980), as when phosphate is depleted, a microorganism may produce metabolites which are antagonistic to other organisms (Vögtil, Chang et al. 1994), or to enhance survival under stress (Maplestone, Stone et al. 1992). Phosphate control of primary and secondary metabolism is mediated by the two component system PhoR-PhoP.

Carbon and nitrogen source also play a role in regulation of primary and secondary metabolism. Descriptions of genes regulated simultaneously by more than one nutrient source are limited. An example of the interaction between phosphate and carbon source includes the regulation of PstS. This phosphate-binding protein accumulates in *Streptomyces*

cultures grown in the presence of various sugars under phosphate limitation conditions (Díaz, Esteban et al. 2005; Esteban, Díaz et al. 2008). For nitrogen metabolism, at a transcriptional level the main regulator is GlnR (Reuther and Wohlleben 2007). Under nitrogen limitation conditions, GlnR activates several genes involved in ammonium transport and metabolism, along with glutamine synthetase genes (Reuther and Wohlleben 2007; Tiffert, Supra et al. 2008). Under phosphate limitation conditions, the expression of some of these genes are increased in a *S. coelicolor* ΔPhoP mutant (Rodríguez-García, Barreiro et al. 2007). This is explained by the fact that when phosphate is low, energy is used to scavenge phosphate and down regulated genes for nitrogen assimilation to equilibrate the ratio of phosphate to nitrogen (Martín, Sola-Landa et al. 2011).

5.11 Summary

To summarise, although the conditions of growth were different *S. coelicolor* grew in a highly similar way in sand microcosms compared to soil microcosms. The production of pigments was observed in sand microcosms after one day of incubation. An Act assay was successful, indicating production of act from day two. An optimised protein extraction protocol successfully extracted proteins from the sand microcosms which were identified by GelCMS. Of the 67 proteins identified, 13 were involved in carbohydrate metabolism, 10 in nitrogen and amino acid metabolism, and four in lipid metabolism. Many of the proteins identified were involved in degradation, stress response and ABC transport. Six secondary metabolism proteins were identified, four from the Act cluster. Seven proteins underwent temporal abundance analysis, including a Bld and an Act protein.

Chapter 6 Final discussion and conclusions

In this chapter the results from the previous chapters are reviewed in a final discussion. The links between the transcriptomic and proteomic results are discussed and the final conclusions of the project drawn. The chapter ends with further research questions.

6.1 Final Discussion

Most studies investigating secondary metabolite production in *Streptomyces*, as well as other bacteria, are undertaken in liquid or on solid media. There is little gene expression data available for *in situ* studies. This study looked at expression in a totally different growth medium, soil. Using soil benefits as it gives an insight of how *Streptomyces* survives under more 'normal' conditions.

The first objective of this study was to identify which *S. coelicolor* secondary metabolite genes are expressed under environmental conditions. In order to achieve this, *S. coelicolor* soil microcosms were established. Growth curves established that *S. coelicolor* follows the same growth pattern in soil and sand as in liquid/solid media, of lag phase, exponential growth and stationary phase, yet over a longer time scale.

Before gene expression profiles were established, a RNA amplification protocol was optimised. This process involved using a T7 promoter sequence attached to random hexamer primer for reverse transcription, producing cDNA with a T7 promoter sequence included. After second strand synthesis, amplified RNA was produced via *in vitro* transcription using a T7 RNA polymerase. The optimised protocol was successful to over 12500x amplification.

The technique of using soil as a growth media proved successful as it uncovered many new and different expression profiles of secondary metabolite biosynthetic gene clusters in *S. coelicolor*. However, it may be noted that findings showed many similarities with data already known from solid/liquid media. This study revealed a wide variety of secondary metabolites being produced in a natural environment, achieving the first aim set out in this study. Many cryptic genes showed novel expression patterns, for example the *rppA* gene from the flaviolin biosynthetic cluster, which had previously been thought to be silent

(Izumikawa *et al* 2003). Also, this study revealed the first evidence of expression of a putative chalcone synthase gene (*sco7671*) from a chalcone biosynthetic cluster. Furthermore, gene expression profiles were established for two lantibiotic biosynthetic genes, which also have not been shown to be expressed in any literature. Finally, *sco6431*, a cryptic peptide synthase gene from a putative NRPS gene cluster was expressed for the first time.

There were some surprising expression profiles of secondary metabolite genes. For example, no expression of the Red biosynthetic gene, although to date no studies have indicated production of Red from *Streptomyces* in soil. Also, the earthy odorant geosmin biosynthetic gene was not expressed, but the alternative early odorant methylisorborneol terpene synthase (*sco7700)* was expressed.

From the siderophore gene expression profiles, it was confirmed that expression of siderophore biosynthetic genes is repressed by the addition of ferric. This study also substantiates the theory that coelichelin and desferrioxamine are co-regulated (Challis and Hopwood 2003). Both biosynthetic genes tested showed the same expression profiles in the sterile soil microcosms and those adjusted with starch plus chitin. An interesting point that came from the coelibactin biosynthetic gene expression profile was that the gene was not completely repressed by the addition of zinc, although it was down-regulated. In fact, expression of the coelibactin biosynthetic gene was almost constitutive. Zinc has been shown to negatively regulate each gene in the coelibactin cluster in NMMP medium (Kallifidas, Pascoe et al. 2010).

The second aim of this study was to elucidate factors affecting expression of these genes under environmental conditions and how nutrient availability and other biologically active organisms affect secondary metabolite gene expression in soil. This study has revealed the effects of changing carbon and nitrogen sources (starch and chitin). For example, the expression of the epi-isozizaene terpene synthase gene (*sco5222*), *scbA* (*sco6266*) and the methylisoborneol terpene synthase (*sco7700*) were starch dependent, whereas the *cpk* type I polyketide synthase (*sco6273*) and the lantibiotic II cyclase (*sco6929*) were chitin dependent. Introduction of *M. anisopliae* spores or *St. kraussei* extract did not result in a significant reorganisation of secondary metabolite gene expression in *S. coelicolor*. As a

result, soil microcosms have been shown to be an important tool for gene expression analysis in combination with a new RNA amplification technique.

Many proteins from the *S. coelicolor* proteome were identified from sand microcosms, and few were quantified. The majority of proteins found were involved in central carbon and nitrogen metabolism, which was expected due to the starch and chitin carbon source, including proteins such as the triosephosphate isomerase, TpiA (SCO1945) involved in glycolysis, the type II citrate synthase, CitA (SCO2736) and the isocitrate dehydrogenase, Idh (SCO7000) involved in the TCA cycle and the agmatinase, speB (SCO2770) involved in amino group metabolism. In addition, the protein TreZ was identified, which functions in trehalose biosynthesis. Trehalose is a protein linked to anhydrobiosis, suggesting that *S. coelicolor* is experiencing water limitation stress. This was also suggested by the expression of the hopanoid phytoene synthase gene (*sco6759*). Hopanoid has a speculative function of alleviating stress in aerial mycelium by diminishing water permeability across the membrane (Poralla et al 2000). Several proteins associated with the oxidative stress response were identified, protecting the organism against reactive oxygen species. These included the superoxide dismutases SodN (SCO5254) and SodF (SCO2633). Also identified was an enzyme that neutralises hydrogen peroxide catalase (SCO0379, KatA), and also a catalase/peroxidase (SCO0560, CpeB), which catalyses the conversion of hydrogen peroxide to water and oxygen. Evidence of phosphate limitation in the soil microcosms was also exhibited by the production of PepN in the sand microcosms.

Several proteins were identified which are involved in secondary metabolic pathways. For example, the Act protein ActVI-ORF3 (SCO5074) was expressed from the beginning of exponential growth through to stationary phase. This correlates with the findings of the transcriptomic data where the Act polyketide synthase (*sco5087*) was found from the very start of exponential growth in soil microcosm amended with starch and chitin. This was confirmed metabolically with Act assays from the sand microcosms. BldKB (SCO5113), a protein considered to be involved with aerial development and morphological differentiation, was also expressed very early in the exponential growth phase, indicating that morphological differentiation is ongoing. From a putative NRPS cluster, a protein of unknown function, SCO6433, was identified in the proteomic sand study, whilst another gene of that cluster, *sco6431*, a peptide synthase, was expressed in soil microcosms.

Furthermore, from the cryptic deoxysugar cluster, an epimerase/dehydratase (SCO0395) was identified whilst another gene from the cluster *sco0381*, was expressed in the soil microcosm.

6.2 Linking transcriptomics and proteomics

At a given moment, the full complement of mRNA in a cell is its transcriptome. This forms the template for protein synthesis resulting in a corresponding protein complement, the cell proteome. Whilst understanding the molecular mechanisms of transcriptomics and proteomics are powerful tools individually, integration of the two studies may be the key to unravelling the complexity of a system. Transcriptomics and proteomics have been implemented in parallel in many studies, with varying degrees of success due to many challenges that hinder progress (Ideker, Thorsson et al. 2001; Chen, Gharib et al. 2002; Griffin, Gygi et al. 2002). Challenges include, from an experimental front, the limitation of experimental design and interpretations as well technological limitations, and from a biological front, differential RNA and protein turnover, post-translational modifications and proteolytic processing events (Hegde, White et al. 2003).

In this study, transcriptomic analyses was limited to secondary metabolite biosynthetic genes, whereas the proteomic study, while incomplete, identified proteins from a wide range of processes. Although soil and sand are very different media, there are instances where the two approaches correlate or support each other. Fig 6.1 is a schematic diagram integrating the findings of the studies presented here. Shown is central carbon metabolism, which provides the precursors for most *S. coelicolor* secondary metabolites, with all the secondary metabolite biosynthetic genes that were identified by transcriptomic studies, along with the proteins identified by the proteomic study.

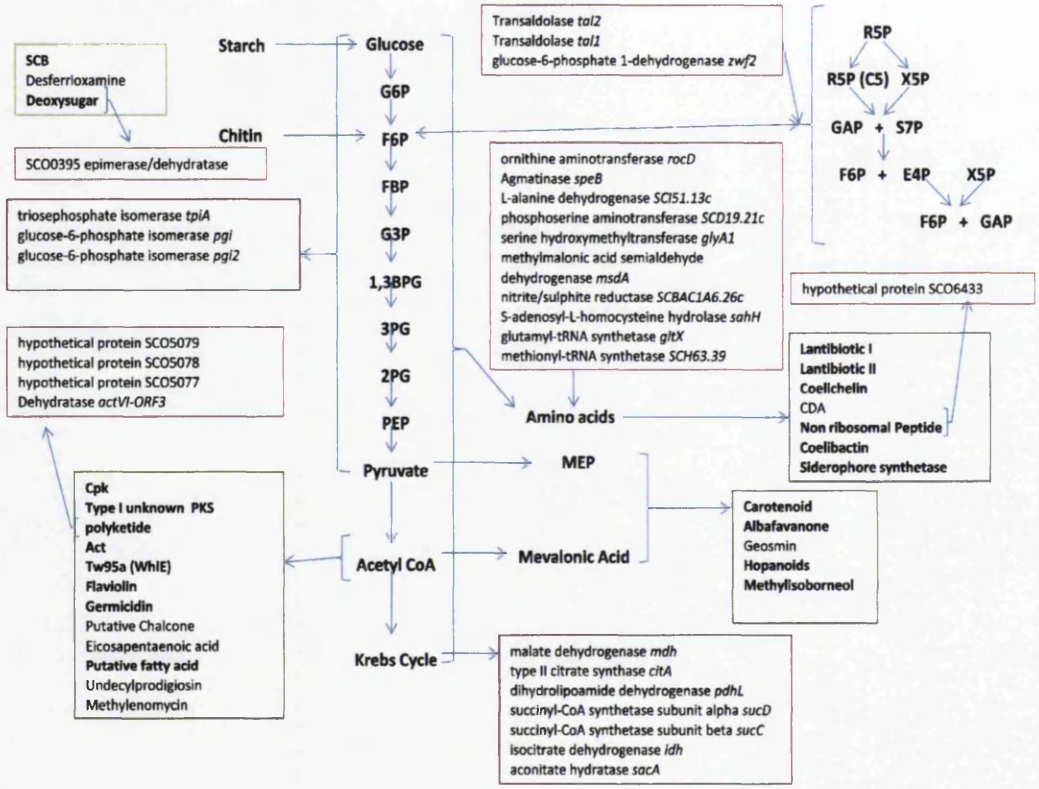


Fig 6.1 Schematic summary diagram of central carbon metabolism with identified proteins and transcripts of secondary metabolite biosynthetic genes. *Red boxes indicate proteins from sand microcosms identified by GeLC-MS, green boxes indicate secondary metabolites (bold indicates that the biosynthetic GOI was expressed in one or more soil microcosms). Blue arrows indicate direction of influence. G6P; glucose-6-phosphate, F6P; fructose-6-phosphate, FBP; fructose-1,6-bisphosphate, G3P; glyceraldehydes-3-phosphate, 1,3BPG; 1,3-bisphosphoglycerate, 3PG; 3-phosphoglycerate, 2PG, 2- phosphoglycerate, PEP; phosphoenolpyruvate, MEP; methylerythritol phosphate, R5P; ribulose-5-phosphate, R5P(C5); riobose-5-phosphate, X5P; xylulose-5-phosphate, S7P; sedoheptulose-7-phosphate, E4P; erythose-4-phosphate.*

6.3 Conclusion

To conclude, in secondary metabolite biology, it the consensus that secondary metabolism commences at the beginning of (or slightly preceding) stationary growth phase. It is interesting therefore that many of the secondary metabolite biosynthetic genes were expressed from the first day of incubation, and also secondary metabolite related proteins were indentified before the onset of exponential growth. Some secondary metabolite genes/proteins were even expressed constitutively. The results of both the transcriptomic data and proteomic data therefore give a new insight into secondary metabolism and its role under more natural conditions.

6.4 Further work

The detailed analysis of the developmental cycle of *Streptomyces* in the soil/sand microcosms may have been achieved by microscopic analysis. The method was tested during the study; however it proved difficult due to the texture and constitution of the media making it difficult to obtain a clear picture of the bacteria. However, it is acknowledge that this would be a useful tool and is a possibility for further investigation for future work or publication.

For future work or the publication of the transcriptomic results detailed in this study, PCR products would need to be sequenced. Although single bands (and therefore seemingly single products) were observed of the correct molecular weight for all the endpoint PCR agarose gels, sequencing would validate that the PCR products were indeed the result of successful amplification from the specific primer pairs.

Due to the interesting and also novel expression profiles of secondary metabolite biosynthetic genes under the different conditions tested in this study, it would be interesting to investigate further environmental factors that may influence expression of these genes (Sole et al 1997). The pH of a media has been shown to effect secondary metabolite expression, and so it may be interesting to find out the effect of changing the pH of the soil on *S. coelicolor.* Also, due to the evidence of stress due to water limitation, it

would be interesting to investigate the effect of soil water levels. These would fluctuate greatly in the natural environment and so would demonstrate expression patterns which may occur naturally. Water levels in soil are also correlated to oxygen limitation and so may have further effect.

For each experiment in the transcriptomic study, the soil was pre-autoclaved producing sterile soil microcosms. All microcosms were kept sterile, except for the microcosms amended with *M. anisopliae* and *St. kraussei*. Further investigation using non-autoclaved, 'untouched' soil would give a further understanding of development in more natural conditions.

Due to the fact that sand and soil are such different media, it is difficult to make links and a correlation between the results. Therefore, it would be advantageous to repeat the transcriptomic work on sand microcosms to give a more rounded discussion from a systems perspective.

It may also be useful to use known mutants for further studies, for example an AbsA mutant, to investigate the regulation of secondary metabolite production in soil/ sand microcosms.

Whilst it would be interesting to search directly for secondary metabolites from the soil or sand microcosms, extraction procedures to date have difficulty due to the properties of soil. Studies have revealed that antibiotics strongly adsorbed to soil particles (Pinck, Holton et al. 1961; Pinck, Soulides et al. 1962). Also, the extremely small amounts of secondary metabolites produced in soil make extraction and concentration of the metabolites a challenge (Williams 1982; Wellington, Marsh et al. 1993). However, this study revealed the possibility of extraction of metabolites from sand microcosms, by the successful extraction and quantification of Act. More recent studies have had positive results using a variety of extraction methods, including the use of acetone for extraction of pseudomonads of 2,4-diacetylphloroglucinol (*Pseudomonas* antibiotic) from rhizocosms for analysis with a HPLC system (Standing, Banerjee et al. 2008). It therefore may be interesting to use sand a media to investigate metabolite extraction from *S. coelicolor* cultures.

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