Detection and Expression of Biosynthetic Genes in Actinobacteria

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Table of Contents

Chapter 2: Materials and Methods

Chapter 3: Results

Chapter 4: Discussion

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Adelaide, May 2008

George Bervanakis

DECLARATIONS

I certify that this Masters thesis entitled “Detection and Expression of Biosynthetic Genes in Actinobacteria” constitutes my own work and has been carried out by myself, unless stated otherwise. The thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief that it does not contain any material previously published or written by another person except where due reference is made in the text. The work described in this thesis was performed by myself while I was enrolled as a Masters of Science student at Flinders University of South Australia, (Biotechnology, School of Medicine), Department of Microbiology and Infectious Diseases, Faculty of Health Sciences.

Signed: _____

Date: _____

Abstract

Most microbial organic molecules are secondary metabolites which consist of diverse chemical structures and a range of biological activities. Actinobacteria form a large group of Eubacteria that are prolific producers of these metabolites. The recurrence of pathogens resistant to antibiotics and a wider use of these metabolites apart from their use as anti-infectives, has been the impetus for pharmaceutical companies to search for compounds produced by rare and existing actinobacterial cultures.

Accessing microbial biosynthetic pathway diversity has been possible through the use of sensitive and innovative molecular detection methodologies. The present study evaluated the use of molecular based screening as a rational approach to detect secondary metabolite biosynthetic genes (SMBG) in uncharacterised natural Actinobacterial populations. A polymerase chain reaction (PCR) approach was selected for ease of application and high sample processivity. Rational designed screening approaches using PCR in the discovery of SMBG, involved identifying common functions in secondary metabolite biosynthetic pathways, such as condensation reactions in polyketide synthesis, genes encoding these functions, and using conserved regions of these genes as templates for the design of primers to detect similar sequences in uncharacterised actinobacteria. Design of primers involved rigorous *in silico* analysis followed by experimentation and validation.

PCR screening was applied to 22 uncharacterised environmental isolates, eight of these displayed the presence of the ketosynthase (*KS*) gene belonging to the type I polyketide synthases and eight contained the ketosynthase (KS_α) gene belonging to the type II polyketide synthases, six of the isolates contained the presence of a presumptive dTDP-glucose synthase (*strD*) gene which is involved in the formation of deoxysugar components of aminoglycoside antibiotics and one isolate contained the presence of a presumptive isopenicillin N synthase (*pcbC*) gene involved in beta-lactam synthesis. Alignments of partially sequenced PCR products from isolates A1488 and A3023 obtained using type II PKS primers showed close similarities with KS_α genes from antibiotic producing actinobacteria. Similarly, alignments of

sequences from isolates A1113 and A0350 showed regions of similarities to *KS* genes from antibiotic producing actinobacteria.

Fermentation techniques were used for inducing expression of secondary metabolites from the uncharacterised actinobacteria isolates. By using antimicrobial guided screening it was determined that most of the isolates possessed the capacity to produce antimicrobial metabolites. Dominant antagonistic activity was detected against Gram positive bacteria and to a minor extent against fungi. Optimal fermentation liquid media were identified for certain isolates for the production of antimicrobial metabolites. Two alternative fermentation methods; solid-state and liquid-oil fermentations were evaluated to improve secondary metabolite production in the uncharacterised isolates. Solid-substrate fermentation showed that it could induce a complex metabolite pattern by TLC analysis, however this pattern varied according to the substrate being used. Liquid media supplemented with refined oils, showed a positive response indicated by higher antibacterial activities detected.

Evaluation of semi-purified organic extracts identified two isolates A1113 and A0350 producing similar antimicrobial metabolites as detected by HPLC/UV/MS, a literature database search of similar compounds containing the same molecular weight identified the compound as belonging to the actinomycin group of compounds. A complex metabolic pattern was identified for isolate A2381, database searching identified some of the compounds as having similar molecular weights to actinopyrones, trichostatins, antibiotics PI 220, WP 3688-5 and YL 01869P.

Drug discovery screening can serve to benefit from PCR detection of biochemical genotypes in initial screens, providing a rapid approach in identifying secondary metabolite producing capabilities of microorganisms prior to the commencement of costly and time consuming fermentation studies. Additionally the identification of biochemical genotypes allows a directed approach in using fermentation media designed to induce biosynthetic pathways of specific classes of compounds.

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

AA	amino acid
ACP	acyl carrier protein
ACV	δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine
ARS	Agriculture Research Service
AT	acyl transferase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AU	absorbance units
BA	bioassay agar
BDH	British Drug House
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSN	broth supernatant
C	carbon
CLF	chain length factor
cm	centimeters
CoA	coenzyme A
CU	codon usage
Da	Dalton
DDBJ	DNA Database of Japan
DEBS	deoxyerythronolide B synthase
DEX	dextrin
DNA	deoxyribonucleic acid
6DOHS	6-deoxysugars
DSM	German Culture Collection
EDTA	ethylenediamine tetra-acetic acid
EMBL	European Molecular Biology Laboratory
EMS	electrospray mass spectrometry
ESI	electrospray ionisation source
EtAc	ethyl acetate
EtOH	ethanol
g	gram
gDNA	genomic deoxyribonucleic acid
h	hour
IPNS	isopenicillin N synthase
kb	kilobase
KR	ketoreductase
KSα	ketosynthase alpha
LC-MS	liquid chromatography-mass spectrometry
LSA	linseed, safflower, and almond crushed seeds
ME	mycelial extracts
MeOH	methanol
min	minutes
mg	milligram
mAU	milli-absorbance units
ml	millilitres

µl	microlitres
µM	micromolar
MS	mass spectrometry
MSA	multiple sequence alignment
mM	milli molar
m/z	mass-to-charge ratio
nm	nanometer
NA	nucleic acid
NRRL	Northern Regional Research Laboratories Culture Collection
^{o}C	degrees Celsius
OLV	olive Oil
ORF	open reading frame
pcbAB	δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine synthase
pcbC	isopenicillin N synthase
PCR	polymerase chain reaction
PDA	Photo Diode Array
PK	Polyketide
PKS	Polyketide Synthatase
R_f	relative mobility
rpm	revolutions per minute
RP-HPLC	reverse-phase high performance liquid chromatography
rRNA	ribosomal ribonucleic acid
σ	sigma
SAF	safflower oil
SBM	soya bean meal
SDS	sodiumdodecylsulphate
SI	sucrose inorganic salts medium
SM	secondary metabolites
SMBG	secondary metabolite biosynthetic genes
SmF	submerged fermentation
SOY	soya oil
SSF	solid-substrate fermentation
spp.	species
SUN	sunflower oil
TLC	thin-layer chromatography
TBE	tris-borate buffer
T_m	melting point temperature
TSB	Tryptic Soy Broth
UV-Vis	Ultra-Violet Visible
VO	vegetable Oil
v/v	volume per volume
WAL	walnut oil
w/v	weight per volume
YME	Yeast-Malt Extract Agar

Aims of the Project

1) Identify biochemical genotypes in a range of actinobacteria cultures from different environments, which are related to the productivity of secondary metabolites.

2) Induce the expression of secondary metabolites from actinobacteria, with an emphasis on genera that are poor producers.

Experimental Objectives

1) To develop and implement PCR screening methodologies for the detection of biosynthetic genes in actinobacteria.

2) To formulate a fermentation media that exhibits broad range applicability for the production of secondary metabolites.

Null Hypothesis

1) The presence of biosynthetic genes in an actinobacterial strain indicates the presence of a biosynthetic pathway and that the organism may have the capability to produce a specific secondary metabolite under defined fermentation conditions.

2) The ability of an actinobacterial strain to produce secondary metabolites is not influenced by the isolation source.

Chapter 1: Introduction

Section 1: General Aspects of Actinobacteria

1.1.1 Description of the genetic, biochemical and morphological features of Actinobacteria

Actinomycetes now referred to as actinobacteria, are Gram-positive, spore-forming soil bacteria, belonging to the order *Actinomycetales* (Goodfellow, 1988), characterised by the formation of substrate and aerial mycelium on solid media and possessing a high guanine plus cytosine content of DNA (60-70 mol %). Actinobacteria contain circular genomes that transcribe 3300 or more genes. A majority of these genes encode large coding sequences which are utilised during complex morphological differentiation and secondary metabolite biosynthesis (Hopwood *et al*., 1985). In addition to circular plasmids, actinobacteria possess linear plasmids which are large extrachromosomal DNA elements, implicated in the transfer of secondary metabolite biosynthetic genes and antibiotic resistance genes (Kinashi, 1994).

1.1.2 Industrial Relevance of Actinobacteria

The plethora of chemical diversity generated from microbial products has been the main contribution to the discovery of bioactive compounds in industrial screening programs (Bérdy, 1992). Actinobacteria are a major group of microorganisms that are prolific producers of secondary metabolites (SM), many of which are bioactive compounds. They are major sources of these compounds and provide over two-thirds of naturally occurring antibiotics (Bérdy, 1995), with a diverse range of pharmacologic and agricultural uses (Lechevalier, 1988; Sanglier, 1993).

The versatility of actinobacteria is reflected in their biotechnological applications which has seen their use in 1) production of commercially important enzymes (Peczynska-Czoch & Mordarski, 1988), 2) bioremediation of industrial wastes (Lacey, 1988) and more recently 3) in the production of recombinant (human) proteins (Binnie *et al*., 1997).

The high cost of discovering novel microbial compounds has lead to commercial attention focusing on efforts to reduce these costs by screening novel groups of actinobacteria, and re-screening existing culture collections for a multitude of new

uses (Kurtböke, 2000). An innovative approach makes use of current molecular techniques to rationally design hybrid molecules by genetically modifying biosynthetic genes, to achieve specific biological functions (Khosla, 1998). Additionally, actinobacteria are important sources of novel genes encoding enzymes which are involved in catalytic reactions forming complex structures such as the triple intramolecular carbon-carbon bonds seen in the molecular structures of the insecticidal compounds of the spinosyns rarely seen in other macrolide compounds (Waldron *et al*., 2001).

1.1.3 Sources of Actinobacteria Secondary Metabolite Diversity

Secondary metabolites, also known as idiolites, are microbial compounds often possessing complex chemical structures, which result from long enzymatic pathways. They are produced under specific conditions, usually after the growth phase has ended, in submerged culture. Secondary metabolites are produced by restricted taxonomic groups of organisms and are usually formed as mixtures of closely related members of a chemical family.

Phenomenal biochemical pathways produced by diverse actinobacteria isolated from unique natural environments, have been shown to produce bioactive compounds which exert their influence by processes that are not compromised by existing multi-drug resistance pathways (Capon, 1998). Some actinobacteria are more prevalent or suited to particular environments than others (Jiang & Xu, 1993; Kurtböke & Wildman, 1998). Terrestrial habitats have been the major source of SM producing microorganisms. However, marine habitats are providing an alternative source yielding a diverse range of metabolites exhibiting novel structures (Jensen & Fenical, 1994). A number of key genera are adapted to symbiotic commensalism and extreme environments and these groups have received little attention because they are difficult to isolate and culture (Strobel & Long, 1998). In addition, Jiang & Xu (1993) showed that a great deal of actinobacterial diversity exists in extreme environments. By understanding the ecological roles of rare actinobacteria, isolation procedures will be better suited to cultivating these rarer or novel genera thus enhancing microbial diversity (Suzuki *et al*., 1994).

1.1.4 Evolution of Biosynthetic Pathways and their Relation to Secondary Metabolite Production

The occurrence of similar secondary metabolites in unrelated organisms, have lead to speculation that the biosynthetic pathways leading to these products were acquired through direct transfer of corresponding genes (Boucher & Doolittle, 2000). There is strong evidence for this speculation in the case of beta-lactams, which are produced by actinobacteria often and ascomycete fungi, where they share identical biosynthetic pathways (Brakhage, 1998) and have enzymes possessing a highly conserved protein sequence (Ogawara, 1996). A theory supporting this transfer of genes known as the endosymbiont theory proposes that during evolution whole organisms, such as bacteria or cyanobacteria with their complete genetic makeup and metabolism, were incorporated into early eukaryotic cells and developed into mitochondria and chloroplasts. This suggests that once a useful biochemical pathway has been developed the secondary metabolite pathway can be transferred to other organisms. Evidence to support this theory is exemplified by the enzyme family amidinotransferases, where the actinobacterial gene encoding this enzyme is related to the rat glycyl amidinotransferases (Piepersberg, 1997).

1.1.5 Classification of Actinobacteria

The rediscovery of actinobacteria while screening for novel compounds has undoubtably been a burden on drug screening programs, in terms of the cost and increasing discovery times. Utilisation of a predictive and stable classification system for actinobacteria can increase the efficiency and prospects of isolating novel compounds. Traditional means of classification have relied on morphological and physiological properties. It was not until the development of chemotaxonomy, which involved the study of the chemical variation in living organisms (Goodfellow & Minnikin, 1985) that actinobacteria could be properly classified into appropriate genera and species. However, the discriminating power of chemical criteria vary between taxa (O'Donnell, 1988). The subjectivity of chosen characters produced small databases which often led to misclassification or groupings of dissimilar actinobacteria.

Pioneering studies by Carl Woese's group, was the turning point in the way bacterial systematics was assessed. His studies focused on ribosomal RNA; the 5S, 16S and 23S molecules. These ubiquitous molecules essential for protein synthesis in living organisms are genetically stable and present in high copy number (Woese, 1987). Although these molecules are highly conserved, they exhibit great variation in regional sequence conservation. These conserved regions are used as regions for primers to amplify the gene using the polymerase chain reaction (PCR) technique and the products sequenced; alignments of rRNA sequences are performed and used to construct phylogenetic affiliations between microorganisms (Stahl & Amann, 1991). rRNA genes provide useful target sites for designing DNA probes for differentiating between species or subspecies of bacteria (Stackebrandt *et al*, 1992; Wellington *et al*., 1992).

1.1.6 Actinobacterial Genetic Diversity and Drug Discovery

Molecular techniques are commonly employed in efforts to establish which geographical areas contain maximum actinobacterial diversity (Takizawa *et al*., 1993). Other applications which have seen the use of the assessment of genetic diversity in actinobacteria is in the area of dereplication. The random amplified polymorphic DNA (RAPD)-PCR method has been used to eliminate duplicates in actinobacterial screening programs by distinguishing commonly isolated bacteria from their respective DNA banding profiles (Anzai, 1994; Roberts & Crawford, 2000).

1.1.7 Activities of Actinobacteria in the Natural Habitat

In the natural environment actinobacteria have significant roles in ecosystem sustainability. They degrade lignocellulosic plant residues and recycle nutrients back into the environment (Crawford, 1988). Also, they are important biological control agents in the control of fungal diseases and also enhance plant growth (Lechevalier, 1988). Apart from their beneficial uses, actinobacteria possess few negative attributes such as being opportunistic pathogens in animals, humans and plants. Furthermore, actinobacteria are involved in the spoilage of hay, cereal grains, paper and plastics (Lacey, 1988).

Section 2: Biology of Secondary Metabolism and the Discovery of Secondary Metabolites in Actinobacteria

1.2.1 Microbial Secondary Metabolism

1.2.1.1 Secondary Metabolite Production

Microbial derived secondary metabolites can be metabolic intermediates or end products from intricate and often lengthy biosynthetic pathways. Secondary metabolites may be found in various species in disparate genera or families and a variety of metabolites can be expressed from a single species under different environmental conditions. Secondary metabolites are derived from the precursors and energy generated through primary metabolic pathways (Figure 1). The SM groups commonly distributed in nature are the polyketides, terpenes, steroids, shikimic acid and alkaloids (Herbert, 1989). The diversity of structures generated are a result of modifications and combinations of reactions from primary metabolic pathways. Most secondary metabolites are low molecular weight compounds having molecular masses less than 1500 Daltons.

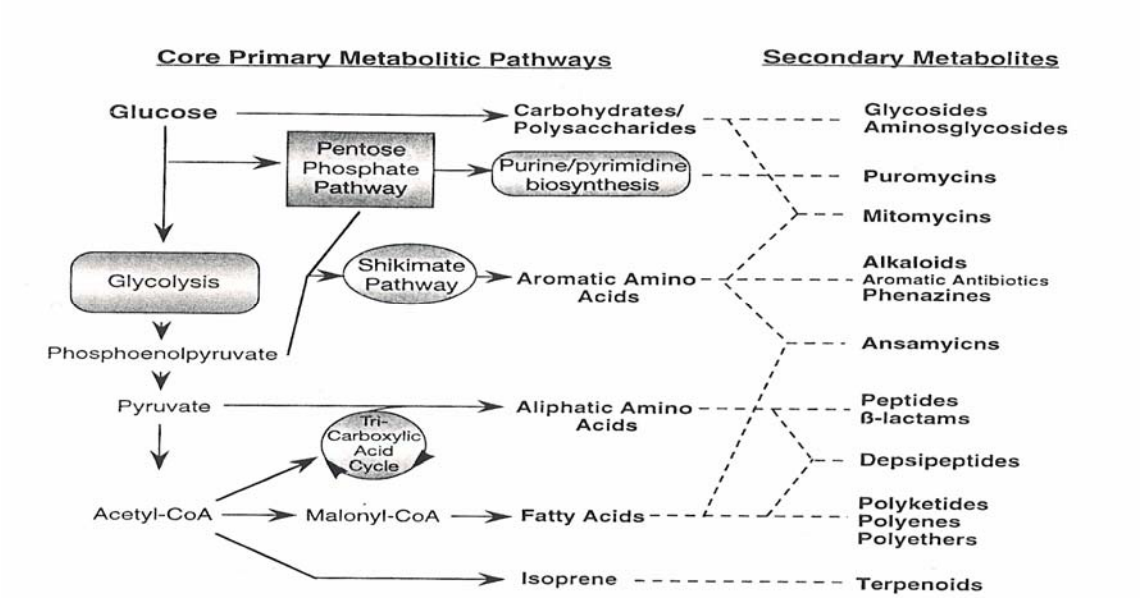


Figure 1. Primary metabolic pathways leading to the formation of secondary metabolites (adapted from August *et al*., 1999).

The biosynthesis of secondary metabolites in actinobacteria involves the following sequence of events; 1) uptake of nutrients into the cell and conversion intointermediates of primary metabolism, 2) accumulation of primary metabolites and signalling molecules induces secondary metabolite production, 3) primary

metabolites branching-off into the pathway particular for a specific secondary metabolite. Several primary metabolic pathways have been identified as sources of precursors for synthesis of secondary metabolites. These are: fatty acid metabolism (acetate and propionate for e.g. polyketide biosynthesis), carbohydrate metabolism (hexose, pyruvate), 4) the production of these secondary metabolites is regulated by pathway specific genes that determine the onset of secondary metabolite production (Hodgson, 2000).

1.2.1.2 Functions of Secondary Metabolites

The production of secondary metabolites serve a number of useful functions benefiting the organisms producing them. Firstly, they act as chemical agents in destroying other microorganisms and increase the fitness and the survival of the producing organism in the natural environment (Demain, 1995a). Certain secondary metabolites aid in metal transport providing metal ions in a soluble form which can be readily utilised by microorganisms (Neilands, 1995). Other uses of secondary metabolites include effectors of differentiation in sporulating bacteria (McCann & Pogell, 1979).

1.2.1.3 Resistance Mechanisms and Secondary Metabolite Secretion

The degree of susceptibility to self-inhibition from secondary metabolites in actinobacteria is governed by the resistance mechanisms being elicited (Méndez & Salas, 2001). These mechanisms can either occur concurrently during idiophase or triggered by sublethal levels of the SM. Secondary metabolite producing actinobacteria possess a range of defensive mechanisms which allows them to protect themselves from their own metabolites. These mechanisms are described as follows; (a) enzymatic detoxification of the antibiotic (Figure 2; route 1), (b) alteration of the antibiotics normal target in the cell (Figure 2; route 2), and (c) modification of the permeability to allow antibiotic to be pumped out of the cell and restrict its re-entry [Figure 2; route 3] (Cundliffe, 1989). (d) cytoplasmic proteins acts to sequester the secondary metabolite (Sheldon *et al*., 1999; Wilson & Cundliffe, 1999), (e) suppressing antibiotic synthases during rapid growth (Vining, 1990). Other suicide avoidance mechanisms include the final biosynthetic step is located on the cell membrane, production of the SM is during idiophase, feedback inhibition/repression. Resistance is tightly regulated with biosynthetic genes and this is evident from their close proximity to one another on chromosomal DNA (Figure 3).

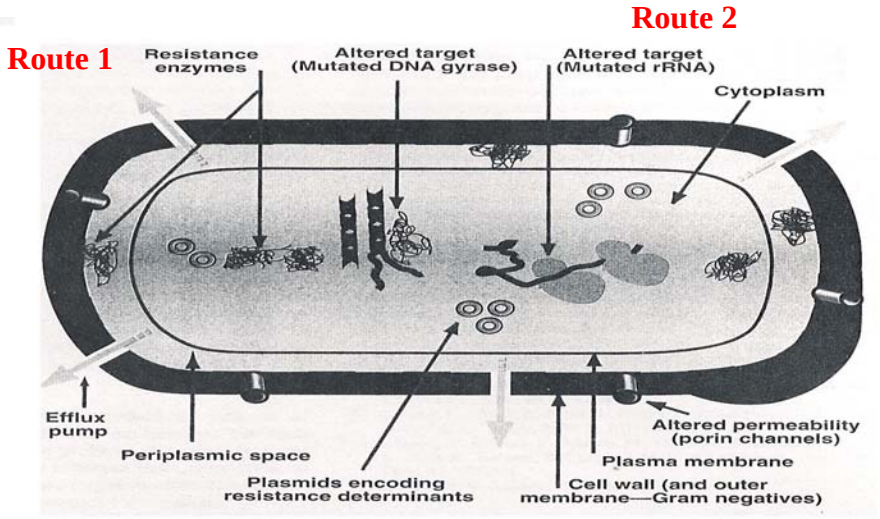


Figure 2. Schematic representation of a bacterial cell eliciting resistance mechanisms. The resistance mechanisms are represented by arrows containing a route number (see above text for explanation; adapted from Kotra *et al*., 2000).

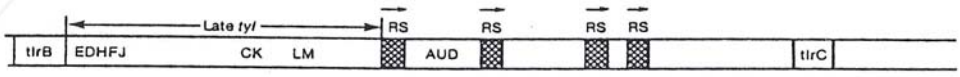


Figure 3. Clustering of biosynthetic and resistance genes, a segment of *Streptomyces fradiae* genome containing tylosin biosynthetic genes (EDHFJ) and tylosin resistance genes [tlrB,tlrC] (adapted from Seno & Baltz, 1989)

1.2.2 Microbial Screening for Secondary Metabolites

In the search for novel microbial metabolites, a number of rational screens have provided an effective means in detecting secondary metabolic products. Targeted screens based upon mechanisms of action have detected metabolites with the desired bioactivity these are either a known compound or have uncovered novel structural classes [Table 1] (Franco & Coutinho, 1991; Higashide, 1995; Silver & Bostian, 1990; Osada, 1995).

Table 1. Screening assays used for assessing bioactive metabolites (White *et al*., 1986).

Screen Type	Method
Antibacterial	Agar Diffusion Agar Plate Assay Organism
Anticoccidial	Primary Chick Kidney Cell Culture Oocysts
Anticancer	Biochemical Inducing Assay (BIA)
Antiviral	Antibacteriophage Assay
Enzyme Inhibitors	Ligand-receptor competition assay

The classical whole-cell well agar diffusion assay has been the conventional approach used in the screening of secondary metabolites which has usually been conducted in a random fashion. The major limitations of this assay is that these test methods are used repeatedly using similar target organisms and common SM classes are often re-discovered and are restricted to the search only for antiinfectives (Grabley *et al*., 1999). Although incorporating new target organisms has lead to the discovery of new compounds (Higashide, 1995), in actinobacteria the rediscovery rate is 99 % (Zähner & Fiedler, 1995). Once a microorganism shows the producing capacity for secondary metabolites, time consuming taxonomic studies are often required in identifying and characterising the microorganism which are costly and labour intensive (Ōmura, 1986). Figure 4 depicts a flow chart of the screening process from the potential producer species to the recognition of a new bioactive metabolite.

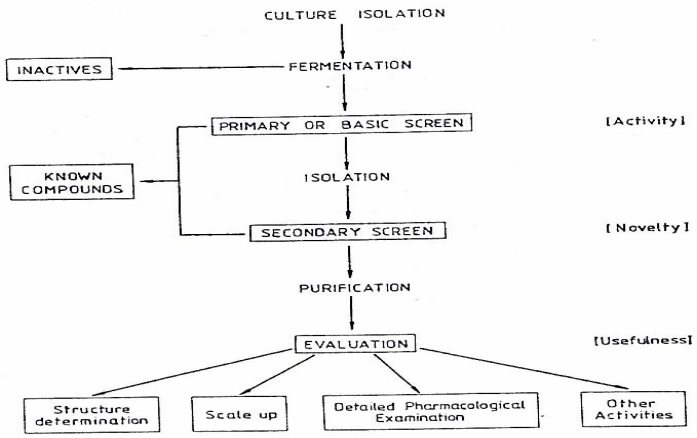


Figure 4. Screening process for a new bioactive microbial metabolite (Bérdy, 1989).

Rational selection of microorganisms by chemical or genetic fingerprinting is providing a way to exclude previously isolated organisms from screening programs and proving to overcome the problems of dereplication (Colquhoun *et al*., 2000; Brandão *et al*., 2002). Alternative approaches to classical or modifications to SM screening have identified a number of ways that could lead to the discovery of new compounds. These approaches include (i) re-evaluation and further development of secondary metabolites that have already been commercially introduced; (ii) evaluation of known antibiotics not used for human therapy; (iii) searching new secondary metabolites using new test methods, novel microorganisms and varying culture conditions; (iv) focus on isolates from unusual or little-explored ecosystems;

(v) utilisation of combinatorial biochemistry approaches in cloning SM biosynthetic genes from a producing strain and introducing it into another producing strain producing a similar compound; (vi) directed evolution in accelerating enhanced enzymatic activity and broader substrate specificity. (Ōmura, 1986; Lazzarini *et al*., 2000; Zähner & Fiedler, 1995; Bull *et al*., 2000; Strohl, 1997). In order for the advancement of SM screening programs, new lead substances are required which can be chemically transformed in which the bioactivity and pharmacological properties are modified to suit particular therapeutic needs (Verpoorte, 1998; Bull *et al*., 2000).

1.2.2.1 Rapid identification of microbial metabolites

Microbial bioactive compounds have proven elusive in industrial screening programs, as culture conditions are not always well defined (Table 2) and often incapable of inducing appropriate biosynthetic pathways. Furthermore, there is a limited capacity of detectable systems being unable to selectively identify the effector compounds. Traditionally thin layer chromatography (TLC) has been used to determine the metabolic fingerprint in response to effects of culture conditions on SM production. However, the current approaches used by Zahn *et al* (2001) show that electrospray mass spectrometry (EMS) is a effective and more accurate approach used in determining the metabolic fingerprint of strains and identifying culture conditions inducing the expression of secondary metabolites. The culture conditions used in the screening for SM production are often the conditions that permit optimum growth, as both are mutually exclusive.

1.2.2.2 Chemical screening using chromatography and spectroscopy

Chemical screens such as high performance liquid chromatography (HPLC) and TLC are effective at identifying known compounds and their congeners. However, they are limited in their usefulness only to known classes of compounds which allows for identification or group allocation of an unknown compound at an early stage in screening (Fiedler, 1993). However, coupling HPLC with diode array detection, mass spectrometry (MS) or nuclear magnetic resonance spectrometry (NMR) have been efficient methods used for screening and identifying microbial metabolites from fermentation broths (Abel *et al*., 1999; Higgs *et al*., 2001). When LC-NMR/MS is used in conjunction with biological activity assays new leads can be detected in a very

time effective manner (Siegal *et al*., 1999). A novel approach used for the discovery of biologically active microbial secondary metabolites from both crude or pure extracts is known as biomolecular-chemical screening. This method combines TLC and reactivity of metabolites using various staining reagents with binding to biomolecules like DNA (Maier *et al*., 1999).

Table 2. Variation of culture conditions (adapted from Zähner & Fiedler, 1995)

Nutrient broth

- use of nutrient broth which prevents carbon, nitrogen and phosphorus repression, if possible
- use of nutrient broths with an excess of carbon and limited nitrogen supply and vice versa
- medium supplementing towards the end of growth phase • nutrient broth deficient in trace elements

Physical conditions

- temperature, possible shift in temperature towards end of log phase
- pH including pH shift during culture • pO_2 • pCO_2 • osmotic values

Collection of metabolites using adsorber resin

- or ion exchanger during fermentation or removal of metabolites by dialysis, for example using a membrane fermenter

Creation of stress conditions

- osmotic stress • stress through heavy metals • stress through inhibitor supplement

Microbial conversions of certain parent substances

- incorporation of modified modules using deliberate fermentation
- incorporation of modified modules using mutasynthesis
- microbial transformation of biologically active parent substances

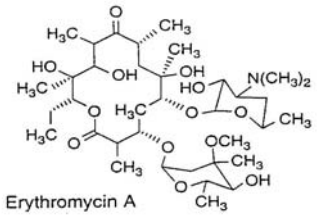
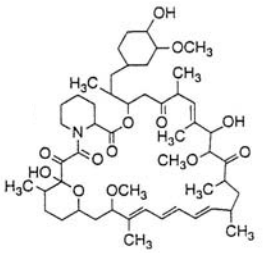
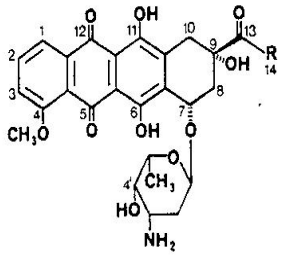
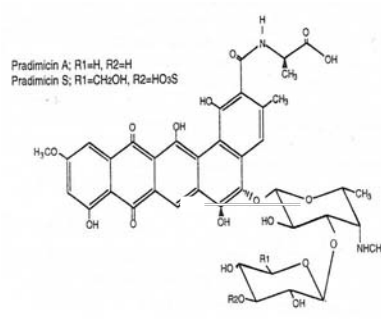
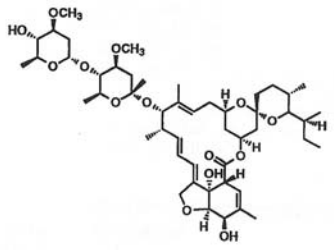
Section 3: Secondary Metabolites Produced by Actinobacteria

The focus of this study is concerned with the molecular screening of polyketide, β-lactam and aminoglycoside deoxysugar biosynthetic pathway genes in a group of environmental actinobacterial isolates. A characteristic feature shared by secondary metabolites (SMs) derived from these pathways is that they are synthesized by common early biosynthetic steps. In the following paragraphs details will be presented on each of these pathways and their respective genes controlling these pathways and the secondary metabolite biosynthetic genes (SMBGs) that were selected to be incorporated in this study.

1.3.1 General Aspects of Polyketides

Polyketides are a ubiquitous class of SMs that are commonly produced by actinobacteria (Table 3). The economic importance of this class of compounds in the case of the anthracycline, doxorubicin (Adriamycin) used as a antitumor drug alone was $156 million in the year 1993 (Strohl, 1997). The polyketides are composed of two classes found in actinobacteria. These include the aliphatic (Type I) and aromatic (Type II) polyketides. The type I polyketides, which include macrolides and polyethers, are synthesized by polyketide synthase modules that consist of several domains with defined functions responsible for the catalysis of one cycle of polyketide chain elongation (Figure 6). These type I polyketides are built from a wide range of simple carbon building blocks (acetates, propionates etc) and are extensively reduced. The type II polyketides which include anthracyclines and isochromanequinones are synthesized by polyketide synthases that catalyze sequential decarboxylative condensation between the starter and extender units to yield a linear poly-β-ketone intermediate (Figure 8B) This intermediate undergoes reduction, aromatization or cyclization to form polycyclic aromatic structures which are further modified by tailoring enzymes which are responsible for the various biological activities (Katz & Donadio, 1993; Khosla & Zawada, 1996).

Table 3: Polyketide compounds produced by actinobacteria.

Chemical Structure	Name of compound/Class/Producer	Biological Activities
 Erythromycin A	Erythromycin/Aliphatic/ *Saccharopolyspora erythraea*	Antibacterial
	Rapamycin/Aliphatic/ *Streptomyces hygroscopicus*	Immunosuppressant/ Antifungal/ Antitumor
	Daunomycin/Aromatic/ *Streptomyces* sp. Strain C5	Anticancer
 Pradimicin A; R1=H, R2=H Pradimicin S; R1=CH_2OH, R2=HO_3S	Pradimicin/Aromatic/ *Actinomadura hibisca*	Antifungal
	Avermectin/Aliphatic/ *Streptomyces avermitilis*	Antiparasitic

1.3.1.1 Polyketide Biosynthesis Pathway

The key enzyme reactions in the polyketide biosynthetic pathway are shown in figure 5, the initiation of polyketide biosynthesis begins with carboxylic acid extender units which may consist of acetate, propionate or butyrate. The carboxylated extender units are transferred from coenzyme A (CoA) to the 4'-phosphopantetheine arm of the acyl carrier protein [ACP] by the actions of the acyltransferase [AT] (Figure 5; stage 1). The polyketide chain is attached via a thioester linkage to a cysteine residue in the active site of the ketosynthase [KS]. A linear chain is assembled, whereby each carboxylic acid incorporates two carbon atoms carrying a β-keto group, a condensation reaction occurs which is catalyzed by ketosynthase [KS] (Figure 5; stage 2), the β-carbonyl can be subjected to all, part, or none of a series of steps catalyzed by a ketoreductase [KR], dehydratase [DH], and enoylreductase [ER] (Figure 5; stage 3). Following condensation cycles, the polyketide chain is released from the PKS via the action of the thioesterase [TE] (Figure 5; stage 4) (Hopwood & Sherman, 1990).

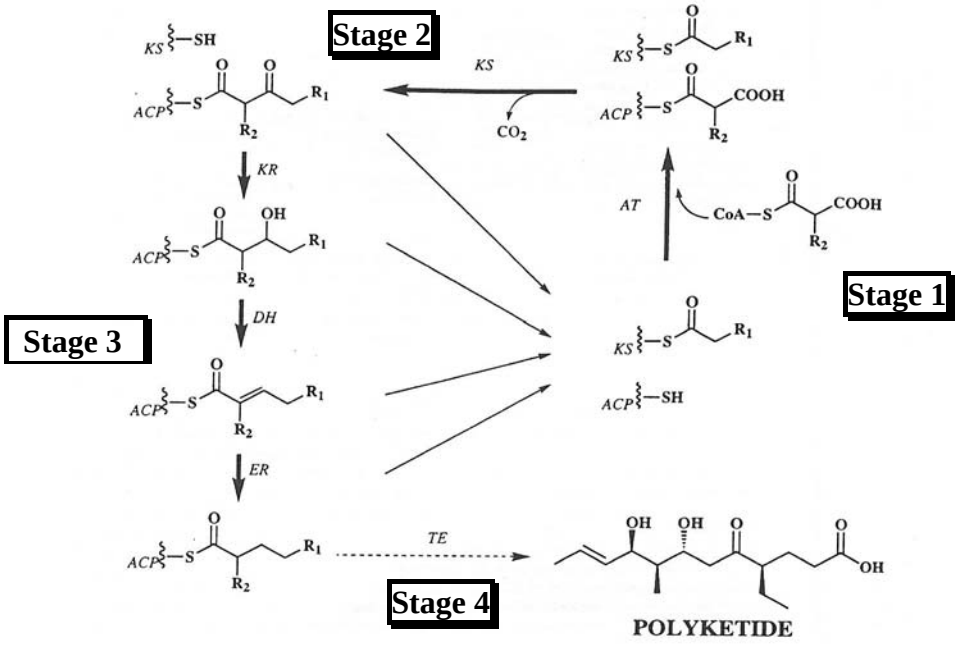


Figure 5. Illustration depicting the key enzyme reactions in polyketide biosynthesis. Condensation begins by the attachment of the polyketide chain to a ketosynthase (KS). An acyltransferase (AT) transfers the carboxylated extender unit from CoA the acyl carrier protein (ACP). The specificity of the AT determines the choice of extender unit (R_2). The solid line arrows in the centre of the biosynthetic scheme, indicate the degree of β-ketoreduction can vary at any given carbonyl. The polyketide chain is released from the PKS by a thioesterase [TE] (adapted from Khosla, 1998).

1.3.1.2 Polyketide Synthetases (PKSs)

Actinobacteria contain two distinct types of polyketide synthases, either type I PKS (multifunctional), which contain a single large protein encoded by a single gene that contains the entire set of catalytic functions (Figure 6), or a type II PKS (monofunctional), which contain several separate proteins that perform specific catalytic functions [see figure 8A] (Hutchinson & Fuji, 1995).

1.3.1.3 Biosynthetic Gene Clusters of Type I Polyketide Synthases

A characteristic feature of type I PKS genes is that they contain large open reading frames encoding giant (>3000 amino acids) multifunctional proteins (Cortes *et al*., 1990). Within these proteins are specific catalytic centers known as "modules" which are present as single copies and contain specific active sites which determine the biochemical order of substrate selection for chain-elongation steps and the degree of β-ketoacyl reduction of each extension step (Figure 6). The regions between the modules have been implicated to serve as structural supports maintaining correct formation for the catalytic process (Katz, 1997; Staunton & Wilkinson, 1999).

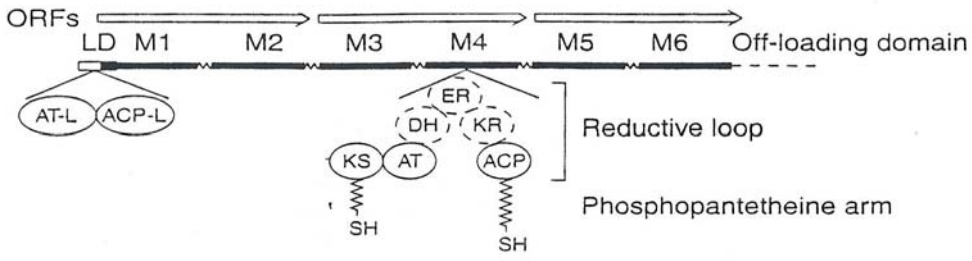


Figure 6. General modular organisation of the type I PKS. Abbreviations (LD) loading domain; (M) modules; (ORFs) open reading frames; (AT) acyl transferase; (ACP) acyl-carrier-protein; (KS) ketosynthase; (DH) dehydratase; (ER) enoyl reductase; (KR) ketoreductase (adapted from Lal *et al*., 2000).

The AT domain selects the type of extender unit (acetate or priopionate) to be incorporated into the growing chain; the KS domain catalyses the condensation reaction and the ACP domain tethers the polyketide chain to the PKS between condesation cycles and accepts the extender unit from the AT domains in readiness for the next condensation event. The keto ester produced by these core domains is processed by optional modifying reductive domains (ER, KR and DH). The extent to which β-reduction occurs is determined by the number of reductive domains present. The loading module which precedes the first module is responsible for activating the starter unit and making it available to the PKS (Lal *et al*., 2000; Katz *et al*., 1997).

In this study a fragment from the KS gene was targeted to be amplified by PCR, as it is a common gene present in all modular PKS clusters (Figure 7). Amplification of short DNA fragments from modular PKS clusters has provided a accurate approach in matching them to a PKS domain. Furthermore, it has been estimated that sequencing 300 – 500 bases provides sufficient amino acid sequence identity to identify a fragment as part of a modular PKS gene (Santi *et al*., 2000). The KS gene is highly conserved containing between 60-75% identity and 75-84% similarity at the DNA sequence level over the whole domain in the erythromycin PKS and avermectin PKS (MacNeil *et al*., 1993). The KS gene contains a region that encodes a highly conserved motif GPXXXXXTACSS which is required for the formation of a thioester linkage to the growing acyl chain (Motamedi *et al*., 1997). Another SMBG of type I PKSs which could be targeted includes the AT gene, which incorporates starter units. The AT gene contains 33-54% identity and 54-71 % similarity at the DNA sequence level over the whole domain between erythromycin PKS and avermectin PKS (MacNeil *et al*., 1993).

An example of a typical type I PKS is that of erythromycin PKS, which is encoded by three genes, designated *eryAI*, *eryAII*, and *eryAIII* (Figure 7A). The three genes encode polypeptides that are over 3300 amino acids in length named DEBS (deoxyerythronolide B synthase). DEBS is composed of modules that contain combinations of KS, AT, KR, DH, ER, and ACP, as well as a thioesterase (TE) at the end of the DEBS3 module (Katz, 1997). The KS-AT-(DH-ER-KR)-ACP modular organization is a common feature found in most type I PKS genes (Figure 7). Similarly to the DEBS, *rapPKS* consists of three multifunctional proteins designated RAPS1 (8,563 amino acids), RAPS2 (10,222 amino acids) and RAPS3 (6,260). However these polypetides contain more than two modules each, as compared to only two found in DEBS (Figure 7B).The loading module consists of three domains. The first domain is Lig (ligase), the second is a ER and thirdly an ACP. The first domain catalyzes the conversion of a carboxylic acid to an active acyl derivative for the transfer to the ACP. The final product is attached to the ACP as a thioester for the transfer to the KS domain for the first chain extension module. Chain termination is performed by the pipecolate incorporating enzyme encoded by the gene *rapP* (Schwecke *et al*., 1995).

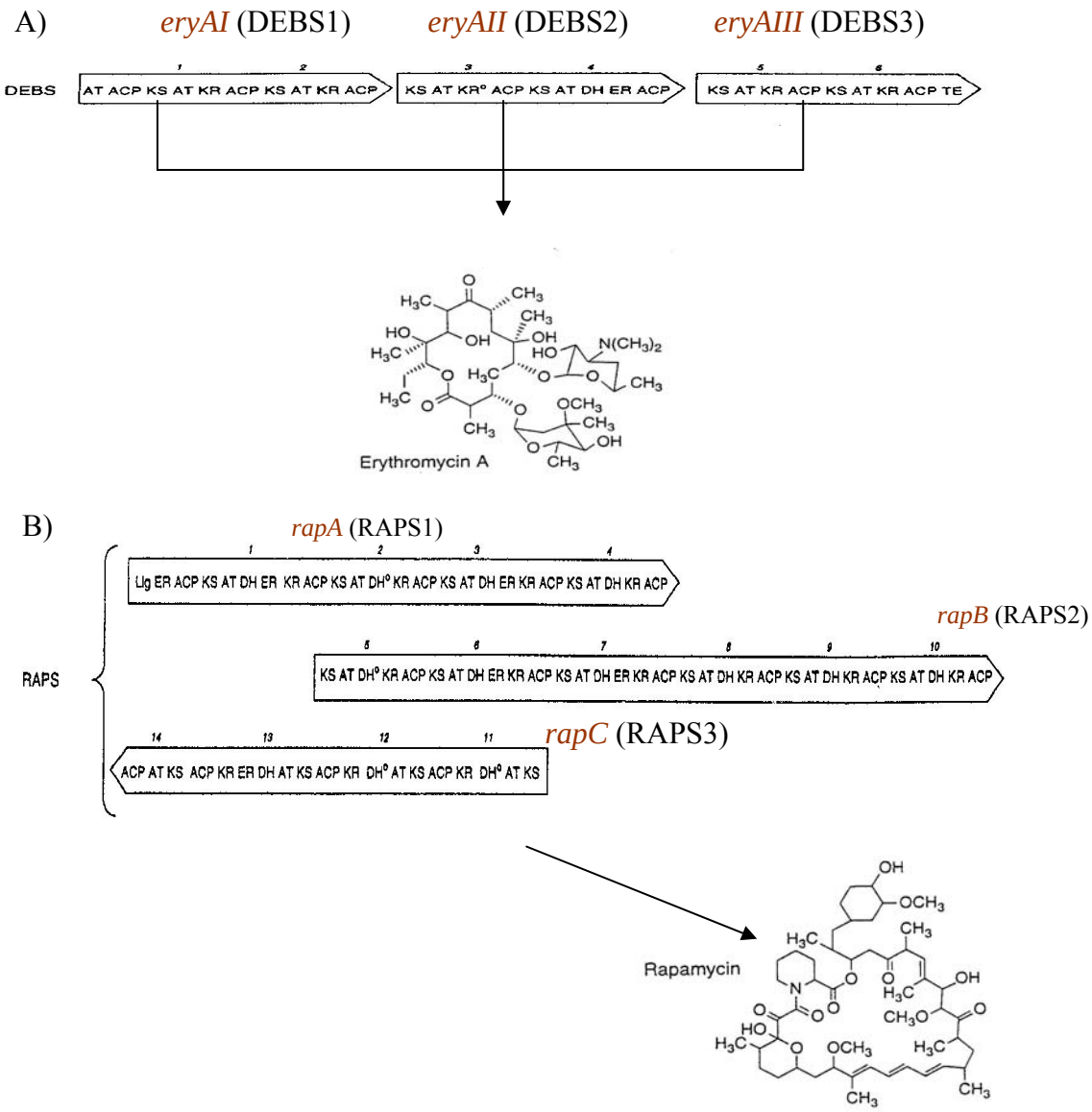


Figure 7. Modular organization of two polyketide synthases. (A) Gene cluster of erythromycin consisting of three genes *eryAI, eryAII, eryAIII,* which encode 6-deoxyerythronolide-B synthase [DEBS] (B) Gene cluster of rapamycin containing the three genes *rapA*, *rapB* and *rapC* which encode the rapamycin polyketide synthase [RAPS] (adapted from Katz, 1997).

1.3.1.4 Biosynthetic Gene Clusters of Type II Polyketide Synthases

A characteristic feature of type II PKS genes is their high amino acid sequence homology and conserved gene organization (Figure 9). As part of the gene organization three core open reading frames (ORFs) of requisite genes known as the "minimal" PKS need to be expressed for *in vivo* aromatic polyketide biosynthesis to occur (Figure 8A). The minimal PKS include the bifunctional domain ketosynthase/acyltransferase (KS/AT), chain length factor (CLF) and acyl carrier protein (ACP) (McDaniel *et al*, 1994). Additional ORFs encoding ketoreductase

(KR), aromatase (ARO) and cyclase (CYC) introduce chemical structural diversity to the polyketide structure (Alvarez *et al.*, 1996). The gene clusters also contain transcriptional activator or regulatory genes, as well as self-resistance genes (Figure 9).

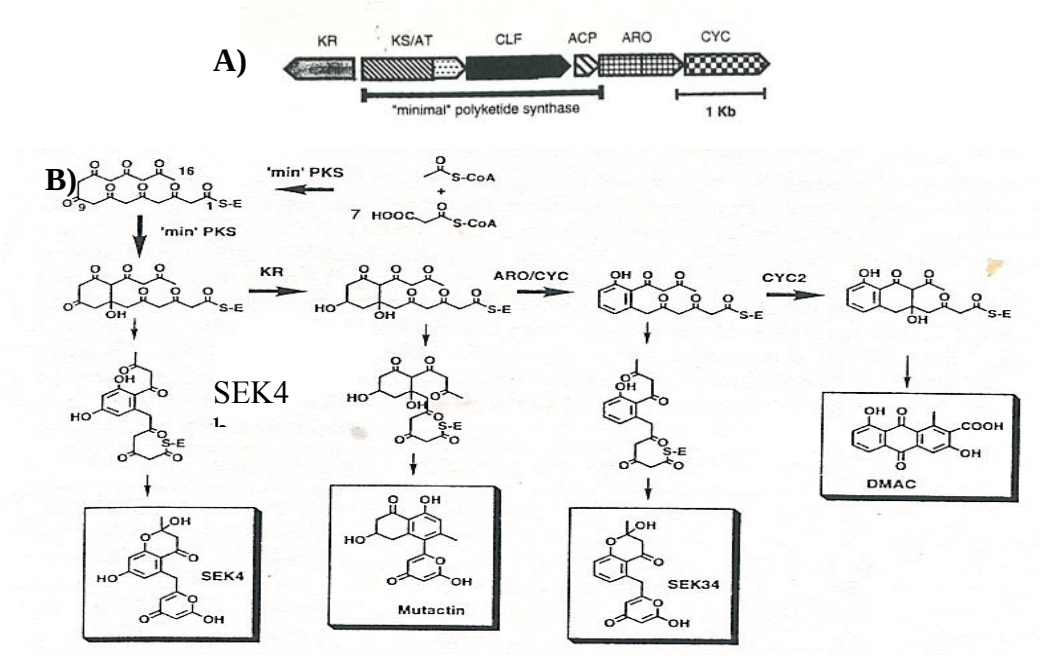


Figure 8. Aromatic polyketide biosynthesis of the gene products from the actinorhodin PKS cluster. (A) Presence of the minimal PKS is sufficient for the production of two 16-carbon polyketides, SEK4 and SEK4b.(B) The nascent octaketide chain undergoes ketoreduction (catalyzed by the KR), aromataization of the first ring (catalyzed by the didomain ARO/CYC) and a second cyclization (catalyzed by the second ring cyclase (CYC2). (adapted from Zawada & Khosla, 1997).

Aromatic polyketide biosynthesis begins with a primer unit loading on to the condensing β-keto acyl synthase (KS; figure 8B). An extender unit is then transferred to the pantetheinyl arm of the acyl carrier protein (ACP; figure 8B). The KS catalyzes the condensation between ACP-bound malonate and the starter unit. Extender units are added sequentially until the polyketide chain reaches a desired chain length determined by the chain length factor. The ketoreductase can then catalyze reduction of the C-9 carbonyl (AT; figure 8B). Aromatase and cyclase (ARO/CYC; figure 8B) then catalyzes the aromatisation of the first ring in reduced polyketides. The second ring undergoes a C-5/C-14 cyclization by the actions of an cyclase in reduced polyketides. The primary in vivo product of the complete actinorhodin PKS is 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC). In the absence of

some of these subunits, shunt products are produced such as SEK4, mutactin (Figure 8B).

In this study the KS/AT gene was targeted as it is common and essential for the synthesis of all aromatic polyketides (Figure 9). The KS/AT genes have been shown to contain between 60 – 85 % identities at the amino acid level in actinobacteria (Piecq *et al*., 1994; Ye *et al*., 1994). Other components of aromatic PKS that could be targeted include KR which is used iteratively as chain building proceeds (Hopwood *et al*., 1985). Additional targets could also include the ARO/CYC which contain highly homologous protein sequences of the N-terminal halves in aromatic PKS (Zawada & Khosla, 1997).

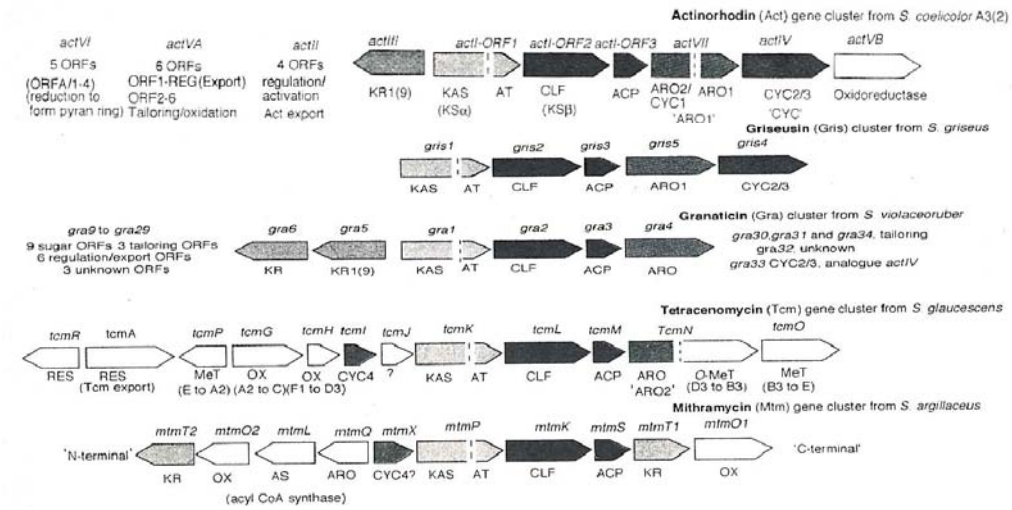
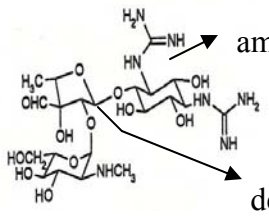
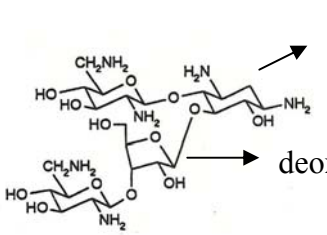


Figure 9. Structural organization of aromatic polyketide synthase genes of various secondary metabolites produced by actinobacteria. Abbreviations: (KAS) 3-oxoacyl ACP synthase; (AT) acyl transferase; (CLF) chain length factor; (KR) ketoreductase; (ACP) acyl carrier protein; (CYC) cyclase; (ARO) aromatase; (MeT) methyl transferase; (REG) transcriptional activator and regulatory genes; (RES) self-resistance gene; (OX) oxidation (adapted from Rawlings, 1999).

1.3.2 Biosynthesis of Deoxysugar Aminoglycoside Antibiotics

Aminoglycoside (AG) antibiotics are compounds which contain aminosugars and often an aminocyclitol ring (Table 4). They are broad spectrum antibiotics, effective against both Gram positive and Gram negative bacteria (Liu & Thorson, 1994). Deoxysugar components are usually attached to specific positions on the aminocyclitol ring and serve to modulate biological functions. Deoxysugars are defined as carbohydrates in which one or more of the normally (e.g., glucose) occurring oxygen atoms are deleted (i.e., replaced by hydrogen, thiosugars, halogens, aminosugars or nitrososugars). The biosynthesis of 6-deoxysugar AG antibiotics has not been well defined with segments of the biosynthetic pathway only beginning to be characterised (Johnson and Liu, 1998). Figure 10B shows the biosynthetic pathway of the 6-deoxyhexose containing AG streptomycin. The first step involves the conversion of glucose-1-phosphate into dTDP-D-glucose by the action of dTDP-glucose synthase. The next catalytic step involves dTDP-D-glucose 4,6 dehydratase which converts dTDP-D-glucose into dTDP-D-4-keto-6-deoxyglucose [Figure 10B; step 2]. Further catalysis by 5- or a 3,5 epimerase directs dTDP-D-4-keto-6-deoxyglucose into D or L series of 6-deoxyhexoses [Figure 10B; step 3]. Subsequent modifications can be achieved by further deoxygenations, *O*-methylations, transaminations or reduction (Johnson & Liu, 1999).

Table 4. Aminoglycoside antibiotics produced by actinobacteria.

Chemical Structure	Name of compound/Producer	Biological Activities
	Streptomycin *Streptomycin griseus*	**Antibacterial**
	Neomycin *Streptomyces fradiae*	**Antibacterial**

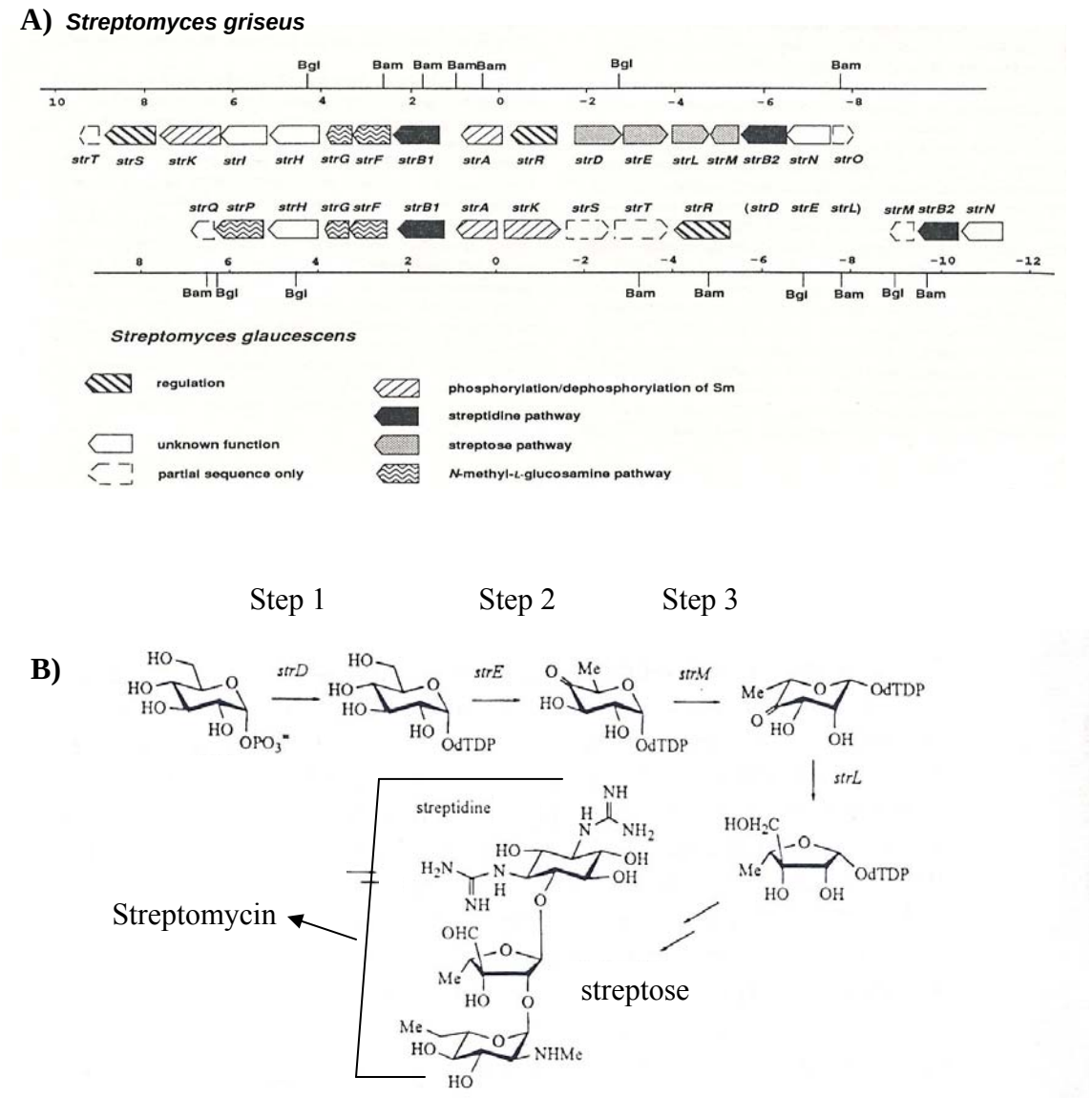


Figure 10. A) Streptomycin (*str*) gene clusters of *S. griseus* N2-3-11 and *S.glaucescens* GLA.0. *strB1* and *strDELM* represent biosynthetic genes; *strA* and *strK* represent resistance genes; *strR* and *strS* represent regulatory genes (adapted from Distler *et al*., 1992). **B)** Streptomycin biosynthetic pathway in *Streptomyces griseus*. The genes involved in streptomycin biosynthesis are labelled as *strD* (dTDP-glucose synthase); *strE* (dTDP-glucose-4,6-dehydratase); *strM* (dTDP-4-keto-6-deoxyglucose 3,5-epimerase); *strL* (dTDP-dihydrostreptose synthase) (adapted from Johnson & Liu, 1999).

1.3.2.1 Aminoglycoside Deoxysugar Biosynthetic Genes in Actinobacteria

In actinobacteria the AG deoxysugar biosynthetic genes are found in a clustered arrangement on chromosomal DNA (Figure 10A). The order of genes are functionally mixed, meaning that they are not arranged in pathway-specific operons (Distler *et al*., 1992). Genes coding for dTDP-glucose synthase (*strD*) and dTDP-D-glucose 4,6 dehydratase (*strE*) are commonly found in AG deoxysugar antibiotics

(Stockmann & Piepersberg, 1992; Decker *et al*., 1995; Hyun *et al*., 2000) and it is believed that these two genes form a transcriptional unit due to the presence of the stop codon of dTDP-glucose synthase genes overlapping the start codon of the dTDP-D-glucose 4,6 dehydratase genes (Kirschning *et al*., 1998).

In this study the *strD* gene was targeted as it is commonly found in aminoglycoside desoxysugar producing actinobacteria (Stockman & Piepersberg, 1992). The *strD* gene exhibits 60 – 70 % amino acid identities with corresponding genes in actinobacteria (Gaisser *et al*., 1997; Stratmann *et al*., 1999). Additional targets could have also included *strE, strM and strL genes* which are also common in the production of 6-deoxyhexose constituents in aminoglycoside antibiotics (Stockmann & Piepersberg, 1992). An interesting aspect of the *strE* gene is that it has been shown to be more closely related between actinobacterial species than to dehydratases from species of other orders (Decker *et al*., 1996). The *strE* gene found in actinobacteria have been shown to contain between 55 – 65 % amino acid identities (Gaisser *et al*., 1997; Stratmann *et al*., 1999).

1.3.3 β-lactam Antibiotics

The discovery made by Alexander Fleming in 1929, that *Penicillium* cultures produced an antibiotic known as penicillin was the first microbial SM developed as a chemotherapeutic agent (Rolinson, 1995). Penicillins belong to the β-lactam class of naturally occurring antibiotics produced by microorganisms. Fungal species synthesize the majority of β-lactams (Brakhage,1998). However, actinobacteria are prolific producers of certain classes of β-lactams these include the clavams, cephamycins, carabapenams and monobactams. β-lactams are classified into five groups (Table 5). All are produced by a similar biosynthetic pathway and contain a general structure of a 4-membered β-lactam ring (Figure 11).

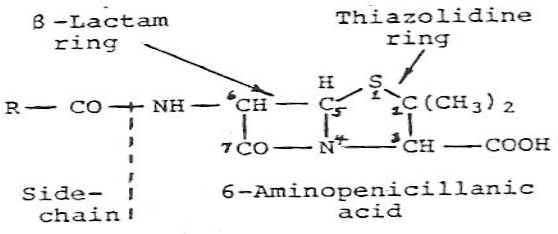


Figure 11. The general structure of β-lactams (adapted from Demain & Elander, 1999).

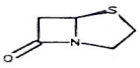
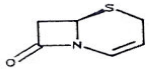
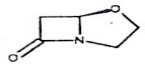
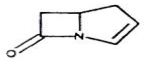
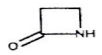
Class of β-lactam	Structure	Fungi	Bacteria	
			Gram-positive	Gram-negative
Penam		*Aspergillus* *Penicillium* *Epidermophyton* *Trichphyton* *Polypaecilum* *Malbranchea* *Sartorya* *Pleurophomopsis*		
Cephem		*Cephalosporium* *Anixiopsis* *Arachnomyces* *Spiroidium* *Scopulariopsis* *Diheterospora* *Paecilomyces*	*Streptomyces* *Nocardia*	*Flavobacterium* *Xanthomonas* *Lysobacter*
Clavam			*Streptomyces*	
Carbapenem			*Streptomyces*	*Seratia* *Erwinia*
Monobactam			*Nocardia*	*Pseudomonas* *Gluconobacter* *Chromobacter* *Agrobacter* *Acetobacter*

Table 5. Classes of β-lactams and their respective producing microorganisms (adapted from Aharonowitz *et al*., 1992).

1.3.3.1 β-Lactam Biosynthetic Genes

A characteristic feature of actinobacterial β-lactam antibiotics is that they share key reactions that are common in their biosynthetic pathways (Figure 13). In actinobacteria β-lactam genes are in a clustered arrangement and consist of the early *pcbAB* gene which is closely linked to the *pcbC* gene (Figure 12) and is consequently the only shared stages in the β-lactam pathway (Smith *et al*, 1990). The genes *lat*, *pcbAB* and *pcbC* are in a conserved transcriptional orientation and are sequentially arranged on the chromosome in the same order as the first steps in cephamycin C biosynthesis (Figure 12).

The *pcbAB* gene which encodes the enzyme ACV synthase is commonly found in the early stages of the biosynthetic pathway of all β-lactam producing actinobacteria. This enzyme functions to bind activated L-amino acids and epimerasize L-valine to polymerase the amino acid to make δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine (ACV) [Figure 13; step 1]. The nucleotide sequence identities found within actinobacterial *pcbAB* genes is approximately 37 %. Following the orientation of transcription, the *pcbC* gene is found next to the *pcbAB* gene and is another common early biosynthetic pathway gene. The *pcbC* gene encodes the enzyme isopenicillin N synthase, which

catalyzes the oxidation of the linear tripeptide to form the bicyclic isopenicillin N, formation

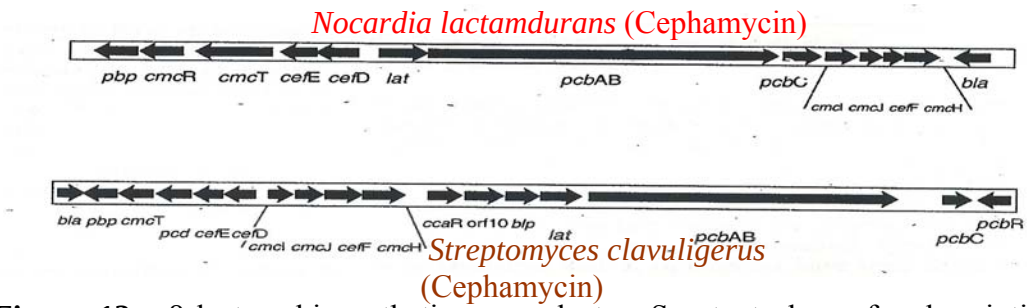


Figure 12. β-lactam biosynthetic gene cluster. See text above for description of genes adapted from Martín, 1998).

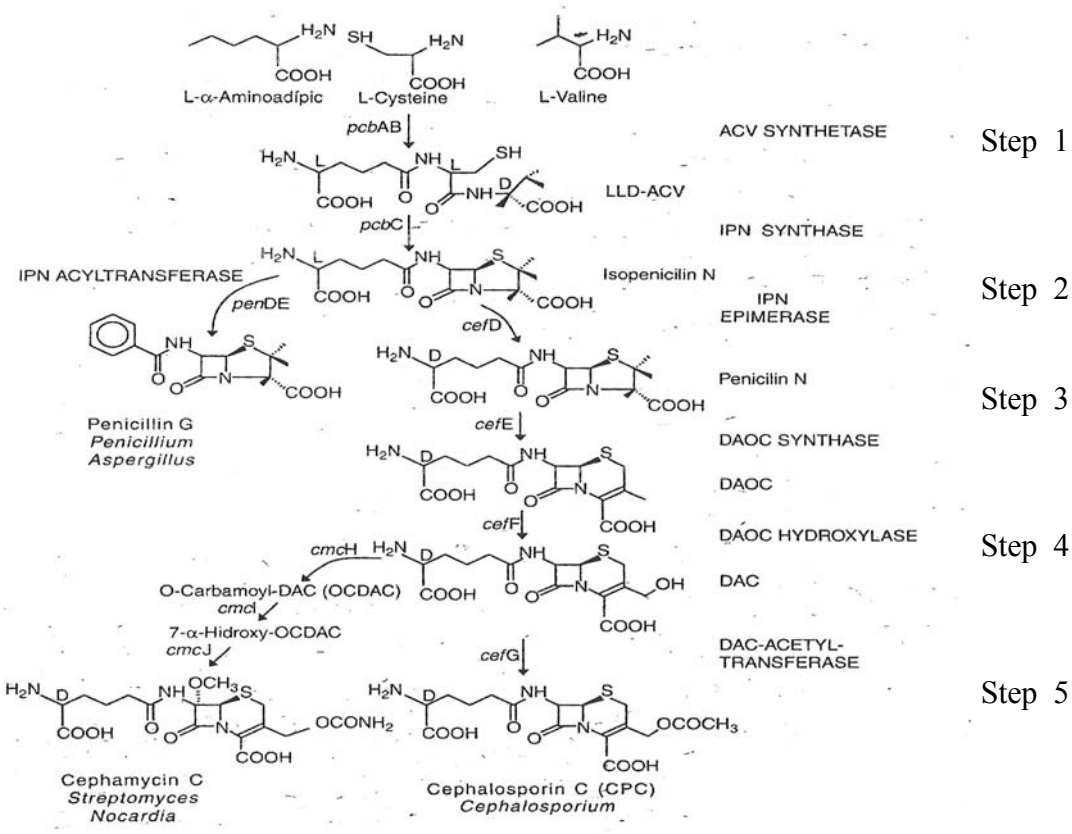


Figure 13. Illustration depicting the key enzymatic reactions in the β-lactam biosynthetic pathway. See above text for explanation (adapted from Martín, 1998).

of the β-lactam and thiazolidine fused rings [Figure 13; step 2]. The nucleotide sequence identities within actinobacterial *pcbC* genes is 79 – 85 % (Aharonowitz *et al*., 1992). Other key reactions include the isomerization of the L-α-aminoadipyl side chain to the D configuration which is catalyzed by an epimerase [Figure 13; step 3]. The *cefD* gene which encodes this epimerase and is transcribed in the opposite orientation to the *pcbAB* and *pcbC* genes, has been shown to contain 71 % nucleotide

sequence identities between *Streptomycete* spp. (Aharonowitz *et al*., 1992). The *cefE* gene encodes the enzyme deacetoxycephalosporin C synthase (DAOCS). The DAOCS enzyme catalyzes the oxidation reaction converting the five-membered thiazolidine ring of penicillin into six-membered dihydrothiazine ring [Figure 13; step 4].. The *cefF* gene encodes the enzyme deacetylcephalosporin C hydroxylase which performs a hydroxylation reaction forming deacetylcephalosporin C (DAC) [Figure 13; step 5].

In this study the *pcbC* gene was targeted. The high DNA and protein sequence homologies of this gene consists of between 79 – 85 % nucleotide and 58 – 64 % amino acid sequence identities. The high nuceleotide sequence homologies has enabled the efficient isolation of the *pcbC* gene in β-lactam producing actinobacteria. (Aharonowitz *et al*., 1992; Sim & Loke, 2000). Other β-lactam biosynthetic genes that could have been screened include *pcbAB* gene which contains 48 % nucleotide sequence identity between actinobacteria producing β-lactam antibiotics.

Section 4: Gene Expression in Actinobacteria

1.4.1 Regulation of Gene Expression in Actinobacteria

The biosynthesis of certain secondary metabolites are regulated by specific sets of structural genes. In general, these genes are clustered on the chromosome in the order in which the biosynthetic reaction will proceed. Regulatory genes (listed in Table 6) are juxtaposed to these biosynthetic genes which control specific SM biosynthetic pathways. The expressivity of genes to changing environmental conditions, eg. nutritional imbalance, leads to the activation or suppression of secondary metabolite biosynthetic genes [SMBG] (van Wezel *et al*., 1997; Narberhaus, 1999; Vicente *et al*., 1999). Pathway-specific regulators listed in table 6 play crucial roles in controlling numerous secondary metabolite biosynthetic genes. Figure 14 depicts the regulatory events leading to the formation of a SM in a bacterial cell.

Table 6. Examples of genes for pathway-specific regulators in the biosynthesis of secondary metabolites in actinobacteria.

Genes for pathway-specific regulators	Comments	References
brpA	*Streptomyces hygroscopicus* mutant blocked in bialaphos production and accumulation of *bap* transcripts.	Anzai et al., 1987
mmyR	Mutant overproduces methylenomycin	Chater, 1992
redD	Mutants cause loss of undecylprodigiosin production	Takano et al., 1992
actII-ORF4	Mutants cause loss of actinorhodin production. Accumulation if *act* transcripts reduced in mutants.	Fernández-Moreno et al., 1991
strR	Required for expression of at least one *str* gene in *Streptomyces griseus.*	Distler et al., 1992
dnrI	*Streptomyces peucetius* mutant blocked in daunorubicin production	Stutzman-Engwall et al., 1992

1.4.1.1 Transcription

Prokaryotic genes are regulated at the level of transcription enabling bacteria to alternate gene expression expediently and economically (Shinkawa, 1996). Gene expression systems for secondary metabolite biosynthesis in actinobacteria have

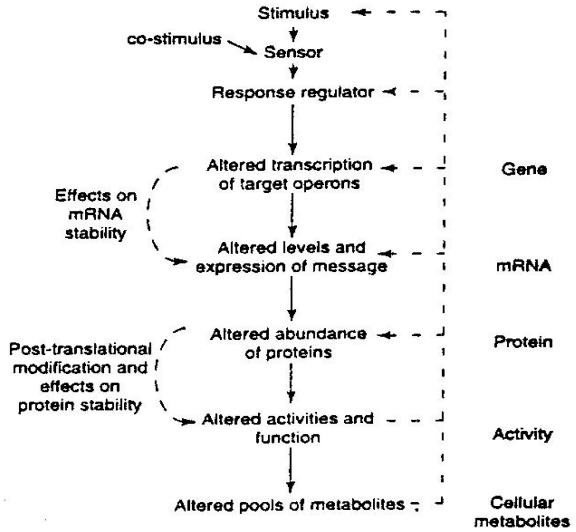


Figure 14. Diagram depicting the regulatory network in response to a stimulus and the changes accompanying a cells activities and functions. The cell first sensors one or more stimuli. The sensor communicates the stimuli to a response regulator, resulting in altered transcription of target operons, altered levels of messenger ribonucleic acids (mRNAs), changes in the synthesis of proteins and alterations in activities and functions. Each of these stages can be affected by feedback, as indicated by dashed arrows (adapted from VanBogelen *et al*., 1999).

been shown to contain multiple forms of RNA polymerases. RNA polymerases play integral roles in determining which genes to transcribe and at what rates (Ishihama, 1997). Promoter recognition in bacteria requires that RNA polymerase associates with a sigma (σ) factor to form a holoenzyme (Buttner *et al*., 1990; Kang & Roe, 1998). Most bacteria contain multiple forms of σ factor, activation of these factors is elicited by specific signals or stress conditions (Helmann, 1999).

Several Streptomycete promoters have been identified from a variety of sources, which have high A + T content compared with coding sequences. Actinobacteria contain several classes of promoters, some of which resemble *E.coli* promoters in specific nucleotide regions 10 to 35 bp before the start point of transcription. A distinctive feature of actinobacteria is that they contain two or more transcription start sites and associated promoter sequences. This feature is believed to aid in the differential expression of genes in different phases of the growth cycle when the major RNA polymerase may have different promoter specificities (Seno & Baltz, 1989). Additionally altered gene expression patterns are achieved by the regulatory system known as the stringent response (Bascarán *et al*., 1991). The system is activated in the event of nutritional limitation in which guanosine 3'-diphosphate-5'-diphosphate (ppGpp) and guanosine 3'-triphosphate-5'-diphosphate (pppGpp) are synthesised causing an intracellular accumulation of these compounds. The accumulation of these compounds causes instable initiation complexes at promoters for stable RNA-synthesis, causing a reduction of the total rate of RNA synthesis and other cellular metabolites (Strauch *et al*., 1991; Takano & Bibb, 1994; Pfefferle *et al*., 1995).

Certain compounds have been shown to regulate secondary metabolite biosynthesis at the level of transcription, such as A-factor (2-(6'methylheptanoyl)-3R-hydroxymethyl-4-butanolide) which alleviates repressor proteins and allows biosynthetic genes to be transcribed (Miyake *et al*., 1990). The two compounds ML-236B and phenobarbitol have been shown to act synergistically, effecting the transcriptional regulation of the cytochrome $P450_{sca}$ gene and influencing the production of pravastatin, a cholesterol lowering drug (Watanabe & Serizawa, 1998).

1.4.1.2 Codon Usage (CU)

Codon usage (CU) of genes in actinobacterial coding sequences exhibit strong bias for G or C in the third position and in the first position, but no obvious preference in position two. This codon bias is significant as it is possible that codon preference may reflect the relative abundance of particular charged tRNA species and that this might be a means of regulating the expression of certain biosynthetic genes (Wright & Bibb., 1992). This finding is supported by Leskiw *et al*. (1991) who found that mutational loss of tRNA that translates UUA leucine codons prevents the production of secondary metabolites. The bias towards G and C in the degenerate position of amino acid codons has greatly facilitated the construction of effective DNA probes based upon amino acid sequences of protein products (Fishman *et al*., 1987).

Section 5: Genetic Factors Affecting the Production of Secondary Metabolites

1.5.1 Plasmid Instability

Plasmids harbouring SM biosynthetic genes have been known to undergo a phenomenon known as genetic instability where frequent deletions arise, which can be spontaneous/induced causing a decrease/loss in SM production (Thomas *et al*., 1991; Dary *et al*. 1992) or a favourable deletion leading to an increase in SM production (Cullum *et al*., 1988). Often the mutants generated by genetic instability decrease the rate of SM production by altering gene expression patterns (Matsushima & Baltz, 1996).

1.5.2 Induced Mutagenesis

Mutagenesis involves the alteration of genes causing an altered state of function. In SM producing actinobacterial strains, mutagenesis can either be used to abolish unwanted intermediates or to increase yields of a desired product (Queener *et al*., 1978., Ivanova *et al.*, 1995). By inducing mutations by UV light exposure, cold storage or chemical treatments such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or ethidium bromide it has been possible to identify biosynthetic genes on chromosomal DNA (Baltz, 1986; Crameri *et al*., 1986; Adamidis *et al*., 1990; Volff *et al*., 1993; Ikeno *et al*., 1996), the genes that block biosynthesis are cloned and sequenced and placed in plasmids to determine the extent to which they control

biosynthesis (Table 7). The limitation of suitable plasmids has hampered efforts in determining the effects of multiple mutations in polyketide synthases (McDaniel *et al*., 1999). In some cases generation of unexpected structures are produced, due to expression of previously latent downstream genes or upstream genes due to inserted fragment.

Table 7. Mutagenesis of secondary metabolite producing actinobacteria.

Mutant strain	Type of mutagenesis	Gene effected	Effects on secondary metabolism	References
NSA205	Chromosomal deletions	*ADS205**	Blocking	Schauner et al.,. 1999
S. coelicolor C542	Induced mutations ^	*absA*	Blocking	Adamidis et al., 1990

* ADS205 Amplified DNA Sequence
^ UV light & chemical exposure

Recent efforts by McDaniel *et al*. (1999) have shown that by selectively inducing single or multiple mutations in the catalytic site of polyketide synthases, the synthesis of unnatural compounds are produced (which could by chemical or natural).

Section 6: Molecular Techniques used to Detect Secondary Metabolite Biosynthetic Genes

Current advances in molecular detection techniques are providing valuable screening tools in determining the taxonomic diversity of antibiotic producing microorganisms and determining the biosynthetic capabilities of microorganisms (Dalbøge & Lange, 1998; August *et al*., 1999). The application of the polymerase chain reaction (PCR) technique has been successfully used to selectively amplify biosynthetic genes using degenerate primers, independent of culture conditions and shown to provide a reliable indication of actinobacteria able to produce secondary metabolites of an expected group, or novel compounds (Seow *et al*., 1997; Morris *et al*., 1999). Alternative molecular techniques which have also been successful include the use of DNA probes, to screen genomic or cDNA libraries for biosynthetic genes to identify candidate secondary metabolite producing clones (Stockmann & Piepersberg, 1992; August *et al*., 1999).

1.6.1 DNA Homology of Secondary Metabolite Biosynthetic Genes (SMBG) in Actinobacteria

Similar SM biosynthetic pathways have been shown to be present among different microorganisms. These include both the polyketide (Santi *et al*., 2000) and β-lactam pathways (Kook & Nam, 1997). Secondary metabolite biosynthetic genes (SMBG) for the polyketide pathway from various microorganisms have been shown to contain significant levels of nucleotide sequence similarity with each other (Bibb *et al*., 1989; Fernández-Moreno *et al*., 1992). By using biosynthetic genes as probes or as templates to design primers for PCR, it has been possible to identify genes of similar function in different actinobacterial producers of secondary metabolites (Table 8).

Determination of nucleic acid sequence similarities of SMBG between and within species, will enable the evaluation of whether nucleic acid detection systems can be developed for the preferential detection and/or isolation of SMBG in specific groups of microorganisms.

1.6.2 Detection of Secondary Metabolite Biosynthetic Genes in Actinobacteria using Heterologous DNA Probes

Genes with common regions of DNA homology involved in biosynthetic pathways of secondary metabolites, allow the detection of similar genes in different microorganisms. Thus, these 'signature' sequences are useful tools in designing oligonucleotide probes to specific regions within microbial genomes in determining the presence or absence of the gene. In this way correlations can be made between the presence of a gene and establishing the capability of actinobacteria in producing a secondary metabolite class. By using this approach several microbial screening groups have successfully detected corresponding secondary metabolite biosynthetic genes in actinobacteria (Table 8). Particular attention has focused on constructing probes from amino acid sequences from biosynthetic enzymes and probing gene libraries to identify enzymes (Cox *et al*., 1986).

Malpartida *et al.* (1987) demonstrated that by using components of polyketide synthases as probes: namely the *actIII* which codes for a reductase and the *actI* which codes for a condensing enzyme, displayed positive signals among polyketide known and unknown producing actinobacteria.

Other approaches which have proved useful in the detection of entire biosynthetic clusters, includes the use of resistant determinants as probes (Cortes *et al*., 1990). Often in probing experiments, resistance genes are simultaneously identified with biosynthetic gene clusters in antibiotic producing actinobacteria. A novel strategy using a collection of perfect hybridisation probes were obtained by random sequencing small fragments from the genomic DNA of a polyketide producing microorganism (Santi *et al*., 2000). In this way only those fragments containing PKS genes were retained, thus providing sufficient coverage of the presence of a representative fragment from any PKS gene cluster in the genome of the microorganism. In addition, the authors could predict the frequency that the PKS fragment would be present in the genome library by taking into account the size of the target PKS gene cluster and the size of the microorganism's genome.

An important feature taken into consideration when cloning SMBG from the same species, is that probes often used are derived from SMBG for a structurally related compound (Gibbons *et al*., 1992; Dairi *et al*., 1999). Table 8 shows secondary metabolite biosynthetic genes that have been cloned using DNA probes. It is clearly evident that a number of SMBG have been detected using heterologous probes. In particular the *actI* and *actIII* probes derived from the actinorodin PKS genes has been useful in the detection of similar PKS genes in different species of actinobacteria producing a diverse class of secondary metabolites (Malpartida *et al*., 1987).

Table 8: DNA Probes used in the detection of SMBG in *Actinobacteria* spp.

SMBG Group	Actinobacteria	Compound Class	Probe	Reference
Type I Polyketide Synthase	*Saccharopolyspora* spp.	Macrolide	eryPKS, *ermE*	Stanzak et al., 1990
	Saccharopolyspora erythraea	Macrolide	*erm*E	Cortes et al., 1990
	S. longisporoflavns	Polyether	AT DEBS3	Cooper et al., 1992
	S .cyaneogriseus	Macrolide	AT	Gibbons et al., 1992
	S. FR-008 sp.	Polyene Macrolide	pab, eryPKS	Hu et al., 1994
	S. coriofaciens	Macrolide	5c	Kuczek et al., 1994
	S. antibioticus	Macrolide	eryA	Swan et al., 1994
	S. hygroscopicus	Macrolide	eryA	Schwecke et al., 1995
	S. caelestis	Macrolide	PKS	Kakavas et al., 1997
	*S. sp.*MA6548	Macrolide	fkbD	Motamedi et al., 1997
	S. hygroscopicus	Macrolide	m5 eryPKS	Ruan et al., 1997
	S. sp. MA6548	Macrolide	fkbA	Motamedi et al, 1997
	S. venezuelae	Macrolide	type I PKS(HP)	Xue et al., 1998
	S. natalensis	Polyene Macrolide	rapPKS	Aparicio et al., 1999
	S. noursei	Polyene Macrolide	KS-ACP	Brautaset et al., 2000
	Amycolatopsis mediterrianae S699	Ansamycin	rifR	Doi-Katayama et al., 2000
	S. antibioticus	Macrolide	oleP	Shah et al., 2000
	M. megalomicea	Macrolide	KS2 eryDEBS	Volchegursky et al., 2000
	S. hygroscopicus var. *ascomyceticus*	Macrolide	rapP, fkbM	Wu et al., 2000
	Saccharopolyspora spinosa	Macrolide	KS	Waldron et al., 2001
	Actinosynnema pretiosum	Ansamycin	rifK & AHBA	Yu et al., 2002
Type II Polyketide Synthase	*Streptomyces* spp.	Isochromanequinone Anthracyclines	actI & actIII	Malpartida et al., 1987
	S. arenae	Isochromanequinone	actI	Brünker et al., 1999
	S. cinnamonensis	Polyether	actI & actIII	Arrowsmith et al., 1992
	S. curacoi	Curamycin	actI	Bergh & Uhlén, 1992
	S. murayamaensis	Angucycline	actI	Gould et al., 1998
	Actinomadura hibisca	Angucycline	KS & AT*	Dairi et al., 1997
	Actinomadura verrucosospora	Angucycline	KS & AT	Dairi et al., 1999
	Saccharopolyspora hirsuta 367	Glycopeptide	actI	Le Gouill et al., 1993
	Kibdelsporangium aridum	Glycopeptide	actI & actIII	Piecq et al., 1994
	S .roseofulvus	Isochromanequinone	actI & actIII	Bibb et al., 1994
	S. griseus	Benzoisochromanes	actI & actIII	Yu et al., 1994
	S. peucetius	Anthracycline	*tcm*KLN	Grimm et al., 1994
	S. venezuelae	Angucycline	actI & actIII	Han et al., 1994
	S. sp. Strain C5	Anthracycline	actI & actIII, PKR	Ye et al., 1994
	S. fradiae Tü2717	Angucycline	tcmKC	Decker & Haag, 1995
	S. nogalater	Anthracycline	actI & acm	Ylihonko et al., 1996
	S. violaceoruber Tü22	Benzoisochromanes	actI & actIII	Sherman et al., 1989
	S. olivaceus Tü2353	Napthacenequinones	tcmG	Rafanan et al., 2001
	S. nogalater	Anthracycline	nogPKS	Torkkell et al., 2001

Table 8: DNA Probes used in the detection of SMBG in *Actinobacteria* spp.

SMBG Group	Actinobacteria	Compound Class	Probe	Reference
6 - Deoxysugar	*Streptomyces* spp.	Aminoglycosides Macrolides	strD, strE, strLM, strH	Stockmann & Piepersberg, 1992
	S . argillaceus	Anthracycline	strD, strE, strM ,actI & actIII	Lombó et al., 1996
	S. rishiriensis	Aminocoumarin	strE	Wang et al., 2000
	S. spheroids	Aminocoumarin	strE	Steffensky et al., 2000
	S. viridochromogenes Tü57	Orthosomycin	strE	Gaisser et al., 1997
	S. violaceoruber Tü22	Benzoisochromane	strE	Bechthold et al., 1995
	Actinoplanes sp. 50/10	Acarbose	strE, AS2,AS5	Stratmann et al., 1999
	S. cyanogenus S136	Angucycline	strE	Westrich et al., 1999
	S. antibioticus	Macrolide	strDEM	Aguirrezabalaga et al., 2000
	S. globisporus	Enediyne	strE	Liu & Shen, 2000
	S. noursei	Polyene macrolide	gdhA	Zotchev et al., 2000

***KS - authors designed probes based on active site of ketosynthase and acyltransferase enzymes from type II polyketide synthases.**
AHBA = 3-amino-5-hydroxybenzoic acid involved in shikimate biosynthesis
lat = lysine 6-aminotransferase
IPNS = Isopenicillin N synthase
PKR = bifunctional cyclase/dehydratase
ACMS = actinomycin synthetases
NGDH/gdhA = dNTP-glucose 4,6 dehydratase involved in deoxysugar biosynthesis
NGS = dNTP-glucose synthase involved in deoxysugar biosynthesis

Similarly the *strE* and *strD* probes derived from the streptomycin deoxysugar genes have also been efficient in cloning corresponding genes in various actinobacteria producing different compounds. The erythromycin PKS genes have proved to be efficient in the cloning of corresponding PKS genes in macrolide producing actinobacteria. However, in certain cases we see that PKS genes from similar classes of secondary metabolites are the preferred option for cloning, such is the case for ansamycin producing actinobacteria using rifamycin (ansamycin compound) PKS as the probe (Yu *et al.*, 2002).

1.6.3 Detection of secondary metabolite biosynthetic genes using the polymerase chain reaction (PCR)

The major focus of this study is concerned with the molecular screening of natural actinobacteria isolated from soil and using PCR in predicting bioactive populations. Degenerate PCR has provided an attractive option in the recovery of secondary metabolite genes in actinobacteria species and other microorganisms (Seow *et al.*, 1997; Nicholson *et al.*, 2001). Degenerate primer design involves performing a

muliple sequence alignment (MSA) of protein sequences of SMBG's and designing degenerate oligonucleotide primers to conserved regions. A key feature incorporated into the selective isolation of SMBG from actinobacteria is the G/C bias found in the third codon position of actinobacteria genes which decreases the degeneracy and thus increasing the prospects of the target gene being amplified (Wright & Bibb, 1992).

1.6.3.1 PCR screening of SMBG in Natural Actinobacterial Populations

Metsä-Ketelä *et al*. (1999) conducted a study showing that by using a degenerate PCR approach they could successfully amplify a KS_α fragment of approximately 0.6 kb corresponding to the minimal PKS cluster. Subsequent screening of unidentified *Streptomyces* species isolated from soil samples showed that 76% contained the presence of this KS_α fragment. Furthermore they were shown to produce the expected anthracycline products. In a similar study Seow *et al.* (1997) used degenerate PCR to amplify a 1.5 kb product corresponding to the KS_a and ACP fragments in the minimal PKS cluster. Interestingly, the primers designed in this case incorporated SMBG known to produce octaketides. Screening of two uncharacterised *Streptomyces* species isolated from soil with these primers amplified the expected band and sequence analysis indicated similar nucleotide sequences with the KS_α–ACP region. Thamchaipenet *et al.* (1997) used a similar approach by using a specifically designed set of degenerate primers to amplify a 1.4 kb fragment corresponding to the ketoacyl synthase and acyltransferase genes of type I PKS in actinobacteria. Screening of *Streptomyces* spp. isolated from composite soil with these primers amplified this band in most of the *Streptomyces* tested, though there was no data on the amount of isolates tested.

An alternative approach used by Morris *et al*. (1999) using degenerate PCR and amplifying type II PKS genes directly from total DNA extracted from the rhizosphere and bulk-soil. Distinct nucleotide sequences were reported to be obtained from *Streptomyces* isolated from soil. In another study, Seow *et al*. (1997) isolated DNA from a forest soil sample and amplifying the expected 1.5 kb product, however subsequent cloning and transformant experiments revealed that only 10 % of transformants contained the correct sequence. Niebla-Perez and Wellington (1997) indicated that degenerate PCR can also be used to amplify the *pcbC* gene that encodes

the isopenicillin N synthase directly from DNA extracted from soil samples. In another study by Krallis and Kirby (1998) showed that by using non-degenerate primers designed for amplification of a internal fragment of the *pcbC* gene, this fragment could be detected in all known β–lactam producing actinobacteria but could not be detected in environmental isolates shown to contain the presence of this fragment using DNA probes.

1.6.3.2 PCR cloning and screening of SMBG in Actinobacteria

Cloning SMBG have been well developed for *Streptomyces* spp. (Hopwood *et al*., 1985; Smokvina *et al*., 1990). However, in non-streptomycete actinobacteria, new cloning systems have to be developed (for specific actinobacteria genera) (Hasegawa, 1991; Lal *et al*., 1998; Dairi *et al*., 1999). A characteristic feature of secondary metabolite biosynthetic genes are that they are clustered as contiguous regions on the chromosome (or, rarely, in plasmids) in actinobacteria. This feature has greatly aided in the ease in which biosynthetic genes are cloned and analysed.

PCR cloning has been well adapted to the cloning of SMBG from genomic DNA libraries of actinobacteria [Table 9] (Loke *et al*., 2000). The design of degenerate and non-degenerate primers has been adapted to meet the DNA sequence preferences of actinobacterial SMBG. In the detection of dTDP- glucose 4,6 dehydratase gene Decker *et al*. (1996) designed specific degenerate primers to encompass nucleic acid sequences from different actinobacteria genera producing different classes of antibiotics. In this way all SMBG nucleotide sequence combinations of this gene would have been taken into consideration. This approach proved to be successful in correlating the presence of the PCR fragment with the structure of the dexysugar moiety. Similarly, Hyun *et al.* (2000), used degenerate PCR to amplify the dTDP-glucose synthase gene from different actinobacterial species and subsequently cloning *spcD* and *spcE* genes involved in biosynthesis of deoxy-sugar moieties in *S.spectabilis*. Kuczek *et al*. (1997) used a PCR approach with non-degenerate primers to clone a 0.45 kb fragment corresponding to KS and AT. The translated amino acid retrieved revealed 31-44% identities with other type I PKS.

Table 9: PCR Screening for SMBG in *Actinobacteria* spp.

Actinobacteria	Biosynthetic Genes	Source	Reference
S.coelicolor A3(2)	Type I PKS[a]	gDNA library	Kuczek et al., 1997
S. caelestis	Type I PKS	gDNA library	Kakavas et al., 1997
S. antibioticus	Type I PKS	gDNA library	Shah et al., 2000
S.hygroscopicus.var ascomyceticus	Type I PKS	gDNA library	Wu et al., 2000
S. venezuelae	Type I PKS	gDNA library	Xue et al., 2000
Streptomyces spp.	Type II PKS	Soil	Metsa-Ketela et al, 1999
Streptomyces spp.	Type II PKS	Soil	Seow et al., 1997
Streptomyces spp.	Type II PKS	Soil	Morris et al., 1999
Streptomyces spp.	Type II PKS	cDNA	Philaniappan et al., 1999
Streptomyces spp.	Type II PKS	cDNA	Ziermann and Betlach, 1999
Actinomycete spp.	NGDH*	gDNA library	Decker et al., 1996
S.antibioticus Tü99	NGDH	gDNA library	Jin-Cheol et al., 1999
S. globisporus C-1027	NGDH	gDNA library	Liu and Shen, 2000
Actinobacteria spp.	NGS~	cDNA	Hyun et al., 2000
Streptomyces spp.	*pcbC* ^	cDNA	Krallis and Kirby, 1998
S. sulfonofaciens	*pcbC*	cDNA	Niebla et al., 1999
Amycolatopsis mediterranei	Glycosyltransferase	gDNA library	Pelzer et al., 1999
S.venezuelae ISP5230	Halogenase	gDNA	Piraee et al., 2002
Actinobacteria spp.	Aminotransferase	Soil	Nagaya et al., 2005

[a] PKS = indicates one or more polyketide synthase genes
* NGDH = dNTP-glucose 4,6 dehydratase involved in deoxysugar biosynthesis
~ NGS = dNTP-glucose synthase involved in deoxysugar biosynthesis
^ *pcbC* = isopenicillin N synthase gene involved in β-lactam biosynthesis

1.6.4 Accessing Secondary Metabolite Diversity from Uncultured Soil Microorgansims

The culturability of a number of terrestrial microorganisms present in soil habitats has proven difficult due to a lack of understanding of the physiology and the inability of current techniques to culture these microorganisms (Amann *et al*., 1995; Rondon *et al*., 2000). Accessing the metabolic diversity without culturing microorganisms can be achieved by using a shotgun cloning approach (Figure 15) which involves isolating

soil DNA, cloning it into a culturable organism and screening the resultant clones for the production of new secondary metabolites (Handelsman *et al*., 1998). The major challenges facing the application of this technology to soil DNA is maintaining the large size (70-120kb) of the DNA fragments while removing nonDNA soil material that inhibits cloning and expressing the soil DNA in suitable heterologous hosts (MacNeil *et al*., 2001).

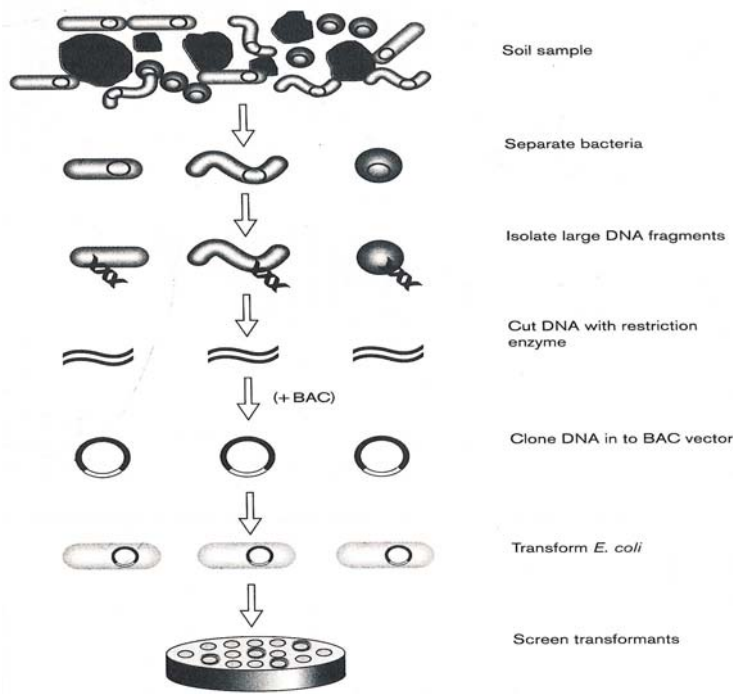


Figure 15. Cloning soil DNA for isolating new biosynthetic pathways for the synthesis of bioactive molecules from noncultured soil microorganisms. Step 1. Separation of intact bacteria from soil, and then DNA extracted directly from the bacteria; Step 2. DNA is cut using a restriction enzyme and cloned into a bacterial artificial chromosome (BAC); Step 3. transformation of E.coli cells with BAC vector; Step 4. BAC clones screened for biological activity and for the production of novel secondary metabolites (adapted from Handelsman *et al*., 1998).

Section 7: Production of Secondary Metabolites

The production of secondary metabolites in actinobacteria can be initiated or enhanced by the manipulation of fermentation conditions (Ōmura & Tanaka, 1984). The need to manipulate fermentation conditions has arisen due to the minimal quantities of SM produced by natural actinobacterial cultures, which may not be sufficient for further down-stream chemical and biological activity characterisation

studies. The most common SM production methods involve liquid (submerged) and solid-state fermentations. In the following paragraphs a description is given of the fermentation conditions and their influences in secondary metabolite production by actinobacteria.

1.7.1 Physical Conditions Affecting Secondary Metabolite Production

Of primary consideration in submerged fermentation of secondary metabolites involving filamentous microorganisms such as fungi and actinobacteria is the fermentation vessel (Whitaker, 1992). For actinobacteria shake flasks are commonly used with angular baffles, these indentations aid in the efficient mixing and in increased oxygen transfer (Katzer *et al*., 2001). Adequate oxygen supply is one of the most frequent problems associated with the use of shake flasks (Büchs, 2001).

1.7.2 Submerged Fermentation Conditions Affecting Secondary Metabolite Production

The expression of secondary metabolism in actinobacteria is dependent on a number of biochemical, physical and environmental parameters (Table 10). Additives to liquid media which can be broadly applied to elicit or enhance the production of a wide range of secondary metabolites is not known. However, indications are that compounds that limit the release of easily assimilatable nutrients or trap inhibitory substances promote the enhancement and induction of novel secondary metabolites by trapping inhibitory substances. These compounds include natural zeolites such as magnesium phosphate which form complexes with ammonium salts (Shapiro, 1989; Masuma *et al*., 1983). Similarly, it has been established that organic nitrogen sources, such as soy-based media can be used to induce the expression of certain classes of secondary metabolites in different actinobacteria (Hessler *et al*., 1997; Gouveia *et al*., 1999).

Table 10. Factors affecting secondary metabolism

Medium Composition	Fermentation Conditions
Carbon source	pH
Nitrogen source	Temperature
Inorganic phosphate	Oxygen Transfer
Inorganic salts	Carbon Dioxide
Trace metals	Agitation
Precursors	Oxidation-Reduction Potential (E_h)
Inhibitors	Rheology
Inducers	Osmotic Pressure
	Ionic Strength
	Fermentation Vessel

1.7.2.1 Components of fermentation media

Two forms of liquid media can be used in the production of secondary metabolites, these include chemically defined (synthetic) or undefined (natural, complex). Inexpensive complex media are usually employed in commercial fermentations which give higher fermentation yields at a lower cost (Dahod, 2000). However, complex media contain ill-defined components which lead to lot-to-lot variability and may alter expression profiles of metabolites in actinobacteria (Zhang and Greasham, 1999).

1.7.2.2 Macronutrients

1.7.2.2.1 Carbon Source

Carbon sources tend to display species-specific variation in actinobacteria for cell growth and production of secondary metabolites (Dekleva *et al*., 1985; Demain, 1989; Platas *et al*., 1999). The repressive negative effects of certain carbon sources on secondary metabolite production is due to carbon catabolite repression. It occurs in many actinobacterial species [Tables 11 and 12] (Stülke & Hillen, 1999). Rapidly assimilated carbon sources such as glucose are good for growth, but it represses the formation of enzymes involved in the biosynthesis of secondary metabolites (Table 11). Polyalcohols (e.g. glycerol), polysaccharides (e.g. starch), oligosachharides (e.g. lactose) and oils (e.g. soybean, methyloleate) are often used as preffered non-repressing carbon sources as the carbon source is released more slowly by hydrolysis (Trilli, 1990; Demain & Fang, 1995).

Table 11. Inhibition of secondary metabolism by nutrients

Secondary Metabolite	Producer	Nutrient Inhibitor	Reference
Streptomycin	*Streptomyces griseus*	Glucose	Demain, 1989
Kanamycin	*Streptomyces kanamyceticus*	Mannose, Lactose	Demain, 1989
Thienamycin	*Streptomyces cattleya*	Glutamine	Paress and Streicher, 1985
Candicidin	*Streptomyces griseus*	Phosphate	Martin, 1989

Glycerol is well known for its stabilising effect on secondary metabolites, biosynthetic enzymes and causing increased SM yields in actinobacteria (Arroyo *et al*., 2000; Chen *et al*., 2002). Alternative less expensive carbon sources which have

been used to increase SM yields, have included the use of sesame, groundnut and coconut oil for anthracycline production (Arun & Dharmalingam, 1999).

Table 12. Secondary metabolite synthases whose production is repressed by various carbon sources (Demain, 1989)

Secondary Metabolite	Enzyme	Repressing nutrient	Actinobacteria
Actinomycin	Phenoxazinone synthase	Glucose	*Streptomyces antibioticus*
	Tryptophan pyrrolase	Glucose, glycerol	*Streptomyces parvulus*
Cephamycin	Deacetoxycephalosporin C synthetase (expandase)	Glucose	*Nocardia lactamdurans*
Kanamycin	*N*-Acetylkanamycin amidohydrolase	Glucose, mannose, fructose, maltose, lactose	*Streptomyces kanamyceticus*
Neomycin	Phosphatase	Glucose	*Streptomyces fradiae*
Puromycin	*O*-Demethylpuromycin *O*-methyltransferase	Glucose	*Streptomyces alboniger*
Streptomycin	Mannosidostreptomycinase	Glucose, dextrin galactose, mannose	*Streptomyces griseus*
Tetracycline	Anhydrotetracycline oxygenase	Glucose	*Streptomyces ambofaciens*

1.7.2.2.2 Nitrogen Source

Organic nitrogen sources are often used in SM fermentations, as these compounds can be broken down into smaller units that are transported into bacterial cells, e.g. amino acids and ammonia (NH_4^+). Ammonia (as the NH_4^+ ion) is the preffered inorganic nitrogen source in actinobacterial SM fermentations, where it is added as ammonium sulfate $(NH_4)_2SO_4$ or ammonium chloride [NH_4Cl] (Dunn, 1985).

The presence of an excessive easily assimable nitrogen source exerts a repressive effect causing a decrease in the levels of secondary metabolites, mainly caused by ammonium salts and amino acids (Ōmura & Tanaka, 1984). Repression is exerted on the enzymes involved in SM biosynthesis. However, this can be alleviated by the use of complex nitrogen sources such as peptones or soybean meal (Bhattacharyya *et al*., 1998; Demain & Fang, 1995).

1.7.2.2.3 Phosphate Source

A number of secondary metabolites produced by actinobacteria are known to be influenced by inorganic phosphate (PO_4^{3-}) regulation (Table 13). The mechanisms that have been implicated in phosphate regulation include: (a) phosphate favours primary metabolism; a shift down in primary metabolism derepresses secondary metabolism (Drew & Demain, 1977); (b) phosphate shifts carbohydrate catabolic

pathways; (c) phosphate limits synthesis of the inducer of the SM pathway (Martín, 1989); (d) phosphate inhibits the formation of SM precursors (Martín, 1977); (e) phosphate inhibits or represses phosphatases necessary for SM biosynthesis; (f) phosphate suppresses SM production by depriving the cell of an essential metal (Martín *et al*., 1989). In liquid media SM biosynthesis is repressed or inhibited by PO_4^{3-} concentrations above 1 mM whereas in solid media higher concentrations of 10 to 25 mM are required (Martín, 1989). The synthesis of biosynthetic enzymes are affected by PO_4^{3-} at the transcriptional level [Table 13] (Reeve & Baumberg 1998). In some instances, excess of glucose and phosphate act synergistically causing repression of SM biosynthesis (Lounès *et al*., 1996).

Table 13. Phosphate-regulated enzymes involved in secondary metabolite biosynthesis (Adapted from Martín *et al*., 1994).

Secondary Metabolite	Producing Organism	Target Enzyme	Mechanism of regulation*
Candicidin	*Streptomyces griseus*	*p*-Aminobenzoate synthase	R
Cephamycin	*Streptomyces clavuligerus*	Deacetoxycephalosporin C^	I
		Isopenicillin N synthase^	I
Neomycin	*Streptomyces fradiae*	Neomycin phosphate phosphotransferase	R

* R, repression; I, inhibition

^ Enzymes involved in cephamycin biosynthesis are less sensitive to phosphate control (concentrations of more than 25 mM phosphate are required to observe phosphate control) than are other secondary metabolite biosynthetic enzymes (usually sensitive to less than 5 mM phosphate).

1.7.2.2.4 Sulphur, Potassium, Magnesium Sources

Sulphur is a component of proteins and prosthetic groups (-SH) of some biosynthetic enzymes and coenzyme A. Actinobacteria producing secondary metabolites containing sulphur atoms such as cephamycin and cyclooctasulfur have preferences towards the source of sulphur they utilise. Amino acids such as L-cysteine and L-cystine have been effectively used in enhancing yields of cyclooctasulphur in *S. albulus* though inorganic sulphur salts such as sodium sulfite and sodium thiosuphate suppressed production (Hayashi *et al*., 1985). Conversly, inorganic salts were good sources of sulphur for cephamycin biosynthesis in *S. clavuligerus* and *S. lactamdurans*, whereas amino acids sources were not effective (Romero *et al*., 1984). A useful inorganic sulphur source for SM fermentations is ammonium sulphate $[(NH_4)_2SO_4]$ which can be used concomitantly as the nitrogen source (Dunn, 1985). Inorganic potassium K^+ cation is a cofactor of some SM biosynthetic enzymes and is

involved in many transport processes. It is usually added as an inorganic K salt, e.g. K_2SO_4, $KHPO_4$ or KH_2PO_4. Magnesium is a important activator of SM synthases and enzyme cofactor. Addition of magnesium ($MgSO_4.7H_2O$) has been shown to enhance the production of the macrolide antibiotic mycinamicin. The optimum effect of magnesium depends on the carbon concentration (Egorov, 1985).

1.7.2.3 Inorganic and Organic Micronutrients

Micronutrients (or trace elements) influence the quantity and in some cases the types of secondary metabolites produced by actinobacteria (Weinberg, 1989). Trace elements are essential for biological processes to occur, as exemplified by their use in enzyme reactions (Iwai & Omura, 1982; Jung *et al*., 2002). However, excess quantities of trace elements can be toxic and high concentrations of elements such as chromium (Cr^{2+}) and manganese (Mn^{2+}), exhibit inhibitory activity against secondary metabolism, thus a narrow range of the amounts of trace elements is beneficial for the production of secondary metabolites (Weinberg, 1989).

Four important transition metals are essential for secondary metabolism to proceed, these include iron (Fe), zinc (Zn^{2+}), cobalt (Co^{2+}) and Mn^{2+} (Weinberg, 1989; Bushell & Gräfe, 1989). Iron an important catalyst for SM synthases is involved in redox processes (Egorov, 1985). Iron deficiency has been shown to increase the production of polyketide antibiotics in *Streptomyces* spp. (Coisne *et al*., 1999; Bechet & Blondeau, 1998). Conversely, iron has been shown to suppresses desferrioxamine and streptomycin (Asai & Shimabara, 1951; Schupp *et al*., 1988). The zinc ion is important for the formation of SM synthases, plays a catalytic role in RNA-polymerases and promotes the biosynthesis of some antibiotics [streptothricin, candicidin] (Weinberg, 1989; Keeraptipibul *et al*., 1984; Liu *et al*., 1975). The zinc ion has its effects on carbohydrate, nitrogen and phophorus metabolism in some organisms and is involved in oxidation-reduction process (Egorov, 1985). Co^{2+} plays important roles in the biosynthesis of antibiotics, such as the production of gentamycin, spiramycins, cumermycin A, and phosphonomycin (Weinberg, 1989). It is usually added as $CoCl_2.6H_2O$ (Egorov, 1985). Mn^{2+} is a cofactor of many SM synthases and has been used to increase the production of SM such as monensin

produced by *S. cinnamonensis* and tobramycin produced by *S. cremeus* (Stark *et al*., 1968; Motkova *et al*., 1982).

The effects of certain trace elements are not exerted unless they are used in combinations (Weinberg, 1989). An example is the use of Zn and Fe in chloramphenicol production where the incorporation of these combined minerals lead to a five fold increase in production of the metabolite as compared to Zn or Fe used independently (Table 14). Calcium (Ca^{2+}) and copper (Cu^{2+}) have also been shown to have a synergistic effect leading to an increase in vancomycin production in *Amycolatopsis orientalis*. The high productivity was shown to be due to Ca^{2+} altering cell permeability leading to increase in intracellular vancomycin being egressed and Cu^{2+} increasing the activity of biosynthetic enzymes in the cells (Jung *et al*., 2002).

Table 14. The effects of iron and zinc on the production of chloramphenicol by *Streptomyces venezuelae* (adapted from Gallicchio & Gottlieb, 1958).

Culture medium Glycerol-lactate plus: Iron (100 μM)	Zinc (100 μM)	Mycelial dry weight (mg/ml)	Chlorophenicol (μg/ml)
0	0	1.132	<5
+	0	1.936	<5
0	+	0.983	<5
+	+	2.399	30

Inorganic salts such as sodium chloride (NaCl) at the concentrations of 0.5 % or less have been been shown to increase antibiotic production (Rake & Donovick, 1946). However, larger concentrations greater than 2 % usually have a inhibitory effect on SM production (Iwai & Ōmura, 1981). Organic compounds such as vitamins, metabolic intermediates, and enzyme inhibitors such as dimethyl sulfoxide, esters, benzyl thiocyanate and barbital have been shown to increase SM production in streptomyces (Chen *et al*., 2000; Mejía *et al*., 1998; Takenaka *et al*., 1998; Weinberg, 1989; Watanabe & Serizawa, 1998).

1.7.2.4 Temperature Shifts

Temperature is a major environmental factor that influences the expression of SM biosynthetic genes and decay rates of secondary metabolite products (Votruba & Vaněk, 1989). The growth temperature is independent of the temperature for SM production (Figure 16). Generally the range for optimum growth temperature is as wide as 20 degrees but the temperature for optimum production of secondary

metabolites is narrow, between 5-10 degrees (Iwai & Ōmura, 1982). In the case of streptomycin production by *Streptomyces griseus*, an increase of 1°C at the threshold temperature results in an 80% reduction in antibiotic production (Dunn, 1985). Secondary metabolite producing actinobacteria are mesophilic, with growth and secondary metabolism production being optimal at 28°C (Iwai & Ōmura, 1982). Furthermore, thermophilic actinobacteria found in extreme environments with higher temperatures requirements at 40°C have been shown to produce novel secondary metabolites (James *et al*., 1991).

Temperature dependence can be used in the regulation of expression of metabolites. Kuznetsov *et al*. (1984) found that subjecting the antibiotic producing *S. galbus* to two different temperatures, could be used to control the type of secondary metabolite being produced.

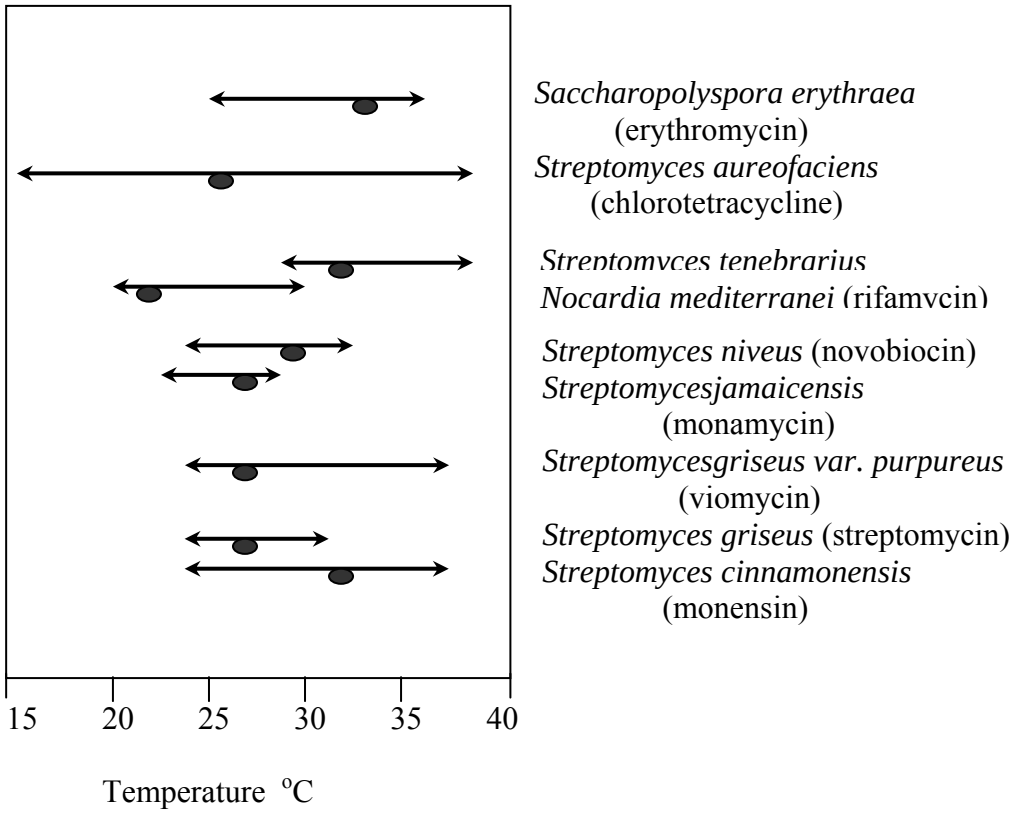


Figure 16. The influence of temperatures permitting vegetative growth on secondary metabolism. Ovals represent temperature at which maximal yield of secondary metabolite is obtained; arrow tips represent temperatures at which minimal yields of secondary metabolite are obtained (adapted from Weinberg, 1974)

1.7.2.5 pH Effects

The pH of the medium affects the growth rate, mycelial morphology and secondary metabolism in actinobacteria (Braun & Vecht-Lifshitz, 1991). Actinobacteria possesses a wide pH optimum for growth (usually between 6 and 8) while secondary metabolism can only tolerate a narrow range within 0.2 pH units (James *et al*, 1991; Chen *et al*., 1999). Studies by Hayakawa *et al*. (1995), indicate that by increasing the pH value from 5.5 to 7.5 members of the rare actinobacterial genus *Microbispora* spp. displayed increased antimicrobial activity.

Intracellular pH (pH_i) of most microorganisms is maintained near neutrality, independent of medium pH. However, as the hydrogen ion gradient across the cytoplasmic membrane increases, the cell is forced to direct its resources towards maintaining the desired intracellular pH, possibly diverting energy away from SM biosynthesis (Forage *et al*., 1985). Consequently, Corvini *et al.* (2000) showed that pH_i is a important parameter in establishing optimal conditions for the excretion of secondary metabolites.

1.7.2.6 Dissolved Oxygen (DOC)

Secondary metabolite producing actinobacteria are obligate aerobes, thus providing cells with adequate supplies of oxygen is critical for respiration to proceed effectively (Liefke *et al*., 1990). A major obstacle in submerged fermentations is the difficulties in delivering sufficient oxygen to bacterial cells due to its low solubility in aqueous media and limitations in gas-liquid mass transfer (Dick *et al*., 1994; El-Enshasy *et al*., 2000). However, a novel approach such as that use by Elibol and Mavituna (1997) making use of perfluorocarbons which are oxygen carriers and have a oxygen solubility of 10-20 times higher than that of water, lead to increases in antibiotic yields.

The major effect that oxygen has on SM biosynthesis is that it can induce or repress enzyme systems which catalyse the incorporation of oxygen into organic molecules (Forage *et al*., 1985; Yegneswaran & Gray, 1991; Stanbury *et al*., 1995). Kaiser *et al*. (1994) showed that by increasing the DOC during production of the antibiotic manumycin, they obtained an increase in the yield of manumycin as well as detected new metabolites which were manumycin derivatives. Furthermore, Pfefferle *et al*. (2000) showed increased SM production in *Streptosporangium* strains by using

controlled excess DOC throughout the SM fermentations. Oxygen limitation has been shown to abolish SM biosynthesis in *Amycolatopsis orientalis* and *S. clavuligerus* (Dunstan *et al*., 2000; Ives & Bushell, 1997).

1.7.2.7 Precursors, Cofactors and Nucleotides

The availability of metabolite precursor and cofactor levels influence the regulation of secondary metabolites (Kheton *et al*., 1999). As many biosynthetic enzymes have a low substrate specificity, analogues can be added as precursors and these can be incorporated to give hybrid products (Jacobsen *et al*., 1997; Brown *et al*., 1999). An example of precursor directed biosynthesis is that of efrotomycin by *Nocardia lactamdurans,* the rate limiting step in the biosynthesis of this metabolite is the avalability of uracil, the precursor of the pyridone moiety of this metabolite. The different types of molecules serving as precursors have included short chain fatty acids which have been shown to enhance SM production (Ohno *et al*., 1980; Untrau-Taghian *et al*., 1995; Cruz *et al*., 1999), purines and nucleotides, such as nicotinamide-adenine-dinucleotide (NADH), adenosine and guanosine phosphates, pyridoxal 5'phosphate which have been implicated in the regulation of SM biosynthesis (Gräfe *et al*., 1994).

1.7.2.8 Enzyme Inhibitors and Repressors of Secondary Metabolism

A severe consequence of the production of secondary metabolites is the inhibition or repression of their biosynthetic enzymes (Table 15). Other contributing factors to enzyme inhibition include certain organic acids and amino acids which when added to media, repress the production of specific biosynthetic enzymes (Iwai & Ōmura, 1982).

Table 15. Enzyme Inhibitors of Secondary Metabolite Production in Actinobacteria.

Secondary Metabolite	Enzyme Inhibitor	Enzyme Effected	References
Streptomycin	Chloramphenicol	Amidinotrasferase	Martin and Demain, 1980
Actinomycin	Chloramphenicol	Phenoxazione Synthase	Katz and Weissbach, 1962
	Puromycin		
Chloramphenicol	*p*-aminophenylalanine	Arylamine Synthase	Jones and Westlake, 1974
Candicidin	Rifampin	Candicidin Synthetase	Liras et al., 1977

1.7.2.9 Autoregulators

Low molecular weight hormone-like signal substances, known as autoregulators can control the production of secondary metabolites (Table 16). This effect is exerted by either the repression or inhibition of secondary metabolite synthases and enzymes supplying biogenetic precursors (Shapiro, 1989). These autoregulators in many instances are able to restore simultaneously secondary metabolite production in blocked mutants of producing actinobacteria (Trilli, 1990).

Table 16. Autoregulators effecting secondary metabolite production in actinobacteria.

Autoregulator Type	Autoregulator	Autoregulator group	Effects on secondary metabolism	References
Antibiotic	S-component of Virginiamycin	peptidolactone	antibiogenisis	Biot, 1984
Non-antibiotic	A-factor	γ-butyrolactone	antibiogenisis	Horinouchi, 1985
Proteinaceous Factors	Factor-C	-	altering biosynthetic pathways	Biro et al., 1980
Nucleotide analogues antibiotics	tunicamycin	pyrimidinoid	antibiogenisis	De Carvalho and Molinari, 1983

1.7.2.10 Miscellaneous Factors Influencing Secondary Metabolite Production

Osmotic stresses such as high sugar or salt conditions have been shown to control SM production (Elibol & Mavituna, 1998). Incompatibilities of medium components can lead to precipitation of macro- and micronutrients particularly Mg^{2+} and PO_4^{3-}. This precipitation of media components are unavailable to the cell and so deficiencies can occur. Controlling the concentrations of trace elements is achieved by chelation, which act as metal ion buffers maintaining a constant supply at physiological concentrations to the growing cells. The amount of chelating agent used in a fermentation is usually empirically determined, the types of chelating agents used in media affects the rate of SM production in chemically defined media. EDTA (as a disodium salt) is a popular choice and in complex media citrate or nitrilotriacetic acid is commonly used (Melzoch *et al*., 1997).

Inoculum types (spores or vegetative cells), concentrations and morphology significantly effects SM production and varies for individual actinobacterial cultures (Elibol *et al*., 1995; Neves *et al*., 2001; Jonsbu *et al*., 2002). In the case of the polyene

antibiotic producing strains *S. griseocarneus* and *S. natalensis* the use of spore inoculums showed an increase in polyene yields as compared to the use of vegetative cells, additionally spore concentrations of 2×10^7 - 10^8 spores/ml were found to be optimal (Cruz *et al*., 2000; El-Enshasy, 2000). Conversely, late exponential growth phase mycelia was used as the inoculum source for the beta-lactam producing strain *S. clavuligerus*, which caused a increase in the production of clavulanic acid and shortened the duration of the fermentation (Neves *et al*., 2001).

The use of controlled-release polymers such as ethylene-vinylacetate containing ammonium chloride (NH_4Cl) has been used to efficiently release NH^+_4 to *S. clavuligerus* and avoiding the repressing effects of high NH^+_4 and evidently leading to a increase the production of cephalosporin (Lübbe *et al*., 1985).

1.7.3 Solid State Fermentations

Alternative fermentation systems such as solid state fermentations (SSF) are being adapted to the production of secondary metabolites (Robinson *et al*., 2001). SSF is characterised by containing the following properties (1) a fermentation process occurring on solid porous matrices which can either be natural materials (such as rice, wheat bran) or an inert support (e.g. polystyrene, vermiculite) impregnated with a liquid medium; (2) low moisture contents (usually at 12 %) of solid matrices and (3) occurring in a natural state (Raimbault, 1998; Ooijkaas *et al*., 2000). The mycelial morphology of actinobacteria which are used for SM production, is advantageous in out competing unicellular microorganisms for colonisation of solid substrates and for efficient utilisation of nutrients. Additionally the solid matrices enable the actinobacterial cultures to develop and differentiate along gradients. Table 17 lists the adaptation of SSF technology in the production of secondary metabolites by actinobacteria.

Table 17. Application of SSF to secondary metabolites produced by actinobacteria

Secondary Metabolite	Actinobacteria	Substrate	Reference
Monensine	*Streptomyces cinnamonensis*	Barley/Oats	León, 1989
Tetracyclines	*Streptomyces rimosus*	Sweet potato residue	Yang and Ling, 1989
Actinorhodin	*Streptomyces coelicolor*	Nutrient Agar	Barrios-González et al., 1990
Aureomycin	*Streptomyces aureofaciens*	Wheat bran	Segura et al., 1993
Oxytetracycline	*Streptomyces rimosus*	Corn-cob	Yang and Swei, 1996
Cephamycin C	*Streptomyces clavurigerus*	Raw wheat	Kota and Sridhar, 1998
Kasugamycin	*Streptomyces kasugaensis*	Celite beads	Kim et al., 2001
Selamectin	*Streptomyces lydicus* SX-1298	Polyester mesh	Pacey et al., 2001

The advantages that SSF technology has over submerged fermentations is that often sterile conditions are not required, more stable SM products are produced in higher yields, energy requirements are low, with easier downstream processing measures, and lower capital costs. The disadvantages of SSF include heat dissipation problems, lack of adequate methods for agitation of solids, difficulties in addition of nutrients, scale-up, lack of availability of sensors for determining microbial growth and fermentative parameters, and lack of sufficient data on physiology and genetics of SSF strains to enable optimisation of SM production (Barrios-González & Mejía, 1996; Sato & Sudo, 1999).

Section 8: Genetic Modification of Biosynthetic Genes and the Development of Novel Compounds

The creation of new secondary metabolites has been facilitated by genetic manipulation techniques which modify existing biosynthetic pathway genes. The detection and cloning of SMBG is continually required to meet the demands of combinatorial biosynthesis in the production of novel secondary metabolites. In the following paragraphs the application of genetic manipulation strategies employed for the generation of hybrid or novel secondary metabolites will be described.

1.8.1 Genetic manipulation of secondary metabolite producing actinobacteria

Secondary metabolites are produced from a series of enzymatic reactions that show a relaxed substrate specificity. Exploiting this inherent property by genetic manipulation of secondary metabolite biosynthetic genes encoding these enzymes has led to the generation of novel compounds (Salas and Mendez, 1998). The genetic

engineering strategies used for modifying SMBG include; (1) gene transfer: (2) gene replacement; (3) chromosomal insertion; (4) Homologous recombination; (5) DNA Shuffling and (6) silent gene induction.

1.8.2 Metabolic Engineering of Secondary Metabolite Producing Actinobacteria

Metabolic engineering is defined as the directed improvement of product formation or cellular properties through modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology (Stephanopoulos, 1999). Rational strategies have focused on the tightly regulated first biosynthetic steps which control the metabolic flux through SM biosynthetic pathways, major emphasis has been placed on directed genetic alteration of genes which has lead to the overproduction of a SM or alteration of a pathway to produce a new product that could either be a intermediate or a modified end product (Piepersberg, 1994; August *et al*., 1999). The strategies used in metabolic engineering of secondary metabolites, (i) include supplying precursors to control rate of product synthesis and amounts of different analogs; (ii) preferential specificity of certain starter units of biosynthetic pathways; (iii) incorporation of synthetic intermediates; (iv) control of chain length and cyclization; (v) and induced post-modification reactions (Chartrain *et al*., 2000). Table 18 outlines the metabolic engineering strategies employed for secondary metabolite production. Manipulation of gene regulators or activators can also be used to improve the flux rates through secondary metabolic pathways (Charter, 1990).

In cases where the biosynthesis of a SM is completely defined biochemically and genetically, flux balance models can be used to dictate the changes needed to shift the metabolic pathway utilization from producing biomass towards producing higher yields of the SM of interest (Neaimpoor & Mavituna, 2000; Varma & Palsson, 1994). Improvements of general cellular properties, such as the ability to withstand hypoxic fermentation conditions (Cedrone *et al*., 2000), as well as inducing constitutive expression of resistance genes preventing self-inhibition of the actinobacteria and enhancing secondary metabolite productivity (Crameri *et al*, 1986).

The generation of novel compounds can also be achieved by using secondary metabolite biosynthetic genes cloned into plasmid vectors and heterologously expressed in different actinobacteria or other microorganisms, which have a relaxed substrate specificity or possessing similar structures sharing parts of their biosynthetic pathways (Kealey *et al*., 1998; Salas & Mendez, 1998). Factors influencing heterologous production of secondary metabolites include: (i) correct posttranslation modification ;(ii) broad range of substrates and supply of substrates coordinately regulated with SM biosynthesis when required; (iii) intracellular factors such as chaperones that ensure correct folding and assembly of SMBG; (iv) transmembrane transporter proteins such as the ATP binding cassette transporters which are required for the export of secondary metabolites; (v) and self-resistance mechanism/s to inhibit the effect of the SM on the heterologous host (Pfeifer & Khosla, 2001). The benefits of expressing genes in a heterologous host are that easier genetic manipulation with regard to enhancement of commercial production and recombination of genes for related pathways to produce new natural products. Chary *et al.* (2000) demonstrated that by using two strong heterologous promoters from *S. griseus* transferred into *N. lactamdurans* lead to the overexpression of the rate limiting *lat* gene and subsequently increased the yield of the production of cephamycin C. A limitation of heterologous expression is that gene clusters larger than 40 kb cannot be accommodated into cosmid cloning vectors and 100 kb for bacterial artificial chromosomes (BAC) vectors (Sosio *et al*., 2000b).

Combinatorial biosynthesis is defined as the production of hybrid secondary metabolites or analogues resulting from novel combinations of genes, achieved by the introduction of genes from one organism into another host organism (Hutchinson, 1997; Tang and McDaniel, 2001). The requirements for the production of hybrid secondary metabolites are that firstly, the availability of two or more known or newly discovered microorganisms that produce secondary metabolites whose biosynthesis has a common feature. The second requirement is that the biosynthetic pathway must be fully characterised, and thirdly a gene-enzyme connection must be established (Huchinson, 1999). Combinatorial biosynthesis can be approached in two ways to generate a diverse range of new compounds with new activities. The first of these includes cloning of single or multiple genes encoding structural modification reactions, such as those encoding methyltransferases and reductases that modify basic

structures in the producing microorganism (Hopwood *et al*., 1985). The second approach includes cloning or mixing and matching of core biosynthetic enzymes, yielding either new compounds containing features from different secondary metabolites or completely new core molecules (Bartel *et al*., 1990; Cane *et al*., 1998). This approach was elegantly demonstrated by Yoon *et al* (2002) whereby novel macrolides were produced by replacing pikromycin PKS in *S. venezuelae* with heterologous modular PKSs in recombinant strains and consequently creating novel natural-product structural diversity.

The chemical diversity able to be generated by combinatorial biosynthesis of SMBGs is providing libraries of novel natural products, and is providing an additional approach to the discovery of new chemical entities (McDaniel *et al*., 1999). Improvements to genetic engineering techniques is providing a means to creating further chemical diversity in a less tedious manner, this approach is exemplified by the recent findings of Xue *et al*. (1999) demonstrating that multiple plasmids carrying known mutations in biosynthetic genes combined with heterologous expression can be used to generate large libraries of SM productive clones. This, alleviates the laborious single plasmid approach and provides an efficient means of producing novel natural products.

Exploitation of the natural genetic makeup of actinobacteria, in particular manipulating SMBG through combinatorial biosynthesis is providing attractive strategies in combating the declining rate of the discovery of novel secondary metabolites and the establishment of compound libraries with virtually a unlimited supply of new molecules to be screened. The continual discovery of novel or related SMBG from actinobacteria and other microorganisms is pertinent in providing an even wider selection of biosynthetic genes to be incorporated into combinatorial approaches for the generation of novel secondary metabolites.

Chapter 2: Materials and Methods

Section 2.1: General Microbiological Methods

2.1.1 Actinobacterial cultures: Selected SM producing actinobacterial pure cultures were obtained from the American Type Culture Collection (ATCC), German Culture Collection (DSM) and from the Agricultural Research Service (ARS/NRRL) (see Table 18), and were used for validation and comparison studies against the Cerylid cultures. Table 19 lists twenty-two environmental actinobacterial isolates which are from the Cerylid Biosciences (Melbourne, Australia) culture collection. These cultures are referred to in the text as environmental isolates due to isolation from an environmental source. It is these isolates which were screened to evaluate their capabilities to produce secondary metabolites.

Table 18: Actinobacterial pure cultures

Secondary Metabolite Class	Secondary Metabolite	Microorganism	Strain Number
Polyketide Synthase Type I	Milbemycin	*Amycolata autrotrophica* subsp. *canberrica*	ATCC35203
	Erythromycin	*Micromonospora rosaria*	ATCC29337
	Nargenicin	*Nocardia argentinensis*	ATCC31306
	Erythromycin	*Saccharopolyspora erythrae*	DSM40517
	Avermectin	*Streptomyces avermitilis*	NRRL8165
	Tylosin	*Streptomyces fradiae*	NRRL2702
	Rapamycin	*Streptomyces hygroscopicus*	ATCC29253
Polyketide Synthase Type II	Mithramycin	*Streptomyces argillaceus*	ATCC12956
	Actinorhodin	*Streptomyces coelicolor A3(2)*	M145^
	Tetracenomycin	*Streptomyces glaucescens*	DSM40922
	Griseusin	*Streptomyces griseus*	DSM40236
	Nogalamycin	*Streptomyces nogalater*	ATCC27451
	Daunomycin	*Streptomyces peucetius*	ATCC29050
	Oxytetracycline	*Streptomyces rimosus*	DSM40260
Beta- Lactams	Nocardicins	*Nocardia uniformis* subsp. *tsuyamanensis* strain R-4	ATCC21806
	Thienamycin	*Streptomyces cattleya*	NRRL8057
	Cephalosporin	*Streptomyces clavuligerus*	DSM738
	Clavulanic Acid	*Streptomyces clavuligerus*	NRRL3585
	Cephamycin C	*Streptomyces griseus*	NRRL3851
Aminoglycosides	Neomycin	*Micromonospora chalcea*	NRRL3222
	Fortimicin	*Micromonospora olivasterospora*	ATCC21819
	Gentamicin	*Micromonospora purpurea*	DSM43036
	Streptomycin	*Streptomyces griseus*	DSM40236

^ Culture obtained from Sir David Hopwood, John Innes Institute, Norwich, England.
DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany)
ATCC – American Type Culture Collection
NRRL – Northern Regional Research Laboratories (Culture Collection)

Table 19: Cerylid environmental actinobacterial isolates

Isolate Number	Genus*	Location of Isolate Obtained
1. A0371	*Streptomyces*	Cow paddock, VIC
2. A2010	*Streptomyces*	Walsh River Burke Rd, QLD
3. A2360	*Streptomyces*	Walker Hill, QLD
4. A0350	*Streptomyces*	Sediment, Townsville Port, QLD
5 .A2226	*Streptomyces*	Shadow soil over granite, QLD
6. A2381	*Streptomyces*	Lamb range Douglas creek, QLD
7. A1215	*Streptomyces*	Koomboolomba, QLD
8. A2056	*Streptomyces*	Open eucalypt forest, QLD
9. A3675	*Streptomyces*	Under native grass, VIC
10. A0096	*Streptomyces*	Track, N of Rocky Pt. Rd. NT
11. A1113	*Streptomyces*	Plant material and wood chips, VIC
12. A1488	*Modestobacter*	Olinda-Dandenongs, VIC
13. A1664	*Geodermatophilus*	Cape York, QLD
14. A3020	*Asiosporangium*	Cape Ferguson (sediment)
15. A3023	*Verrucosispora*	Cape Ferguson (sediment)
16. A2702	*Micromonospora*	Eucalyptus woodland, QLD
17. A2376	*Micromonospora*	Open eucalyptus forest, QLD
18. A3014	*Micromonospora*	Mt Fox, QLD
19. A0347	*Couchioplanes*	Australian Antarctic Territory, Antartica
20. A3771	*Couchioplanes*	Port Melbourne, VIC
21. A1990	*Nocardioides*	Tall eucalyptus forest, QLD
22. A2834	*Nocardioides*	Eucalyptus pauciflora tablelands, QLD

***** The Cerylid environmental actinobacterial isolates were assigned to appropriate genus groups following 16SrDNA sequencing carried out by Cerylid Biosciences.

2.1.2 Maintaining Cultures (solid medium): Type strains and environmental cultures were maintained for long, medium and short term storage. In the case of long term storage two loopfuls of agar cultures were immersed in a sterile 20% v/v glycerol solution and cryopreserved in a –80^{o}C freezer (Wellington & Williams, 1978), in the medium term (up to 3 months) cultures were maintained on Yeast-Malt Extract (YME) Agar slants containing (g/L) Yeast Extract 4, Malt Extract 10, Glucose 4, Agar 16, pH 7.2 and held at 27^{o}C, for the short term (2-4 weeks) cultures were maintained on their respective agar plates and held at 27^{o}C.

Section 2.2: Primer Design For Actinobacteria-Specific Secondary Metabolite Biosynthetic Genes

2.2.1 *In Silico* Analysis of Nucleotide Sequences

Computational analysis of nucleotide sequences involved using WebANGIS (www.angis.org.au). This software package was utilised for extracting information from sequence databases and conducting a large number of procedures and genome analysis tasks outline below. A stepwise procedure was followed in the design of

appropriate primers, as a number of crucial parameters were tested prior to the commencement of any polymerase chain reaction (PCR) experiments. Figure 17 depicts a flow diagram of the set of procedures carried out at each step of the primer design process.

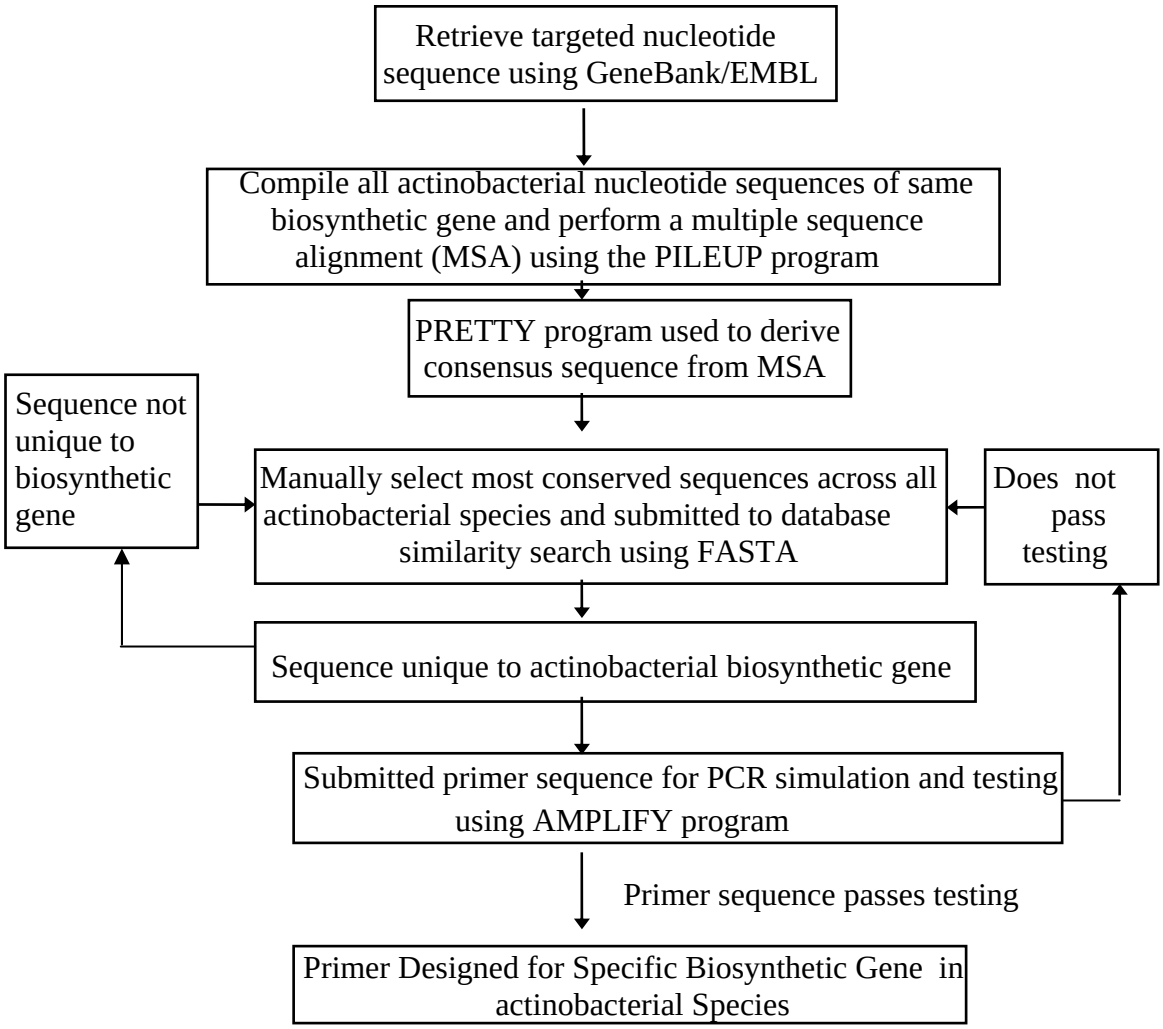


Figure 17. Flow chart depicting the strategy employed for selecting and validating appropriate primer sequences.

2.2.1.1 Retrieval of Nucleotide Sequences from Databases

GeneBank® (National Institute of Health) http://www.ncbi.nlm.nih.gov/Genbank/, the European Molecular Biology Laboratory (EMBL) http://www.embl-heidelberg.de/ and ENTREZ Protein Sequences www.ncbi.nlm.nih.gov/entrez/ databases were searched to extract all available nucleotide and protein sequences corresponding to selected biosynthetic genes. A number of these sequences have been published and readily available (Tables 32, 37, 40 and 44).

The retrieval of nucleotide sequences involved using a corresponding accession number which was available from the literature and entered into a Browse Code option available in the WebAngis software or alternatively a Query Search option was selected which involved entering key terms. The output of the search provided a list of all available sequences corressponding to that query. Appropriate sequences were selected for further *in silico* analysis.

2.2.1.2 PILEUP Multiple Sequence Alignment (MSA) Program

Following the retrieval of nucleotide or protein sequences, a comparative analysis of the sequences was performed of the same biosynthetic gene in different species of actinobacteria using the PILEUP program. A selection of suitable sequences were aligned to generate a multiple sequence alignment (MSA) by using program default values.

2.2.1.3 PRETTY Consensus Sequence Program

Proceeding the MSA, the PRETTY program was used to determine the regions of the biosynthetic genes of low variability to establish the location of the consensus sequence. MSA were submitted to the PRETTY program and a default setting of 80% conservation was extracted. These conserved regions or consensus sequences were targeted as sites for the design oligonucleotide primers.

2.2.1.4 Database Similarity Of Primer Sequence Using the FASTA Program

Following the selection of suitable primers, the FASTA http://www.ebi.ac.uk/fasta3/ database search program was used to determine the specificity of these primers and published primers. The two criterion used to select suitable primers were that the primers matched with the gene of interest and were specific for actinobacteria species.

2.2.1.5 Calculating Annealing Temperatures of Primers

The annealing temperature of newly designed primers were calculated using oligonucleotide calculators which are available as freeware, such as www.microbiology.adelaide.edu.au/learn/index.htm.

2.2.1.6 *In Silico* PCR Experiments using AMPLIFY simulation program

The Macintosh program AMPLIFY version 1.2 (Engels, 1993) was used for simulating and testing the designed primers for use in polymerase chain reactions, against the template genome. PCR simulation experiments were used to indicate whether a single or multiple amplified products will be obtained. Primers failing the default tests, incorporated into the program, do not produce any products.

2.2.1.7 Selection of Primers for Screening

Primers that were designed specifically to detect the selected secondary metabolite biosynthetic genes in actinobacteria are shown in Table 20, these primers were used to screen the Cerylid environmental actinobacterial cultures. Published primers were also used in this study (Table 21).

Table 20. Primer sequences and predicted lengths of PCR amplification products.

Primer*	Sequence of primers	Gene recognised	Product size (bp)
act04(f)	GATGGTCTCCACCGGCTGC	Ketosynthase (*KSα*)	
act06(r)	GTCTCGTGGCGGTCGTTCTGC		480
ole01(f)	CTTCGACGCCGCCTTCTTCGGGAT	Ketosynthase (*KS*)	
ole01(r)	CTGCGTATGCCCGATGTTCGACTTC		840
pcb03(f)	CGAGTCCTGGTGCTACCTGAACC	Isopenicillin N Synthase (*pcbC*)	
pcb03(r)	TCATCGACACGTCCAGGTGGTC		355
strD01(f)	CTTCGCCATGTATCTCGGCGACAA	dTDP-glucose synthase (*strD*)	
strD01(r)	TGCCGGTGTCCTTCCAGTAG		370

* (f) and (r) forward and reverse primers, respectively. Note that primers are in the 5' - 3' orientation

Table 21. Primer sequences and predicted lengths of PCR amplification Products.

Primer*	Sequence of primers	Product size (bp)	Reference
KSM	GCSTCCCGSGACCTGGGCTTCGACTC		Liu &
ATM	AGSGASGASGAGCAGGCGGTSTCSAC	750	Shen, 2000

* (f) and (r) forward and reverse primers, respectively. Note that primers are in the 5' - 3' orientation
- Mixed base code: S = (GC)

Section 2.3: Molecular Biology Methodologies

2.3.1 Extraction of Bacterial DNA

The total genomic DNA content was extracted from isolated cultures following a modified published protocol (Rainey *et al*., 1992). An additional final purification step was incorporated into the protocol to ensure purity of the DNA.

The processing of the cultured isolates for DNA extraction involved scraping actinobacterial mycelium from the surface of a 10 day-old colony on yeast extract-malt extract agar (2 loopfuls) and incubating the culture in 400 μl saline-EDTA extraction buffer (0.15M NaCl, 0.1M EDTA, pH 8) with added lysozyme (10 mg/ml) (Sigma Chemical Co. St. Louis, MO., Cat. No. L7651) at 37^{o}C and then digesting the bacterial cells with two proteolytic enzymes, lysozyme and 1 % (w/v) proteinase K (Sigma Chemical Co. St. Louis, MO., Cat. No. P2308) in the presence of 25 % (w/v) SDS (Sigma Chemical Co. St. Louis, MO., Cat. No. L5750) and saline-EDTA buffer, causing lysis of the cell. Following incubation a phenol-chloroform extraction was performed followed by a ethanol precipitation step (Sambrook *et al*., 1989). Proceeding the ethanol precipitation of DNA a final purification step was carried out with the Prep-A-Gene DNA Purification kit (BioRad, Hercules, CA., Cat. No. 732-6011). The DNA sample was loaded onto a ion-exchange resin where it bound, while the contaminants were washed out and the DNA was eluted off with water. Storage of the DNA extracts were kept at –20^{o}C.

2.3.2 PCR Reaction Conditions

The 5X reaction buffer consisted of 50 mM Tris HCI pH [8.3], 250 mM KCI, 7.5mM $MgCI_2$ and 0.2mM of each deoxynucleotide triphosphates (Boehringer and Mannheim). Each PCR reaction was overlayed with 40 μl of mineral oil (Sigma Chemical Co. St. Louis, MO., Cat. No. M-5904). The PCR was performed using a Perkin Elmer 480 Thermal Cycler (Perkin Elmer, Norwalk. USA).

2.3.2.1 General PCR Conditions for Designed Non-Degenerate Primers

Table 22: PCR reaction components for amplification with the designed primers shown in table 20.

Reaction Components	Concentration	Components of a typical 25 μl reaction (μl)
5X buffer	5X	5
Water	-	16
Primer forward	400 ng	1
Primer reverse	400 ng	1
Taq Polymerase	2 units	1
DNA	50 – 150 ng	1
Total		25

Table 23: PCR cycling profile for amplification with the designed primers shown in table 20.

PCR Step	Temperature (^{o}C)	Time (mins)	Cycles
Initial Denaturation*	94	8	1
Denaturation	94	1	30
Annealing	65	1	
Extention	72	2	
Final Extention	65	1	
	72	10	1
Soak^	25	-	-

* A hot start was performed at beginning of PCR cycling, whereby Taq polymerase was added after temperature of PCR machine reached greater than 90^{o}C.
^ The PCR program ends at the Soak step where the PCR reaction stops and is incubated at 25^{o}C until ready for gel electrophoresis

2.3.2.2 Degenerate PCR Conditions for Type I PKS

Separate reaction and amplification conditions for degenerate PCR were applied to amplify the 0.75 kb product representing the ketosynthase (*KS*) gene as specified in the original report (Liu & Shen, 2000). Only in the presence of 20 % glycerol was a PCR product of the correct size produced as specified in the original published method (Liu & Shen, 2000). Absence of 20% glycerol yielded no amplification product.

Table 24: PCR Reaction Components for Type I PKS genes.

Reaction Components	Concentration	Components of a typical 50 μl reaction (μl)
Glycerol	20%	10
DMSO	5%	2.5
5X buffer	5X	10
Water	-	23.5
Primer forward	400 ng	1
Primer reverse	400 ng	1
Taq Polymerase	2 units	1
DNA	50 - 150 ng	1
Total		50

Table 25: PCR Cycling Profile for amplification of Type I PKS genes.

PCR Step	Temperature (^{o}C)	Time	Cycles
Initial Denaturation	94	5 min	1
Denaturation	94	45 sec	
Annealing	60	1	30
Extention	72	2	
Final Extention	72	7	1
Soak^	25	-	-

^ The PCR program ends at the Soak step where the PCR reaction stops and is incubated at 25^{o}C until ready for gel electrophoresis

2.3.2.3 PCR Controls

The PCR technique has the potential to amplify single copies of DNA in a sample, including contaminants (Ortona *et al*., 1996). Therefore, the necessary precautions were taken for routine PCR, that is using dedicated pipettors for PCR and prepared PCR mixtures away from the amplification area. Attention was given to appropriate controls for successful interpretation of results. Controls listed below were incorporated in every experiment to avoid false-positive or negatives. These included:

Positive control 1: template DNA of type strains known to possess target nucleic acid sequences.

Negative control 1: omission of template DNA from reaction mix, to evaluate if spurious products are produced from reaction components.

External control: 16S rDNA amplified in parallel with the test template DNA, to confirm that the DNA was amplifiable (Table 26).

Controls implemented for validation studies, included evaluating the primers with genomic DNA from non-producing polyketide strains, where no amplified products were visualised as expected.

Table 26: Sequences of oligonucleotides primers used for 16SrDNA*

Primer	Nucleotides Sequence	Gene recognised	Product size (bp)	Reference
27f	GAGAGTTTGATCCTGGCTCAG	16S rDNA	750	Lane (1991)
765r	CTGTTTGCTCCCCACGCTTTC			Damiani *et al.* (1997)

* Numbering of primers based on *E.coli* numbering

2.3.2.4 Agarose Electrophoresis of PCR Products

PCR products were separated on a 1.5 % molecular biology grade agarose gel (Scientifix, France., Cat#9010B) and PCR purified products were separated on low gelling temperature agarose gel (Sigma Chemical Co. St Louis, MO., Cat# A-3038) these gels were separated on a horizontal gel system in 1X TBE buffer (54g Tris-HCI, 27.5g Boric Acid, 20 ml 0.5 mM EDTA pH [8] per litre) and run at 100 V for 30 mins using a BioRad PowerPac300 system. The PCR products were visualised after staining with ethidium bromide (100 ug/ml), washed and photographed under UV light. The AmpliSize^{TM} Molecular Ruler 50-2000 bp (BioRad, Hercules, CA., Cat # 170-8200) was used to establish the size of the PCR products. Five microlitres of PCR reaction was mixed with 2 μls of loading buffer prior to loading onto gel.

2.4 Analysis of sequenced amplified PCR products

Direct sequencing of the amplified products involved performing a preparative PCR reaction and purifying the PCR product using the Wizard PCR purification system (Promega, Madison, WI., Cat # A7170). The purified PCR products were submitted for direct sequencing to the Haematology Department at the Flinders Medical Center, carried out by Mr Rob Skinner.

Initial analysis of the sequenced products involved determining if the correct sequence had been amplified. This was performed by subjecting the amplified sequence to a FASTA sequence similarity search. Once it was determined that a similar nucleotide sequence corresponding to the targeted SMBG of interest had been

isolated, the sequence was translated into the corresponding amino acid sequence using the Translate program (Wisconsin Package, 10.0, GCG via WebAngis). Verification of the correct translated protein was performed by conducting a FASTA sequence similarity search. In the case where a reverse primer was used for sequencing, the nucleotide sequence was reversed and complemented using the Reverse program (Wisconsin Package, 10.0, GCG via WebAngis) and the resulting sequence was evaluated as previously described. The nucleotide chromatograms were visualised using the software package CHROMAS (Version 1.45).

Section 2.5 Phylogenetic Analysis

The nucleotide and protein sequences incorporated in the construction of the phylogenetic trees were retrieved from the two databases GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and EMBL (http://www.ebi.ac.uk/embl/). The sequences were uploaded in FASTA format. The nucleotide sequences obtained from the sequenced SMBG's in this study were converted into FASTA format, this conversion is necessary for further processing by sequence analysis programs. Sequences were aligned across specific regions which included only the secondary metabolite biosynthetic gene under investigation, these alignments were performed using the ClustalW alignment program (http://www.ebi.ac.uk/Tools/clustalw2/) (Thompson *et al.*, 1994). The output of the ClustalW multiple sequence alignment was incorporated into the program TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview) which was used to generate phylogenetic trees from it. The imaging application program Windows Paint was used to edit and print the phylogenic trees.

Section 2.6 Extraction and Processing of Actinobacteria Culture Extracts

A general extraction procedure adopted in this study (Figure 18) involved extracting metabolites from the whole broth with methanol (MeOH) or ethyl acetate (EtAc). The broth supernatant fraction following centrifugal separation was also retained for analysis. Following solvent extraction the fractions were tested in well-type assays to determine if they contained biological activity. Fractions containing bioactivity were further analysed using bioautography to determine the active component. Inactive

cultures were analysed using thin layer chromatography for secondary metabolite production.

2.6.1 Actinobacteria agar metabolite extraction: Extraction of metabolites from actinobacterial cultures grown on YME or ½ PDA agar was carried out using two solvents separately, methanol HPLC grade (BDH, Cat. No. 15250) and ethyl acetate analytical grade (BDH, Cat. No. 10108). Actinobacterial cultures were grown on ½ strength potato dextrose agar (PDA) which consisted of (g/L) Potato Dextrose Agar powder 19.5, Agar 7.5, Cycloheximide 50 mg/ml, Nystatin 50 mg/ml, pH 7.2 or YME for 7 - 10 days. The agar was removed from the petri dish, chopped into small fragments, and placed into 50 ml centrifuge tubes and immersed in 15 ml of MeOH or EtAc. Tubes were capped and placed onto a orbital mixer for 4 hrs at room temperature. Following incubation the extracts were filtered through Whatman® No.1 filter paper 70 mm diameter (Cat. No. 1001070) to remove solids. Clear filtrate was collected and analysed in antimicrobial assays (section 2.7).

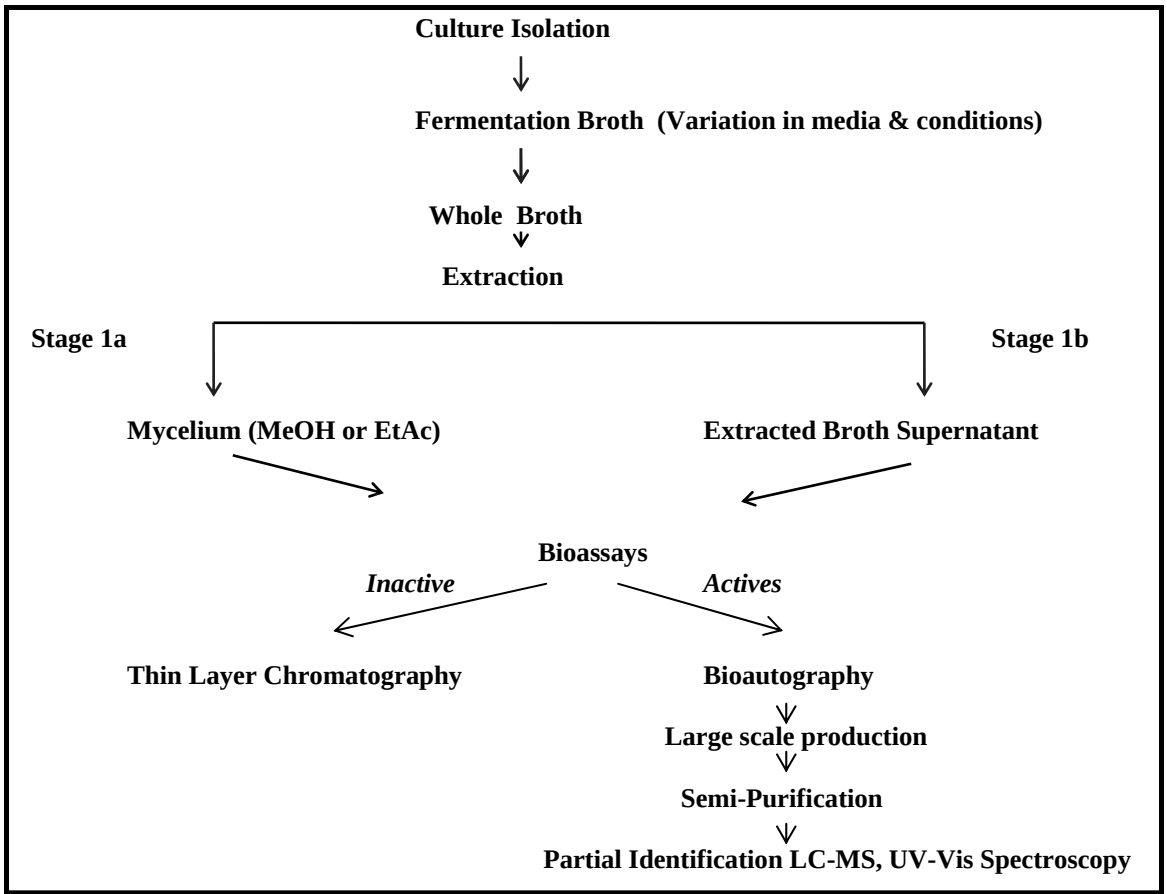


Figure 18. Schematic flow diagram of the isolation of microbial bioactive secondary metabolites.

2.6.1.1 Small scale actinobacterial fermentation metabolite extraction and processing

Aliquots (1 ml) of actinobacterial fermented culture grown in various production media were centrifuged at 4000 rpm for 20 min to pellet the mycelium. After centrifugation the broth supernatant was separated out by siphoning it off with a micropipette and it was kept for testing. 200 μls of MeOH or EtAc was added to the pelleted mycelium to extract metabolites. Following addition of MeOH or EtAc to the mycelium a vortex mixer was used for thorough mixing and then left standing for 30 min. Following this incubation time the mycelium extract was centrifuged as above, and the MeOH or EtAc mycelial extract was siphoned off with a micropipette. Both the broth supernatant and MeOH or EtAc mycelial extract were tested for bioactivity (section 2.7).

2.6.2 Large scale production and recovery of antimicrobial metabolites

Genetic and bioassay directed screening identified a number of the environmental actinobacterial cultures as producers of antimicrobial metabolites (Table 55). Three of these cultures were selected for large-scale production and purification studies. These cultures were A0350, A1113 and A2381. Using optimal fermentation conditions, identified in section 3.7 for each of the cultures, the fermentation was scaled up to 1 liter. In order to partially identify and characterise the active constituents from these metabolite producing cultures, a semi-purified organic extract was recovered using the conditions outlined below. A general purification regime was applied to each of the cultures to produce a suitable quantity (>1mg) of semi-purified organic extracts for chemical characterisation studies. The purification regime involved using 1 litre of actinobacterial fermented culture, dispensed into 200 ml centrifuge bottles and centrifuged at 4000 rpm for 30 min at 4^{o}C to pellet the mycelium. The broth supernatant was placed into a separating funnel and extracted twice with an equivalent volume of EtAc to obtain the non-polar extractable metabolites. The mixture was allowed to stand for 1 hr until two layers were visible, the top organic layer and an extracted broth supernatant bottom layer. An intermediate layer formed which consisted of globules was also apparent. This globule layer was passed through non-absorbent cotton wool (Smith+Nephew, Aus., Cat. No.131578A) and sodium sulphate

added (BDH, Cat.No. 30223) to breakup the globules. Both the broth supernatant and organic layers were tested for antibacterial activity (see section 2.7).

2.6.3 Concentration of the Extract

Clarified organic extract was dispensed into a 1 litre round bottom flask and subject to a rotary evaporator. The settings of the rotary evaporator were speed at 4 m/s and temperature set at 45^{o}C. The organic solvent was evaporated off, a powder was collected. Filtrate of the MeOH or EtAc extract were placed in 10 ml glass test tubes and concentrated in a Savant CENTRIVAP™ (LABCONCO, Kansas City, Missouri) until the MeOH or EtAc was evaporated off. Following concentration the extracts were freeze-dried using the FTS Systems Maxi-Dry freeze drier having the settings at 300 millitor and –70^{o}C for 6 hr.

Section 2.7: Bioassays of Secondary Metabolites

2.7.1 Plug Type Bioassay

Screening the actinobacterial cultures for the production of antimicrobial substances was carried out by extruding 6-mm plugs from 8 day actinobacterial cultures using a stainless steel cylinder cork borer (6-mm inner diameter, 8-mm outer diameter, and 10-mm length). The plug was transferred to bioassay agar (BA) plates which consisted of (g/l) Beef extract 4, Peptone 4, Glucose 2.5, Sodium Chloride 3, Agar 15, pH 7.0 seeded with 2% (v/v) inoculum of test cultures (see table 29), grown in Tryptic Soy Broth (TSB) culture containing (g/l) tryptic soy broth powder 30 at pH 7.2 to an optical density of 0.1 at 600_{nm}. Optical measurements were performed on a UV-Visible Spectrophotometer SHIMADZU Model UV-160A. Bioassay agar plates were incubated at 37^{o}C and zones of inhibition recorded after 2-4 days.

Table 27: Test Cultures for Bioassays

Test Culture	Test activity
Candida albicans ATCC10231	Antifungal
Micrococcus luteus	Anti-bacterial (gram positive)
Staphyloccocus aureus	Anti-bacterial (gram positive)
Bacillus pumilus	Anti-bacterial (gram positive)
Escherichia coli ATCC25922	Anti-bacterial (gram negative)

2.7.2 Well Type Bioassay

Screening was also performed using the conventional well type bioassay, 6-mm wells were extruded from BA plates seeded with test cultures. 30 μls aliquot of resuspended extract, fermentation broth supernatant or mycelial extract were pipetted into the wells. The plates were then incubated at 37^{o}C and zones of inhibition recorded after 2 – 4 days.

2.7.3 Bioautography

Thin Layer Chromatography (TLC) plates were assayed to identify active bands. TLC plates were placed face down on bioassay agar plates seeded with a test culture for 30 minutes to allow transfer of metabolites. The pattern of the bands were traced onto tracing paper, so as to establish the position of the active bands. Following incubation the TLC plate was removed and the plates incubated for 2-4 days at 37^{o}C and zones of inhibition recorded. 0.1% Tetrazolium Blue Chloride (SIGMA# T-4375) was sprayed over the top of the agar so as to clearly visualize these zones.

Section 2.8: Fermentation of Secondary Metabolites

Preliminary screening for antimicrobial activities in liquid media was conducted to determine (1) which conditions were optimal for fermentative expression of bioactive metabolites, (2) to make a comparison of solid and liquid fermentations and (3) to determine optimal fermentation conditions for selected environmental isolates.

2.8.1 Small Scale Submerged Shake-Flask Fermentations

Actinobacterial cultures were grown on PDA or YME plates for 7-10 days and used as inoculum into 50 ml of IM22 inoculum medium. Following 3 days of fermentation at 27^{o}C, 120 rpm on a Orbital shaker (Ratek, Australia) or Innova 2300 platform shaker (New Brunswick Scientific). 2 ml of inoculum medium IM22 was transferred to baffeled 250 ml erylemeyer flasks containing 50 ml of selected production media as shown in table 28. Fermentations were conducted over a 10 day period, with samples taken at days 2, 5, 7 and 10 for biological activity assessment.

Table 28. Production media used for the secondary metabolite studies.

Medium	Main Ingredients (per liter)
248P	Glucose 20g, Soyabean meal 10g, $CaCO_3$ 2g, K_2HPO_4 0.5g, $CaCl_2.6H_2O$ 1mg
SI	Sucrose 20g, $CaCO_3$ 2.5g, KNO_3 1g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.5g, NaCl 0.5g
153	Glucose 20g, Peptone 5g, Beef Extract 5g, $CaCO_3$ 3g
153m	Glucose 20g, Peptone 5g, Beef Extract 5g, $CaCO_3$ 3g, NaCl 5g, Yeast Extract 3g
153+Glycerol	Glycerol 20g, Peptone 5g, Beef Extract 5g, $CaCO_3$ 3g
153m+ Glycerol	Glycerol 20g, Peptone 5g, Beef Extract 3g, $CaCO_3$ 3g, NaCl 5g, Yeast Extract 3g
Dextrin	Dextrin 20g, Soyabean meal 10g, Yeast Extract 2g, $FeSO_4.7H_2O$ 1mg
IM25	Sucrose 40g, Soyabean meal 25g, NaCl 2.5g, $CaCO_3$ 2.5g,$CuSO_4.5H_2O$ 5mg, $MnCl_2.4H_2O$ 5mg, $ZnSO_4.5H_2O$ 5mg
IM22	Glucose 15g, Soyatone 15g, Pharmamedia 5g, $CaCO_3$ 2g, NaCl 5g

2.8.2 Small Scale Solid State Fermentations (SSF)

Solid substrate media (see table 29) were dispensed into 250 ml Erlenmeyer flasks which were then autoclaved for 1 hr at 121^{o}C. Following autoclaving, sterile liquid supplements were added to serve as a nutrient source and to maintain the moisture content, table 31 indicates the processing of the individual solid substrates. 2 ml of seeded IM22 inoculum was transferred to baffeled 250 mL Erlenmeyer flasks containing various types of selected SSF media as shown in table 29 and were mixed by gently tapping on the palm of the hand. The flasks were maintained at 27^{o}C ± 2^{o}C on a static platform. Flasks were then harvested on day 10. The solid substrate was extracted with 30 - 50 ml ethyl acetate (Ajax Chemicals).

Table 29. Solid Substrate Fermentation Media

Solid Substrate	Pretreatment	Amount of Solid Substrate (g)	Liquid Supplements
Burghul	NT	10	5 ml ddH2O
Barley Flakes	NT	10	5 ml ddH2O
Rice	Soaked in sterile water overnight, macerated[d]	10 [a]	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Corn	Soaked in sterile water overnight, macerated	10 [a]	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Natural Unprocessed Wheat Bran	NT	10	4.5 ml Trace Salts [b] 5.0 ml LF42[c]

Vermiculite	NT	1.2	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Perlite	NT	1.2	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Whole Grain Oats	Soaked in sterile water overnight, macerated	10[a]	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Whole Grain Rye	Soaked in sterile water overnight, macerated	10 [a]	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
Psyllium Husk	NT	10	4.5 ml Trace Salts [b] 5.0 ml LF42[c]
LSA Mix*	NT	10	4.5 ml Trace Salts [b] 5.0 ml LF42[c]

*LSA = Linseed, Sunflower Kernels, and Almonds
^ NT = not treated
[a] wet weight
[b] Trace Salts Solution added: 0.1g Sodium Tartrate., 0.01g $ZnSO_4.5H_2O$., 0.01g $FeSO_4.7H_2O$., 0.1g KH_2HPO_4., 0.1g $MgSO_4.7H_2O$., 0.2g Yeast Extract made up to 1000 ml with distilled water and sterilised.
[c] LF42 Solution: 5g Yeast Extract., 5g Peptone., 5g Soyaflour., 40 ml glycerol solution., 2g Soluble Starch., 2g $CaCO_3$., 5g NaCl made up to 1000 ml with distilled water and sterilised.
[d] maceration was carried out using a hand held kitchen blender.

2.8.3 Liquid Fermentations Supplemented with Refined Oils

The two liquid fermentation media IM25 and 153m, which had been shown to be useful for the production of antimicrobial compounds were further evaluated with the addition of refined oils. 1.5 g of each refined oil was added to 50 ml of fermentation media IM25 or 153m to achieve a final concentration of 3 % w/v. Inoculum of the actinobacterial environmental isolates A0347, A2702 and A3675 was obtained from YME plates which were grown for 7 days. The duration of the fermentation was carried out over 10 days, at 27^{o}C, at the speed 120 rpm on a Innova 2300 platform shaker (New Brunswick Scientific). Following the end of the fermentation the samples were processed and assayed as in section 2.7.

Section 2.9: Physicochemical Characterisation Methods Used to Elucidate Semi-Purified Fermented Extract

Chemical characterisation methods at early stages of the purification process included analytical techniques such as Thin Layer Chromatography (TLC) and Ultra-violet-

visible (UV-Vis) spectrophotometry which provided useful indications in establishing a purification process.

2.9.1 Thin Layer Chromatography (TLC)

TLC was used to separate and isolate active constituents from fermentation broth mixtures. 20 µl of microbial extract was spotted onto an aluminium backed pre-coated silica gel plate (60 F_{254} 20 x 20 cm, 0.2 mm layer thickness plates Merck, Cat. No. 5554) and air dried using a handheld hairdryer; Plates were eluted with a solvent system consisting of Ethyl Acetate: Methanol (90:10) with a drop of 28% Ammonia solution. TLC plates were run for 10-15 min. In an airtight glass tank, pre-saturated with solvent vapors, containing the eluting solvent system. Bands were visualised under 254_{nm} and 365_{nm} wavelength UV light and their respective R_f values calculated.

2.9.2 Ultraviolet-Visible Spectrophotometry

An evaluation of the UV-Vis spectrum of the microbial extracts at different levels of purity were carried out. To determine the chromophores present in the metabolites. Optical measurements were performed on a UV-Visible Spectrophotometer SHIMADZU Model UV-160A. The spectrum was scanned over the desired wavelength range of 200_{nm} to 600_{nm}.

2.9.3 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC): In the current study, organic extracts were subjected to RP-HPLC (Table 30) and peaks were further analysed by recording their UV absorbance across the wavelengths 200_{nm} to 400_{nm}. In addition, eluting fractions were collected at 0.25 min intervals and subjected to antimicrobial assays. Both the RP-HPLC and antimicrobial assays was carried out in the laboratory of the industry partner Cerylid Biosciences.

Table 30: RP-HPLC conditions and parameters

Conditions	Specification and Parameters
System	WATERS 2960
Column	Xterra MS (WATERS) C_{18} Reverse phase 4.6 x 50 mm, 2.5 µm diam.
Solvents	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient Profile	0 to 100% solvent A in B 17 mins 100% solvent B 20 mins
Flow Rate	1 $ml.min^{-1}$
Detection	Photodiode array (PDA), Elutions monitored at 200_{nm} to 400_{nm}
Sample volume	10 µl

2.9.4 HPLC/UV Mass Spectrometry (MS)

In the current study HPLC/UV/MS was used to identify the molecular weights of the active compounds using the parameters given in table 31. This analysis was carried out in the laboratory of the industry partner Cerylid Biosciences.

Table 31: HPLC/UV MS conditions and parameters

Conditions	Specification and Parameters
System	Finnigan LCQ iontrap mass spectrometer
Column	Xterra MS (WATERS) C_{18} Reverse phase 2.1 x 50 mm, 2.5 μm diam.
Gradient Profile	0 % solvent A in B to 100 % solvent B 70 mins
Flow Rate	100 $\mu l.min^{-1}$
Detection	Electronspray ionisation source (MS), UV-Visible (HPLC)
Sample volume	10 μl

2.9.5 Literature Searching of Natural Product Databases

Querying natural product literature databases was conducted to ascertain if the natural product extracted from the microbial source was a known compound. Using the chemical and physical characteristics obtained from HPLC/UV/MS, matching compounds were identified against the Chapman and Hall dictionary of natural products (DNP). This analysis was carried out in the laboratory of the industry partner Cerylid Biosciences. In addition, UV-Vis spectroscopy profiles were compared against common UV-Vis spectra in the literature.

Chapter 3: Results

Section 3.1: Development of a Polymerase Chain Reaction Based Screening Assay for the Detection of Secondary Metabolite Biosynthetic Genes

3.1.1 Primer design and PCR screening assay for actinobacterial type II polyketide synthases (PKS)

Nucleic acid and amino acid sequence accession numbers of all available type II PKS actinobacterial genes that were retrieved from the databases and incorporated into the design of the primers act04f and act06r are presented in table 32.

Table 32: Type II PKS genes with respective nucleic acid and amino acid sequences incorporated into the design of the act04f and act06r PCR primers.

Class/ Gene	Secondary Metabolite Biosynthetic Gene	Actinobacteria	Amino Acid Accession Number	Nucleotide Accession Number
Type II Polyketides /Ketosynthase	Pradimicin (PKS)	*Actinomadura verrucosospora*	BAA82309	AB019690
	Pradimicin (PKS)	*Actinomadura hibisca*	BAA23144	D87924
	Ardacin (ardIORF1)	*Kibdelosporangium aridum*	AAA67433	L24518
	Napthocyclinone (ncnA)	*Streptomyces arenae*	AAD20267	AF098965
	Mithramycin (mtmP)	*Streptomyces argillaceus*	CAA61989	X89899
	Tetracyclines (aur2A)	*Streptomyces aureofaciens*	BAA92278	AB024976
	Anthracycline (KS)	*Streptomyces bobili*	BAA92272	AB019690
	Chlorotetracyline	*Streptomyces aureofaciens*	BAB12566	AB039379
	Ciclacidine (KS)	*Streptomyces capoamus*	BAA92274	AB024972
	Actinorhodin (actIORF1)	*Streptomyces coelicolor A3(2)*	CAA45043	X63449
	Aclacinomycin (aknB)	*Streptomyces galilaeus*	BAA92273	AB024971
	Tetracenomycin (tcmK)	*Streptomyces glaucescens*	CAA33369	X15312
	Griseusin (gris1)	*Streptomyces griseus*	CAA54858	X77865
	Nogalamycin (KS)	*Streptomyces nogalater*	BAA92283	AB024981
	Nogalamycin (snoa1)	*Streptomyces nogalater*	CAA12017	AJ224512
	Daunomycin (dpsA)	*Streptomyces peucetius*	AAA65206	L35560
	Platenomycin (KS)	*Streptomyces platensis*	BAA92279	AB024977
	Rhodomycin (KS)	*Streptomyces purpurascens*	BAA92276	AB024974
	Oxytetracycline (ORF1)	*Streptomyces rimosus*	CAA80985	Z25538
	Ravidomycin (orf1)	*Streptomyces rochei*	BAA87907	AB021222
	Steffimycins (KS)	*Streptomyces steffisburgenesis*	BAA92281	AB024979
	Frenolicin (frnL)	*Streptomyces roseofulvus*	AAA19616	L26338
	Panamycin (KS)	*Streptomyces tauricus*	BAA92275	AB024973
	Jadomycin (jadA)	*Streptomyces venezuelae*	AAB36562	AF126429
	Jadomycin (KS)	*Streptomyces venezuelae*	BAA92282	AB024980
	Anthracycline (KS)	*Streptomyces albus*	BAA92280	AB024978
	Monensin (KS)	*Streptomyces cinnamonensis*	CAA77596	Z11511
	Daunomycin (KS)	*Streptomyces* sp. strain C5.	AAA87618	L34880
	Aclacinomycin (KS)	*Streptomyces galilaeus*	AAF70106	AF257324
	Granaticin (gra-orf1)	*Streptomyces violaceoruber* Tü22	CAA34369	X16300

The PCR assay designed for screening Type II PKS genes took advantage of the consistent orientation of the β-ketoacyl synthase (KS_α) gene, which is one of three

genes that constitute the minimal PKS required to create the carbon skeleton of the polyketide molecule (McDaniel *et al*., 1994). The PCR primers were designed according to the consensus sequences of 29 amino acid and 26 nucleic acid sequences derived from multiple sequence alignments of actinobacterial KS_α genes available in the GenBank/EMBL databases at that time (Figures 20 and 21). From these 26 KS_α nucleic acid sequences 24 were from *Streptomyces* spp, and two from non-streptomyces species these were from a *Actinomadura* spp. and from a *Kibdelosporangium* spp. The primers designed were used to amplify a 0.47 kb product corresponding to an internal fragment from within the KS_α gene (Figure 19). The design strategy employed embedding a 19 base pair forward primer (act04f) in the active site of the KS_α gene at nucleic acid position 595-614 (numbers correspond to *Streptomyces coelicolor* GenBank accession number: X63449), the corresponding 21 base pair reverse primer (act06r) was positioned at 1048-1069 flanking the KS_α gene (Figures 20 and 21).

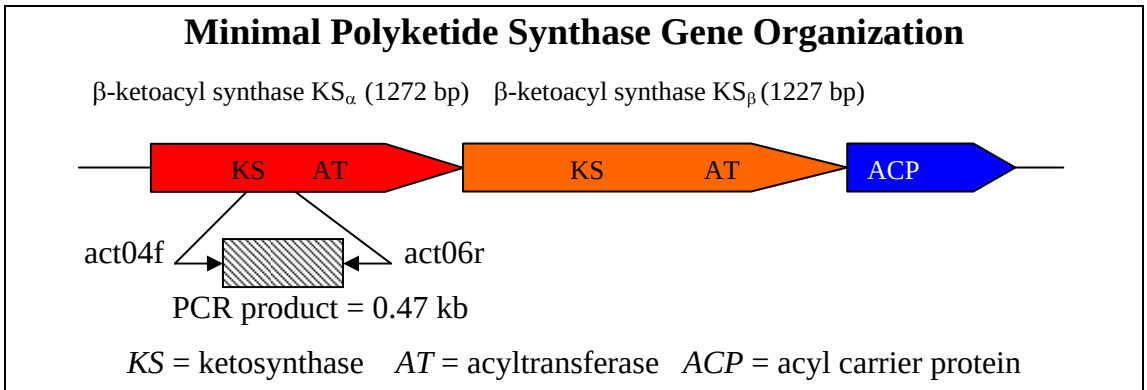


Figure 19. Illustration depicting the conserved minimal PKS gene organization in Type II PKS. Design strategy used to obtain a internal fragment within the KS_α gene from uncharacterised environmental actinobacterial isolates.

Evaluation of the amino acid alignment of the act04f primer sequence, indicated that the 5' region is variable (Figure 20 & Table 33). The act04f primer has the sequence MVSTGC, in the amino acid sequence alignment with other actinobacteria it is evident that two degenerate codons occur at positions 1 and 4. Codon 4 is the critical codon at the 3' end however three possible amino acids occur at that position across different actinobacterial species these include Aspartic Acid (GAT/C), Alanine (GCA/T/C/G) and Tyrosine (TAT/C). In this case a degenerate primer covering all these possible combinations would have been more appropriate. Partial nucleotide

sequence homology can be a problem particularly at the 3'end of the primer which is used for initial recognition of annealing and if the first seven bases at the 5' terminus are not conserved then efficient binding does not occur and either non-specific products are produced as seen in figure 22 (lanes 6,11 & 12) or the primer does not recognise the template and there are no amplification products. Evaluation of the reverse primer (act06r) sequence alignments indicated that across different actinobacteria a highly conserved sequence QND(X)HET (Figure 20 & Tables 33) which can be used to construct a universal primer for actinobacteria.

Table 33. Relative amino acid positions and codon degenerecies of act04f and act06r primers within the sequence.

Primer	Amino Acid Position	Actual Primer Amino Acid Sequence	*Streptomyces* Amino Acid Sequence[a]	Amino Acid Sequence~	Amino Acid Sequence*
act04f	170 - 175	MVSTGC	VVSTGC	(X)VS(X)GC	(X)(X)STGC
act06r	323 - 329	QNDRHET	QNDRHET	QND(X)HET	QNDRHET

(X) = variable codon
[a] *Streptomyces* consesus sequence for targeted regions
~ *Streptomycete* and *Non-Streptomycete Actinobacteria species*
* Non - *Streptomycete Actinobacteria species* only

Table 34. Relative nucleic acid positions of act04f and act06r primers and variable nucleotides within the sequence

Primer	Nucleic Acid Position	Nucleic Acid Sequence~	Nucleic Acid Sequence*
act04f	595 – 614	NNNNNTNTCNNCNGGNTGC	GNTGNTCTCCACCGGCTGC
act06r	1048 - 1069	GCAGAACGACNNNCACGAGAC	GCAGAACGACCGGCACGAGAC

(N) = variable base
~ *Streptomycete* and *Non-Streptomycete Actinobacterial species*
* Non- *Streptomyces species only*

Validation of the specificity of the act04f and act06r primers was performed by direct sequencing of the amplified 0.47 kb product from the actinobacterial type strain *Streptomyces nogalater* (ATCC27451), known to contain the KS_{α} gene (Torkkell *et al*., 2001). Confirmation that the correct sequence had been amplified was verified using a gapped-FASTA search that the nucleotide and the translated amino acid sequence were similar to known Type II PKS sequences from actinobacterial species.

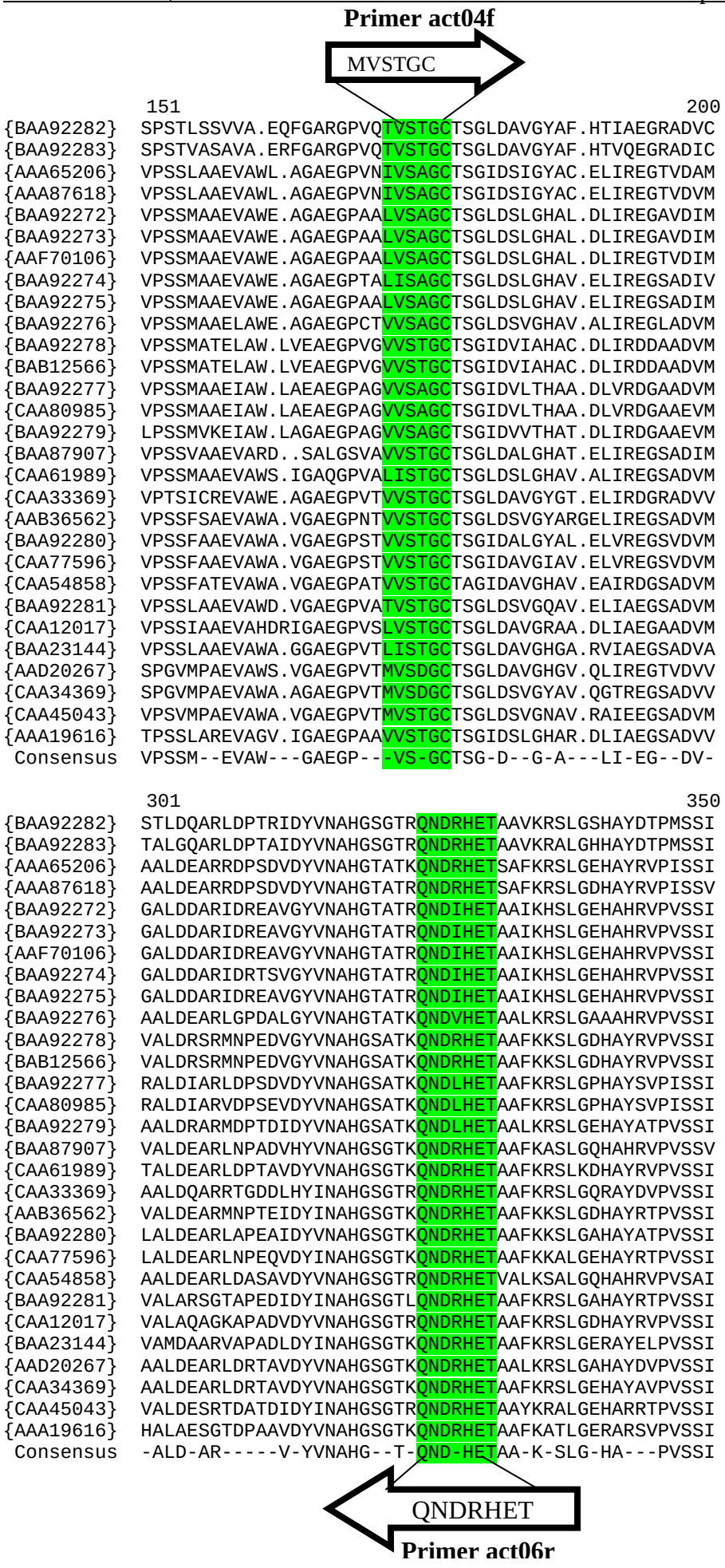

Primer act04f
MVSTGC
151
200
{BAA92282} SPSTLSSVVA.EQFGARGPVQTVSTGCTSGLDAVGYAF.HTIAEGRADVC
{BAA92283} SPSTVASAVA.ERFGARGPVQTVSTGCTSGLDAVGYAF.HTVQEGRADIC
{AAA65206} VPSSLAAEVAWL.AGAEGPVNIVSAGCTSGIDSIGYAC.ELIREGTVDAM
{AAA87618} VPSSLAAEVAWL.AGAEGPVNIVSAGCTSGIDSIGYAC.ELIREGTVDVM
{BAA92272} VPSSMAAEVAWE.AGAEGPAALVSAGCTSGLDSLGHAL.DLIREGAVDIM
{BAA92273} VPSSMAAEVAWE.AGAEGPAALVSAGCTSGLDSLGHAL.DLIREGAVDIM
{AAF70106} VPSSMAAEVAWE.AGAEGPAALVSAGCTSGLDSLGHAL.DLIREGTVDIM
{BAA92274} VPSSMAAEVAWE.AGAEGPTALISAGCTSGLDSLGHAV.ELIREGSADIV
{BAA92275} VPSSMAAEVAWE.AGAEGPAALVSAGCTSGLDSLGHAV.ELIREGSADIM
{BAA92276} VPSSMAAELAWE.AGAEGPCTVVSAGCTSGLDSVGHAV.ALIREGLADVM
{BAA92278} VPSSMATELAW.LVEAEGPVGVVSTGCTSGIDVIAHAC.DLIRDDAADVM
{BAB12566} VPSSMATELAW.LVEAEGPVGVVSTGCTSGIDVIAHAC.DLIRDDAADVM
{BAA92277} VPSSMAAEIAW.LAEAEGPAGVVSAGCTSGIDVLTHAA.DLVRDGAADVM
{CAA80985} VPSSMAAEIAW.LAEAEGPAGVVSAGCTSGIDVLTHAA.DLVRDGAAEVM
{BAA92279} LPSSMVKEIAW.LAGAEGPAGVVSAGCTSGIDVVTHAT.DLIRDGAAEVM
{BAA87907} VPSSVAAEVARD..SALGSVAVVSTGCTSGLDALGHAT.ELIREGSADIM
{CAA61989} VPSSMAAEVAWS.IGAQGPVALISTGCTSGLDSLGHAV.ALIREGSADVM
{CAA33369} VPTSICREVAWE.AGAEGPVTVVSTGCTSGLDAVGYGT.ELIRDGRADVV
{AAB36562} VPSSFSAEVAWA.VGAEGPNTVVSTGCTSGLDSVGYARGELIREGSADVM
{BAA92280} VPSSFAAEVAWA.VGAEGPSTVVSTGCTSGIDALGYAL.ELVREGSVDVM
{CAA77596} VPSSFAAEVAWA.VGAEGPSTVVSTGCTSGIDAVGIAV.ELVREGSVDVM
{CAA54858} VPSSFATEVAWA.VGAEGPATVVSTGCTAGIDAVGHAV.EAIRDGSADVM
{BAA92281} VPSSLAAEVAWD.VGAEGPVATVSTGCTSGLDSVGQAV.ELIAEGSADVM
{CAA12017} VPSSIAAEVAHDRIGAEGPVSLVSTGCTSGLDAVGRAA.DLIAEGAADVM
{BAA23144} VPSSLAAEVAWA.GGAEGPVTLISTGCTSGLDAVGHGA.RVIAEGSADVA
{AAD20267} SPGVMPAEVAWS.VGAEGPVTMVSDGCTSGLDAVGHGV.QLIREGTVDVV
{CAA34369} SPGVMPAEVAWA.AGAEGPVTMVSDGCTSGLDSVGYAV.QGTREGSADVV
{CAA45043} VPSVMPAEVAWA.VGAEGPVTMVSTGCTSGLDSVGNAV.RAIEEGSADVM
{AAA19616} TPSSLAREVAGV.IGAEGPAAVVSTGCTSGIDSLGHAR.DLIAEGSADVV
Consensus VPSSM--EVAW---GAEGP--VS-GCTSG-D--G-A---LI-EG--DV-
301
350
{BAA92282} STLDQARLDPTRIDYVNAHGSGTRQNDRHETAAVKRSLGSHAYDTPMSSI
{BAA92283} TALGQARLDPTAIDYVNAHGSGTRQNDRHETAAVKRALGHHAYDTPMSSI
{AAA65206} AALDEARRDPSDVDYVNAHGTATKQNDRHETSAFKRSLGEHAYRVPISSI
{AAA87618} AALDEARRDPSDVDYVNAHGTATRQNDRHETSAFKRSLGDHAYRVPISSV
{BAA92272} GALDDARIDREAVGYVNAHGTATRQNDIHETAAIKHSLGEHAHRVPVSSI
{BAA92273} GALDDARIDREAVGYVNAHGTATRQNDIHETAAIKHSLGEHAHRVPVSSI
{AAF70106} GALDDARIDREAVGYVNAHGTATRQNDIHETAAIKHSLGEHAHRVPVSSI
{BAA92274} GALDDARIDRTSVGYVNAHGTATRQNDIHETAAIKHSLGEHAHRVPVSSI
{BAA92275} GALDDARIDREAVGYVNAHGTATRQNDIHETAAIKHSLGEHAHRVPVSSI
{BAA92276} AALDEARLGPDALGYVNAHGTATKQNDVHETAALKRSLGAAAHRVPVSSI
{BAA92278} VALDRSRMNPEDVGYVNAHGSATKQNDRHETAAFKKSLGDHAYRVPVSSI
{BAB12566} VALDRSRMNPEDVGYVNAHGSATKQNDRHETAAFKKSLGDHAYRVPVSSI
{BAA92277} RALDIARLDPSDVDYVNAHGSATKQNDLHETAAFKRSLGPHAYSVPISSI
{CAA80985} RALDIARVDPSEVDYVNAHGSATKQNDLHETAAFKRSLGPHAYSVPISSI
{BAA92279} AALDRARMDPTDIDYVNAHGSATKQNDLHETAALKRSLGEHAYATPVSSI
{BAA87907} VALDEARLNPADVHYVNAHGSGTKQNDRHETAAFKASLGQHAHRVPVSSV
{CAA61989} TALDEARLDPTAVDYVNAHGSGTKQNDRHETAAFKRSLKDHAYRVPVSSI
{CAA33369} AALDQARRTGDDLHYINAHGSGTRQNDRHETAAFKRSLGQRAYDVPVSSI
{AAB36562} VALDEARMNPTEIDYINAHGSGTKQNDRHETAAFKKSLGDHAYRTPVSSI
{BAA92280} LALDEARLAPEAIDYVNAHGSGTKQNDRHETAAFKKSLGAHAYATPVSSI
{CAA77596} LALDEARLNPEQVDYINAHGSGTKQNDRHETAAFKKALGEHAYRTPVSSI
{CAA54858} AALDEARLDASAVDYVNAHGSGTRQNDRHETVALKSALGQHAHRVPVSAI
{BAA92281} VALARSGTAPEDIDYINAHGSGTLQNDRHETAAFKRSLGAHAYRTPVSSI
{CAA12017} VALAQAGKAPADVDYVNAHGSGTRQNDRHETAAFKRSLGDHAYRVPVSSI
{BAA23144} VAMDAARVAPADLDYINAHGSGTKQNDRHETAAFKRSLGERAYELPVSSI
{AAD20267} AALDEARLDRTAVDYVNAHGSGTKQNDRHETAALKRSLGAHAYDVPVSSI
{CAA34369} AALDEARLDRTAVDYVNAHGSGTKQNDRHETAAFKRSLGEHAYAVPVSSI
{CAA45043} VALDESRTDATDIDYINAHGSGTRQNDRHETAAYKRALGEHARRTPVSSI
{AAA19616} HALAESGTDPAAVDYVNAHGSGTKQNDRHETAAFKATLGERARSVPVSSI
Consensus -ALD-AR-----V-YVNAHG--T-QND-HETAA-K-SLG-HA---PVSSI
QNDRHET
Primer act06r

Figure 20. Comparison of the amino acid sequences of KSα genes. The 29 protein sequences were compared by the PILEUP method. Respective accession numbers are shown in brackets after the species name. *S.coelicolor* (CAA45043)., *S.nogalater* (BAA92283)., *Streptomyces* sp. strain C5 (AAA87618)., *S.galilaeus* (AAF70106)., *S.steffisburgenesis* (BAA92281)., *S.albus* (BAA92280)., *S.platensis* (BAA92279)., *S.aureofaciens* (BAA92278)., *S.albofaciens* (BAA92277)., *S.aureofaciens* (BAB12566)., *S.purpurascens* (BAA92276)., *S.tauricus* (BAA92275)., *S.capoamus* (BAA92274)., *S.galilaeus* (BAA92273)., *S.bobili* (BAA92272)., *S.griseus* (CAA54858)., *S.glaucescens* (CAA33369)., *S.violaceoruber* (CAA34369)., *S.peucetius* (AAA65206)., *S.rimosus* (CAA80985)., *S.argillaceus* (CAA61989)., *S.venezuelae* (AAB36562)., *S.venezuelae* (BAA92282)., *S.nogalater* (CAA12017)., *A.hibisca* (BAA23144)., *S.rochei* (BAA87907)., *S.roseofulvus* (AAA19616)., *S.cinnamonensis* (CAA77596)., *S.arenae* (AAD20267).

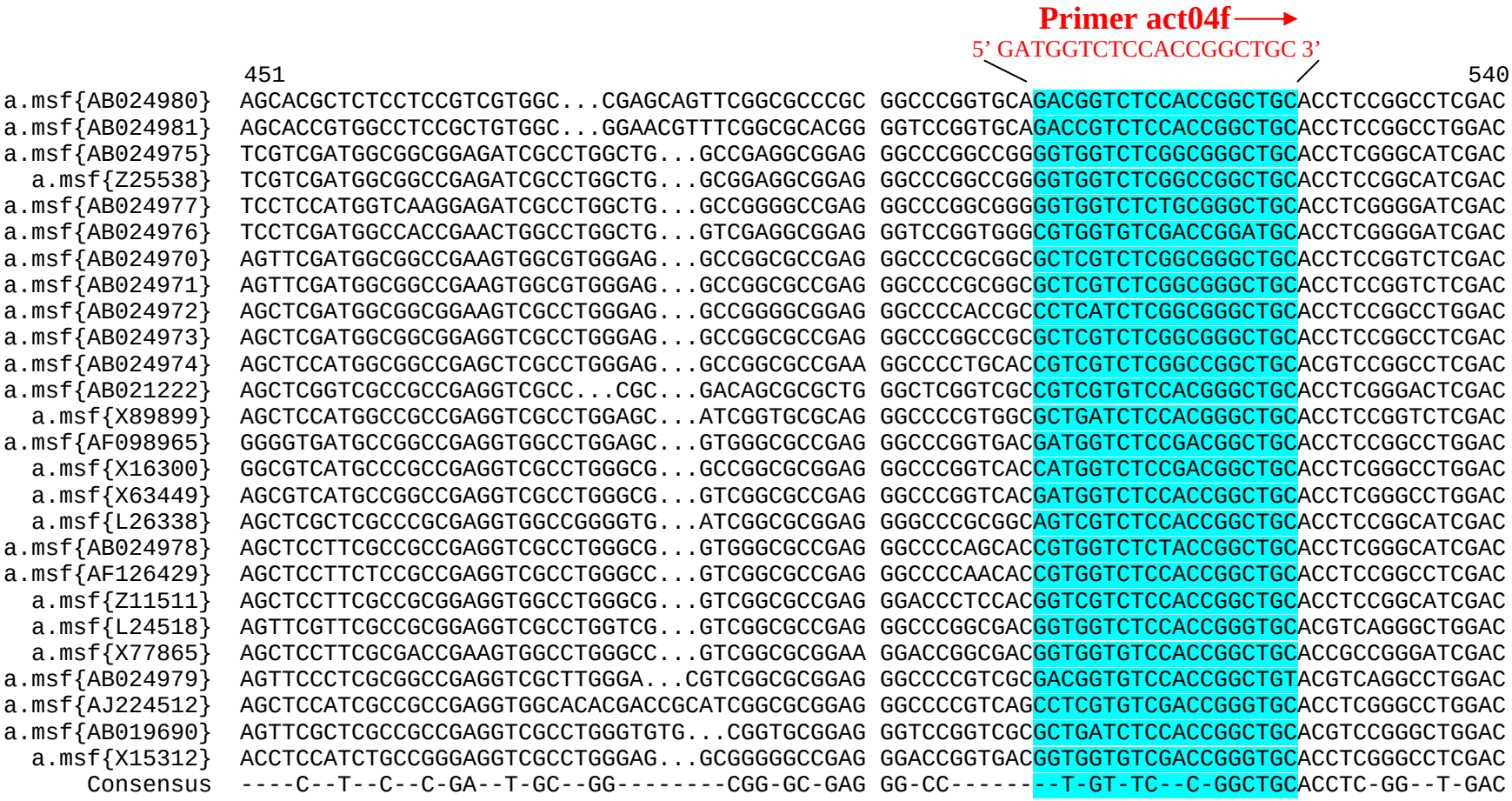


Figure 21. Comparison of the nucleic acid sequences of KSα genes. The 26 sequences were compared by the PILEUP method. Respective accession numbers are shown in brackets after species name. *S.coelicolor* (X63449)., *S.nogalater* (AB024981)., *S.steffisburgenesis* (AB024979)., *S.albus* (AB024978)., *S.platensis* (AB024977)., *S.aureofaciens* (AB024976)., *S.albofaciens* (AB024975)., *S.purpurascens* (AB024974)., *S.tauricus* (AB024973)., *S.capoamus* (AB024972)., *S.galilaeus* (AB024971)., *S.bobili* (AB024970)., *S.griseus* (X77865)., *S.glaucescens* (X15312)., *S.violaceoruber* (X16300)., *S.rimosus* (Z25538)., *S.argillaceus* (X89899)., *A.verrucosospora* (AB019690)., *S.venezuelae* (AB024980)., *S.venezuelae* (AF126429)., *S.nogalater* (AJ224512)., *S.rochei* (AB021222)., *S.roseofulvus* (L26338)., *S.cinnamonensis* (Z11511)., *S.arenae* (AF098965)., *K.aridum* (L24518).

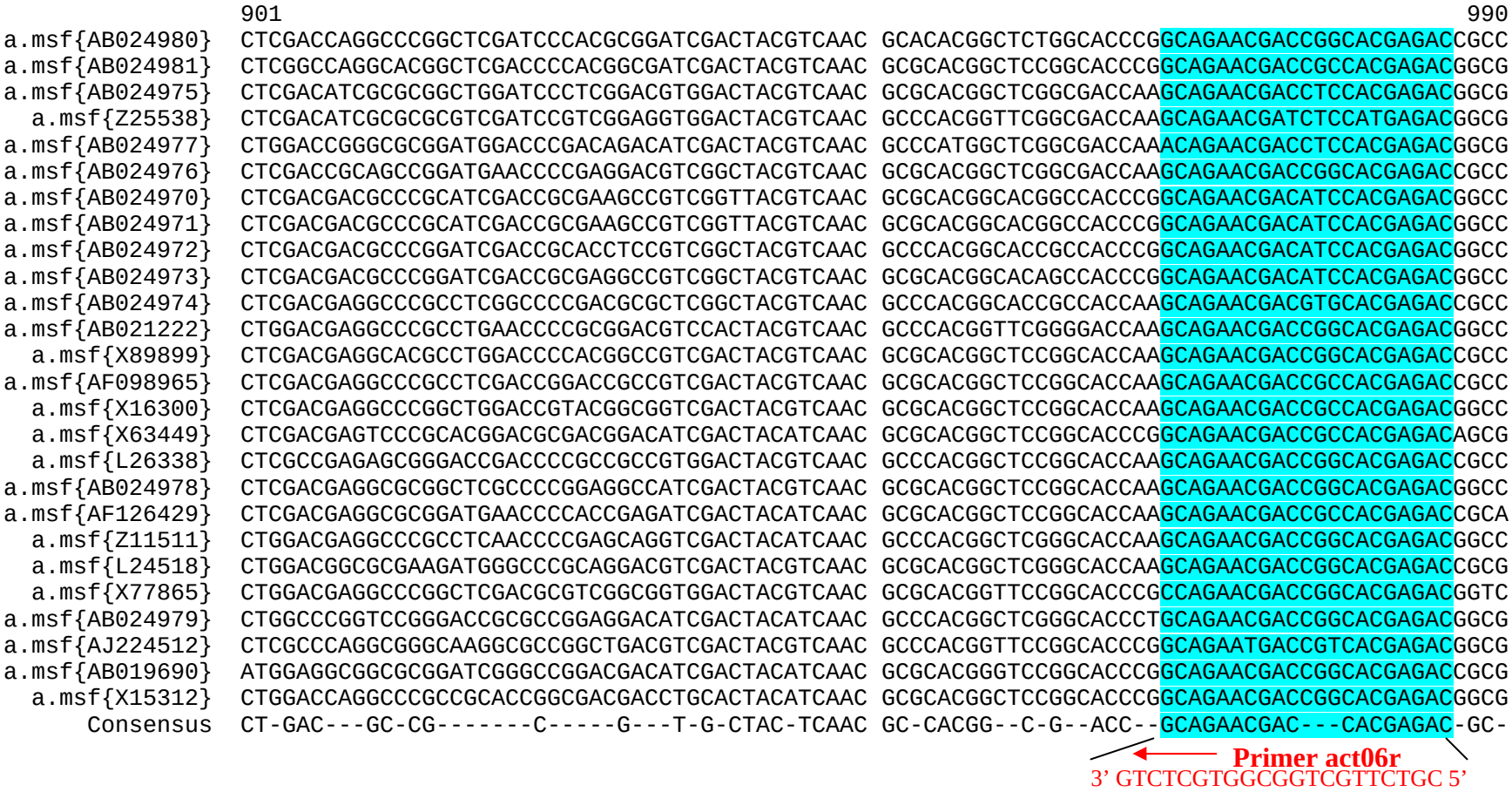


Figure 21. Comparison of the nucleic acid sequences of KSα genes. The 26 sequences were compared by the PILEUP method. Respective accession numbers are shown in brackets after species name. *S.coelicolor* (X63449)., *S.nogalater* (AB024981)., *S.steffisburgenesis* (AB024979)., *S.albus* (AB024978)., *S.platensis* (AB024977)., *S.aureofaciens* (AB024976)., *S.albofaciens* (AB024975)., *S.purpurascens* (AB024974)., *S.tauricus* (AB024973)., *S.capoamus* (AB024972)., *S.galilaeus* (AB024971)., *S.bobili* (AB024970)., *S.griseus* (X77865)., *S.glaucescens* (X15312)., *S.violaceoruber* (X16300)., *S.rimosus* (Z25538)., *S.argillaceus* (X89899)., *A.verrucosospora* (AB019690)., *S.venezuelae* (AB024980)., *S.venezuelae* (AF126429)., *S.nogalater* (AJ224512)., *S.rochei* (AB021222)., *S.roseofulvus* (L26338)., *S.cinnamonensis* (Z11511)., *S.arenae* (AF098965)., *K.aridum* (L24518).

A total of 450 bp from the amplified fragment was sequenced and a portion of this sequence was translated into 148 amino acids. A gapped–FASTA search of both the nucleotide and translated amino acid sequence confirmed that the sequences corresponded to the KS_α gene.

Validation studies confirmed that the amplified DNA sequence using the act04f and act06r primers corresponded to the KS_α gene. It was anticipated that by using these primers in PCR assays similar KS_α sequences could be amplified from the Cerylid environmental actinobacterial isolates. In twenty of the twenty-two isolates screened with the KS_α primers, eight of the isolates amplified the 0.47 kb product (Figure 22). Direct sequencing of the 0.47 kb amplified product from the two isolates, A1488 and A3023, verified that KS_α similar sequences had been isolated (Table 35). A total of 450 bp of the amplified fragment was able to be sequenced from isolates A1488, and 366 bp from isolate A3023. A gapped-FASTA database similarity search using the nucleotide sequence revealed a high degree (67-87%) of sequence similarity from both sequenced products isolated from the environmental isolates to known and putative actinobacterial KS_α gene sequences (Table 35). The translated amino acid sequence 152 amino acids, from isolate A1488 was highly similar (62-72%) to KS_α genes from aromatic PKS actinobacteria. It was shown that amongst the actinobacterial species containing the highest matches of the amino acid sequence from A1488, were shown to be with corresponding KS_α genes in *S. curacoi*, *S. fradiae* and *A. hibisca*, which are known to produce angucycline antibiotics (Decker & Haag, 1995; Dairi *et al*., 1997). In the case of isolate A3023 where a minor portion of 118 amino acid was translated, the deduced sequence showed similarities ranging from 50-90%. The highest matches were shown to be with corresponding KS_α genes in *S. curacoi* and *S. halstedii* which produce polyketide derived spore pigments and antibiotics (Bergh & Uhlén, 1992; Blanco *et al*., 1992).

Multiple sequence alignments of the partial amino acid sequence from the amplified internal KS_α fragment from isolate A1488 with corresponding KS_α sequences in other actinobacteria showed that it contains characteristic conserved domains of the protein sequence for the KS_α gene (highlighted in blue in Figure 23), and is in accordance

with the consensus sequence for the KS_α protein (Fernández-Moreno *et al*., 1992). The MSA with the partial sequence obtained from isolate A3023, was also shown to be in accordance with the KS_α gene consensus sequence.

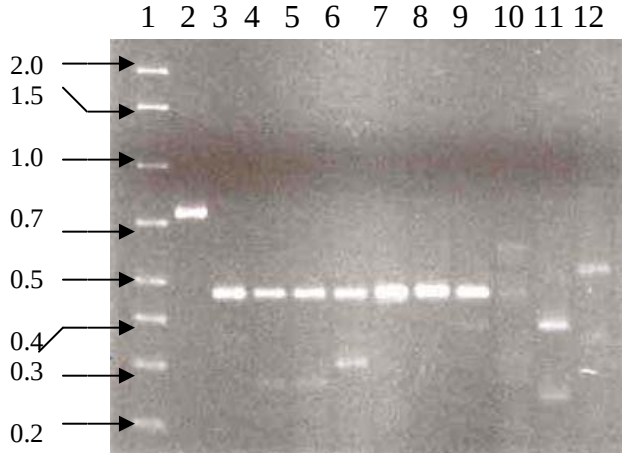


Figure 22. PCR amplification of 0.47 kb KS_α internal fragment by PCR from environmental actinobacterial isolates. Lane 1: Molecular weight marker in kilo bases (kb). Lane 2: Positive control 16SrDNA 27f and 765r primers with *S.coelicolor* genomic DNA., Lane 3: A1113., Lane 4: A3675., Lane 5: A2226., Lane 6: A2010., Lane 7: A3023 Lane 8: A1488., Lane 9: A2056., Lane 10: A3014., Lanes 11 and 12, non-specific amplification products from isolates A2834 and A2360.

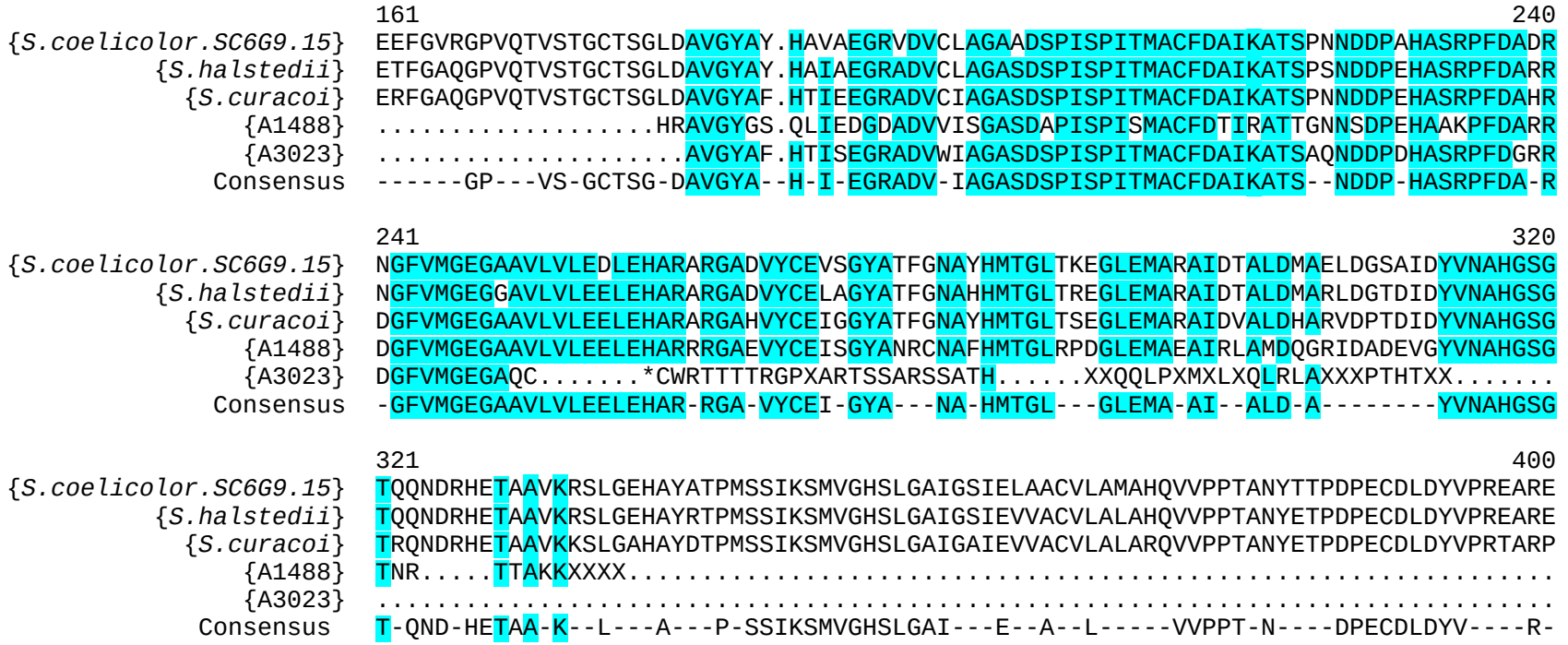


Figure 23. Multiple sequence alignment of β-Ketoacyl synthase (KS_{α}) genes from type II PKS of spore pigment genes in actinobacteria *S. coelicolor* SC6G9.15 (SWISS-PROT # P23155;*whiE*); *S. halstedii* (SWISS-PROT # Q05356;*sch1*); *S. curacoi* (SWISS-PROT # Q02578;*curD*) with amplified environmental KS_a genes from environmental actinobacteria isolates A1488 and A3023.

Table 35: Cerylid actinobacterial isolates A1488 and A3023 nucleotide and translated amino acid sequence, percentage similarity with aromatic PKS genes using gapped-FASTA database search.

		% Similarity			
Actinobacteria	**PKS Gene**	**A1488**		**A3023**	
		Amino Acid	**Nucleic Acid**	**Amino Acid**	**Nucleic Acid**
A. hibisca^	ORF1	68	76	70	74
A. verrucosospora	PKS	67	73	66	70
K. ariduim[a]	ardIORF1	71	74	70	69
Sacch. hirsta[b]	orf4	67	75	60	70
S. arenae	ncnA	65	70	67	70
S. albofaciens	KS	66	74	64	76
S. albus	KS	68	74	55	69
S. antibioticus	simA1	62	74	71	72
S. argillaceus	mtmB	63	70	70	72
S. argillaceus	mtmP	65	70	66	70
S. aureofaciens	aur2A	62	74	68	72
S. aureofacens	TcsD	70	74	61	70
S. cinnamonensis	KS	71	73	65	68
S. capoamus	KS	72	74	50	70
S. coelicolor	actIORF1	68	71	69	71
*S. coelicolor**	ORFIII whiE	69	74	72	77
S. bobili	KS	70	73	66	70
S. collinus	rubA	66	76	50	76
S. collinus	pksA-ORF1	71	74	70	68
*S. curacoi**	curA	71	77	90	80
S. cyanogens	lanA	65	75	70	73
S. galilaeus	aknB	70	72	66	70
S. glaucescens	tcmK	72	74	55	69
*S. halstedii**	sch1	70	74	85	78
S. fradiae	urdA	67	74	72	67
S. maritimus	encA	70	72	63	70
S. nogalater	KS	72	74	85	87
S. nogalater	snoa1	69	72	67	69
S. platensis	KS	68	71	62	71
S. purpuras	KS	70	73	69	70
S. rimosus	ORF1	65	70	41	79
S. rochei	orf1	66	72	55	76
S. roseofulvas	frnL	69	73	66	69
S. sp. *PGA6*	pgaA	63	74	62	67
S. tauricus	KS	71	73	65	69
S. steffisburgensis	KS	70	74	66	71
S. venezuelae	jadA	66	77	74	76
S. violaceoruber	gra-orf1	67	72	67	69

* genes involved in spore pigment color development
^ *Actinomadura*, [a] *Kibdelosporangium*, [b] *Saccharopolyspora*

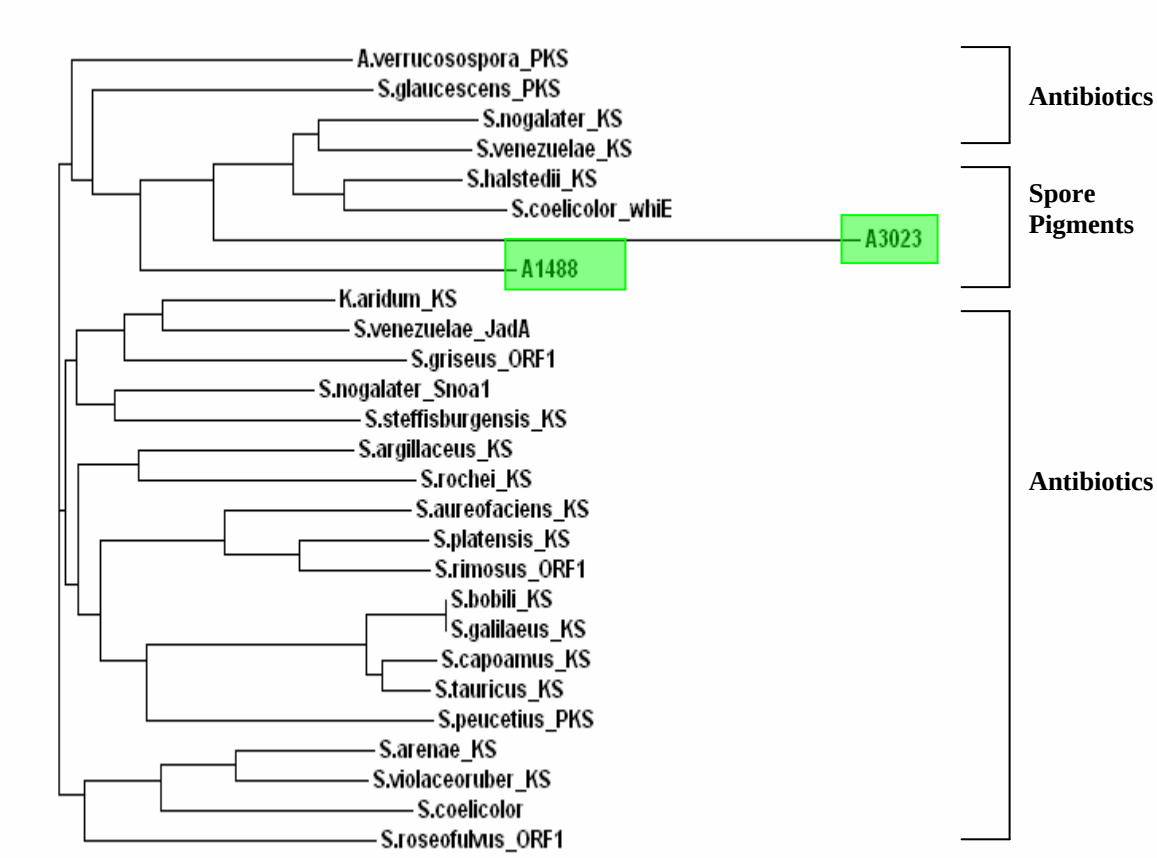


Figure 24. Unrooted neighbour-joining phylogenetic tree constructed from amino acid sequences of KSα genes fragments from type II polyketide synthases. The amino acid sequence accession numbers for the reference strains are as follows; *A. verrucosospora* (BAA82309); *S. glaucescens* (CAA33369); *S. nogalater* (BAA92283); *S. venezuelae* (BAA92282); *S. halstedii* (AAA02833); *S. coelicolor whiE* (CAA39408); *Kibdelosporangium aridum* (AAA67433); *S. venezuelae* (AAB36562); *S. griseus* (CAA54858); *S. nogalater* (CAA12017); *S. nogalater* (BAA92281); *S. argillaceus* (CAA61989); *S. rochei* (BAA87907); *S. steffisburgenesis* (BAA92278); *S. platensis* (BAA92279); *S. rimosus*(CAA80985); *S. bobili* (BAA92272); *S. galilaeus* (BAA92273); *S. capoamus* (BAA92274); *S. tauricus* (BAA92275); *S. purpurascens* (BAA92276); *S .peucetius* (AAA65206); *S. arenae* (AAD20267); *S. vioaceoruber*Tü22 (CAA34369); *S. coelicolor* (CAA45043); *S. roseofulvus* (AAA19616) and amplified *KS*α from the Cerylid isolates A3023 and A1488. The sequences from the reference strains where obtained from GenBank.

The amino acid sequences of 24 known antibiotic type II PKS gene fragments and 2 spore pigment type II PKS gene fragments were used as a basis for classification of the Cerylid isolates. The most clearly separated groups contain genes involved in the biosynthesis of antibiotics (Figure 24). Another identifiable group is the group that is from the spore pigments. Both of the Cerylid isolates A3023 and A1488 have been placed on unique branches by the analysis in the spore pigment group. The type II polyketide synthase phylogenetic tree also shows that the KS gene amplified fragment from isolates A3023 and A1488 are closely related to each other.

3.1.2 PCR Screening Assay for Modular Polyketide Synthases

To evaluate the extent to which modular polyketide synthases exist in the Cerylid actinobacterial cultures, initial PCR evaluations were conducted using designed non-degenerate PCR primers (ole01f and ole01r) to amplify the KS region (Table 20). However, after applying all the standard PCR conditions used for the other SMBG primers, these primers were shown to be of limited use as they only amplified the correct size band of 0.84 kb in one of the four actinobacterial pure cultures tested and known to contain modular PKS. The one positive result was detected in the rapamycin producer *Streptomyces hygroscopicus* ATCC29253, and no products were detected in *Saccharopolyspora erythreae, S. avermitilis* and *S. fradiae*. The PCR result may be partially explained by the amino acid and nucleotide sequences being slightly variable at the 3' end of both the forward and reverse primers across different actinobacterial species (Tables 38 & 39). However the amino acid sequence of the forward primer ole01f is identical to the *Streptomyces* consensus sequence and with further optimisation of the PCR conditions could be useful for incorporating the sequence into a specific primer for this SMBG in streptomyces species (Table 38).

Table 37: Secondary metabolite biosynthetic gene nucleic acid and amino acid sequences incorporated into the design of the ole01f and ole01r PCR primers.

Class/ Gene	Secondary Metabolite(SMBG)	Microorganism	Amino Acid Accession #	Nucleotide Accession #
Type I Polyketides /Ketosynthase	Rifamycin (PKS)	*Amycolopsis mediterranei*	CAA11035	AMM22302
	Macrolide (mycAORF1)	*Micromonnospora griseorubida*	BAA76543	AB017641
	Erythromycin (eryA)	*Saccharopolyspora erythraea*	CAA39583	X56107
	Oleandomycin (oleA1)	*Streptomyces antibioticus*	AAA19695	L09654
	Avermectin (aveA2)	*Streptomyces avermitilis*	BAA84474	AB032367
	Niddamycin (nidA1)	*Streptomyces caelestis*	AAC46024	AF016585
	Tylosin (tlyG)	*Streptomyces fradiae*	AAB66504	SFU78289
	Rapamycin (PKS)	*Streptomyces hygroscopicus*	CAA60460	X86780
	Pimaricin (pimS1)	*Streptomyces natalensis*	CAB41040	SNA132221
	FK506 (fkbA)	*Streptomyces Sp. MA6548*	AAC68815	AF082100
	Pikromycin (pikAII)	*Streptomyces venezuelae*	AAC69329	AF070138
	Spiramycin (KS)	*Streptomyces albofaciens*	BAA92277	AB024975

An alternative strategy was adopted by using published degenerate primers that are specific for the *KS* and *AT* regions of modular PKS genes in actinobacteria (Liu & Shen, 2001). Validation of the specificity of these primers was performed by direct sequencing of the amplified 0.75 kb product from the actinobacterial pure strain *Saccharopolyspora erythraea* (DSM40517), known to contain the *KS* gene (Cortes *et al*., 1990). A partial nucleotide sequence totaling 600 bp of the amplified fragment

was obtained from *S. erythraea* and a portion of this sequence, totaling approximately 90 amino acids was translated. A gapped-FASTA search revealed that the nucleotide and amino acid sequence was shared similarities to known modular PKS genes and their products.

Table 38. Relative amino acid positions and codon degenerecies of ole01f and ole01r primers within the sequence.

Primer	Amino Acid Position	Actual Primer Amino Acid Sequence	*Streptomyces* Amino Acid Sequence[a]	Amino Acid Sequence~	Amino Acid Sequence*
ole01f	59 - 66	FDAAFFGI	FDAAFFGI	FDA(X)FFG(X)	FDAGFFG(X)
ole01r	330 - 338	KSNIGHTQ	KSNIGHTQ	KSN(X)GH(X)(X)	KSNIGH(X)(X)

(X) = variable codon
[a] *Streptomyces* consesus sequence for targeted regions
~ *Streptomycete* and *Non-Streptomycete Actinobacteria species*
* Non - *Streptomycete Actinobacteria species* only

Table 39. Relative nucleic acid positions of the ole01f and ole01r primers and variable nucleotides within the sequence.

Primer	Nucleic Acid Position	Nucleic Acid Sequence~	Nucleic Acid Sequence*
ole01f	474 - 498	NTTCGACGCNGNNTTCTTCGGNAT	NTTCGACCGCNGGNTTCTTCGGGAT
ole01r	1298 - 1323	AGNCNAACATCGGNCANNNNNAN	AGNNNAACATCGGNCACNNNNAN

(N) = variable base
~ *Streptomycete* and *Non-Streptomycete Actinobacterial species*
* Non- *Streptomyces species only*

In the PCR screening assay with the degenerate primers, the 0.75kb product was obtained with eight of the Cerylid cultures tested (Figure 25). Direct sequencing of the amplified products was performed on two isolates A0350 and A1113 using the reverse primer, ATM, which showed that partial sequences were obtained, and translated into respective amino acid sequences, encoded a protein similar to KS (Figure 26). The remaining six PCR products were not sequenced due to lack of time. It was anticipated that by using the portion of the amplified sequence and translating it into the amino acid sequence, the Cerylid cultures could be assigned as containing putative *KS* genes. A partial nucleotide sequence totaling 280 bp of the amplified fragment was sequenced from isolate A1113 and 390 bp from isolate A0350. The translated amino acid sequence of isolate A1113 comprising 90 amino acid and isolate A1113 containing 128 amino acid showed sequence similarity to the protein product

of the *KS* genes of *S. avermitilis* and *S. erythraea*. Database gapped-FASTA sequence similarity search showed that both amplified fragments corresponded to protein sequences of known and putative *KS* genes from antibiotic-producing actinobacteria (Table 40). All of the 750 bp sequence could not be sequenced and only segments of the DNA sequences could be obtained from the two cultures A1113 and A0350 which indicates that direct sequencing using only the one primer ATM may have not been sufficient and that both primers may have been required. DNA sequences obtained from the sequencing carried out on the other six putative mPKS that were amplified in the actinobacterial cultures lacked fidelity and could not be aligned or translated sufficiently to make any justifiable comparisons.

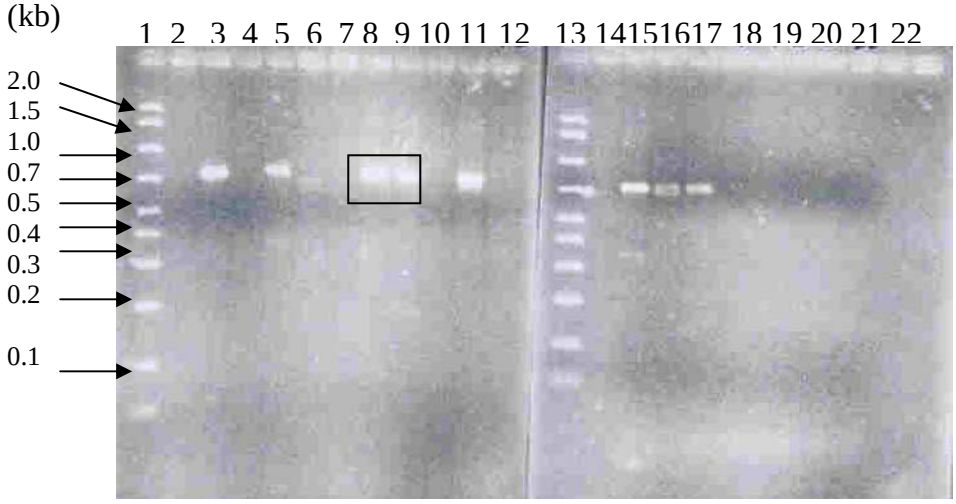


Figure 25. Amplification of 0.75 kb Ketosynthase fragment using degenerate PCR primers from the Ceylid cultures. Lane 1: Molecular weight marker in kilobases (kb). Lane 2: Blank., Lane 3: A3675., Lane 4: A2010., Lane 5: A1113., Lane 6: A3023., Lane 7: A2834 Lane 8: A347., Lane 9: A2702., Lane 10: A3771.,Lane 11: A350 Lane 12: A3014., Lane 13: Molecular weight marker in base pairs (bp). Lane 14: Blank., Lane 15: A2226., Lane 16: A2381., Lane 17: A2360.,Lane 18: A096., Lane 19: A1488., Lane 20: A2376., Lane 21: A1990., Lane 22: A2056. The boxed area indicates that PCR products of the expected size were produced however were very faint and not clearly seen in the photograph.

Table 40: Comparison of PCR amplified KS gene from Cerylid cultures A0350 and A1113 sequence percentage similarity with mPKS genes using the FASTA database search.

		% Similarity			
Actinobacteria[§]	**PKS Gene**	**A0350**		**A1113**	
		Amino Acid	**Nucleic Acid**	**Amino Acid**	**Nucleic Acid**
A. mediterranei	*PKS*	50	70	61	77
M. griseorubida	*mycAORF1*	47	68	72	75
M. inyoensis[a]	*PKS*	69	72	76	77
M. megalomicea. subsp.*nigra*	*megAII*	49	70	64	77
*S. erythraea**	*eryA*	55	71	67	80
*S. spinosa**	*spnC*	51	66	61	74
S. albus	*PKS*	73	75	74	76
S. antiibioticus	*oleAI*	48	71	58	60
S. avermitilis	*aveA2*	55	71	59	75
S. bluensis	*PKS*	70	73	59	62
S. caelestis	*nidA1*	49	69	63	77
S. cinnamonensis	*PKS*	48	70	59	77
S. coelicolor	*SC2C4.04C*	52	73	63	74
S. griseus	*PKS*	46	68	58	79
S. hydroscopicus	*PKS*	52	72	65	73
S. kanamyceticus	*PKS*	52	66	64	78
S. natalensis	*pimSO*	65	68	68	71
S. noursei.	*nysI*	53	70	60	74
S. rimosus	*PKS*	60	65	71	74
S. rochei	*lkm*	70	73	60	75
S. sp. MA6548	*fkbA*	67	70	75	76
S. venezuelae	*pikAII*	53	71	64	79
S.viridochro-mogenes	*aviM*	65	68	66	72
S. netropsis^	*PKS*	58	60	59	66

[§] All *Streptomycete* species are indicated by the letter S., non-*Streptomycetes Actinobacteria* are depicted in the following manner * *Saccharopolyspora.*, ^ *Streptoverticillium.*, [a]*Micromonospora*

Multiple sequence alignments of partial amino acid sequence translated from the amplified DNA sequence from isolates A0350 and A1113, indicated that they also contained conserved domains from *KS* genes (purple coloured boxes figure 26).

Figure 26. Multiple sequence alignment of the ketosynthase (*KS*) gene from secondary metabolite producing actinobacteria *M. sagamiensis* (AAD43312); *Streptoverticillium netropsis* (Q9XCE8); *M. griseorubida* (BAA76543); *S. avermitilis* (BAA84474); *S. erythreae* (CAA39583); *S. spinosa* (AAG23264) and amplified *KS* from the Cerylid environmental actinobacteria A1113 and A0350.

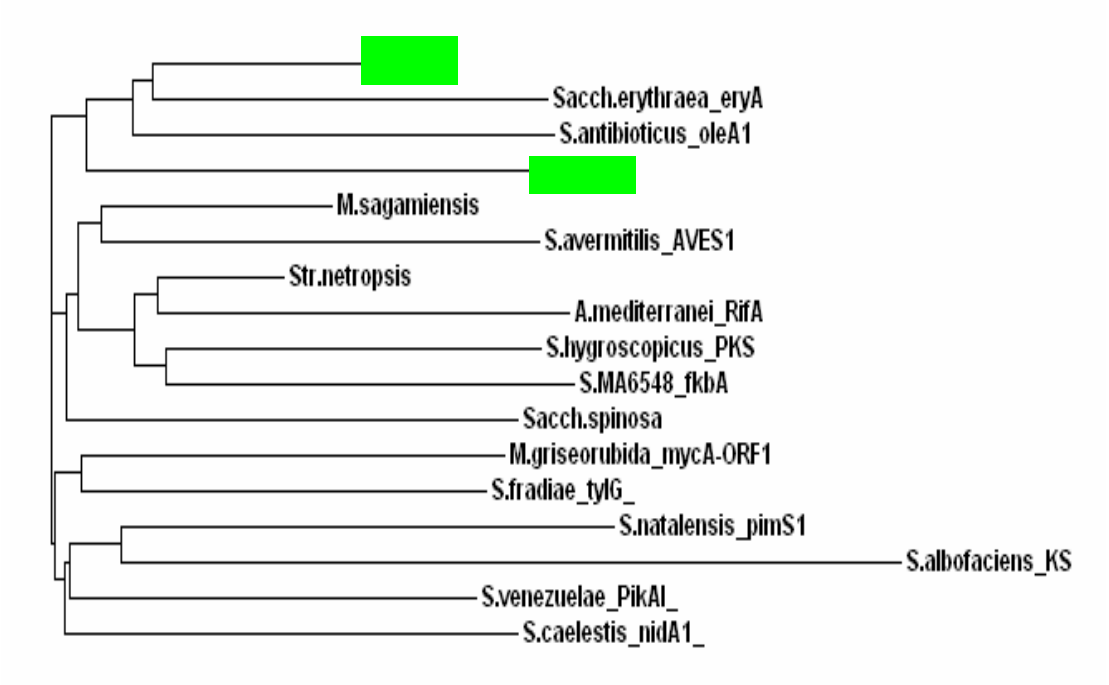


Figure 27. Unrooted neighbour-joining phylogenetic tree constructed from amino acid sequences of KS genes fragments from type I polyketide synthases. The amino acid sequence accession numbers for the reference strains are as follows; *A. mediterranei* (AAC01710); *S. hygroscopicus* (CAA60460); *S. sp.MA6548* (AAC68815); *S. natalensis* (CAB41040); *S. antibioticus* (AAA19695); *M.sagamiensis* (AAD43312); *Streptoverticillium netropsis* (Q9XCE8); *M. griseorubida* (BAA76543); *S.avermitilis* (BAA84474); *S. erythreae* (CAA39583); *S. fradiae* (AAB66504); *S. spinosa* (AAG23264); *S. caelestis* (AAC46024); *S. venezuelae* (AAC69329) and amplified *KS* from the Cerylid isolates A1113 and A0350. The sequences from the reference strains where obtained from GenBank.

The amino acids encoded by 15 known macrolide PKS antibiotic gene fragments were used as a basis for classification of the Cerylid isolates. The phylogenetic tree resolved sequences into four major groups (Figure. 27). The A1113 sequence is similar to *Sachharopolspora erythrae eryA* the PKS responsible for the production of erythromycin, while the A0350 sequence forms a distinctive branch in the phylogenetic tree.

3.1.3 PCR Screening Assay for dTDP-Glucose Synthase

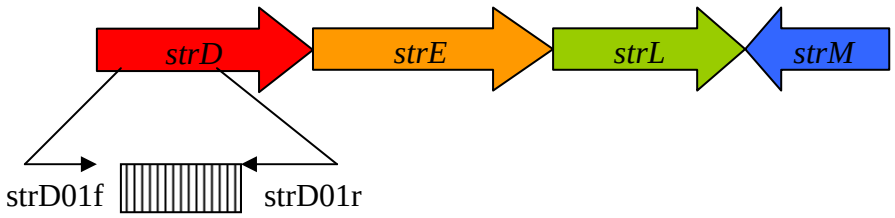
Secondary metabolites such as aminoglycosides often contain deoxygenated hexose sugar components that are essential for biological activity (Liu & Thorson, 1994). A common early enzyme dTDP-glucose synthase involved in the biosynthesis of all types of 6-deoxyhexose was chosen to design PCR primers for screening assays. The PCR primers were designed according to the consensus sequences derived from multiple sequence alignment of 15 actinobacterial dTDP-glucose synthase gene sequences available in the GenBank and EMBL databases. From these 15 sequences, 13 were from *Streptomyces* spp., and two from non-streptomyces these included 1 from a *Micromonospora* sp. and 1 from an *Actinoplanes* sp (Table 40).

Table 40: dTDP-glucose synthase genes with their respective nucleic acid and amino acid sequences used in the design of the strD01f and strD01r PCR primers.

Class / Gene	Secondary Metabolite / (SMBG)	Actinobacteria	Amino Acid Accession Number	Nucleic Acid Accession Number
Deoxyhexose (dTDP – glucose synthase)	Acarbose (acbA)	*Actinoplanes sp. SE50/110*	CAA77210	Y18523
	Fortimicin (fotD)	*Micromonospora olivasterospora*	AAD31892	AF144041
	Oleandomycin (oleS)	*Streptomyces antibioticus*	AAD55453	AF05579
	Mithramycin (mtmD)	*Streptomyces argillaceus*	CAA71846	Y10907
	Oleandomycin (orf8)	*Streptomyces antibioticus* Tü99	AAF59934	AF237894
	Blensomycin (blmD)	*Streptomyces bluensis*	AAD28517	AF126354
	Tylosin (urdG)	*Streptomyces fradiae*	AAA21343	U08223
	Streptomycin (strD)	*Streptomyces glaucescens GLA.0*	CAA07386	AJ006985
	Streptomycin (strD)	*Streptomyces griseus*	CAA68514	Y00459
	Kanamycin (knaD)	*Streptomyces kanamyceticus*	AAD31891	AF144040
	Granaticin (gra-orf16)	*Streptomyces violaceoruber*	AAA99940	L37334
	Nogalamycin (snogJ)	*Streptomyces nogalater*	AAF01820	AF187532
	Paromomycin (prmD)	*Streptomyces rimosus f. paromomycinus*	AAD31893	AF144042
	Spectinomycin (spcD)	*Streptomyces spectabilis*	AAD31796	AF128272
	Avilamycin (aviD)	*Streptomyces viridochromogenes* Tü57	CAA72714	Y11985

The 24 base pair forward primer (strD01f) at position 326 – 350 nucleotides (numbers correspond to *S. griseus* GenBank accession number: Y00459) and the 20 base pair reverse primer (strD01r) at position 677 – 697 nucleotides (Figure 26). FASTA sequence similarity searches of the GenBank/EMBL with the designed primers revealed matching dTDP-glucose synthase gene sequences from other actinobacterial species, ranging from 87 – 100 %. PCR screening experiments using the designed primers amplified the predicted 0.37 kb product size in the two actinobacterial species

Streptomyces and *Micromonospora*, known to contain deoxysugar moieties (Figure 28).



StrD = dTDP-glucose synthase *strE* = dTDP-glucose-4-6-dehydratase *strL* = dTDP-dihydrostreptose synthase *strM* = dTDP-4-keto-6-deoxyglucose 3,5-epimerase

Figure 28. Illustration depicting the organization of the deoxyhexose genes.

Evaluation of the strD01f primer amino acid sequence alignments indicates a highly conserved sequence across different actinobacterial species *XXXXLGDN* [X position represents codon degeneracy's] (Table 41). This sequence could be used to design a primer to screen for the *strD* gene in actinobacteria. The MSA of the primers indicated that they were biased towards *Streptomyces* species (Table 41).

Table 41. Relative amino acid positions and codon degenerecies of the strD01f and strD01r primers in the sequence.

Primer	Amino Acid Position	Actual Primer Amino Acid Sequence	*Streptomyces* Amino Acid Sequence[a]	Amino Acid Sequence~	Amino Acid Sequence*
strD01f	111 - 118	FAMYLGDN	FVMYLGDN	(X)(X)M(X)LGDN	(X)(X)M(X)LGDN
strD01r	182 - 187	YGRTPA	YLFTPA	Y(X)(X)(X)(X)(X)	Y(X)Y(X)(X)D

(X) = variable codon
[a] *Streptomyces* consesus sequence for targeted regions
~ *Streptomycete* and *Non-Streptomycete Actinobacteria species*
* Non - *Streptomycete Actinobacteria species* only

Table 42. Relative nucleic acid positions of the strD01f and strD01r primers and variable nucleotides within the sequence

Primer	Nucleic Acid Position	Nucleic Acid Sequence~	Nucleic Acid Sequence*
strD01f	326 - 350	CNTNGNNNTGNNNCTNGGNGACAA	NNTNNNNNTNNNNCTNGGCGACAA
strD01r	677 - 697	No Consensus[a]	N/C

(N) = variable base
N/C - means that there are no corressponding sequences for a comparison to be made
[a] No consensus implies that sequences were highly variable and no conserved regions were detected
~ *Streptomycete* and *Non-Streptomycete Actinobacterial species*
* Non- *Streptomyces species only*

Despite this the strD01f and strD01r primers bias towards *Streptomyces* species produced partial bands of expected size in both the *Micromonospora* species tested. This indicated that a similar sequence was present in the *Micromonospora* species genome (Figure 29). Additional extra bands of unexpected size were detected which may have resulted from partial homologies between primers and template DNA (Figure 29:lane 4).

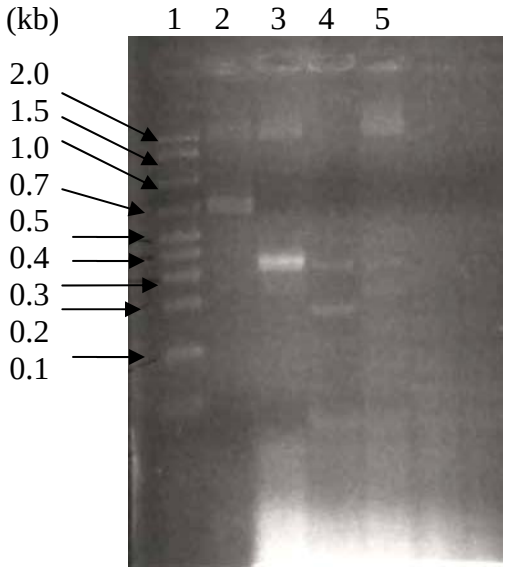


Figure 29. Agarose gel electrophoresis of 0.37 kb strD segment amplified DNA from aminoglycoside positive actinobacterial type cultures. Lanes 1: Molecular weight marker in kilobases (kb). Lane 2: *S. coelicolor* M145 DNA amplified 16SrDNA region. Lanes 3: *Streptomyces griseus* (DSM40236), Lane 4: *Micromonospora purpurea* (DSM43036), Lane 5: *Micromonospora olivasterospora* (ATCC21819).

Direct sequencing of the amplified 0.37 kb product from the pure strain *Streptomyces griseus* DSM40236, known to contain the *strD* gene (Distler *et al.*, 1987) confirmed that the correct gene had been amplified. A Gapped-FASTA database search revealed that both the nucleotide and translated amino acid sequences derived from this type strain showed sequence similarities (58 – 83%) and (54 - 80%), respectively, to known dTDP-glucose synthase genes and their product in actinobacteria (Table 43).

The PCR product obtained with the strD01f and strD01r primers indicated that putative dTDP-glucose synthase genes could be detected using this primer pair for the Cerylid isolates. This was indicated by the PCR products of the predicted length

which were obtained with six of the Cerylid isolates (Figure 30). A qualitative assessment only could be made based on the presence of the amplified band, as no direct sequencing was performed due to the lack of time.

Table 43: Comparison of nucleotide and amino acids from amplified dTDP-glucose synthase gene from *Streptomyces griseus* DSM40236 Type Strain with dTDP-glucose synthase from different actinobacterial strains. Percentage similarity obtained using the FASTA database search.

		% Similarity	
Actinobacteria[1]	**dTDP-glucose synthase gene**	*Streptomyces griseus* DSM40236 **Nucleic Acid**	**Amino Acid**
Actinoplanes sp. *SE50/110*	*acbA*	60	58
M.olivasterospora^	*fotD*	59	56
*S.spinosa**	*Gtt*	62	60
S.antibioticus Tu99	*oleS*	73	69
S.argillacens	*mtmD*	76	69
S.bluensis	*blmD*	83	77
S.collinus	*napG*	59	54
S.cyanogenus S136	*lanG*	72	61
S.fradiae	*urdG*	72	60
S.glaucescens	*StrD*	81	79
S.griseus	*strD*	82	78
S.kanamyceticus	*knaD*	72	80
S.peucetius	*dnrL*	70	67
S.rimosus	*prmD*	71	63
S.rishiriensis	*couV*	59	57
S.spheroides	*novV*	58	55
S.violaceoruber	*gra-orf16*	69	55
S.viridochromogenes	*avid*	71	62

[1] All *Streptomycete* species are indicated by the letter S., non-*Streptomycetes Actinobacteria* are depicted in the following manner * *Saccharopolyspora.*, ^*Micromonospora.*

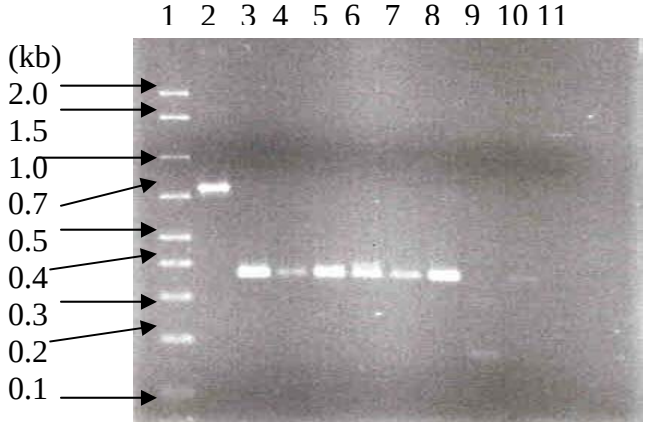


Figure 30: Amplification of 0.37 kb glucose dTDP glucose synthase fragment by PCR from environmental actinobacterial cultures. Lane 1: Molecular weight marker in kilobases (kb). Lane 2: Positive control 16SrDNA 27f and 765r primers.,Lane 3: A2010., Lane 4: A1113., Lane 5: A2707., Lane 6: A350., Lane 7: A2056., Lane 8: A371., Lane 9: A1990., Lane 10: A096., Lane 11: A2834.

3.1.4 PCR Screening Assay for Isopenicillin N Synthase Gene

Isopenicillin N synthase (IPNS) is a essential enzyme which catalyzes the formation of the lactam ring in the biosynthetic pathway of β-lactam antibiotics. PCR primers were designed according to the consensus sequence within the *pcbC* genes encoding isopenicillin N synthases, this consensus sequence was derived from the multiple sequence alignment from the seven actinobacterial *pcbC* genes available in the GenBank/EMBL database at that time, six *pcbC* gene sequences were derived from *Streptomyces* spp. and a *pcbC* gene sequence was from *Amycolatopsis* sp. which is a non-streptomyces species (Table 44).

Table 44: Isopenicillin N synthase genes with their corresponding nucleic acid and amino acid sequence accession numbers incorporated into the design of the pcbC03f and pcbC03r PCR primers.

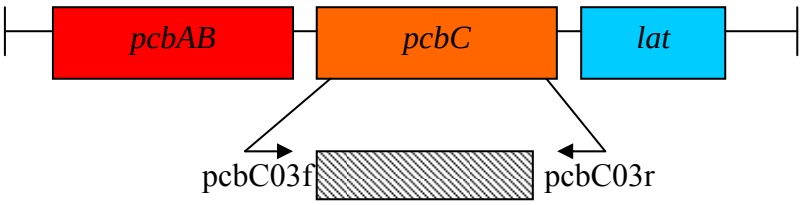
Class / Gene	Secondary Metabolite / (Secondary Metabolite Biosynthetic Gene)	Actinobacteria	Amino Acid Accession Number	Nucleic Acid Accession Number
β-lactams (Isopenicillin N synthase)	Nocardicins (*pcbC*)	*Amycolatopsis lactamdurans*	CAA40562	X57310
	Thienamycin (IPNS)	*Streptomyces cattleya*	BAA11234	D78166
	Clavulanic Acid (IPNS)	*Streptomyces clavuligerus*	CAA00131	A01132
	Cephamycin C (*pcbC*)	*Streptomyces griseus*	CAA38431	X54609
	Cephalosporins (IPNS)	*Streptomyces jumonjinensis*	AAA26772	M36687
	Cephalosporins (IPNS)	*Streptomyces microflavus*	AAA26771	M22081
	SF2103(*pcbC*)	*Streptomyces sulfonofaciens*	AAD30553	AF141676

The 23 base pair forward primer (pcbC03f) was positioned at 305-328 nucleotides (numbers correspond to *S. griseus* GenBank accession number: X54609) and a 22 base pair reverse primer (pcbC03r) at position 648-670 nucleotides (Figure 31). The expected 0.35 kb product was amplified from the two β-lactam producing actinobacterial pure strains within the *pcbC* gene (Figure 32). Matching nucleotide sequences in the GenBank and EMBL databases with the sequences of the designed primers revealed high similarities ranging from 80-100 % for corresponding *pcbC* genes in actinobacterial species, as expected as this gene contains greater than 80 % similarities at the nucleotide level (Shiffman *et al*., 1988).

Specificity assays conducted on the β-lactam producing actinobacterial species indicated that only PCR products of the correct size were obtained from *Streptomyces*

species (Figure 32) which is reflected in the sequence bias of both primers and indicating that the primers are highly specific for *Streptomyces* species (Table 45).

Gene Organization of Beta Lactam Synthase Genes



PCR product = 0.355 kb

pcbAB = δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine *pcbC* = Isopenicillin N synthase

lat = lysine aminotransferase

Figure 31. Illustration depicting organization of the β-lactam genes in actinobacteria. PCR amplification of internal fragment within the *pcbC* gene.

A positive control was included in the assays (Lane 2: Figure 32) which indicated that the PCR reactions components were working properly and that an amplification product could be produced. The pcb03f primer amino acid sequence across the actinobacterial species in the MSA indicated that it was highly degenerate (Table 45) containing the following sequence *XSXXYXN* (X position represents codon degeneracy's) and may not be suitable for PCR screening of different actinobacterial β-lactam producers. However this primer could be used to selectively detect novel *Streptomyces* species containing the *pcbC* gene.

Table 45. Relative amino acid positions and codon degenerecies of pcbC03f and pcbC03r primers within the sequence.

Primer	Amino Acid Position	Actual Primer Amino Acid Sequence	*Streptomyces* Amino Acid Sequence[a]	Amino Acid Sequence~	Amino Acid Sequence*
pcbC03f	102 - 108	ESWCYLN	ESFCYLN	(X)S(X)(X)Y(X)N	N/C
pcbC03r	216 - 223	DHLDVSMI	DHLDVSMI	(X)HL(X)VSMI	N/C

(X) = variable codon

N/C - means that there are no corresponding sequences for a comparison to be made

[a] *Streptomyces* consesus sequence for targeted regions

~ *Streptomycete* and *Non-Streptomycete Actinobacteria species*

* Non - *Streptomycete Actinobacteria species* only

Table 46. Relative nucleic acid positions of pcbC03f and pcbC03r primers and variable nucleotides within the sequence

Primer	Nucleic Acid Position	Nucleic Acid Sequence~	Nucleic Acid Sequence*
pcbC03f	305 - 328	NNAGTCCTNNNNNTACCNNAACC	N/C
pcbC03r	648 - 670	GNCCANNTNGNNGTNTCGATGAT	N/C

(N) = variable base
N/C - means that there are no corressponding sequences for a comparison to be made
~ *Streptomycete* and *Non-Streptomycete Actinobacterial species*
* Non- *Streptomyces species only*

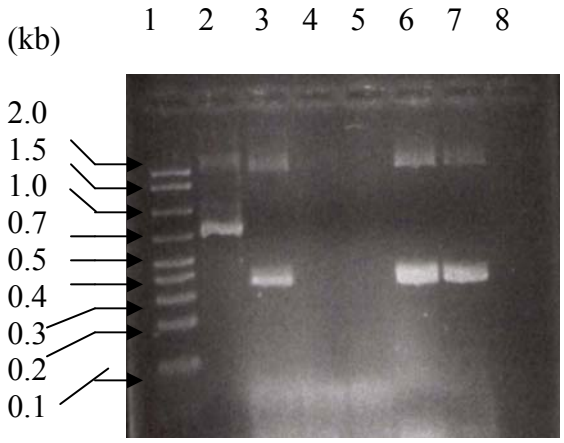


Figure 32 . Agarose gel electrophoresis of 0.35 kb isopenicillin N synthase (*pcbC)* segment amplified DNA from β-lactam positive actinobacterial type cultures. Lane 1: Molecular weight marker kilobases (kb). Lane 2: *Streptomyces coelicolor* M145 DNA amplified 16SrDNA region. Lanes 3: *Streptomyces cattleya* (NRRL8057), Lane 4: *Nocardia uniformis* subsp. *tsuyamanensis* (ATCC21806). Lane 5: *Streptomyces clavuligerus* (NRRL3585). Lane 6 & 7 *Streptomyces griseus* (NRRL3851). Lane 8: negative control (no template DNA).

Direct sequencing was successfully performed on the amplified 0.35 kb product from the two β-lactam producing actinobacterial type strains *Streptomyces griseus* NRRL3851 and *Streptomyces cattleya* ATCC8507, known to contain the IPNS gene (Gracia-Dominguez *et al*., 1991; Wang & Li, 1996). Both the nucleotide and translated amino acid sequences derived from both type strains showed the highest similarities with the corresponding IPNS gene. Table 47 shows that the sequence

similarities for *Streptomyces griseus* was 98 - 99% respectively and the IPNS sequence from *Streptomyces cattleya* showed 96 – 98 % respectively. The sequenced IPNS genes also matched with known *pcbC* genes in a number of actinobacteria (Table 47).

Table 47: Comparison of nucleotide and amino acid sequences from isopenicillin N synthase (*pcbC*) gene with sequenced *Streptomyces griseus* NRRL3851 and *Streptomyces cattleya* ATCC8507 Type Strains. Percentage Similarity obtained using the FASTA database search.

	% Similarity			
Actinobacteria	*Streptomyces griseus NRRL3851*		*Streptomyces cattleya ATCC8507*	
	Nucleic Acid	**Amino Acid**	**Nucleic Acid**	**Amino Acid**
N. lactamdurans	78	77	77	76
S. argenteolus	83	77	83	81
S. cattleya	82	78	98	97
S. fimbriatus	87	87	77	73
S. griseus	99	98	77	74
S. hygroscopicus	89	90	80	78
S. heteromorphus	89	89	82	79
S. jumonjinensis	82	76	82	81
S. lipmanii	78	73	78	69
S. panayensis	91	90	81	73
S. sulfonofaciens	74	39	73	64
S.viridochromogenes	87	89	80	76
S. wadayamensis	87	84	79	76

The amplification product and correct sequence obtained using the primer pair pcbC03f and pcbC03r in the pure cultures indicated that corresponding presumptive *pcbC* genes could be detected in the Cerylid isolates. Screening for presumptive *pcbC* genes in the Cerylid isolates resulted in the amplification of the expected 0.35 kb band from one of the Cerylid isolates (A2360). Unexpected high molecular weight products in the 0.4 – 0.55 kb range were amplified in four Cerylid isolates A2010, A1113, A3023, A0347. Due to time constraints, verification of the amplified products by DNA sequencing was not carried out.

Table 48. Summary of PCR screening on environmental isolates*.

Isolate #	Type I PKS	Type II PKS	Deoxysugar	Beta-Lactam
A0096	-	-	+	-
A0347	+	-	+	-
A0350	+	-	+	-
A0371	-	-	+	-
A1113	+	+	+	-
A1488	-	+	-	-
A1664	-	-	-	-
A1990	-	-	-	-
A2010	-	+	+	-
A2056	-	+	+	-
A2226	+	+	-	-
A2360	+	-	-	+
A2376	-	+	-	-
A2381	+	-	-	-
A2702	+	-	+	-
A2834	-	-	-	-
A3014	-	+	-	-
A3023	-	+	-	-
A3675	+	+	-	-
A3771	+	-	-	-

\+ *PCR Positive amplification of the correct sized product*
\- *PCR Negative no amplification of the correct sized product*
* Shaded boxes indicate confirmation of correct PCR product was carried out by partial sequencing.

3.2 Chemical screening for Secondary Metabolites in Actinobacteria

3.2.1 Bioassays of Environmental Actinobacterial Cultures

Environmental actinobacterial cultures which were identified as containing either one or more secondary metabolite biosynthetic genes (SMBG) by PCR, were subjected to biological and chemical assays to determine their ability to produce secondary metabolites. A large proportion of secondary metabolites possess antimicrobial activities (Hutchinson & Fuji, 1995), hence antimicrobial assays were conducted.

3.2.1.1 Plug Type Antimicrobial Assays

Results of the initial screening for *in vitro* antimicrobial activity of agar plugs of 7 – 10 day old cultures of the environmental actinobacterial cultures, are shown in Table 49.

Table 49. Antibacterial and antifungal activities of extracts from the Cerylid Environmental Actinobacteria Cultures: plug and well type diffusion assays.

Extract	**Test organisms**					
	S. aureus		*B. pumilus*		*C. albicans*	
	P.T.*	W.T.^	P.T.	W.T.	P.T.	W.T.
A0096	-	+	+	-	-	-
A0347	-	+	+	-	+	-
A0350	+	+	+	+	+	+
A0371	-	+	-	-	-	-
A1113	+	+	-	+	+	+
A1488	-	-	-	-	-	-
A1664	-	+	-	-	-	-
A1990	+	+	+	-	-	-
A2010	-	+	-	-	-	-
A2056	-	+	+	-	-	-
A2226	-	+	+	-	+	+
A2360	-	+	+	-	-	-
A2376	-	-	-	-	-	-
A2381	-	+	-	+	+	-
A2707	-	+	+	-	-	-
A2834	+	-	-	-	-	-
A3014	-	+	+	-	-	-
A3023	+	+	-	-	-	-
A3675	-	+	+	-	+	+
A3771	+	+	+	-	-	-

* P.T. = Plug Type Assay; ^W.T. = Well Type Assay

\- = Not Active (No Inhibition)

\+ = Active (Inhibition)

Of the twenty cultures screened, 70 % contained antibacterial activity and 30 % contained antifungal activity. No antimicrobial effect was detected against *E. coli*.

3.2.1.2 Well Type Antimicrobial Assays of Metabolites Extracted from Agar

The well-type assay involved testing metabolites extracted with methanol from 7-10 day old cultures grown on YME agar. Table 49 indicates that a higher proportion (85%) of the cultures were active against *S. aureus* and *B. pumilus* which represent Gram positive bacteria than compared with that of *C. albicans* (15%) which represent fungi. No antagonistic effect was detected against the gram negative *E. coli* bacteria.

The metabolite producing isolates were subjected to further studies using liquid fermentation media (section 3.2.2) and cultures having no SMBG or antimicrobial activities were subjected to thin layer chromatography studies (section 3.2.4).

3.2.2 Small Scale Liquid Fermentations of Antimicrobial Producing Actinobacteria

3.2.2.1 Well -Type Antimicrobial Assays of Metabolites Extracted from Liquid Fermentation Media

Eighteen environmental actinobacterial cultures containing antimicrobial activities on solid agar media, were grown in liquid production media. Nine different liquid production media were evaluated (Table 28). Table 50 shows the results of the bioactivity of six environmental actinobacteria. Antimicrobial activities were detected in both the broth supernatant and in the methanol (MeOH) extracted mycelium, as indicated by the zones of inhibition (Table 50). Higher antimicrobial activities were obtained with liquid media compared with solid agar media. Mycelial extracts from strains A0350, A1113 and A2381 were active against *S. aureus, M. luteus* and *B. pumilus* and *C. albicans*, whereas other mycelial extracts showed inhibition activities only against *S. aureus, M. luteus* and *B. pumilus* (A0347, A2707) or *C. albicans* (A3675). Controls incorporating pure MeOH solution indicated it had no antimicrobial activity. Naldixic Acid was incorporated as a positive control which produced zones of inhibition and served as an indicator of the proper functioning of the antimicrobial screen.

Table 50: Fermentation analysis, depicting the antimicrobial activities for supernatant and mycelial extracts of the Cerylid environmental actinobacterial isolates.

Isolate Number	Zones of Inhibition*						Optimal Fermentation Medium~	Optimal Fermentation Time (hr)
	Supernatant			Mycelial Extract				
	S. aureus	*M. luteus*	*C. albicans*	*S. aureus*	*M. luteus*	*C. albicans*		
A0350	++	+++	+	++	+++	+	Dextrin	240
A1113	++	+++	++	+++	+++	++	153	240
A2381	++	+++	++	+++	+++	+++	Dextrin	240
A3675	N/D	N/D	+	N/D	N/D	21.5	153m	168
A0347	N/D	++	N/D	N/D	++	N/D	IM25	240
A2707	N/D	++	N/D	N/D	++	N/D	IM25	240

* Zones of Inhibition: + 8 – 15 mm, ++ 16 – 25 mm, +++ >25 mm, - no inhibition
N/D: no zones of inhibition detected
~ ingredients for fermentation media described in the materials and methods (Table 28)

The optimal fermentation time was determined by taking samples of the medium at 24 hour intervals and analyzing the broth supernatant and mycelial extract in well type antibacterial assays (section 2.7.2). The different types of liquid production media influenced the chemical expression of metabolites (Table 50). Fermentation production media which showed enhanced or induced antimicrobial activities included Dextrin, 153 and IM25. In other cases where the same culture was inoculated into different liquid production media antimicrobial activity was reduced or not detectable. This effect was observed in SI and 153m liquid media.

3.2.3 Improving Antimicrobial Metabolite Production in Low Yielding Actinobacteria Cultures

3.2.3.1 Solid-Substrate Fermentations (SSF)

Actinobacterial cultures exhibiting low antimicrobial activity in liquid media fermentations were also subjected to solid-substrate fermentations, to determine if the productivity of metabolites could be enhanced. Table 51 indicates that inoculating low yielding actinobacterial cultures into solid state media either suppressed or enhanced antimicrobial activities depending on the fermentation conditions. Only isolate A2707 culture showed an increased antimicrobial effect due to the solid substrate.

Table 51: Comparison of fermentation conditions for bioactive metabolite screening^.

	Fermentation Medium*		
Environmental Isolate Number	**Liquid[a]**	**Solid Substrate**	**Liquid-Oil**
A3675	+	+	+++
A0347	++	+	+++
A2707	+	++	+++
No Inoculum Control	-	-	-

^ well type assays measured against *M.luteus*
* Zones of Inhibition: + 8 – 15 mm, ++ 16 – 25 mm, +++ >25 mm, - no inhibition
[a] Liquid medium used was IM25

In order to further investigate the productivity of culture A2707 using SSF, a survey of various solid substrates was carried out to explore the effects of the different solid substrates or variations thereof for enhancement of metabolite production. The solid substrates that were selected for this study consisted of the most commonly used substrates used in solid substrate fermentations these consisted of natural materials which serve both as a support and nutrient source and a commonly used inert support which serves as an anchor point for microorganisms (Barrios-González and Mejía, 1996). As depicted in table 52 different solid substrates affected antibacterial activity and metabolic profile. From the grains used in this study culture A2707 produced well on linseed, sunflower kernels (LSA), corn (C), rye (RY), rice (R), wheat bran (WB) and whole oats (WO). The inert material, perlite did not prove to support the production of the antimicrobial compound produced using the grains. The majority of the antimicrobial activity was shown to be in the solid substrates with the addition of mineral and trace element liquid supplements such as solutions LF42 and SF29 however there is only a small difference between the two. It is evident that the presence of antimicrobial activity is influenced by the availability of nutrients, this is seen with whole oats and burghal which were impregnated with water no antimicrobial activity is detected however when liquid supplements were added antimicrobial activity is detected. Solid substrates supplemented with only water in most cases showed suppressed anti-bacterial activity but showed a complex metabolic profile shown by thin layer chromatography (Table 52).

Table 52. Characterisation of ethyl acetate extracted metabolites from isolate number A2707 on different solid-substrate fermentation media.

Media Type*	Well-Type Assay^	TLC Number of Bands	Extract Colour
WSF29	++	4	Yellow tinge
WBYLF42	++	3	Yellow
WBYH_20	++	3	Yellow
WOH_20	-	8	Yellow tinge
WOSF29	++	4	Orange
RSF29	++	5	Clear
RH_20	++	2	Yellow
RLF42	++	5	Yellow
RYSF29	++	5	Yellow
RYLF42	++	7	Pale Yellow
RYH_20	++	6	Yellow
BYH_20	++	3	Yellow
BYLF42	++	2	Yellow
BYSF29	++	3	Pale Yellow
PH_20	-	ND	Clear
PLF42	++	2	Clear
PSF29	-	ND	Clear
PHSF29	++	3	Yellow Tinge
PHLF42	+	4	Clear
CH_20	++	4	Yellow
CLF42	++	5	Yellow
LSAH20	++	1	Orange
BH_20	-	7	Yellow
BSF29	++	5	Yellow
BLF42	++	6	Yellow

* Fermentation media key:
H_20 (water)
Liquid Solutions: LF42 (trace elements), SF29 (minerals)
Solid Substrate: W = Wheat Bran., WBY = Whole Barley., WO = Whole Oats., R= Rice., RY = Rye., BY = Barley., P = Perlite., PH = Psyllium Husk., C = Corn., LSA = Linseed, Sunflower Kernals, Almonds., B = Burghal
^ Antimicrobial activity was measured against *M. luteus*.
^ Zones of Inhibition: + 8 – 15 mm, ++ 16 – 25 mm, +++ >25 mm,
- no inhibition, ND = not detected

The EtAc extract color was also recorded to observe if any color changes occurred as a result of the extraction process. The EtAc extract of culture A2707 which has an orange appearance in liquid media produced two colorations on solid media either yellow or clear extracts (Table 52). The yellow EtAc extracts were the more bioactive and expressed a greater number of metabolites than the clear extracts.

3.2.3.2 Liquid Fermentations Supplemented with Refined Oils

Actinobacterial cultures showing low antimicrobial activities in liquid media were fermented in media supplemented with refined oils to determine their usefulness as additives in enhancing liquid fermentations. In the case of cultures A2707 and A0347, higher activities were detected against the same pathogen when grown in oil-supplemented fermentations than in liquid media alone (Tables 50 and 53). With isolate A3675 incorporation of refined oils resulted in additional antibacterial activity not detected in liquid media alone. A summary of the effect of addition of specific refined oils on the production with 3 cultures is listed in table 53. The refined oils tested in this study exhibited an enhancing effect from low yielding metabolite producing actinobacterial liquid fermentations (Table 53). A limitation of this preliminary screening experiment was that extracts from the uninoculated media containing the refined oils were not obtained, thus the anti-bacterial activity attained cannot conclusively be said to be due to the oils as the oils themselves are known to contain antimicrobial activities.

Table 53. Well-Type anti-bacterial assay of mycelium extracted metabolites from actinobacterial liquid-oil fermentations after 10 days incubation.

	Zones of Inhibition^		
Medium Type*	**A3675**	**A2707**	**A0347**
IM25 + No Inoculum	-	-	-
IM25 + VO	+++	+++	+++
IM25 + SUN	+++	+++	+++
IM25 + OLV	+++	++	++
IM25 + HAZ	++	+++	++
IM25 + WAL	++	+++	++
IM25 + SAF	++	+++	+++
IM25 + SOY	++	++	+++
IM25 + ALM	++	+++	++

*Fermentation Media Key: IM25 Liquid Media, VO = Vegetable Oil., SUN = Sunflower Oil., OLV = Olive Oil., HAZ = Hazelnut Oil., WAL = Walnut Oil., SAF = Safflower Oil., SOY = Soya Oil., ALM = Almond Oil.

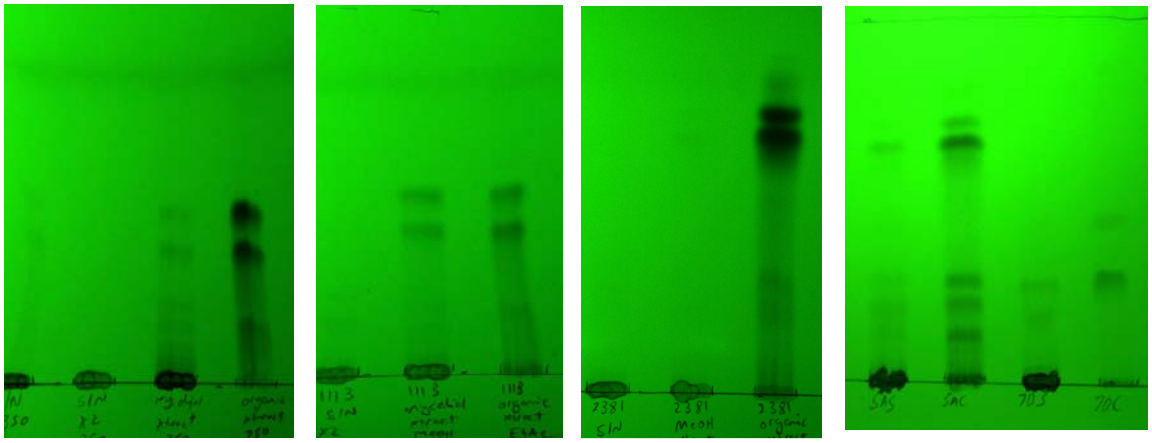
^ Zones of Inhibition: + 8 – 15 mm, ++ 16 – 25 mm, +++ >25 mm,
- no inhibition

^ Activity was measured against *M. luteus*

3.2.4 Thin Layer Chromatography (TLC) of Antimicrobial Metabolites from Fermented Actinobacterial Cultures

3.2.4.1 Liquid Fermentations

As a result of preliminary antimicrobial screening optimal fermentation medium was selected for each actinobacterial culture based on the strongest antibacterial activity. Extracted fractions from these actinobacterial cultures were collected and further analysed using thin layer chromatography to obtain a chemical metabolic banding profile. Three fractions were obtained these consisted of the broth supernatant, methanolic and EtAc extracts of the mycelium As shown in figure 33, metabolites were present in the MeOH or EtAc extracts. The EtAc extracts from isolates A0350, A1113 and A2707 in lanes 4, 7 and 12 respectively, displayed a banding pattern that was stronger in intensity than those extracted from the broth supernatant (lanes 1, 5, and 8) and methanol (lanes 3 & 6), indicating that EtAc was better suited for extraction of these metabolites. Similar chemical banding profiles were present in the MeOH extracted mycelium from isolates A0350 and A1113, indicating that the same compounds were present in the mycelium (lanes 3 & 6), however the banding intensity was not as strong as that seen in the EtAc extracts (lanes 4 & 7). In certain extractions, metabolites were only extractable using EtAc for isolate A2381 (lane 10), indicating a selective isolation step which can be incorporated into the purification process for this class of compound.



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane 1: Dextrin fermentation medium with A0350 isolate (Broth Supernatant)
Lane 2: Dextrin fermentation medium with A0350 isolate extracted twice with Ethyl Acetate

Lane 3: Mycelial extract of A0350 isolate with methanol
Lane 4: Organic ethyl acetate extract of A0350 isolate
Lane 5: Fermentation medium #153 with A1113 isolate (Broth Supernatant)
Lane 6: Mycelial extract of A1113 isolate with methanol
Lane 7: Organic ethyl acetate extract of A1113 isolate
Lane 8: Dextrin fermentation medium with A2381 isolate (Broth Supernatant)
Lane 9: Dextrin fermentation medium with A2381 isolate extracted twice with Ethyl Acetate
Lane 10: Organic ethyl acetate extract of A2381 isolate
Lane 11: Fermentation medium#IM22 with A2707 isolate (Broth Supernatant)
Lane 12: Mycelial extract of A2707 isolate
Lane 13: Fermentation medium#IM22 with A2360 isolate (Broth Supernatant)
Lane 14: Mycelial extract of A2360

Figure 33. Thin Layer Chromatography of Fermented Actionbacterial Cultures. TLC on pre-coated silica gel plates ($60F_{254}$ 20 X 20 cm, 0.2 mm thickness). Ethyl Acetate:Methanol (90:10) solvent system.

By using the methanol:ethyl acetate (90:10) solvent system it was evident that there were common metabolic products produced, this was represented by bands at various retention times (R_f) seen amongst the seven actinobacteria tested. All of the isolates produced polar compounds as indicated by their small R_f values. Bands 1,2 and 4 may represent a common chemical species (Table 54). However, certain isolates contained unique banding profiles, such as bands 3 and 6-9.

Table 54. Chemical metabolic banding profile of compounds using TLC visualised under short wavelength fluorescent light at 254_{nm} and biological active fractions from mycelial extracts.

Isolate Number	R_f values of bands								
	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9
A0350	0.11*	0.21*	-	0.45*	0.53*	-	-	-	-
A1113	0.16	0.26	-	0.43*	0.54*	-	-	-	-
A2381	-	0.22	-	0.43	-	0.61*	0.68*	-	-
A2707	0.19	0.26	0.30	-	-	-	-	0.7	0.75
A3675	0.13	-	0.31	0.46	-	-	-	-	-
A2360	-	0.24	-	0.42	-	-	-	-	-
A0347	0.11	0.20	-	-	-	-	-	-	-

*** Antimicrobial active bands as determined by bioautography in section 3.2.5**

3.2.5 Bioautography of Antimicrobial Metabolites

Environmental actinobacterial isolates exhibiting antimicrobial activity were further analysed using bioautography to identify the active fractions. Figure 34 shows antibacterial activity of the broth supernatant of isolates A0350 and A1113 (lanes 1 and 3). These results indicate that partial extractions of fermentation broth supernatants of isolates were achieved with EtAc require further extractions to completely extract all the active metabolites. For culture A2381 the active fractions from the fermented broth supernatant were extracted completely with EtAc, as indicated by the absence of clear zones in the broth supernatant. The active fractions within the extract were clearly visible and correlated with the R_f values obtained by TLC separation (Table 54). The remaining cultures in table 54 were not tested using bioautography due to lack of time, however bioassays indicate that the majority contain antimicrobial activity and the banding profiles indicate that metabolites were produced.

Figure 34. Bioautogram of Extracted Metabolites from Fermented Actinobacteria Cultures. *Lane 1*: Broth supernatant media#153m with A0350 isolate., *Lane 2*: Mycelial extract of A0350 isolate., *Lane 3*: Broth supernatant media#153m with A1113 isolate., *Lane 4*: Mycelial extract of A1113 isolate., *Lane 5*: Broth supernatant Dextrin media with A2381 isolate., *Lane 6*: Mycelial extract of A2381 isolate.

Table 55: Comparison of the results of the metabolite screening strategies employed in this study.

Environmental Isolate Number	PCR Assay	Antimicrobial Assays^	TLC Assay
A1113	+	+	+
A0350	+	+	+
A2381	+	+	+
A3675	+	+	+
A1488	+	-	-
A2360	+	+	+
A2010	+	+	-
A2226	+	+	+
A3771	+	+	-
A2707	+	+	+
A3023	+	+	+
A2834	-	+	+
A0347	+	+	+

^ Antimicrobial assays include both agar plugs and well type assays

3.2.6 UV-Vis Spectroscopy of Semi-Purified Antimicrobial Organic Extract

Semi-purified organic extracts were evaluated using UV-Vis spectroscopy to determine if any of the metabolites contained chromophoric functional groups. Two of the extracts from actinobacterial cultures A0350 and A1113 exhibited similar UV spectral properties, with characteristic peaks [observed between 300_{nm} and 400_{nm} with peaks spanning approximately 20_{nm} apart] (Figures 35B and C) indicative of polyene compounds (Figure 35A). It was also observed that in the UV spectrum of these extracts an additional peak in the 400_{nm} – 500_{nm} range was also evident, which could indicate the presence of an extended chromophore, seen in aromatic polyketides (Figure 35E).

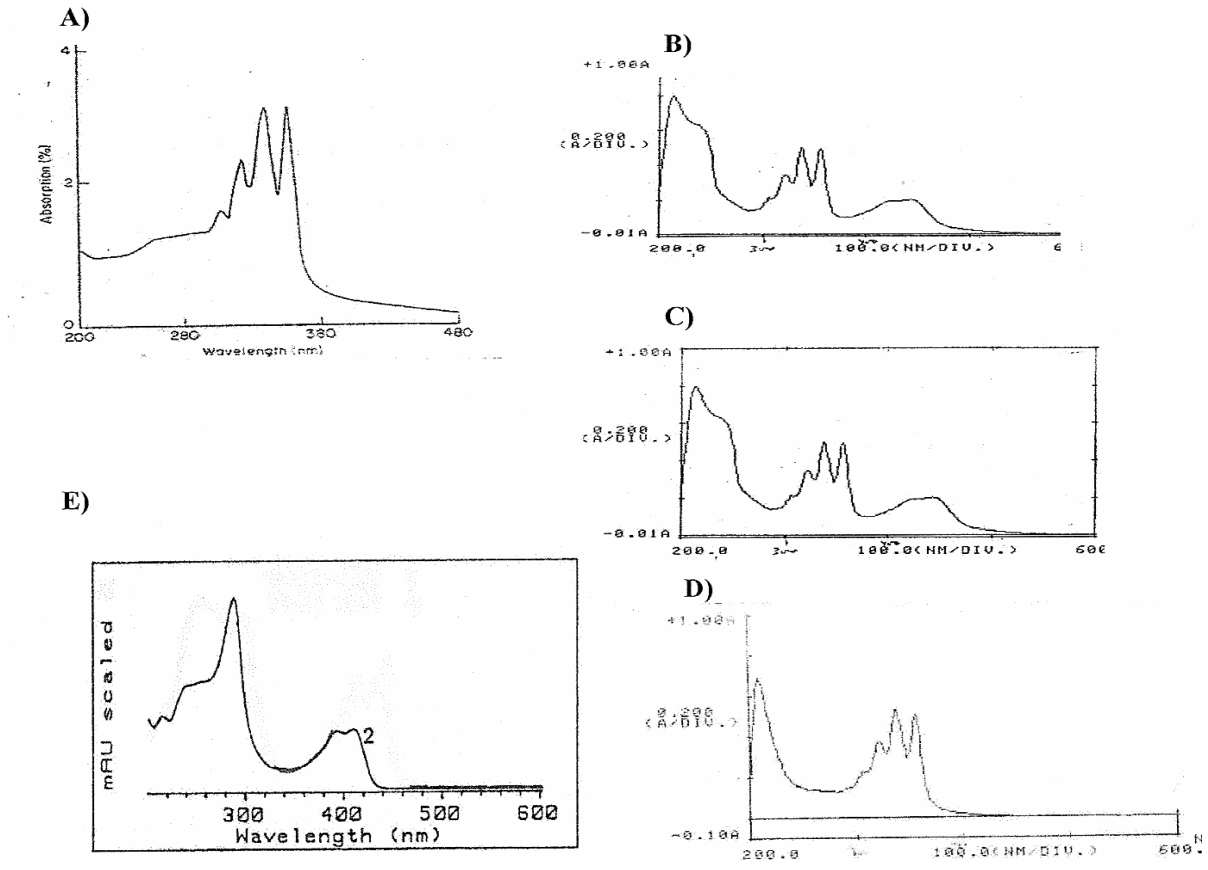


Figure 35. Spectrogram of antimicrobial semi-purified organic extract.
A) Spectrum of purified polyene antibiotic (Hacene *et al*., 1994)., B) Semi-purified organic extract of A0350 isolate., C) Semi-purified organic extract of A1113 isolate., D) Semi-purified extract of A3675., E) Tetracenomycin C (Decker & Haag, 1995).

The UV-Vis spectra of the actinobacterial extracts showed a number of chemical species absorbing at a various wavelengths (Table 56). The λ_{max} is an important characteristic in identifying the class of compound being investigated as certain compounds are known to absorb at certain wavelengths. This information was retained to form a chemical profile of the unknown compound and perform a database search in section 3.2.10.

Table 56. Maximum absorption wavelength λ_{max} detected from semi-purified extracts using UV-Vis Spectroscopy.

Isolate Number	Wavelength λ_{max} (nm)
A0350	257, 310, 329, 338, 356, 425, 475
A1113	257, 310, 329, 338, 356, 425, 475
A2381	257, 425
A2707	257
A3675	257, 310, 329, 338, 356
A2360	257
A0347	257

3.2.7 Reverse Phase High Performance Liquid Chromatography of Semi-Purified Antimicrobial Organic Extracts

In order to further characterize the chemical species in the organic extracts from the actinobacterial cultures reverse-phase high performance liquid chromatography (RP-HPLC) was employed with Photo Diode Array (PDA) detection. The chromatogram shown in figure 36 of A1113 and A0350 indicates that there are two distinctive components present at retention times 11.68 and 11.86 mins (Figure 36A) containing identical chromophores (Figure 36B) with maximum absorbance at approximately 240 nm.

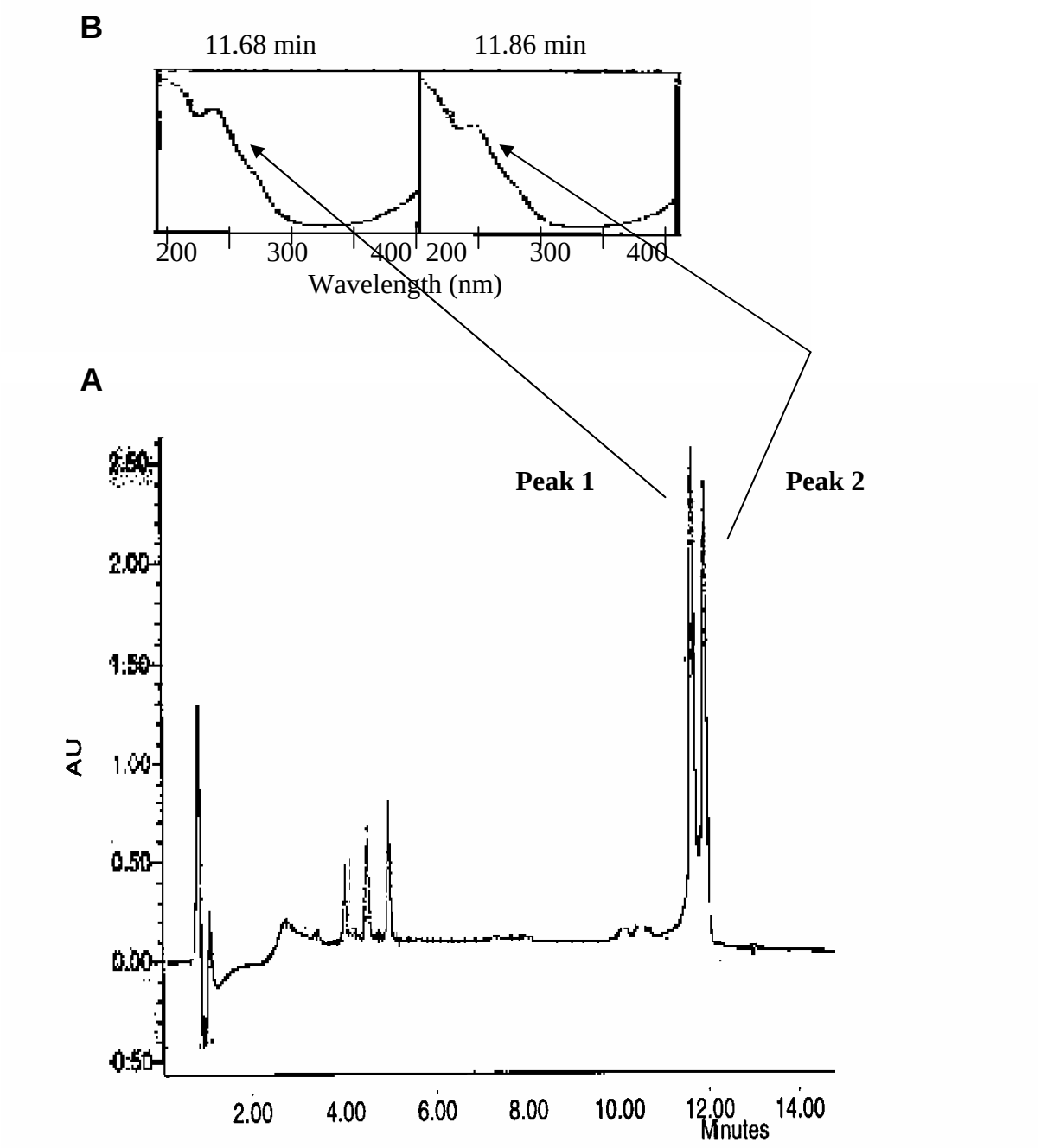


Figure 36. (A) HPLC elution profile of organic extracts obtained from isolates A1113 and A0350. (B) UV spectra of peak 1 (retention time, 11.68) and peak 2 (retention time 11.86).

3.2.8 Determination of antibacterial activity of HPLC fractions from Organic Extracts

Fractions corresponding to the HPLC peaks were collected and assayed using whole cell antibacterial activity screens by the industry partner. Extracts from A0350 and A1113 showed similar chromatographic profiles exhibiting two main peaks **1** and **2** with retention times 11.6 and 11.9 minutes respectively (Figure 36), both exhibiting the same chromaphore (UV-Vis Spectra). Fractions recovered from 11.0 to 12.5 minutes from extract A0350 showed strong antibacterial activity against *Staphylococcus aureus* achieving 98% inhibition in the whole cell antibacterial screen. In extract A1113 two antibacterial activities were recovered from fractions collected from 11.0 to 12.5 minutes. Strong inhibition was detected against *S.aureus* achieving 100 % inhibition and at 11.5 minutes strong inhibition against *Streptococcus pneumoniae* achieving 100% inhibition. A number of peaks were detected in both chromatograms in the UV-Vis region from extracts A0350 and A1113, exhibiting distinct chromophores however no antibacterial activity was detected (Figure 36). A number of peaks were detected in the chromatogram of extract A2381 exhibiting different chromophores. Fractions recovered from between 14 and 18 minutes in the non-polar region of the chromatogram showed strong antibacterial activities against *S.aureus* achieving 100 % inhibition and *S.pneumoniae* 100% inhibition. No antibacterial activity was recorded in the course of screening the Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* for all the extracts tested.

3.2.9 Electrospray Ionisation High Performance Liquid-Chromatography Mass-Spectrometry (ES HPLC-MS) of Organic Extracts

LC-MS analysis in the negative mode revealed that both extracts A0350 and A1113 contained two major peaks at 26.2 and 26.5 minutes, these dominant peaks yielded molecular ions at m/z 1253 to 1255 in the negative ion mode. The compounds did not ionize well in the positive ion mode. LC-MS analysis in the negative ion mode of extract A2381 showed the presence of multiple peaks. The two major compounds showed peaks at 25.53 and 26.3 with respective molecular ions in the negative ion mode at m/z 425.5 and m/z 385.3 (Table 57).

Table 57. Summary of mass-spectrometry

Extract / Peak Number		HPLC-MS (Retention Time, Negative Ion Mode)	ESI (m/z)
A0350	1	22.23	1253.4
	2	26.35	1255.7
A1113	1	26.24	1253.4
	2	26.34	1254.5
A2381	1	25.53	385.3
	2	26.36	425.5

3.2.10 Literature search of natural product database of organic extract physico-chemico characteristics

The physico-chemico characteristics obtained from the organic extracts analysed by chromatographic separation with UV-Vis detection and spectroscopy techniques, as well as the biological activity (antibacterial) were compiled and submitted to the compound database search program Chapman and Hall Dictionary of Natural Products (DNP). From the searches conducted in the database a number of possible matches to known compounds were retrieved (Table 58).

Table 58. Compound database matches with organic extracts.

Extract Number	Match in Database	Type of Compound	Source
A0350	Actinomycin D	Chromopeptide	*Actinomyces* spp.
	Antibiotic DJ400B	Heptane antibiotic	*Streptomyces surinam*
A1113	Actinomycin D		
	Antibiotic DJ400B		
A2381	Aureonuclemycin	Nucleoside antibiotic	*Streptomyces aureus suzhouneusian*
	Antibiotic YL 01869P	-	*Streptomyces* sp.
	Actinopyrones	Polyene	*Streptomyces pactum*
	Antibiotic PI220	-	*Streptomyces* sp. *A8056*
	WP-3688-5	Angucycline antibiotic	*Streptomyces phaenochromogenes*
	Trichostatin A		*Streptomyces hygroscopicus*

Chapter 4: Discussion

4.1 PCR screening assays for detecting biosynthetic capability in environmental actinobacteria

The distribution of microorganisms in nature is influenced to a great extent to the surrounding physico-chemical environment, which in turn effects the metabolism of microorganisms to adapt to change (Finlay *et al*., 1997). Actinobacteria which are major components of the soil microbiota have been isolated from diverse natural habitats (Xu *et al*., 1996; Groth *et al*., 1999) and many exhibit the metabolic capability to produce a diverse array of bioactive secondary metabolites (Osada, 1995; Sanglier *et al*., 1993). The chemical diversity generated by actinobacterial species is unparalled to that observed in any other microorganism. Molecular based screening has become an efficient means of accessing this chemical diversity through the detection of secondary metabolite biosynthetic genes (SMBG) in bacterial genomes (Seow *et al*., 1997). The application of gene homology based screening in actinobacteria was first demonstrated by Hopwood *et al*. (1985) who showed that a homologous probe corresponding to the antibiotic actinorhodin biosynthetic gene, could be used to detect similar biosynthetic gene clusters in actinobacterial species. The cloning of SMBG has been facilitated by using conserved domains of SMBG as DNA probes or as templates for the design of PCR primers (Julien *et al*., 2000; Brautaset *et al*., 2000). In this study PCR-mediated screening was used to determine the prevalence of secondary metabolite biosynthetic genes in natural actinobacterial populations isolated from the Australian environment. Appropriate design of conserved non-degenerate primers, specific for SMBG permitted the detection of amplifiable DNA corresponding to presumptive SMBG fragments. Screening with multiple sets of PCR primers targeting individual SMBG of the four major classes of secondary metabolites in actinobacteria allowed for the detection of biosynthetic capabilities of the environmental isolates to be determined.

The diverse chemical structures generated by actinobacteria are derived from common biosynthetic pathways. These consist of the aromatic and aliphatic polyketides, 6-deoxysugars and beta-lactam pathways. In this study, attention was focused on these four biosynthetic pathways and their associated SMBG encoding synthases which are involved in catalytic reactions in these biosynthetic pathways. These synthases commonly occur in the early phases of SM biosynthetic pathways and form common

intermediatery chemical structures from which a number of diverse secondary metabolites are derived. The DNA and protein sequences of these synthases are highly conserved across different actinobacteria genera, and DNA homology-based detection methodologies have been successfully adapted in cloning SMBG and determining the biosynthetic origins of secondary metabolites in different actinobacteria (Metsä-Ketelä *et al*., 1999; August *et al*., 1999; Miao *et al*., 2001).

4.1.1 Design of PCR primers

The validity of the PCR assays involved applying various controls. A negative control (blank) containing all the reaction components except the actinobacterial DNA was implemented in all PCR assays to ensure that the PCR reactions were contaminant-free and that no spurious amplifiable products appeared. The specificity of the primer sets were tested against DNA extracted from two known non-producing SM bacteria (*Escherichia coli* and *Bacillus pumilus*), no amplifiable product was obtained in both cases. All DNA extracted from the actinobacterial pure strains and Cerylid isolates were tested by 16S rDNA PCR using actinobacterial-biased primers to ensure that the DNA was amplifiable and to avoid false-negative results. The designed primer sets were tested against actinobacterial pure cultures known to contain the respective SMBG, and all primers sets except ole01f/ole02r were effective in amplifying the correct sized product in two or more pure strains. Direct DNA sequencing of amplified fragments of SMBG was performed to ensure that the correct sequence was obtained.

4.1.2 Type II Polyketide Synthase

Aromatic polyketides are synthesized by monofunctional proteins, which contain catalytic activities, of which the β-ketoacyl synthase, which is encoded by the KS_α gene, is involved in condensation reactions. The highly conserved KS_α gene sequence was used to design non-degenerate primers, which were used in the screens conducted in detecting similar DNA sequences in actinobacterial pure cultures and the Cerylid actinobacterial isolates. Multiple sequence alignments of amino acid and corresponding nucleic acid sequences of the KS_α gene revealed several highly conserved regions. Two regions were chosen as suitable target sites for primer development as shown in figures 20 and 21, these two regions passed the required

testing for primer design (chapter 2 section 2.2). Application of the act04f and act06r primers in PCR screening experiments using actinobacterial pure cultures amplified the predicted 0.47kb product, in two of the three streptomyces tested. However in the third streptomycete *S. argillaceus* a faint band in the expected area was observed, which may indicate that only partial sequence similarity was able to be achieved. The presence of unexpected low molecular weight products in the molecular weight range of 0.2 – 0.3 kb was also detected (Figure 22). These products may be due to the presence of similar sequences as actinobacteria are known to contain multiple copies of PKS genes or could be non-specific products.

Partial sequencing of the amplified products from the actinobacterial Cerylid isolates A1488 (*Modestobacter*) and A3023 (*Actinoplanete*) showed that the DNA sequence obtained from the isolates showed a high degree of DNA sequence similarity (73-77 % and 66-80 %, respectively) with aromatic KS_α genes from different actinobacterial species (Table 35). Alignment of the deduced protein sequences translated from the two nucleotide sequences A1488 and A3023 Cerlylid cultures, to known KS_α domain peptide sequences from different actinobacteria genera indicated that approximately 39 - 47 % similarity from the respective isolates were similar to that of aromatic KS_α genes (Figure 23). The translated sequence obtained from isolate A1488 showed that it contains characteristic conserved domains of the protein sequence for the KS_α gene (highlighted in blue in Figure 23), and is in accordance with the consensus sequence for the KS_α protein (Fernández-Moreno *et al*., 1997). Comparison of both the amino acid sequences of A1488 and A3023 showed that they share 57 % similarity to each other.

The presence of the KS_α gene in two environmental non-streptomycete actinobacteria is significant in that, the primer sequence chosen was designed from mainly streptomycete KS_α sequences indicating that the primer set act04f/act06r is capable to detect similar KS_α sequences in different actinobacterial species. Further cloning and expression studies of these genes in a suitable host could be used to determine the function and metabolic products of these putative KS_α genes. Indications from antimicrobial testing showed that isolate A3023, contained bioactivity however no activity was detected for A1488. As only antimicrobial activity was tested providing

an indication of metabolite production in isolate A1488 and no chemical expression tests were conducted it cannot be assumed conclusively that this isolate does not produce any metabolites. Detection of KS_α related DNA sequences in environmental actinobacteria has provided evidence for the presence of a aromatic polyketide pathway (Seow *et al*., 1997; Metsä-Ketelä *et al*., 1999), this may be the case for isolate A1488 however fermentation studies may have not been adequate to induce the appropriate pathway.

4.1.3 Type I Polyketide Synthase

The *KS* gene is involved in the biosynthesis of aliphatic compounds such as erythromycin and tylosin. The *KS* gene encodes a condensing enzyme (ketosynthase), which is part of a large polypeptide unit known as a modular PKS. These modular PKS contain all the necessary enzyme activities, present as discrete catalytic domains (Khosla, 1997). The highly conserved *KS* domain containing 60-84% identity over the whole domain between different actinobacterial species (MacNeil *et al*., 1993), was chosen as the target region for the PCR screening assay in this study. Degenerate PCR has provided a useful approach in cloning and detecting modular PKS genes in pure isolates in various microorganisms (Brautaset *et al*., 2000; Nicholson *et al*., 2001), though there have been limited reports concerning the detection of modular PKS genes from natural actinobacterial populations, using non-degenerate or degenerate PCR primers (Thamchaipenet *et al*., 1997).

A non-degenerate primer set ole01f/ole01r was designed which passed all the primer design criterion (chapter 2 section 2.2.1.7) and was shown by computer simulation experiments to amplify a 0.84 kb product. Application of these primers in PCR experiments showed that they were effective in detecting the putative ketosynthase (*KS*) gene fragment in only one of four type cultures tested. A 0.84 kb product was amplified in *S. hygroscopicus* but not in *S. erythreae, S. avermitilis* and *S. fradiae*. A explanation of this result may be due to the partially variable 3' end of the ole1f and ole01r primers (Table 38). The limited detection capabilities of primer set ole01f/ole01r indicated that they were not suitable and were omitted from implementation for SMBG screening.

Published degenerate primers were substituted for non-degenerate primers, and were found to be effective in amplifying DNA corresponding to *KS* genes in different actinobacterial pure cultures when glycerol was incorporated into the PCR reaction mixture (Liu & Shen, 2000). However when glycerol was absent from the reaction mixture no amplification product was obtained, this result was similarly reported in the original paper. Incorporation of the additive glycerol, is known to increase the efficiency and specificity of primers (Gibbs *et al*., 1990).

Amplification products from isolates A0350 (*Streptomycete*) and A1113 (*Streptomycete*) were analyzed further by sequencing which revealed that the isolates shared a high degree of DNA sequence similarity of 66 - 75 % and 60 - 80 % respectively, with the nucleotide sequences of actinobacterial modular KS genes (Table 40). This is in accordance with the percentage DNA similarities shared between modular *KS* genes (Apriciano *et al*., 1999). The partial amino acid sequences deduced from the DNA sequence from isolates A0350 and A1113 displayed regions of similarity to known actinobacterial modular *KS* genes (Figure 26). Isolate A0350 showed the closest amino acid similarity of > 54% corresponding to modular *KS* genes of *S. netropsis*, *S. avermitilis* and *S. erythraea*. In the case of isolate A1113 the deduced amino acid sequence showed the greatest similarity > 65% with that of modular KS genes from *S. erythraea*, *S. natalensis* and *S. hygroscopicus*. Alignment of the indicative protein fragments derived from the two nucleotide sequences of A0350 and A1113, to known modular KS domain peptide sequences from different actinobacterial genera indicated that approximately 40 % of amino acid sequence was similar to that of modular KS (Figure 26). A comparison of both amino acid sequences derived from A0350 and A1113 showed that they share 77 % sequence similarity. It was demonstrated that by using degenerate-PCR with the primer sets KSM/ATM, similar sequences to known modular PKSs could be detected in the Cerylid cultures. As only a partial DNA sequence could be obtained using the reverse primer ATM, a more complete sequence could have been obtained by using both primers which would increase the fidelity and provide a more accurate DNA sequence to read from.

4.1.4 dTDP – glucose synthase

The 6-deoxyhexoses (6DOHs) sugars commonly contribute to the structures of microbial secondary metabolites, which are essential for biological activity (Méndez & Salas, 2001). The gene coding for dTDP-glucose synthases which are involved in common catalytic reactions, whereby glucose-1-phosphate is converted to the first intermediate dTDP-D-glucose in 6DOH biosynthesis (Liu & Rosazza, 1998), was targeted for PCR screening using the conserved non-degenerate primers strD01f/strD01r. PCR screening using these primers amplified the predicted 0.37 kb product, however only in three of the four actinobacteria tested (Figure 29). The 0.37- kb product was amplified in *S. griseus*, *Micromonospora purpurea* and *M. olivasterospora*, but no product was seen in *M. chalcea*. The MSA of the primers indicates that they were biased towards streptomyces species. However the PCR experiments show partial bands of the expected size in both of the *Micromonospora* species tested indicating that a similar sequence is present in the *Micromonospora* species genome (Figure 29). In addition some extra low molecular weight bands that were not expected were detected which may have resulted from partial homologies between primers and template DNA.

PCR has been a common approach for amplifying and subsequent cloning of the dTDP-glucose synthase gene in actinobacterial species (Hyun *et al*., 2000; Stockman & Piepersberg, 1992). The abundance of putative dTDP-glucose synthase genes detected in 8 of 22 of the environmental actinobacteria screened indicates that the isolates contain the 6DOH sugar pathway and have the potential to produce compounds such as aminoglycosides. In certain isolates both the putative dTDP-glucose synthase gene and PKS genes were detected in their genomes, which may indicate the presence of multiple biosynthetic pathways - a common phenomenon in actinobacterial species (Table 48). As DNA sequencing was not carried out on the amplified products, no sequence correlation could be made on the degree of similarities between the amplified putative dTDP-glucose synthase genes and known dTDP-glucose synthase genes. The designed primers were able to detect putative dTDP-glucose synthase genes in the uncharacterised environmental actinobacteria, and could be used to facilitate the detection and cloning of similar genes from natural actinobacterial populations.

4.1.5 Isopenicillin N Synthase

One of the most promising approaches in the search for novel β-lactams have focused on the presence of isopenicillin N synthase (IPNS) which is common to all β-lactam antibiotics (Kralis and Kirby, 1998). The IPNS catalyzes the main reaction to form isopenicillin N and is encoded by the *pcbC* gene. The conserved nature of the DNA and amino acid sequence corresponding to β-lactam biosynthetic genes (Smith *et al*., 1990) has made it possible to utilize PCR-mediated approaches for in detecting *pcbC* genes from natural actinobacterial populations (Kralis and Kirby, 1998; Niebla-Perez and Wellington, 1997; Sim *et al*., 1997). Studies using DNA probes for detection of IPNS gene in β-lactam producing *Streptomyces* have shown that they share more than 70 % amino acid sequence similarity (Shiffman *et al*., 1988). In this study, the *pcbC* gene was selected to design primers. This gene exhibited several conserved regions as was to be expected as this gene contains greater than 80 % sequence similarity at the nucleotide level (Shiffman *et al*., 1988). PCR experiments using primers pcbC03f and pcbC03r amplified the predicted 0.35 kb product, however only two of the four actinobacterial species tested produced the expected amplified product. The 0.35 kb product was amplified in *S.cattleya* and *S.griseus*, but no product was detected in the *Nocardia* species and *S.clavuligerus*. The 0.35 kb amplified product was only obtained from streptomyces species which is reflected in the sequence bias of both primers and indicates that the primers are highly specific (see table 45).

Amino acid sequences translated from the DNA sequence amplified from both β-lactam producing *Streptomycete* spp. type strains showed approximately 70 % sequence similarity (Table 47). The *pcbC* gene was the least abundant SMBG, detected in 1 of 22 of the environmental isolates tested. As no DNA sequencing was carried out on the amplified product in one the positive environmental isolate (A2360), sequence correlation could not be made on the degree of similarities between the amplified DNA and *pcbC* genes. Single PCR products were amplified in the 0.4 – 0.55 kb range using the pcbC01f/pcbC01r primer set in four of the Cerylid cultures; the amplification of these higher molecular weight products may indicate that they possess similar genes.

The success of PCR amplification is dependant on a suitable target sequence with available nucleotide sequences for the design of primers and PCR conditions. In this study the majority of nucleotide sequences of SMBG retrieved from the GenBank/EMBL database were derived from *Streptomyces* species (Table 32,37,40 and 44), and the deduced consensus sequence, on which the design of the primers was based, may have restricted the detection of SMBG in non-*Streptomycete* actinobacteria which have different codon preferences. The designed primers may have displayed codon bias in amplifying only streptomycete SMBG. Strategies which have been used to isolate SMBG having unknown codon preferences include using degenerate primers (Seow *et al*., 1997; Nicholson *et al*., 2001) or heterologous probes (Sosio *et al*., 2000a). It was demonstrated in this study that degenerate-PCR primers appear better suited to environmental screening for SMBG, as was shown in the case of the *KS* gene when non-degenerate primers were substituted for degenerate primers. Codon usage (CU) patterns in *Streptomyces* genes have been established (Wright & Bibb, 1992), but further analysis of the CU patterns of non-streptomycete actinobacterial genes needs to be further evaluated. Evidence for differences in codon usages in actinobacterial genera is exemplified in the case of the aminoglycoside resistance *aph* (aminoglycoside phosphotransferase) gene. It has been shown that the *Micromonospora aph* gene has a lower G or C in the third position in codon triplets, which is significantly lower than in their corresponding *Streptomyces aph* gene (Salauze & Davies, 1991).

Twenty two uncharacterized environmental actinobacterial isolates obtained from the Cerylid culture collection (Melbourne, Australia) were individually screened with the four sets of primers to assess the SMBG diversity amongst the isolates (Table 48). The distribution of the SMBG in the environmental isolates tested were as follows; the ketosynthase gene (*KS)*, representive of aliphatic polyketides, was detected in 8 of the 22 isolates; the β-ketoacyl synthase gene (KS_{α}), representative of aromatic polyketides, was detected in 8 of the 22 isolates; the dTDP-glucose synthase gene representative of deoxysugar aminoglycoside compounds was detected in 6 of the 22 isolates; the least abundant SMBG detected amongst the isolates was the isopenicillin N synthase gene (*pcbC*), representative of the β-lactam antibiotics, which was only detected in 1 of the 22 isolates. In addition, 8 from the 22 isolates contained the

presence of two SMBG (Table 48). This may indicate that the isolate(s) have the potential to produce more than one type of secondary metabolite or that it contains a component of other biosynthetic pathways (Stockmann & Piepersberg, 1992). Of particular interest are isolates A2010, A1113, A2707, A0350 and A2056 which contain both polyketide and deoxysugar genes. Such a profile is indicative of the genetic composition of a number of bioactive microbial metabolites, particularly macrolides and anthracyclines (Staunton & Wilkinson, 1999; Richardson & Khosla, 1999).

PCR screening of the Cerylid isolates showed that ten of the eleven isolates classified as *Streptomycete* strains were positive for containing secondary metabolite biosynthetic genes. Whereas only 6 of the 11 non-*Streptomyces* isolates tested positive for secondary metabolite biosynthetic genes.

The PCR screens applied to the actinobacterial culture collection provided by Cerylid Biosciences (Melbourne, Australia), were benefical in identifying environmental isolates harboring putative SMBG. Thus providing a useful selection criterion in concentrating fermentative efforts on those isolates shown to possess the genetic machinery necessary for the synthesis of certain classes of secondary metabolites. This is a major advantage in screening microbial sources for secondary metabolites, as identifying the biosynthetic capabilities of the isolate prior to the commencement of fermentation studies, offers clues to elucidating production media which can be tailored for the identified class of compounds. Empirical approaches have been continually used for the determination of suitable fermentative media and selecting the most appropriate media where chemical metabolites are expressed (Bu'Lock *et al*., 1982; Zahn *et al*., 2001). Due to the declining rate at which novel secondary metabolites are being discovered using empirical screening (Strohl, 1997), more directed approaches have evolved such as PCR based screens which discriminate productive cultures from redundant ones. Degenerate PCR has been a powerful approach in detecting divergent SMBG in cultured and uncultured microorganisms (Seow *et al*., 1997), however based on the SMBG sequences retrieved from the environmental isolates using the KSM/ATM degenerate primer set highly similar genes could only be detected. Thus, in order to detect novel SMBG this primer set may not be appropriate, or may need to be modified or used in variations of the

degenerate PCR technique (Okuta *et al*., 1998). As screening DNA is becoming more efficient, as is the case with colony PCR whereby cultures are used directly in PCR reactions with no need for lengthy DNA extractions (Sheu *et al*., 2000; Ishikawa *et al*., 2000), detection of SMBG from environmentally isolated microorganisms should become economically feasible for high throughput screening.

4.2 Secondary metabolite production of actinobacteria

4.2.1 Solid Agar

Twenty two environmental actinobacterial isolates from the Cerylid culture collection were initially screened by agar-type antimicrobial assays. Mycelial extracts from isolates grown on yeast-malt extract (YME) solid agar, showed that fungi and gram positive bacteria were sensitive to the secondary metabolites (SM) produced by the Cerylid cultures (Table 49).

4.2.2 Submerged Fermentations

Enhancement of antimicrobial activity was achieved by applying a variety of fermentation techniques and using complex media with known constituents which enhance SM production (see discussion below). Complex media was selected due to the beneficial properties imposed on antibiotic submerged fermentation studies these include, promotion of homogeneous dispersal of mycelial growth, variable chemical expression, faster growth and higher quantities of antibiotics (Dekleva *et al*., 1985; Doull & Vining, 1989; Whitaker, 1992). However the disadvantage of using complex media include difficulty in distinguishing constituents inducing production of a desired SM. Utilisation of general SM screening conditions is a common dilemma posed in many screening programs, the conditions are often empirically determined which requires high technical resources and is costly (Huang *et al*., 1999). However, it is becoming apparent that culture conditions favoring production of SM are being identified and implemented in screening for novel SM (Iwai & Ōmura, 1982; Ōmura, 1986).

4.2.2.1 Carbon Sources

Of the major constituents which are known to influence the efficacy of SM production, include carbon and nitrogen sources (Iwai & Ōmura, 1982). The carbon

sources used in the nine complex media evaluated in this study included, the monosaccharides glucose and glycerol (medium IM22, 248P and 153, 153m, 153+Glycerol, 153m+Glycerol), the disaccharide; sucrose (medium SI and IM25) and the polysaccharide; dextrin (medium DEX). Antibacterial producing environmental actinobacteria, identified from earlier experiments on solid agar, showed that the liquid media DEX, IM25 and medium 153 were suitable for SM screening for individual isolates.

Dextrin proved to be the most appropriate carbon source for eliciting higher production of secondary metabolites in the selected isolates evaluated. This is consistent with methodologies utilizing dextrin to increase the antibiotic productivity in actinobacteria by inducing synthases involved in SM production (Chatterjee & Vining, 1981), in contrast to this observation dextrin used in conjunction with another source of carbon yeast extract which decreased antibiotic production (Benslimane *et al*., 1995). In order to verify the positive effect of dextrin in fermentations, more extensive studies need to be conducted using defined media incorporating statistical design using stepwise discriminant analysis (SDA) in identifying substrates having weak effects and those possessing strong effects in relation to secondary metabolite producing soil actinobacteria (Huck *et al*., 1991), or conducting response surface experimental designs (Bull *et al*., 1990). Glycerol usually used as a alternative carbon source to glucose, at a concentration of 20% w/v was substituted for glucose in complex liquid fermentation media 153 and 153m, this resulted in suppressive effects on SM production. The same effect was shown by Gouveia et al. (2001) using complex medium that high concentrations (20% w/v) of glycerol suppressed antibiotic production. The defined medium SI, containing only sucrose as a carbon source was a poor fermentation medium, however another medium containing sucrose with organic sources of carbon such as IM25 proved to be suitable for SM screening. It has been shown that by incorporating sucrose in complex media osmotic balance is maintained at equilibrium between the cell and the external environment, so that bioconversion of SM efficiently occurs (Elibol & Mavituna, 1998).

4.2.2.2 Nitrogen sources

Slowly utilizable organic nitrogen sources such as soybean meal (SBM) have been shown to benefit antibiotic fermentations, due to the avoidance of immediate

interference of ammonium on synthases of secondary metabolism, which can be generated by rapidly utilizable nitrogen sources such as inorganic ammonium salts (Braña & Demain, 1988). It has been shown that organic nitrogen sources promote the enhancement of polyene type antibiotics (Kim *et al*., 2001). It was shown that SBM had a superior effect in actinobacterial fermentations, however when combined with another natural source of nitrogen YME minimal antimicrobial activity was attained. This may indicate an excessive use of nitrogen sources which is known to repress secondary metabolite production, alternate sources of nitrogen or elimination of one nitrogen source may have alleviated repression (Demain, 1995)

4.2.2.3 Suitability of liquid media for secondary metabolite screening

It was evident that certain media, secondary metabolites exerted antibacterial and anti-fungal activity (dextrin and medium 153) whereas other media exhibited narrow spectrum activity against either bacteria (medium IM25) or fungi (medium 153m). Commonalities between the medium used by Saadoun and Al-Momani (2000) with medium 153 which both contain beef extract-peptone-glucose as the major ingredients has been shown to induce specific antifungal activity, this was evident in our experiments. Directed screens could serve to benefit from the implication of media types such as #153 expressing SM with a desired biological activity, and eliminating cultures not expressing this activity in specific media.

4.2.2.4 Effect of Oil Supplementation to Submerged Fermentations

Further evaluations incorporating novel approaches to improving cultivation conditions for SM screening involved utilizing oil supplementation. In this study all eight different oils showed good antimicrobial activities (>20mm) at tested concentration of 3 % (w/v), however vegetable, safflower and soybean oils produced higher antibacterial activities (Table 53). This observation is in agreement with other antibiotic fermentations where addition of high level supplementation from 2 % to 10 % (w/v) vegetable oils in complex media enhances production of SM (Jia *et al*., 1999). In contrast it has been shown that low level supplementation at a concentration of 0.05 % (w/v) vegetable oils particularly soybean and sunflower oils act as adjuvants improving consumption of carbon sources and increasing production of antibiotics (Jones & Porter, 1998).

4.2.2.5 Duration for chemical expression of bioactive metabolites

An extended duration of actinobacterial fermentations in submerged culture were run over 288 hrs (12 days) instead of the usual average of 120 –240hrs [5 – 10 days] (Iwai & Ōmura, 1982). By using antimicrobial activity as a indicator of expressivity of SM, it was shown that a cultivation period of 240 hrs (10 days) was sufficient for production of antimicrobial compounds in most of the media evaluated (Table 50). As antimicrobial screening was the only parameter tested for secondary metabolite production throughout the duration of the fermentation, this may have not been sensitive enough to detect minor quantities of the compound/s. Thus, further testing by TLC or HPLC-MS may have provided been better suited to detect all secondary metabolites produced by the isolate throughout the fermentation.

4.3 Correlation between genetic screening and antibiotic effects

In certain cases the PCR screening strategy used in this study was able to detect putative SMBG's in the actinobacterial isolates and this was able to be correlated with the antimicrobial activities detected. However this correlation was not encountered with all the isolates. Interpretation of a positive PCR result must be treated with caution as this indicates only that the strain being screened is presumed to posses the genes necessary for the biosynthesis of that type of antibiotic. It does not indicate whether the genes are expressed, nor does it indicate that the strain possess all the biosynthetic genes for that class of antibiotic. Two cases were encountered that did not adhere to this correlation. In the first case isolate A1488 produced a PCR product with the absence of antimicrobial activity, this result may partially be explained by either by the lack of sensitivity of the biological activity screens not been able to detect the activity or the fermentation conditions may have not been favorable to promote the production of the compound. In the second case isolate A2834 did not produce a PCR product, however antimicrobial activity was detected. This result may be explained by the limitation in the sensitivity of the assay as the primers may have not been appropriate to detect the SMBG responsible for the antimicrobial activity or that it may have been a false negative where the strain being screened may possess the biosynthetic genes for the antibiotic, but no PCR product is seen. False negatives in

PCR rections arise when variations in the primer target sequences prevent one or both primers from binding efficiently.

The isolates A1113 and A0350 that produced a PCR product and that were sequenced, from the limited sequence obtained which contained a conserved region spanning 21 amino acids (Figure 26) this region is close to the ketosynthase active site which is responsible for the formation of type I polyketide molecules which are known to be responsible for antimicrobial activities. A prediction into the antimicrobial activities based on the sequence alone could not be made due to the limited DNA sequence obtained. As with the sequence analysis for the type II PKS genes in isolates A1488 and A3023 conserved amino acid regions were identified and showed high similarity with other KSα genes involved in spore pigment production and antimicrobial activities (Figure 23). Once again the limited sequence obtained was not sufficient to predict the possibilities of the presence of antimicrobial activities.

In the pursuit of secondary metabolite genes using direct PCR product sequencing from actinobacteria it has been reported in the literature that mixed sequences are likely to exist (Busti et al., 2006), suggesting that more than one DNA segment has been amplified. In this investigation all the isolates yielded a distinct band of the expected size with all primer sets, however faint bands were also observed. Direct sequencing of the PCR products of the expected size did not indicate the presence of extraneous sequences and the translated DNAs were highly related to known type I and type II PKS sequences. Gene organization is an important characteristic that must be considered when interpreting PCR products amplified with primers targeting type I PKS genes. The modular orientation of these genes consisting of a repetition of similar gene segments within a single gene cluster, PCR may amplify multiple bands which indicates that each amplified band may consist of different sequences originating from a single cluster. Thus the presence of fainter bands observed in the PCR products may indicate the presence of a similar SMBG in the genome of the actinobacterial isolate. As no sequencing was carried out on these fainter bands this proposition cannot be substantiated. In the case of type II PKS genes a gene cluster encodes a single KSa, however some strains possess more than one type II PKS cluster and this is used for the synthesis of spore pigments. Direct sequencing did not

reveal any extra sequences that may have been indicative of the amplification of more than one DNA segment.

4.4 Adaptation of cultivation conditions for secondary metabolite screening

4.4.1 Solid Substrate Fermentations

The metabolic pattern of extracted metabolites from SSF as determined by TLC, showed that barley and whole oats supplemented with water produced the highest number of TLC bands though the EtAc extracts showed no antibacterial activity. This result showed that the type of screening is crucial in determining the bioactive metabolite producing capabilities of microorganisms, often combining a number of screens provides a comprehensive approach (Bérdy, 1989). In addition, it was shown that minimal supplementation with the use of distilled water only of the solid substrates can produce a complex metabolic pattern, however when substrates where supplemented with additional mineral or trace elements a less complex pattern was evident. The interpretation of these results supports the notion that under nutrient deprived conditions variable expression of SM is produced, whereas cultures exposed to nutrient rich conditions only produce certain types or a decreased number of compounds.

Altering cultivation conditions and diversifying substrates in SM screening can induce bioactive compounds and benefit screening efforts. A novel approach to cultivating actinobacteria using solid-state fermentation (SSF) for SM production, has been shown to posses many attractive advantages over conventional liquid fermentations these include; minimal energy input, higher yields in shorter durations of antibiotic production and decreased production costs have made this technology a attractive and economical approach for SM screening groups (Yang &Ling, 1989., Robinson *et al*., 2001). SSF involves incorporating heterogeneous natural solid substrates having low moisture contents (12 %) (Barrios-González & Mejía, 1996., Jermini & Demain, 1989). In the current study, environmental isolates exhibiting low antimicrobial activity on agar media were subjected to SSF using various natural substrates to determine if enhanced production could be achieved. Cultivation of the low yielding isolates on SSF varied in their response in comparison to that of liquid fermentations

for isolate A2707 a increase in antibacterial activity was produced, though isolate A0347 showed a suppressive effect and isolate A3675 was comparable (Table 51).

4.5 Extractability of bioactive metabolites

Liquid-liquid partitioning of secondary metabolites serves two purposes, to recover and concentrate the product from the fermentation broth (Hatton, 1985). Two water-immiscible organic solvents were used in extractability studies. Comparisons of the resulting extractions using TLC indicated that the efficiency of extraction varied with the solvent used. Often SM are selectively purified using specific organic solvents (Schügerl, 1994). Choice of a suitable solvent for SM extractions is determined by the polarity of the compound (Cannell, 1998). Mycelial extractions with MeOH were suitable for most of the isolates studied, however in other cases metabolites were only extractable in EtAc (Figure 33). Furthermore, it was shown that by using a highly polar elution solvent system in TLC that the metabolites were soluble in highly polar organic solvents. As a result of the favourable elution conditions identified for each of the secondary metabolites, further optimization of the recovery process using adsorption resins or using a series of liquid-liquid extractions could be used to selectively purify the metabolite/s of interest (Cannell, 1998).

In order to distinguish from the metabolic profile which of the TLC bands contained biological activity, bioautography was implemented. It was ascertained from the clear zones of inhibition against *S. aureus* detected in the bioautograms that the majority of the activity was derived from organic fractions (Figure 34). The active bands were correlated to bands visualized under short wavelength at 254_{nm} (Table 54), however smearing was evident which may indicate lack of resolution of SM components or incomplete purification. The smearing effect observed hampered efforts in correlating bands with biological activity. An alternative approach to determining if a band is one or a combination of chemical species, involves scraping off the separated components on the TLC plate and solubilising the material in a suitable solvent and subjecting the mixture to HPLC. In this way components in the mixture are resolved and fractions collected, the fractions are then tested in biological activity screens. This combined

approach to chemical screening provides an efficient means in elucidating biologically active fractions in a chemical heterogeneous mixture.

Evaluations determining whether SM were produced intra- or extracellularly, was shown that the antimicrobial compounds were in most cases present in the broth supernatant (BSN) and mycelial extracts (ME). Extraction efficiency of SM is determined by the polarity of solvent and pH (Schügerl, 1994). The solvent behavior of the compounds were shown to be highly polar as they were soluble in both water and organic solvents. Acid-base characteristics of SM extracted in organic solvents, evaluated by small-scale pH extractability studies showed that there was no alteration in the antimicrobial activities or coloration of the extract which may indicate pH dependence of classes of compound such the anthracyclines (Arcamone, 1998). The presence of antimicrobial activity in the BSN may indicate that SM were able to be excreted into the medium, however constituents in the media may have masked some of the antimicrobial activity. Overlooking this masking phenomena can lead to false negatives, thus alternative methods of metabolite production detection was used such as thin-layer chromatography (TLC) which provided a metabolic fingerprint of each strain (Zähner *et al*., 1988). In certain isolates the metabolic banding patterns were identical in the BSN and ME represented by fluorescent common bands on on the TLC plate under UV wavelength of 254_{nm}, however fainter bands were evident in the BSN. The stronger intensities of the metabolic bands in the ME and the presence of similar bands in the BSN, indicated that the compounds were more soluble in organic solvents, and that partial extractions took place (Figure 33).

Zones of inhibition were also detected from the initial points of application of sample on TLC plates, indicating that the solvent system used may not have been adequate for efficient separation of the bioactive compounds. A low-polar solvent system consisting of butanol-acetic acid-water (4:1:1), was evaluated to separate the bioactive compounds however this proved to be unsuccessful.

4.6 Isolation of Bioactive Metabolites

Devising a isolation scheme for the bioactive metabolites produced by the environmental isolates, was devised from the TLC solvent system used where EtAc

was the major organic solvent. The highly polar solvent system, ethyl acetate:methanol (9:1) was successfully used in the separation of compounds by TLC from both fermentation broth and organic fractions (Figure 33). This also indicated that the compounds were soluble in the solvents used. Small scale extractions using both methanol and ethyl acetate showed that compounds were able to be separated. A purification scheme was devised incorporating ethyl acetate as the solvent to be used in solvent extraction. In order to isolate sufficient amounts of the antimicrobial metabolites, a liquid fermentation was scaled-up from 50 mls upto 1 litre to obtain a dry yellow-orange powder from isolates A0350, A1113 and a oily brown residue from isolate A2381. The average recoveries were 4.0 mg of semi-purified extract.

4.7 UV-Vis Spectroscopy scanning of organic extracts

Many microbial metabolites have characteristic absorption spectra in the ultraviolet – visible region, such spectra; (1) can be used for initial identification by comparison with spectral libraries to determine if the compound belongs to a particular chemical class, (2) for identifying components related to a known structure and (3) for monitoring the production of secondary metabolites (Stead., 1998; Bystrykh *et al*., 1996). Properties of classes of compounds such as polyketides, which have absorbance maxima between 205 –280_{nm} or polyenes which absorb between 300 – 380_{nm}, can be used to partially identify constituents in microbial fermentation extracts (Williams & Fleming, 1973). UV spectra of extracts from strains A1113, A0350 and A3675 showed the presence of polyene type compounds, and an anthraquinone chromophore (Rohr *personal commun.*). The presence of this four peak profile has been detected in microbial extracts possessing antifungal activity (Gagoś *et al*., 2001). Interestingly, extract A3675 containing this profile contained only antifungal activity, however extracts A0350 and A1113 containing the extended chromophore showed both antibacterial and antifungal activities (Table 56 and Figure 35). From the detected antimicrobial activities indications are that the chemical species represented by the four peak UV spectrum may be responsible for the antifungal activity. The extended chromophores detected in UV-Vis spectral profiles of A1113 and A0350 (Table 56) are characteristic of aromatic compounds (Doyle *et al*., 1979). UV-Vis spectroscopy of extract A2381 showed a number of highly absorbing chemical species indicated by the bell shaped UV spectra profile in the 250 – 400_{nm} range.

4.8 Analysis of bioactive organic extracts by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) with UV-Visible Diode Array and Electrospray Ionisation-Mass Spectrometric (ESI- MS) detection

Chemical characterisation studies were carried out by chemists at Cerylid Biosciences (Melbourne, Australia), using dedicated analytical instrumentation for natural products. HPLC with UV-Vis diode array detection identified two major peaks constituted the major components of organic extracts A0350 and A1113 (Figure 36) Further analysis by HPLC and ESI-MS/HPLC showed two dominant peaks for both extracts, these two peaks may be either two compounds possessing the same chromophore, or peak 2 may be a derivation product of compound 1. Antibacterial activity against gram-positive bacteria was detected from the fractions collected from these dominant two peaks. LC-MS analysis of the bioactive fractions from both A0350 and A1113 extracts showed that the two dominant peaks yielded molecular ions at a mass-to-charge ratio (*m/z*) 1253 to 1255 in the negative ion mode. A literature search conducted in the Chapman and Hall Dictionary of Natural Products (DNP) database, using the molecular weights from 1254 to 1256 identified the active compounds as being part of the actinomycin group of compounds which are known to be produced by several streptomycete species and over 100 various actinobacteria have been isolated and described (Egorov, 1985). The actinomycins belong to the chromopeptide group of antibiotics, phenoxazinone synthase is a enzyme involved in the synthesis of the actinomycin chromophore (Jones & Hopwood, 1984) which consists of an aromatic structure derived from the polyketide pathway (Figure 37A). PCR screening detected the presence of the aromatic PKS gene which could be involved the production of the actinomycin chromophore. The second closest match in the DNP was a heptane antibiotic DJ400B, figure 37B shows the structure of a example of a heptane antibiotic candicidin D which shows the characteristic long chain bonds which are derived from modular polyketide synthases and attached deoxysugar mycosamine (Hu *et al.*, 1994). Interestingly, PCR screening of culture A1113 indicated that it contained both a modular PKS gene which could be responsible for the formation of the carbon chain structure and a deoxysugar gene which could be involved in the formation of the deoxysugar component.

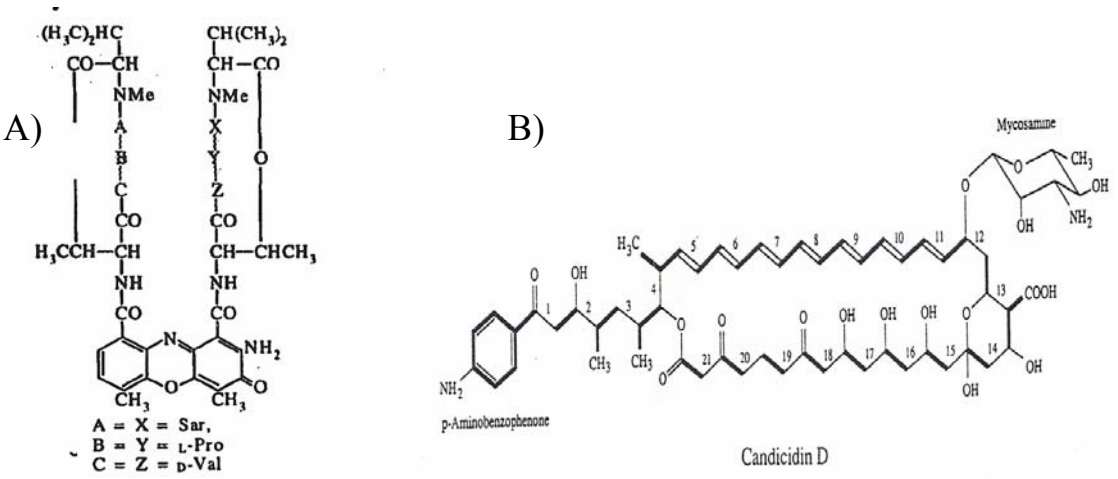


Figure 37. The chemical structure of A) Actinomycin D (Adapted from the Chapman and Hall DNP, 1982-2001), B) Candicidin D an example of a heptaene macrolide, containing 21 carbon bonds (Hu *et al*., 1994).

RP-HPLC analysis of extract A2381 indicated the presence of several compounds, with different chromophores. However antibacterial activity was only detected against gram positive bacteria from peaks collected between 14 to 18 mins. This region of the UV-chromatogram revealed only minor constituents. However, the LC/MS showed the presence of several compounds in the non-polar region of the chromatogram. Peaks 1 and 2 from the chromatogram clearly showed in the ESI-MS trace that they were two compounds with molecular ions in the negative ion mode at *m/z* 385.3 and *m/z* 425.5 respectively. A search of the Chapman and Hall DNP with MW from 425 to 427, and MW from 386 – 387 revealed several possible candidates for the classification of these compounds which included; antibiotic YL 01869P, actinopyrones, antibiotic WP 3688-5 or derivatives of trichostatin, all these compounds are known to be produced by streptomycete species.

Actinopyrone and trichostatin A are polyene antibiotics (Figure 38a and b), modular PKS are involved in the formation of the polyene chain structures (Katz, 1998). PCR screening detected a modular PKS gene in isolate A2381, indicating that it could be responsible for the formation of the polyene structure.

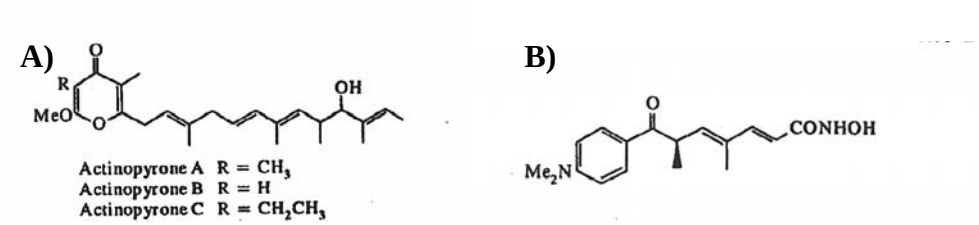


Figure 38. The chemical structure of A) actinopyrone, and B) trichostatin A (Adapted from Chapman and Hall DNP, 1982-2001).

4.9 Conclusions

Prescreening environmental cultures using PCR prior to the commencement of fermentative studies was shown to be a reliable indicator that an isolate contained the capability to produce biologically active secondary metabolite(s) and that all of the isolates contain multiple biosynthetic genes. Screening by conventional PCR with non-degenerate primers was found to be applicable to isolating similar sequences from environmental isolates, however this approach may not be suitable for isolating novel SMBG genes. Degenerate-PCR would be better suited in isolating divergent biosynthetic genes from environmental microbial sources due to the degeneracy in annealing of the primers to similar sequences and can lead to the discovery of novel SMBG (Seow *et al*., 1997).

The degree of SM productivity is reliant on the type of biological screen being implemented (Franco & Coutinho, 1991). Antimicrobial screens were successfully used to detect the highly productive isolates from the less productive isolates. Antifungal metabolites derived from A0350, A1113 and A3675 were shown to contain spectral properties similar to polyene type compounds. Extract A1488 showing no antimicrobial activities, was shown by TLC to express metabolites and contain SMBG by PCR indicating the biosynthetic capability to produce a aromatic polyketide. This observation highlights the importance of using a multi-directional approach to screening and that the type of compound to be discovered is dependent on detection method used for screening.

Realisation of an isolates full potential for producing secondary metabolites, fermentative media design for SM screening needs to well-defined for each individual isolate. It was shown that certain constituents in the liquid media such as dextrin and

glycerol favour antimicrobial activity, whereas liquid media supplemented with various refined oils enhanced antimicrobial activity. Solid substrate fermentations were found to increase the heterogeneity of metabolites expressed as well as increase antibacterial activity. It was established that a general fermentation medium for SM screening could not be identified, as individual isolates had preferred cultivation conditions.

The correlation between antimicrobial activity and TLC bands was successfully performed using bioautography. UV-Vis spectroscopic studies were useful in partially identifying similar chemical species present in the organic extracts. RP-HPLC was used to separate the chemical species present in the microbial extracts it was shown that extracts A1113 and A0350 contained identical chromatograms, with two major peaks adjacent to one another containing the same chromaphore indicating the presence of one compound. LC/MS analysis of organic extracts A0350 and A1113 showed identical chromatograms with molecular ions at *m/z* 1253 to 1255. Based on the molecular weights and UV spectrum of the compounds, and the visible color of the extracts, the active compounds in both extracts appear to belong to actinomycin D or one of it's isomers. Evaluation of A2381 organic extract by RP-HPLC indicated the presence of a number of non-polar compounds which may have been responsible for the antibacterial activity. Two major compounds with molecular ions at *m/z* 425.5 and 385.3 were identified by LC/MS analysis, these compounds could represent a number of compounds such as the actinopyrones, Antibiotic YL 01869P, Antibiotic WP 3688-5 or derivatives of trichostatin. HPLC combined screening of UV-Visible and mass spectrometric detection offers a accurate analysis of constituents in organic extracts and partially characterising compounds. The active compounds identified as a result of chemical screening would need to be produced in larger quantities in order to elucidate the chemical structures.

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Appendix 1: Conference Presentation and Awards

A1.1 Conference Presentation

12th International Symposium on the Biology of Actinomycetes
The University of British Columbia, Vancouver, Canada, August 5 – 9, 2001.

Poster Title: Prescreening for Biosynthetic Genes in Actinomycetes Isolated from the Australian Environment.
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Abstract: In order to optimise the selection of environmental isolates for chemical expression studies, a number of prescreens were developed to detect the presence of genes of common biosynthetic pathways. These PCR-based assays were used to evaluate actinomycetes isolated from various sites in Australia. In one of these prescreens, environmental isolates were screened with type I PKS specific degenerate primers. The amplified 750 bp products from the positive isolates were sequenced and showed homology to corresponding type I PKS genes in the GenBank database. The positive isolates were subjected to submerged and solid state fermentation, and the resultant secondary metabolites were purified and characterised chemically. The results show that PCR screening of environmental actinomycete isolates can be used to eliminate redundant cultures, and focus efforts on development of cultures known to have the biosynthetic potential to produce a specific class of secondary metabolites.

A1.2 Awards

1999-2001: Australian Postgraduate Award (Industry)
Australian Research Council (ARC) – Strategic Partnerships with Industry – Research and Training (SPIRT) grant and
AMRAD Corporation Limited (Australia).