Isolation and Identification of Beta-Lactam Producing Microorganisms Using PCR Based Methodologies

THESIS

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ABSTRACT

The polymerase chain reaction (PCR) was investigated as a potential tool in microbial screening for β-lactam producing organisms. Optimization of PCR conditions and the addition of acetamide to the PCR reaction allowed for the successful amplification of the isopenicillin N synthetase (IPNS) gene in *S. clavuligerus, S. tanashiensis, S. griseus, S. olivaceus, S. lipmanii*, and *S. chartreusis*. PCR was used to produce a radiolabelled probe from *S. clavuligerus* that was used to detect analogous genes in bacteria and fungi. Southern blot and dot blot analysis using the IPNS probe revealed the presence of IPNS-like sequences in seventeen organisms. Fourteen of these sequences belonged to known β-lactam producing organisms; one unidentified soil isolate; and two non-β-lactam producing organisms *viz. S. venezuelae* ATCC 10712 and *S. hygroscopicus* ATCC 21703. The IPNS gene was also detected in a β-lactam producer (*S. chartreusis*) that had lost its ability to produce antibiotic. It would therefore have been overlooked in a conventional antibiotic screening program. The use of PCR, coupled with Southern hybridization and dot blot analysis, increased the sensitivity and specificity of the antibiotic screening procedures and allowed for the investigation of evolutionary relationships between the eukaryotes and the prokaryotes. A preliminary investigation into the potential use of RAPD PCR and protein fingerprinting as tools for solving discrepancies in streptomycete identification was conducted. A variety of streptomycete species that were chosen as being representative of a number of numerical taxonomic classes were amplified using various RAPD primers. Streptomycetes appear to be genetically diverse organisms as was reflected by their RAPD and protein profiles. The application of PCR in an antibiotic screening program showed great potential as a specific and sensitive tool in the detection of β-lactam producers and in the elimination of duplicate strains.

CONTENTS

ABBREVIATIONS

α	Alpha
β	Beta
δ	Delta
μ	Micro
AP-PCR	Arbitrary primed PCR
ATCC	American Type Culture Collection, Rockville, Maryland, USA
bp	Base pair(s)
Ci	Curie
cpm	Counts per minute
CTAB	Cetyltrimethylammonium bromide
DAF	DNA amplification fingerprinting
ddH_2O	Double distilled water
DGGE	Denaturation gradient gel electrophoresis
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ds	Double stranded
DSM	Deutsche Sammlung von Mikroorganismen, Masscheroder Weg, Braunschweig, Germany
ed.	Editor(s)
EDTA	Ethylenediaminetetraacetic acid
eg.	Example
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium Bromide
GC	Guanine, cytosine
Gram$^+$	Gram-positive
Gram$^-$	Gram-negative
HPLC	High-performance liquid chromatography
ISP	International *Streptomyces* Project
kb	Kilobase(s)
m	Milli
MDS	Multi Dimensional Scaling
min	Minute(s)
NMR	Nuclear Magnetic resonance
No(s)	Number(s)
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reacton
PyMS	Curie point pyrolysis mass spectrometry
RAPD	Randomly Amplified DNA
RE	Restriction enzyme

RFLP	Restriction-fragment-length polymorphisms
RiBiTS	*Mse*I fingerprints of the rRNA operon
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sarkosyl	*N*-lauroylsarcosine
SDS	Sodium dodecyl sulphate
sec	Second(s)
sp(p)	Specie(s)
SSC	Sodium chloride/sodium citrate (buffer)
SSCP	Single Stranded Conformation Polymorphisms
syn.	Synonymous
TAE	Tris/acetate (buffer)
T*aq*	*Thermus aquaticus* DNA (polymerase)
TBE	Tris/borate electrophoresis (buffer)
TCA	Trichloroacetic acid
TE	Tris/EDTA (buffer)
TEMED	*N,N,N',N'*-tetramethylethlenediamine
TGGE	Thermal Gradient Gel Electrophoresis
T_m	Melting (or midpoint) temperature
Tris	Tris(hydroxymethyl)aminomethane
Tris/HCl	Tris hydrochloride
U	Units of enzyme activity
UV	Ultraviolet
v/v	Volume/volume
viz.	Namely
w/v	Weight/volume

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Molecular genetics has revolutionised research in bioactive microbial products and in particular the β-lactam antibiotics. Several studies in the past few years have made great contributions to the understanding of the chemical nature, mode of action, and biochemical pathways of the β-lactam antibiotics (Baldwin and Abraham, 1988; Jensen, 1986; Pérez-Esteban *et al.,* 1993). Cloning and genetic manipulations of antibiotic-specific genes has led to a better understanding of β-lactam biosynthetic pathways (Hodgson *et al.,* 1995; Martín and Gutiérrez, 1995), and may lead to new approaches for strain improvement, screening of novel producers and the chemical synthesis of novel antibiotics. The elucidation of antibiotic biochemical pathways may also provide insight into some fundamental biological questions concerning the role and origin of these antibiotics. In the search for new β-lactam antibiotics there are many factors which must be taken into account or an important discovery may be overlooked. Such factors include the screening of cultures capable of producing antibiotic; conditions that allow synthesis of detecable amounts of the said antibiotic; a sensitive detection system that is selective enough to detect what is a likely β-lactam antibiotic (Zimmerman and Stapely, 1983); and the identification and classification of the producing organisms. It would be desirable to shortcut this process by integrating the detection and recognition of the antibiotic produced and the identification of the producing organisms in the initial stages of screening. Powerful molecular tools such as cloning, Southern hybridizations and the polymerase chain reaction (PCR) may now provide the sensitivity, specificity and selectivity that such screening processes require.

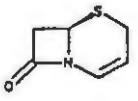
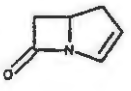
1.1.1 Beta-lactam producing organisms

The discovery of penicillin by Alexander Fleming (1929), led to a revolution against infection. X-ray crystallographic studies of penicillin showed it to contain a fused β-lactam ring (Elander, 1983) which was found to be inhibitory to peptidoglycan synthesis (Demain

and Solomon, 1983). Since peptidoglycan synthesis is a prokaryotic function, β-lactam antibiotics do not pose a threat to mammalian systems. The intrinsic potency and broad spectrum activity of the β-lactam antibiotics has attracted attention in the search for new producers.

Penicillin was initially produced by the filamentous fungi, *Penicillium notatum*, and for many years penicillin isolates were from different strains of *Penicillium*. Despite their primacy, the fungi were found to be a limited source of β-lactam molecules. Subsequently, β-lactam antibiotics have been found to be produced by numerous species of mycelium forming Gram-positive ($Gram^+$) actinomycetes, especially within the subgroups *Streptomyces* and *Nocardia*; as well as a variety of Gram-negative ($Gram^-$) bacteria including *Agrobacterium, Serratia, Gluconobacter, Flavobacterium* and *Xanthomonas* (Cohen *et al.*, 1990).

Table 1.1: Bacterial and fungal producers of β-lactam antibiotics. The β-lactam antibiotics are produced by certain species within the groups of microorganisms indicated below (Aharonowitz *et al.*, 1992).

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*

The filamentous fungi produce penicillins or cephalosporins, whereas the Gram$^-$ producers synthesise clavams, carbapenems, monobactams, as well as cephalosporins (Martín and Gutiérrez, 1995) but do not produce penicillins as end products (Elander 1983). All the above mentioned antibiotics (except the monobactams) contain the β-lactam ring that is fused to a second five- or six-membered ring (Figure 1.1). It is the presence or the absence of the sulphur atom that distinguishes the penicillins and the cephalosporins from other classes of β-lactam antibiotics. The clavams and the carbapenems have oxygen and carbon atoms substituted for the sulphur atom, respectively, and the monobactams possess a single ring (Sykes *et al.*, 1981). Many of the naturally occurring β-lactams have been chemically modified to produce superior antimicrobials such as cephoxitin, cefmetazol and SKF-73678 which are semi-synthetic β-lactams that were derived from cephamycin (Zimmerman and Stapley, 1983). Many streptomycetes were discovered to produce compounds such as clavulanic acid, olivanic acids, MC696-SY2-A and -B, thienamycin, PS-5, PS-6, and -7, and carpetimycins (Zimmerman and Stapley, 1983) which are potent inhibitors of β-lactamases. The combination of β-lactamase inhibitors with β-lactam antibiotics can render treatment more effective especially against β-lactamase producing strains.

Isolation programs are time consuming and expensive, especially in the search for novel antibiotic producers. Eli Lilly and Company Ltd. discovered three new antibiotics after screening 400 000 microorganisms in 10 years (Nelson, 1961: cited in Kinghorn and Turner, 1992) and Merck, Sharp and Dohme discovered one producer in 10 000 organisms (Woodruff and MacDaniel, 1958). Extensive screening of soil for antibiotic producers has significantly reduced the possibility of discovering new producers. It is evident that new screening methods have to be devised that are sensitive, selective and specific.

Microorganisms that produce antimicrobial substances have been isolated from a wide variety of environments and most commonly from soil. The predominant actinomycete group found in soil are the streptomycetes (Williams *et al.*, 1971). Streptomycetes and other actinomycetes are assured a central place in any volume of industrial microorganisms as they have been found to produce two-thirds of the thousands of naturally occurring antibiotics (Hopwood *et al.*, 1989).

Streptomycetes are Gram$^+$, obligate aerobic organisms that produce a variety of hydrolytic enzymes such as amylases, hemicellulases, cellulases, proteases and nucleases, in order to utilise the insoluble inorganic debris as part of their nutrition. They have adapted to living in the soil by growing as a mycelium of branching hyphae that produce enzymes locally and obtain nutrients through the hyphae. There are few cross-walls in the hyphae which aid in the passage of nutrients and enzymes through the colony. After a period of vegetative growth streptomycetes enter a reproductive phase which is a response to nutrient limitation (Williams *et al.*, 1989). The mycelium gives rise to spore-bearing hyphae which serve as a means of dispersal and can survive adverse conditions (Wellington *et al.*, 1992). Antibiotic production is manifested at about the same time that sporulation begins (Charter *et al.*, 1993).

1.1.2 Biosynthesis of β-lactam antibiotics

The first two steps in the biosynthesis of penicillin, cephalosporin and cephamycin classes of β-lactam antibiotics have been found to be common to both prokaryotic and eukaryotic β-lactam producers (Figure 1.1) (Jensen, 1986; Smith *et al.*, 1990a). The first step involves the formation of the cysteine-containing tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) (Arnstein and Morris, 1960; Jensen *et al.*, 1982) by the ACV synthetase complex (van Liempt *et al.*, 1989). This is followed by the conversion of the linear tripeptide ACV to isopenicillin N (IPN) by the enzyme isopenicillin N synthetase (IPNS) (Konomi *et al.*, 1979). From here the pathway diverges to penicillin, cephalosporin and cephamycin. The gene encoding the IPNS gene (*pcbC*) has been cloned from *Penicillium chrysogenum* (Carr *et al.*, 1986; Ramos *et al.*, 1985), *Cephalosporium acremonium*, (syn. *Acremonium chrysogenum; Acremonium strictum*) (Samson *et al.*, 1985); *Aspergillus nidulans* (Weigel *et al.*, 1988), *Streptomyces clavuligerus* (Leskiw *et al.*, 1988); *S. lipmanii* (Jensen *et al.*, 1986); *S. jumomjinensis* (García-Domíniguez *et al.*, 1991); *Nocardia lactamdurans* (Coque *et al.*, 1991); and *Flavobacterium sp.* (Shiffman *et al.*, 1990). All of the *pcbC* genes were isolated using synthetic or heterologous DNA probes that were used to screen by hybridization to genomic libraries of these organisms.

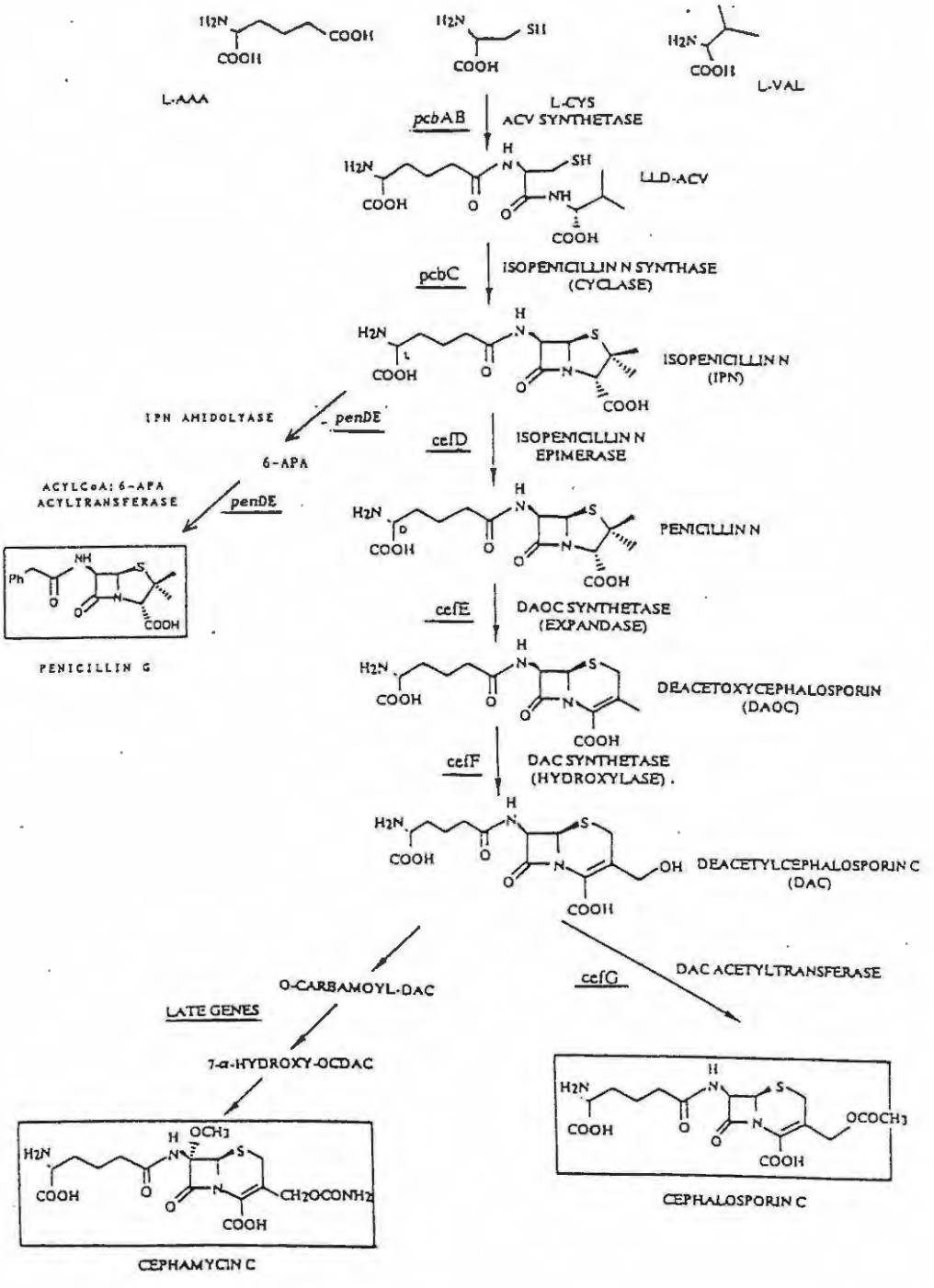


Figure 1.1: Biosynthetic pathway of penicillin, cephalosporin and cephamycin classes of β-lactam antibiotics. Gene designations are underlined (Coque *et al.*, 1991).

Malpartida *et al.*, (1987) was the first to use an antibiotic coding gene, the *act* gene (*act*I and III) as a DNA hybridization probe to hybridize to parts of gene clusters for other polyketide antibiotics. Cross-hybridization studies using the IPNS gene as a probe, has also been used to examine the distribution of β-lactam genes in nature (Shiffman *et al.*, 1988). Positive signals were obtained with producing and some of the non-producing species signifying the presence of the IPNS-like genes (Cohen *et al.*, 1990; García-Domíniguez *et al.*, 1991; Shiffman *et al.*, 1988) which may suggest cryptic or silent antibiotic genes that may have the potential to produce antibiotics. In some cases these silent genes could be activated to produce antibiotic (Adamidis and Champness, 1992; Jones and Hopwood, 1984; Malpartida *et al.*, 1987). For example, cloning of various parts of an antibiotic pathway into an organism which produces a related antibiotic can result in the production of a hybrid antibiotic (Hopwood *et al.*, 1985). Recombinant DNA methods can be used to improve antibiotic production (Skatrund *et al.*, 1989) and with the development of sophisticated vector systems (Bibb *et al.*, 1980; Gormley and Davies, 1991; Gusek and Kinsella, 1992), various enzymes can be cloned (Landman *et al.*, 1991) and manipulated for the production of novel antibiotics.

1.1.3 Origins of the β-lactam biosynthetic genes

The elucidation of evolutionary pathways may provide insight into the genetic exchange that occurs between species, and their potential to produce novel antibiotics. The degree of amino acid or nucleic acid homology between genes can be used as a molecular clock to provide an approximate estimate of the time of divergence. The relative ratios between distance separating the eukaryotes and the prokaryotes was calculated (Landan *et al.*, 1990) using sequence information obtained from nine *pcb*C genes (Aharonowitz *et al.*, 1992) which are represented in Table 1.2. It was found that the sequence similarity between the fungal and the bacterial β-lactam genes with respect to other eukaryotic and prokaryotic genes was exceptionally high (Hensel *et al.*, 1989).

Table 1.2: Percent sequence similarity between nine *pcbC* genes of different β-lactam producing organisms (Kinghorn and Turner, 1992).

	Nocardia lactamdurans	*Streptomyces griseus*	*Streptomyces jumonjinensis*	*Streptomyces lipmanii*	*Streptomyces clavuligerus*	*Flavobacterium* sp.	*Acremonium chrysogenum*	*Penicillium chrysogenum*	*Aspergillus nidulans*
								Amino acids homology (%)	
N. lactamdurans		72.6	77.0	71.5	75.0	59.3	57.3	57.3	59.6
S. griseus	78.8		75.7	73.9	72.0	59.8	53.6	56.0	57.2
S. jumonjinensis	80.9	82.0		69.7	81.5	60.9	60.4	58.4	59.1
S. lipmanii	78.8	82.8	82.0		70.9	55.3	55.6	54.4	57.0
S. clavuligerus	79.0	78.9	84.8	79.3		59.4	56.8	56.6	57.9
Flavobacterium sp.	69.1	69.8	70.1	66.7	69.2		53.6	54.5	55.2
A. chrysogenum	66.5	69.7	70.9	69.8	67.8	67.0		76.6	74.0
P. chrysogenum	64.3	65.5	67.1	67.2	65.2	65.0	76.1		81.3
A. nidulans	63.7	63.0	64.7	65.3	63.8	69.6	71.5	76.2	
			Nucleotides homology (%)						

There is 74%-80% similarity between the fungal IPNS genes and 70%-85% between *Streptomyces* IPNS genes (Shiffman *et al.*, 1988), which is typical for organisms that diverged several million years ago. However, there is 56%-62% similarity between the fungal and streptomycete IPNS genes (Leskiw *et al.*, 1988), which is much higher than is expected of organisms that diverged 2 billion years ago, based on differences in 5S RNA sequences (Hori and Osawa, 1979).

There are two hypotheses as to the unusually high degree of homology between the fungal and streptomycete IPNS sequences. One explanation is that the IPNS gene did not change much in the course of evolution (Martín and Gutiérrez, 1995). However, this hypothesis has many shortcomings as the ratio of the branch lengths between the bacteria and the fungal *pcb*C genes and the distance separating the Gram$^-$ and Gram$^+$ *pcb*C genes (Landan *et al.*, 1990; Pañalva *et al.*, 1990) is much higher than what was obtained from sequence analysis of the 5S rRNA tree (Chen *et al.*, 1984; Hori and Osawa, 1987). In addition the IPNS gene is a secondary metabolism gene, therefore not essential for the survival of the species (Vining, 1992), and the evolutionary drift of the IPNS gene is fairly typical.

A more plausible explanation is that the gene was transferred horizontally well after the divergence of the eukaryotes and the prokaryotes. The horizontal-transfer hypothesis also supports the observation that the streptomycetes, some Gram$^-$ bacteria and a wide variety of fungi are capable of producing β-lactam antibiotics (Smith *et al.*, 1990a). Weigel *et al.* (1988) estimated the transfer as having occurred some 370 million years ago. This is well after the proposed time of the eukaryotic-prokaryotic split which was estimated as 1.8 billion

years ago (Doolittle *et al.*, 1989). The transfer event was probably from the *Streptomyces* to the fungi as the *Streptomyces* have a more elaborate pathway for β-lactam production (Diez *et al.*, 1989; Peñalva *et al.*, 1990). The IPNS gene exists in gene clusters in the streptomycetes (Smith *et al.*, 1990a; Ward and Hodgson, 1993) whereas in some fungi the genes encoding the enzymes are dispersed over several chromosomes (Kovacevic *et al.*, 1989). In addition the IPNS genes lack introns which favour the idea that the transfer took place from the bacteria to the fungi. A comparison of the G+C content of fungal and bacterial IPNS genes suggests that the codon usage in fungal IPNS genes is reminiscent of *Streptomyces* IPNS genes (García-Domíniguez *et al.*, 1991; Peñalva *et al.*, 1990; Samson *et al.*, 1987). This implies that the fungal and the bacterial genes are more closely related to each other than would have been expected based on their rates of evolution. Supporting this evidence is the recent findings of the *cef*E gene of *S. clavuligerus* and *N. lactandurans* that share almost the same sequence similarity as do the *pcb*C genes from these organisms; and the *pcb*AB genes from fungi and bacteria have also been found to have sequence similarity between three repeated domains (Aharonowitz *et al.*, 1992; Landan *et al.*, 1990). If the transfer event took place 370 million years ago, then the *Streptomyces* IPNS genes would be expected to resemble the fungal genes more closely than the *Flavobacterium* IPNS genes, since the $Gram^+/Gram^-$ split occurred around 1.8 billion years ago. Subsequently, the *pcbC* gene from *Flavobacterium* spp. was cloned and sequenced (Shiffman *et al.*, 1990). This information was used to re-examine the gene-transfer hypothesis which now shows the *Streptomycete* and *Flavobacterium* IPNS genes to be closer related than the fungal IPNS genes suggesting that a single transfer event occurred some 1.0-1.5 billion years ago (Cohen *et al.*, 1990) (Figure 1.2).

Evolutionary history indicates that the invasion of land by insects, plants and soil dwelling organisms, approximately 400 million years ago, led to biological diversity and consequently competitive interaction between organisms (Maplestone *et al.*, 1992). The fact that antibiotic production is a complex secondary metabolic function, and is not essential for growth, implies that it was and is still used as a survival mechanism for species (Vining, 1992). Thus, Weigel's proposed time of antibiotic gene transfer between $Gram^+$ to $Gram^-$ (Courvalin, 1994) appears to support evolutionary history. Alternatively, multiple transfer events may have occurred at about the same time between the $Gram^+$ and the $Gram^-$ and

eukaryotic microorganisms (Peñalva *et al.*, 1990). The comparison of amino acid sequences of antibiotic genes may eliminate problems due to codon bias between diverse organisms (Kirby, 1992; Wright and Bibb, 1992) thus may provide a more complete and representative picture of the gene transfer theory.

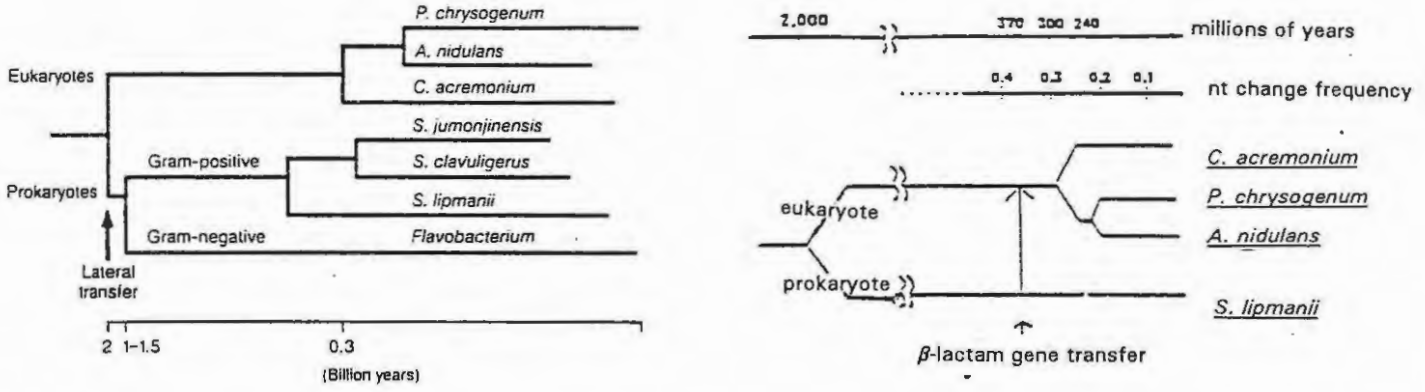


Figure 1.2: Phylogenetic trees of the IPNS genes showing lateral transfer as produced by:
A) Cohen *at al.* (1990).
B) Weigel *et al.* (1988).

1.1.4 Discovery of new antibiotics

Screening for β-lactam antibiotics includes a variety of tests based on the chemistry or biology of the compound such as activity spectra (Selwyn and Bakhtiar, 1980); mobility in paper chromatography (Woodruff *et al.*, 1979); antimicrobial spectrum (Grove and Randall, 1955); inactivation by penicillinase (White *et al.*, 1986); cross-resistance with penicillin; the chemical identity of the degradation products; the lack of antimicrobial activity against *Mycoplasma* strains (Schindler *et al.*, 1986; Selwyn and Bakhtiar, 1980); and observation of morphological changes in the test organism (Spratt, 1975).

Enzyme-based screening procedures include the enzyme linked immunosorbent assay (ELISA) (Klein *et al.*, 1993), for the detection of antibodies to the unmodified β-lactam ring, and assays of the antibiotic's inhibitory effect on a selected enzyme, such as the cell-wall D-

alanine carboxipeptidase (Schindler *et al.*, 1986). The latter method allows for the screening of β-lactam producers from broth. This method involves the conversion of the chromophor-labelled substrate *N*α-acetyl-*N*ϵ-4-(7-nitrobenzofurazanyl)-L-lysyl-D-alanyl-D-alanine ($ANLA_2$) into $ANLA_1$ with one D-alanine residue left by the DD-carboxypeptidase (DDCase). In the presence of a β-lactam antibiotic the DDCase is inactivated and since both $ANLA_1$ and $ANLA_2$ are highly florescent (Cantley *et al.*, 1978; Draper and Gold, 1980) they can be separated by thin-layer chromatography.

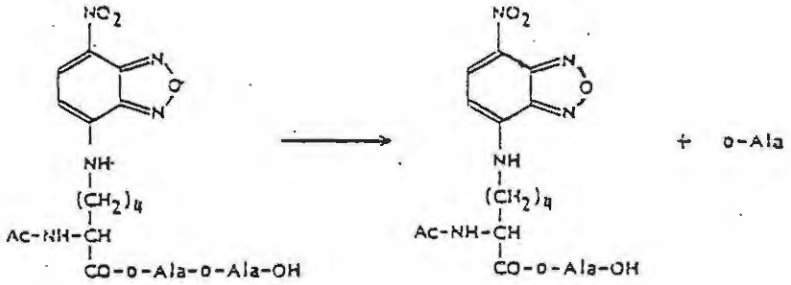


Figure 1.3: The enzymatic conversion of $ANLA_2$ to $ANLA_1$ by DDCase (Schindler and Koning, 1986).

In devising identification schemes, the characterisation tests may vary depending on criteria such as the inoculum size, temperature of incubation, length of incubation, composition of the medium, surface-to-volume ratio of the medium, and the criteria used to identify a negative or positive reaction. Antibiotic producing organisms may be found to produce more than one type of antibiotic which may be found to interfere with the screening process. For example, *S.griseus* may be found to produce streptomycin, candicidins, cephamycins, cycloheximides and several other antibiotics (Hopwood, 1978).

While some of the screening techniques are extremely sensitive, it is desirable to develop alternative techniques to detect antibiotic-biosynthesis genes in microorganisms. Genetic techniques have been shown to have great potential in the isolation and manipulation of antibiotic producers. One such technique that has revolutionised molecular biology is the polymerase chain reaction.

1.1.5 Isolation of antibiotic genes using the Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a method used to amplify a target sequence without the use of microorganisms (Mullis and Faloona, 1987; Saiki *et al.*, 1985). Since the discovery of PCR (Mullis and Faloona, 1987) this technology has been modified for many uses and has revolutionised molecular biology (Guyer and Koshland, 1990); however, it has not been used extensively for the detection of antibiotic coding genes. PCR allows the selection, isolation and amplification of an area of DNA of interest, starting with nanogram quantities of DNA. After 25 cycles of *in vitro* DNA synthesis, a specific region of DNA can be amplified $>10^5$-fold (Farr *et al.*, 1988). Due to the versatility of the technique, large scale information such as sequence data required for population and systematic studies is now possible (Gyllensten, 1989; Kocher and White, 1989; Rainey *et al.*, 1994).

The sensitivity of detecting PCR products can be increased with Southern hybridization or nested PCR (Leys *et al.*, 1994; Sellner and Mackenzie, 1994) especially if there are nonspecific amplification products present. Nested PCR comprises a two step PCR reaction where a first set of primers is used to amplify sequences that are specific to all the organisms screened. This is followed by a second and more stringent round of amplification with primers that are homologous to internal parts of the sequence produced in the first round of amplification. Such techniques could improve the detection of antibiotic genes when PCR-based methods are used.

1.1.6 Classification and identification of antibiotic producing organisms

The search for novel antibiotic producers has placed great emphasis on the identification and taxonomy of new species, especially the streptomycetes. There are three interrelated disciplines into which taxonomy can be divided: descriptive taxonomy, identification and relationships between taxa. Descriptive taxonomy is used as the original recognition and description of new taxa which are not previously known to exist. Identification can be used to assign organisms into groups previously described, whereas phylogenetic reconstruction may be used to investigate the relationships between organisms.

The primary level of information is the complete nucleotide base sequence of the bacterial genome. In practice the determination of the complete sequence of the genome is not

practical, thus various genomic techniques have been developed for the classification of bacteria. These techniques include DNA-DNA hybridizations, DNA-rRNA hybridizations, restriction fragment length polymorphisms (RFLP) and oligonucleotide probe analysis. A second level of information is given by the cellular proteins. At the third and final level, classification can be achieved using various chemotaxonomic markers by chromatographic procedures such as gas chromatography and mass spectrometry.

Despite various investigations which have attempted to provide good classification that is predictive, stable and objective (Austin and Priest, 1986), the taxonomy of *Streptomyces* remained in disarray. Failure by taxonomists to establish a reliable classification for *Streptomyces* led to a proliferation of 'new' species based on a few morphological and cultural properties, with the result that streptomycete taxonomy became a serious problem by the 1960's. In 1964 a study known as the International *Streptomyces* Project (ISP) was established to provide a set of methods that would help classify the genus and related genera (Gottlieb and Shirling, 1967). This study redescribed about 450 species and secured 400 type and neotype cultures. Descriptions were based on spore chain morphology, spore surface ornamentation, colour of spores, substrate mycelium and soluble pigments, production of melanin pigments and utilisation of carbon sources (Williams *et al.*, 1989). However, the history of streptomycete taxonomy remained in disarray (Goodfellow *et al.*, 1992) as it was based on subjectively chosen features, with heavy emphasis on morphology and pigmentation, thus placing species in monothetic groups.

1.1.7 Numerical classification of *Streptomyces*

Eventually, a comprehensive numerical taxonomic survey was attempted by Williams *et al.*, (1983a). Numerical taxonomy requires the examination of a great number of strains for a large number of characters before assigning the test organism to a cluster based on shared features. This has become an invaluable approach to the classification of large heterogeneous taxa such as *Streptomyces* (Williams *et al.*, 1983b), and *Bacillus* (White *et al.*, 1993). Williams *et al.* (1983a) classified a total of 475 *Streptomyces* strains by assembling 139 phenotypic traits that were analysed using the Jaccard (S_J) (Sneath and Sokal, 1973) and Simple Matching Coefficients (S_{SM}) (Sokal and Michener, 1958) and the unweighted arithmetic averages algorithm (UPGMA) (Sneath and Sokal, 1973). Clusters were defined

at the 63% S_J level and 77.5% and 81% S_{SM} level of similarity. The S_J coefficient was used to define the clusters. The *Streptomyces* type-strains were assigned to ten cluster-groups, 23 major clusters, 20 minor clusters, and 25 single member clusters (Figure 1.4).

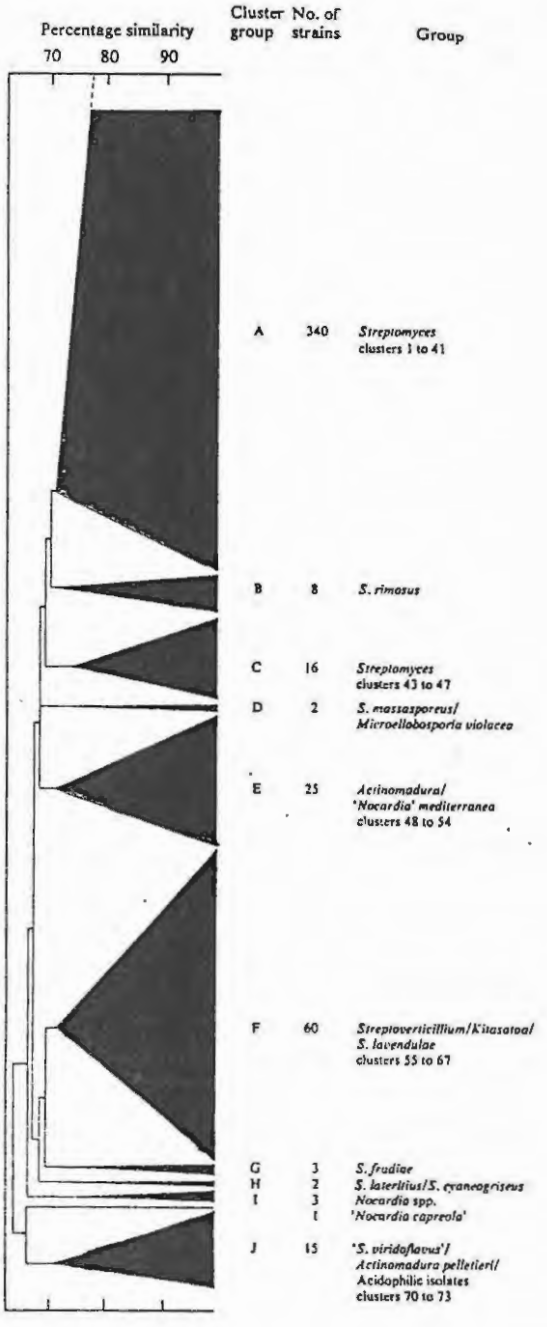


Figure 1.4: Williams *et al.*, (1983a) classification of *Streptomyces* represented as a dentrogram that shows relationships between cluster groups defined at the 70.1% similarity level using the S_{SM}/UPGMA analysis.

The Williams *et al.* (1983a) classification has been found to support chemical, DNA sequencing, and serological data (Goodfellow *et al.,* 1992). Numerical taxonomic procedures have also been successfully applied in the classification of actinomycetes (Goodfellow and Dickinson, 1985; O'Donnell *et al.,* 1993) and related genera such as *Streptosporangium* (Whitham *et al.,* 1993) that were isolated from natural environments.

Databases have been established that can be used to generate frequency matrices for the identification of unknown *Streptomyces* (Clarke *et al.,* 1994; Langham *et al.,* 1989; Williams *et al.,* 1983b) such as the CHARSEP, DIACHAR and MATIDEN programs (Vickers *et al.,* 1984). The identification systems are polythetic and provide a balanced representation of the species. However, a large number of tests are required with some being time-consuming and difficult to read. Although this approach provided a basis for classification, no model for classification has been established.

In contrast to the large biochemical and phenotypic data base of actinomycetes (O'Donnell *et al.,* 1993; Williams *et al.,* 1983a), there is only a limited amount of information on the genetic relatedness of the genus. Researchers have now turned to DNA-based methods in order to resolve discrepancies in streptomycete taxonomy. The degree of congruency between consensus classifications from chemical, phenetic and molecular systematic data needs to be examined in order to refine classification.

1.1.8 Variation at the DNA level

Over time genes may mutate, but it is only certain regions that are accessible to change whilst other regions are preserved by purifying selection. It is the variation that reveals the time and mode of evolutionary change. The organisms genome is the ultimate record of evolutionary history (Woese and Fox, 1977). Certain gene sequences and proteins are seen as 'living fossils' in the sense that they have been conserved for billions of years (Schwartz and Dayhoff, 1978) such as the 5S ribosomal RNA, 16S (Witt and Stackebrandt, 1990) and 23S rRNA (Kim *et al.,* 1990).

More recently attempts have been made to classify *Streptomyces* using molecular approaches that show good congruency to phenetic properties (Williams *et al.,* 1983b); however, rules

have not been defined to date. Such molecular approaches include 16S rRNA cataloguing and sequencing (Sherriff *et al.*, 1994), 5S rRNA sequencing (Park *et al.*, 1991), and RFLP analysis. The study of the 16S ribosomal RNA is particularly useful as it is a genetically stable and highly conserved sequence thus revealing phylogenetic relationships among the prokaryotes (Woese, 1987). 16S rRNA oligonucleotide cataloguing was used to elucidate suprageneric relationships between the actinomycetes and other bacteria (Stackebrandt and Woese, 1981). The interrelationship of the genera *Streptomyces* and *Streptoverticillium* was clarified using 16S rRNA sequences and DNA-16S/23S rRNA hybridizations (Witt and Stackebrandt, 1990) which resulted in the unification of the two genera in an amended genus *Streptomyces*. Partial 16S rRNA and 23S rRNA/DNA sequence analysis (Stackebrant *et al.*, 1992) show streptomycetes to be closely related at the rRNA level (>91%), and thus may fail to differentate between all species. DNA-DNA hybridizations are often used to evaluate genomic relationships between species as they show overall genome similarity. However, DNA-DNA hybridization studies of *Streptomyces* and *Streptoverticillium* did not show phylogenetic depth of phenetic clusters (Witt and Stackebrandt, 1990), as intercluster values were found to be as low as those between clusters. Inconsistencies in DNA-DNA hybridization studies were attributed to 'genetically silent' regions in the genome (Kieser *et al.*, 1992). Recently, studies have shown these regions may be partly the ends of the linear *Streptomyces* chromomsome (Chen, unpublished; Lin *et al.*, 1993) and their repetitive domains represent a limited portion of the genome, therefore they should not contribute a significant amount to inconsistencies in taxonomy.

Amplification of conserved regions of the prokaryotic genome such as the 16S and 23S rRNA genes flanking the spacer region (Leys *et al.*, 1994), followed by RFLP analysis of the resulting fragment, can contribute useful phylogenetic information for *Streptomyces* species. A new method known as RiDiTS was used to group *Streptomyces* stains on the basis of *Mse*I fingerprints of the rRNA operon (Fulton *et al.*, 1995). The *Mse*I restriction sites are found predominately in conserved regions of the 16S and the 23S rRNA genes and can be identified on polyacrylamide gels as intense bands as opposed to the less conspicuous non-rRNA gene fragments. When RiDiTS was compared to 16S sequence analysis, phenotypic classification (Williams *et al.*, 1983a) and DNA-DNA similarity showed some correlation in major cluster groupings but correlation was poor between individual phenotype clusters (Fulton *et al.*,

1995).

1.1.9 Serology

Divergence of proteins between species can also be quantified by serological studies. Application of immunodiffusion techniques such as Ouchterlony double-diffusion (Ridell and Williams, 1983) and indirect enzyme-linked immunosorbent assay (IND-ELISA) (Kirby and Rybicki, 1986) has been applied to streptomycete taxonomy and have shown congruence to phenetic data (Kirby and Rybicki, 1986; Ridell *et al.*, 1986).

1.1.10 Genetic 'fingerprinting' as a taxonomic tool

New tools such as oligonucleotide probing, 'genetic fingerprinting' and the analysis of PCR gene products have made the identification of taxa possible at all levels. The identification of individuals using genetic markers is often referred to as 'genetic fingerprinting'. Methods of 'genetic fingerprinting' analyses available for bacterial identification purposes include RFLP (Vermeulen *et al.*, 1994), random amplified polymorphic DNA (RAPDs) (Barua *et al.*, 1993), pulse-field gel electrophoresis (PFGE) (Cole and Girons, 1994), and ribotyping.

Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990), arbitrary primed PCR (AP-PCR) (Welsh and McClelland, 1991), and DNA amplification fingerprinting (DAF) (Caetano-Anollés *et al.*, 1991), all based on PCR (Welsh and McClelland, 1990) have shown great potential as genetic markers. These techniques differ from the conventional PCR reaction in that the genomic DNA sequences are amplified using primers of arbitrary nucleotide composition; one primer type is employed; and no previous knowledge of the target DNA is required. Each primer amplifies limited portions of the genome, producing a characteristic profile of amplification products. AP-PCR utilises primer similar to PCR primers (18-22bp in length), DAF primers can be 5 base pairs but usually 7-8 base pair primers are used, and RAPD PCR uses 10-mer primers. Theoretically, conserved and highly variable regions of the genome are encompassed by the primers (Caetano-Anollés *et al.*, 1991), however this has not been tested. RAPD PCR has been used to discern genetic variation between plant (Barua *et al.*, 1993; Hsiao and Rieseberg, 1994; Klein-Lankhorst *et al.*, 1991; Roy *et al.*, 1992; van Heusden and Bachmann, 1992; Wilde *et al.*, 1992), animals (Fukatsu *et al.*, 1994; Jones *et al.*, 1994), fish (Bardakci and Skibinski, 1994), insect

(Chapco *et al.*, 1992; Kambhampati *et al.*, 1992) bacterial (Manulis *et al.*, 1994; Rasmussen *et al.*, 1994); virus (van Belkum *et al.*, 1994) and fungal (Cobb and Clarkson, 1993; Fujimori and Okuda, 1994) species, demonstrating the versatility of the technique that can be applied at all levels of life. RAPD PCR offers a number of labour and time-saving advantages not shared by other molecular methods such as RFLP and DNA sequencing analyses.

Chachaty *et al.* (1994) found that there was good agreement between PFGE after restriction with restriction enzymes, RAPDs and ribotyping when used to distinguish between *Clostridium difficile* strains although the level of similarity between the clusters varied between the different methods. Unlike PFGE and ribotyping, PCR-based techniques proved to be faster and easier to implement.

More recently, conserved motifs in bacteria (REP, ERIC, and BOX elements) have been amplified and used as genomic markers (Louws *et al.*, 1994). REP-PCR uses stringent amplification conditions, with primers of 18-22 bp that have high homology to repetitive sequences. The resulting REP-PCR profiles show reduced experimental variation and PCR artifacts, unlike RAPD-PCR. The resulting profile is a fingerprint that corresponds to a specific lineage. Since REP, ERIC and BOX elements are repetitive sequences that are conserved in diverse bacterial species (Lupski and Weinstock, 1992), thus they may be of informative value in discerning the evolution of the bacterial genome. However, the utility of this method may be limited to closely related strains (de Bruijn, 1992). REP-PCR of *Haemophilus influenzae* using ERIC primers at low annealing temperatures were in full agreement with RFLP results, but differed from major outer-membrane protein analysis with SDS-PAGE (Belkum *et al.*, 1994). RAPDs were found to be more sensitive than conventional protein-based multilocus enzyme electrophoretic (MLEE) typing, for detecting differences between closely related *E. coli* strains (Wang *et al.*, 1993).

Streptomycete taxonomy has made limited use of 'fingerprinting' methods which include: i) Restriction fragment length polymorphisms (RFLPs) (Beyazova and Lechevalier, 1993), however this technique is labour intensive, requires the use of radioisotopes, and of large quantities of high quality DNA; ii) Since the *Streptomyces* genome has a high guanine and

cytosine (G+C) content of 69-78 mol% (Strohl, 1992), it is likely that AT-regions are conserved areas of the genome. Therefore, low frequency restriction fragment analysis (LFRFA) was performed using enzymes that would recognise these AT-regions; iii) Streptomycetes form 'chemical fingerprints' when examined by gas chromatography of cellular fatty acids (Garrity *et al.*, 1993) or by curie-point pyrolysis mass spectrometry (PyMS) (Chun *et al.*, 1993). PyMS involves the breaking down of the whole organism in an inert atmosphere, which is then detected and quantified by mass spectrometry. This method has proved to be rapid and reproducible and is of value for the classification and identification of industrially important actinomycetes (Chun *et al.*, 1993).

Although various 'fingerprinting' methods have been utilised for streptomycete taxonomy, no one system has been established that can be used reliably by different laboratories. Congruency between molecular, phenetic and chemical data will eventually lead to better classification systems for streptomycetes.

1.2 CONCLUSION

The success that has been achieved in the screening for naturally occurring β-lactam antibiotics justifies the continuation of such investigations. The greatest limitation to discovering new antibiotics is the sensitivity of the screening systems. Conventional antibiotic screening may overlook many potential antibiotic producers, whereas DNA hybridization screening of the IPNS gene has shown great potential as a sensitive and specific screening system. However, the implementation of hybridization techniques requires the use of a variety of probes obtained by cloning which can be time consuming and laborious.

The search for novel antibiotic producers has lead to the reclassification of many existing species as novel producers, especially amongst the streptomycetes. Classification of *Streptomyces* has been in disarray as one set system of classification has not yet been established. Most classification schemes are either monothetic or require a large amount of information for compilation, making them inappropriate when a large number of samples are to be screened. Thus, the elimination of duplicate strains and the identification of existing and new strains is a rate limiting step in any screening process.

It is becoming increasingly important to derive and integrate consensus classifications from chemical, phenetic and molecular data. New and refined approaches to phylogenetic analysis will aid in better representation of evolutionary and phenetic relationships. The PCR reaction has been shown to be a dynamic molecular tool with wide application; however, it has not been tested in antibiotic screening programs.

1.3 RESEARCH OBJECTIVES

The PCR technique was examined as an alternative method that could be used to target, isolate and amplify the IPNS gene without the need to clone. The utility of the PCR reaction in an antibiotic screening program was examined and evaluated as follows:

i) The development of a sensitive detection system for the screening of potential β-lactam producers, which would involve the development of a PCR-based screen for novel β-lactam producers.

ii) Application of a molecular screen against organisms of established identity and also novel isolates; and the investigation of evolutionary relationships amongst the genes.

iii) The evaluation of RAPD profiles of *Streptomyces* as a potential identification tool in detecting novel isolates in an antimicrobial screening program.

iv) Investigation of the potential contribution of RAPD and protein-fingerprinting to streptomycete systematics.

v) The investigation of current data-handling procedures and their application to RAPD analysis of *Streptomyces* species.

The application of PCR-based molecular methods may lead to a greater understanding of the genetic basis of β-lactam production and may help in the detection of new and superior antibiotics.

CHAPTER 2

ISOLATION, MAINTENANCE AND SCREENING OF ANTIBIOTIC PRODUCING ORGANISMS

An integral part of any antibiotic screening program is the isolation and maintenance of the producing microorganisms. Cultures that were stored as glycerol stocks and lyophilised cultures were more likely to retain their antibiotic producing ability. Isolation of antibiotic-producing cultures involved the obtaining of a mixed or pure culture, followed by their assessment to determine which cultures were actively producing antimicrobial products. Various antibiotic screening methods were tested of which the cross-streak method produced the most reproducible results.

2.1 INTRODUCTION

Various factors have to be considered when screening programs are established in the search for new antibiotics. The first factor of any antibiotic screening program is the isolation of potential producers. Secondly, screening programs require that the test culture be grown under conditions where it will produce a detectable amount of the antibiotic. The third factor to be considered is the selection of a screening system that is sensitive enough to detect antibiotic production and specific enough to select for β-lactam producers. Finally, an identification system is essential in any screening program, where producing organisms can be classified and differentiated from the large number of organisms screened, thus eliminating the rediscovery of existing producers.

2.1.1 Isolation and purification of actinomycetes from soil

The actinomycetes and the *Aspergillales* are the producers of the greatest variety of antibiotics. Actinomycetes have been isolated from various environments including soil, leaf materials, water and marine sediments (Hunter-Cevera, *et al.,* 1986; Schrader and Blevins, 1993; Weyland, 1981). Isolation of actinomycetes has various associated limitations due to their rate of radial growth on media being lower than that of the fungi and their rate of cell production is generally lower than that of bacteria. Except for the thermophilic forms, their growth cycle spans from three to 20 days (Cross, 1989). Therefore, in mixed cultures the actinomycetes are often overlooked due to faster growing organisms such as the fungi. Air drying of the soil for three

to 10 days helps to reduce the contaminating bacterial numbers whilst allowing actinomycete spores to survive. Actinomycete cultures that are contaminated by bacteria and fungi can be purified using the 'streak plate technique' where the actinomycete is streaked onto a medium that is conducive to growth; higher success in separation can be achieved using the pour-plate technique with the inclusion of antifungal antibiotics such as nystatin and cycloheximidine (Hunter-Cevera *et al.*, 1986).

Most actinomycetes are isolated in lean or complex media rather than a rich growth media (Williams and Cross, 1971). However, no one medium will allow the detection of all the actinomycetes in the sample, therefore several media are commonly used. In order to isolate a representative population from a particular ecosystem, various ecological parameters must be considered, including pH, temperature, ionic strength, E_h potential, and substrate concentration. Taking all parameters into account, selective media can be modified to represent the ecosystem being examined.

2.1.2 Screening for antimicrobial activities

Since actinomycetes produce such a plethora of antibiotics, it is necessary to have a rapid, specific, and efficient method of identification, especially a method that does not require prior isolation and purification of the antibiotic. In the search for novel antibiotics, new compounds may occur at very low concentrations, therefore biological tools are generally preferred for screening purposes as they are more sensitive and selective than chemical screening procedures.

It is not possible nor practical to describe all the screening tests currently used. The most common type of microbial screening method for the detection of β-lactam producers is the agar diffusion test (Grove and Randall, 1955; White *et al.*, 1986). Since the discovery of antimicrobial screening the tests have been refined without much alteration. The conventional antibiotic diffusion assay is simple and does not require elaborate apparatus. The test organism is dispensed onto a plate which has been inoculated with a known organism. Following incubation, the production of antimicrobial substances by the test organism can be detected by a zone of inhibition in the growth of the known organism. The tests most commonly employed

are the serial-dilution tests, the plate-diffusion tests and streaking on solid media containing the test organism (Brock and Madigan, 1988; Zimmerman and Stapley, 1983).

Many organisms are producers of β-lactamases and β-lactamase inhibitors which can be utilized in the screening for β-lactam antibiotics (Collatz *et al.*, 1990). The β-lactamases are enzymes that hybrolytically cleave the cyclic amide bond of the β-lactam ring (White *et al.*, 1986). Potent inhibitors of the β-lactamases include the clavams and carbapenems (thienamycins) discovered in *S. clavuligerus* and *S. cattleya*, which are themselves β-lactam antibiotics (Cohen *et al.*, 1990). Assay methods developed to increase the sensitivity of β-lactam detection include: growth inhibition assays which involve β-lactam hypersensitive strains (Aoki *et al.*, 1977); β-lactamase induction assay (Sykes and Wells, 1985); differential growth inhibition assays that involve antibiotic sensitive and resistant strains (Ono *et al.*, 1984); and the β-lactamase stability assay (Aoki *et al.*, 1977; Ono *et al.*, 1984). Although these assay methods may improve the sensitivity and selectivity of the screening procedures, the organisms isolated are still reliant on the culture media and growth conditions selected for screening.

2.2 METHODS

2.2.1 Standard buffers and media

Appendix A and B lists all the standard media and buffers used.

2.2.2 Media and culture techniques

All streptomycete cultures (Table 2.1) were routinely grown on M_3 medium and broth (Appendix A). Broth cultures were grown in 250 ml Erlenmeyer flasks containing 25-50 ml culture and were incubated on an orbital shaking incubator (28°C; 160-200 rpm). Growth of streptomycete cultures in broth required considerable agitation and aeration, in order to give an even, suspended growth. Glass balls were added to agitate the broth cultures to prevent pellet formation due to the intertwining, germination and elongation of the mycelia. A large number of growth sources was required to give a heavy dispersed growth, which was achieved by the

use of a spore inoculum or homogenised vegetative mycelium.

Bacterial cultures that were used as indicator strains in antibiotic assays and were maintained on nutrient agar (NA) plates and nutrient broth (NB) included: *E. coli* DH5α, *E. coli* DH5α/pUC18, *E. coli K12*, *Bacillus subtilus*, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* (obtained from the Rhodes University Culture collection, Grahamstown, SA). *Acremonium strictum* and *A. chrysogenum* were grown on YpSs media at 24°C; *A. persicinum* and *Penicillium chrysogenum* were maintained on PDA at 24°C; and *Aspergillus nidulans* was cultured on AS media at 30°C (Appendix A). Thick agar plates, containing 25 ml of medium, were required for fungal and streptomycete strains as growth was slow (3-30 days) which resulted in eventual medium dehydration. Streptomycete incubations were carried out between 25°C-30°C. Contaminating bacteria were detected by streaking out isolated colonies and examining them for differences in colony colour, size and texture. Turbidity of the broth media was also found to be an indication of bacterial contamination (*Streptomyces* mycelium settles to the bottom of the flask when undisturbed, leaving a clear upper supernatant). Fungal contamination in broths could be checked by dispersing a sample in lactophenol-cotton blue (Williams *et al.*, 1989).

2.2.3 Preservation of cultures

Cultures were preserved using slope and plate cultures, glycerol stocks, and freeze dried cultures (Hopwood *et al.*, 1985).

2.2.3.i Slope and plate cultures

Many of the cultures obtained for analysis were revived from M_3 slopes that had been incubated at room temperature. Some streptomycete strains could be subcultured from old slants whereas more sensitive species were found to regain viability when inoculated in M_3 or YG broth and incubated with shaking at 25°C. Once growth was evident the cells were plated out onto M_3 agar plates. All cultures on M_3 slants and sealed Petri dishes were stored at 4°C. These cultures retained their viability for months provided they had sporulated before they were placed at 4°C.

2.2.3.ii Glycerol cultures

A convenient method employed for preserving streptomycetes for longer periods of time was by detaching spores from aerial hyphae with a loop and placing them in a suspended medium (usually dH_2O) containing 15-20% glycerol. The spore suspension was placed in Eppendorf tubes and stored at -20°C. Such spore suspensions were repeatedly used as inoculum for both DNA and protein extractions.

2.2.3.iii Freeze dried cultures

Most of the streptomycete and fungal strains were revived from freeze dried cultures (Perlam and Kikuchi, 1977). The freeze dried cultures were resuspended in 1 ml M_3 broth and allowed to rehydrate for 2 hours. Subsequently, 100 μl was spread onto M_3 plates. The plates were incubated between 25-30°C until sporulation occurred (usually 5 to 10 days).

2.2.3.iv Storage of fungal cultures

Agar plates and agar slants were inoculated with fungal agar plugs which were incubated at 25°C for 4-10 days. Agar plugs under sterile water proved to be an efficient method for long term preservation of fungal cultures (McGinnis *et al.*, 1974).

2.2.3.v Culturing and preservation of indicator strains

All bacterial indicator strains were maintained on NA plates and liquid cultures in NB broth. Cultures were found to grow equally well on M_3 plates at 37°C. All cultures were frequently subcultured on agar plates and slants to prevent contamination and maintain viable daily working stocks. For long term storage, bacterial stains were maintained as 20% glycerol stocks at -20°C.

2.2.4. Isolation of actinomycetes from the soil

Soil was collected from six areas at the "Ex-Shell" depot in Grahamstown, RSA, during the month of March 1993. This area was chosen as the soil samples were readily available and the soil was considered to be from an extreme environment, therefore it was hoped that a novel antibiotic producer would be isolated. Soil isolation procedures are described by Hunter-Cevera *et al.* (1986). Briefly, soil samples were first air dried for three to 10 days. This step reduced

the vegetative bacterial cells and allowed actinomycete spores to survive. Soil samples collected from various representative sites were pooled, mixed and divided into quarters. The first and the third quarter were pooled together, mixed and divided into quarters again, this procedure being repeated until a small (5 g dry weight) representative sample of the site was obtained. The soil was added to 99 ml of 25% (v/v) soil infusion contained in a 250 ml flask. The sample was incubated at 26°C on a rotary shaker at 150-200 rpm, for 30 minutes. Agar plates were dried to prevent contaminating bacterial growth (Demain and Solomon, 1986). A serial dilution of the soil suspension was prepared in the appropriate diluent and 0.1 ml was spread onto Soil infusion, M_3 and NA agar plates (Appendix A). Plates were incubated at 25°C for four to 10 days. Plates were checked on a daily basis, and streptomycetes were subcultured onto M_3 plates using the streak-plate method to obtain pure cultures[1] (Hunter-Cevera *et al.*, 1986).

2.2.5 Antimicrobial screening

Almost any nutrient agar can be used although the composition of the medium can affect the size and sharpness of the zone of inhibition. M_3 agar plates (Appendix A) were used for the antibiotic assays as it was found to sustain both the test and indicator organisms. All actinomycete strains that were used in antimicrobial screening were grown to stationary phase or until sporulation occurred. Several different antimicrobial activity screening methods were tested:

2.2.5.i. Agar plugs (10 mm diameter) containing the actinomycete strains or the fungi were placed on M_3 plates that had been spread with 100 μl of a 0.1 diluted overnight culture of *E. coli* DH5α, and were then incubated at 37°C. Zones of inhibition were measured and taken to indicate potential antimicrobial activity of the test organism.

2.2.5.ii. The Cup-Plate Method: Wells (10 mm in diameter) that were cut out of M_3 agar and had been seeded with *E. coli*, were filled with 80 μl of actinomycete or fungal

[1] Isolations done by Maria Da Serra, LIRI Technologies, Grahamstown, RSA

broth, and were incubated at 37°C. The test organism had been prepared by diluting an overnight culture to an optical density equivalent to an absorbance of 0.3 at a wavelength of 660 nm. Of this dilute suspension 1 ml was used per 100 ml of the assay agar.

2.2.5.iii The cross-streak method was performed where the actinomycete was streaked in the centre of the plate, and the test strain was streaked perpendicular to the actinomycete. The indicator strains were the *E. coli* β-lactam sensitive strain DH5α, and the β-lactamase producer DH5α/pUC18.

2.2.5.iv Antimicrobial spectra of all the soil isolates were determined by streaking the soil isolate on one half of the plate. The plate was incubated at 30°C until sporulation was evident. Indicator strains: *E. coli* DH5α, *B. subtilis, S. aeruginosa* and *S. aureus* were then streaked at right angles to the soil isolate (Figure 2.1) and the plate was incubated, overnight at 37°C. Antimicrobial activity was indicated by inhibition of growth of the indicator strains adjacent to the streak of the actinomycete. All tests were done in triplicate and an average zone of inhibition was recorded.

2.3 RESULTS

2.3.1 Preservation of cultures

In general, most slant and plate cultures retained their viability for months when stored at 4°C. Fungal cultures retained their viability on agar slants for up to 3 months storage at 4°C. Strains that were maintained as suspensions of spores and mycelial fragments in glycerol (20%, v/v) at -20°C maintained their integrity over a longer period of time than plate or slope cultures.

2.3.3 Antimicrobial screening

Various antimicrobial screening methods that were tested were based on conventional agar diffusion assays. Lysis zones were evident around microorganisms that were producing antimicrobial substances. Agar plugs of streptomycete cultures placed on a plate seeded with the test organism, were found to be less sensitive than the cup-plate method and the cross-streak method. Unlike the streptomycetes, the fungal zones of inhibition were reproducible

between screening tests.

Due to the closer proximity of the test organism to the test solution in the cup-plate method, the zones of inhibition were greater than using agar plugs as the suspended particulate matter did not interfere with the outward diffusion of the antibiotic. However, this method did not give satisfactory reproducibility and there was a greater tendency for contamination to occur.

The rationale behind using a β-lactamase producing strain parallel to an antibiotic sensitive strain in the cross-streak method was to determine potential β-lactam producers. Zones of inhibition between the test organisms, DH5α/pUC18 and DH5α, and the actinomycete where determined. If the test organism was a β-lactam producer the zone of inhibition was expected to be reduced by the β-lactamase that was excreted by DH5α/pUC18. Incubation of the test strains with β-lactamase producing indicator strains did not produce any conclusive results.

The agar-plug method worked well for fungal cultures whereas actinomycete strains produced the most reproducible results with the method illustrated in Figure 2.1.

2.3.4 Isolation of *Streptomyces* from the soil

Various actinomycetes that were isolated from the soil were found to be producers of antimicrobial substances against both Gram$^-$ and Gram$^+$ organisms (Table 2.3). A preliminary screen indicating the potential antimicrobial activity of the various soil isolates was represented with '+' signs (Table 2.3). The cross-streak method was used as it allowed for the simultaneous screening of multiple organisms (Figure 2.1).

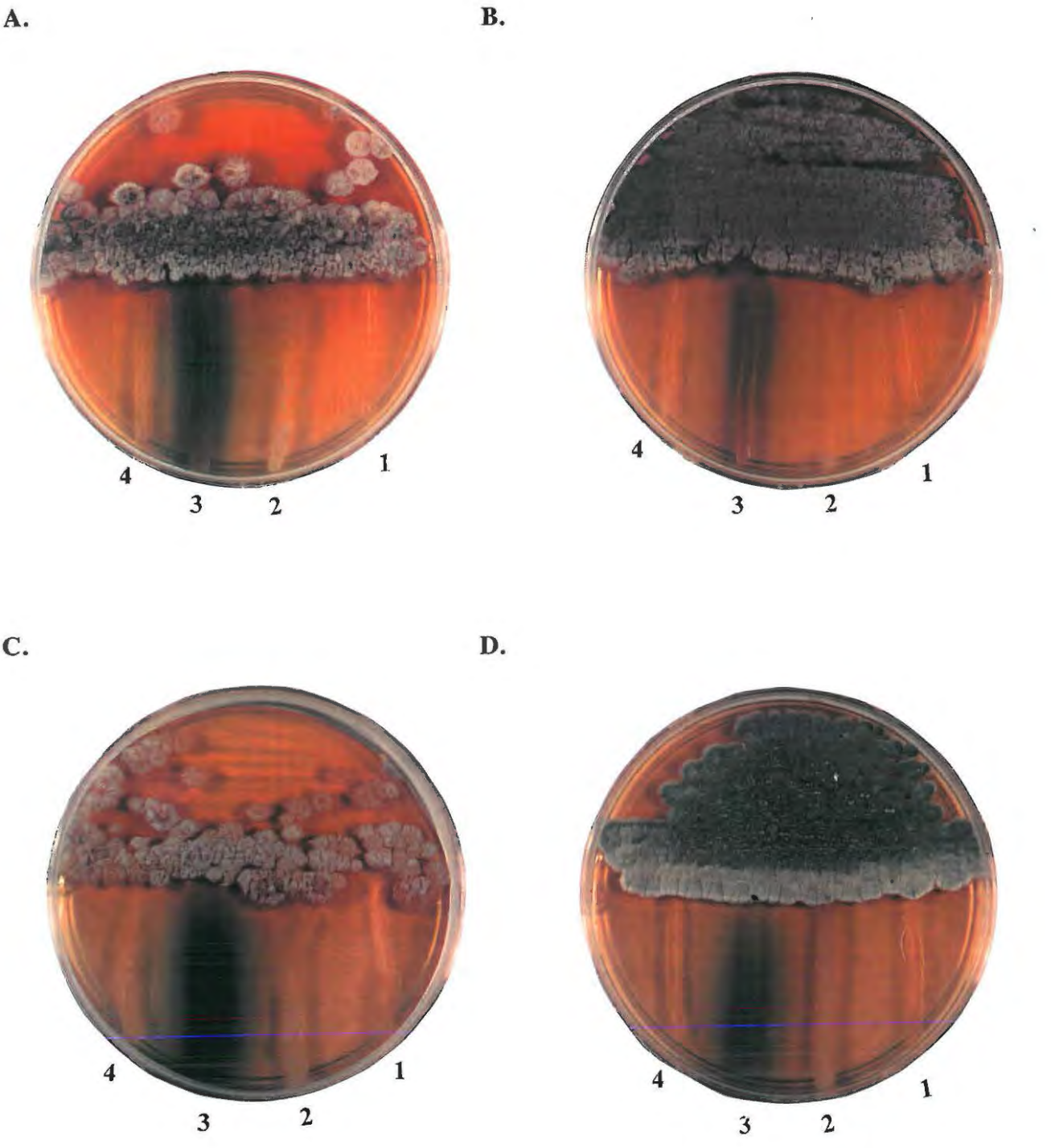


Figure 2.1: Preliminary testing of streptomycete soil isolates for the production of antibiotic. Perpendicular to the producer *(Streptomyces)* the test organisms were streaked in the following order: 1. *E. coli*; 2. *B. subtilis*; 3. *P. aeruginosa*; 4. *S. aureus*. Failure of the test organisms to grow near the *Streptomyces* indicated that the *Streptomyces* produced an antimicrobial active against these bacteria. Plates A, B, C, D correspond to soil isolates (S): S14, S15, S21 and S22, respectively. Zones of inhibition are recorded in Table 2.3.

Table 2.1: Antimicrobial properties of *Streptomyces* strains against *E. coli* (using the 'cross-streak' method). All of the streptomycete cultures that were used for genetic studies.

Streptomyces	Zones‡ of inhibition	Antimicrobials and other metabolites produced	References
S. ambofaciens ATCC 23877	+	spiramycin and congocidin	Williams *et al.*, 1989
S. ambofaciens ATCC 15154	NR	spiramycins I, II and III	Williams *et al.*, 1989
S. antibioticus ATCC 11891	+	actinomycin; oleandomycin	Hopwood and Merrick, 1977; Williams *et al.*, 1989
S. argenteolus ATCC 11009	++++	vitamin B_{12}; carbapenems	Williams *et al.*, 1989; Nakamura *et al.*, 1989
S. aureofaciens ATCC 10762	+	tetracyclines aureomycin chlortetracycline	Crandall and Hamill, 1986; Williams *et al.*, 1989; Häcker *et al.*, 1991.
S. cattleya ATCC 35852	+++	thienamycin; carbapenems cephamycin C	Elander, 1983; Nakamura *et al.*, 1989; Smith *et al.*, 1990b
S. chartreusis ISP 5085; ATCC 14922	-	chartreusin	Kuczek *et al.*, 1994
S. clavuligerus ATCC 27064	++	cephamycin, penicillin N, *O*-carbamoyldaecetyl; cephalosporin C; cephamycin C; clavulanic acid; olivanic acid	Kuczek *et al.*, 1994; Yu *et al.*, 1994; Ward and Hodgson, 1993; Hopwood and Merrick, 1977; Kirby, 1983
S. flavogriseus DSM 40990	+	carbapenems	Nakamura *et al.*, 1989
*S. fradiae**	-	neomicin	Crandall and Hamill, 1986; Hopwood and Merrick, 1977
S. fulvoviridis ATCC 21954	+++	β-lactams, Tylosin, olivanic acid	Kuczek *et al.*, 1994, Kirby, 1983
S. galbus ATCC 14077	+++	streptomycin	Williams *et al.*, 1989
S. griseus ATCC 10137	+++	β-lactams	
S. griseus ATCC 31031	-	β-lactams: deacetoxycephalosporin	Williams *et al.*, 1989
S. griseus ATCC 13968	-	steroids	Williams *et al.*, 1989
S. halstedii ATCC 13449	+	steroids	Williams *et al.*, 1989
S. hygroscopicus ATCC 31039	++	deacetoxycephalosporin	Williams *et al.*, 1989
S. hygroscopicus subsp. sagamiesis ATCC 21703	++++	spectinomycin	Williams *et al.*, 1989
S. lipmanii ATCC 27357	++++	penicillin N, cephamycins, olivanic acid	Weigel *et al.*, 1988; Hopwood and Merrick, 1977
S. olivaceus ATCC 31126	++++	β-lactams; oxytetracycline	Kirby and Rybicki, 1986
*S. rimosus**	NR	chlorotetracycline	Kuczek *et al.*, 1994
S. tanashiensis subs. *cephalomyceticus* ATCC 33159	-	β-lactams, cephalomycin	Williams *et al.*, 1989
S. venezuelae ATCC 10595	+	chloramphenicol	Hopwood and Merrick, 1977
S. venezuelae ATCC 10712	+	chloramphenicol	Crandall and Hamill, 1986

* From Rhodes University culture collection, Grahamstown, SA

‡The zones of inhibition are represented as -: no inhibitory effect; +: <10mm; ++: 10-20mm; +++: 20-30mm; ++++: >30mm. **NR**: not recorded, due to lack or reproducibility.

Table 2.2: Fungal and bacterial β-lactam cultures that were tested for antimicrobial production against *E. coli* DH5α.

Fungal and bacterial cultures	Zones‡ of inhibition	Antimicrobials and other metabolites produced	References
Acremonium strictum DSM 2399/ATCC 20339	++++	β-lactam	-
Acremonium chrysogenum DSM 880/ATCC 11550	+++	cephalosporins	Láiz *et al.*, 1990
Acremonium persicinum DSM 1073/ATCC 34702	++	β-lactam	-
Acremonium persicinum DSM 1052/ATCC 20418	++++	β-lactam	-
Aspergillus nidulans ATCC 28902	++++	penicillin G	Hopwood and Merrick, 1977
Penicillium chrysogenum DSM 895/ATCC 10003	++++	penicillin G,O,V	Hopwood and Merrick, 1977
Flexibacter sp. DSM 3098/ATCC 35103	NR	β-lactam	Sykes *et al.*, 1982
Flexibacter sp. DSM 3589/ATCC 35208	NR	monobactams; formadicins	Singh *et al.*, 1983

[a]For media refer to appendix A; ‡The zones of inhibition are represented as -: no inhibitory effect; +: <10mm; ++: 10-20mm; +++: 20-30mm; ++++: >30mm; NR: not recorded, due to lack or reproducibility.

Table 2.3: Results of antimicrobial plate screening of soil isolates is tabulated below. The zones of inhibition are represented as : +: <10mm; ++: 10-20mm; +++: 20-30mm; ++++: >30mm; and -: no zone of inhibition.

Sample No.	Media of original isolation‡	*E. coli HB101*	*B. subtilus*	*Ps. aeruginosa*	*S. aureus*
		Zones of Inhibition			
S1	SIA	-	-	-	-
S2	SIA	-	-	-	-
S3	SIA	-	-	-	-
S4	NA	-	+++	-	+++
S5	SIA	++++	-	-	+
S6	SIA	++++	-	-	+
S7	SIA	+	++	-	+
S8	SIA	-	-	-	-
S9	SIA	-	-	-	-
S10	SIA	+++	-	-	+
S11	SIA	+++	-	-	+
S12	SIA	-	-	-	+
S13	SIA	-	+++++	+++	-
S14	NA/SIA	+++	+	-	+
S15	SIA	++	++	-	+
S16	NA	-	-	-	-
S17	SIA	+++	+++	-	+
S18	M_3	-	+	-	-
S20	M_3	-	-	-	-
S21	SIA	+++	+++	-	+
S22	SIA	-	-	-	-
S24	SIA	++	+	+	++
S25	SIA	+++++	++++	++	+++
S26	SIA	+++	-	-	+
S27	SIA	+	-	-	+

‡ Media used for the original isolation of the actinomycetes is described in Appendix A.2.4.

2.4 DISCUSSION

Preservation of cultures on slants and on plates, although practical, must be viewed as unreliable. Frequent subculturing of strains onto fresh media can result in the loss of desirable biosynthetic properties and a diminution of the ability to produce aerial mycelia (Williams and Cross, 1971). Storage at 4^0C retarded the process and cultures remained viable for over 3 years without showing any noticeable changes. There is great variability in actinomycetes which is caused by the breakdown or mutations of a heterokaryotic condition on sporulation (Williams and Cross, 1971). Therefore, it is important to use methods of maintaining cultures that reduce the chance of morphological and biochemical changes. Freeze-dried cultures retain their ability to produce spores and pigments, and according to Kutznetsov *et al.* (1962; cited in Williams and Cross, 1971), freeze-dried cultures retained their ability to produce antibiotic better than strains preserved on agar.

2.4.1 Antimicrobial screening

Preliminary antibiotic screening tests were used to determine whether the revived slant cultures had lost their ability to produce antibiotics. Various methods for screening for anti-microbial production by actinomycetes and the fungi were investigated and compared. Antibiotic assays were performed when cultures had grown to stationary phase as antibiotic production is developmentally regulated and is found to coincide with sporulation (Charter, 1984). The 'cross-streak' method was found to be sensitive and reproducible (Tables 2.1, 2.2 and 2.3). This type of preliminary screening was simple and allowed for a large number of indicator strains to be tested simultaneously (Figure 2.1).

The 'cross-streak' method, where the test organism was tested against a β-lactam sensitive strain and a β-lactam resistant strain, was employed to distinguish between β-lactam producing strains and other antimicrobial producing strains. Results were not conclusive as certain actinomycetes produce their own β-lactamase inhibitors (Elander, 1983). For example, the clavams and the carbapenems, that are produced by *S. clavuligerus* and *S. cattleya* respectively, and olivanic acid produced by *S. olivaceus* are both inhibitors of the β-lactamases (Elander, 1983). Thus, the use of β-lactamases as part of the screening process may not always yield valid results when novel producers of unknown etiology are being screened.

2.4.2 Cross-streak method

S. chartreusis, *S. fradiae*, *S. griseus* ATCC 31031, and *S. griseus* ATCC 13968 are know producers of antibiotic; however, they did not show any antimicrobial activity against *E. coli* DH5α (Table 2.1). Continuous subculturing and long term storage on agar slants could have caused *S. chartreusis* to loose its ability to produce antibiotic. Lack of reproducibility between experiments for *S. rimosus*, *S. ambofaciens* ATCC 15154, and *Flexibacter* spp. could also indicate that the test organisms were resistant or not sensitive enough for the particular antimicrobial substance produced. This method gave a preliminary indication of antimicrobial activity but could not be taken as an absolute recording. The use of highly sensitive β-lactam organisms could be used to improve the sensitivity of the screening procedures (Kitano *et al.*, 1977; Sykes *et al.*, 1982).

Failure of the agar plate method to detect low levels of antibiotic is attributed to the choice of test organism and media use. Moreover, many actinomycetes produce a variety of other antibiotics that may interfere with the screening process.

2.4.3 Discovery of β-lactam and β-lactam-like antibiotics

In an attempt to obtain better chemotherapeutics, that are active against $Gram^+$ and $Gram^-$ bacteria, there is a continuous search for naturally occurring β-lactam antibiotics with a novel nucleus or partial structures.

Soil isolates from the "Ex-Shell" depot that were screened for antimicrobial activity (Table 2.3) proved to be abundant sources of antimicrobial products. Further screening would be required to relate these producers in a general group, before identification can be conducted using techniques such as NMR, HPLC, spectroscopy, mass spectroscopy, UV and infrared spectraphotometry. Soil isolates displayed a wide spectrum of antimicrobial activity against Gram- and Gram+ organisms: 44% of the soil isolates showed antimicrobial activity against *E. coli*; 37% against *B. subtilis*; 11% against *P. aeruginosa*; and 52% against *S. aureus*. These results clearly demonstrate actinomycetes as valuable producers of antimicrobial substances. The discovery of novel producers is only limited by the selectivity and sensitivity of the screening process employed.

Although preliminary screening for biologically active compounds from soil dwelling organisms has been reported widely (Holmalahti *et al.*, 1994; Nakamura *et al.*, 1989), there are limitations that may result in valuable producers of antibiotics being overlooked: i) It is not possible to test all microorganisms for production of antibiotics. For example, slow growing organisms may be overlooked or overrun by more robust growers; parasitic organisms may be overlooked as there are no generally applicable test methods to determine the antibiotics that they may produce. ii) Investigators engaged in screening usually focus on a limited number of organisms. iii) Testing conditions also impose various limitations on the detection of novel producers, namely the limited number of indicator strains that can be tested; the time of exposure of these strains to the antibiotic producing strain; and the general culture conditions (Zähner and Maas, 1972). iv) Generally the effectiveness of an antibiotic depends on a number of variables that have to be taken into account when screening for new producers. These variables include: the medium used for testing; the phase and the rate of growth of the organism; the concentration of the antibiotic produced; the population density; and resistance of the test organism to the antibiotic being produced (Zähner and Maas, 1972).

2.5 CONCLUSION

A simple and effective method for the detection of antibiotic production is the cross-streak method. In order to confirm readings a greater number of organisms would have to be screened making this procedure laborious and time consuming. Due to the nature of biological systems, there are many steps in traditional screening techniques which may limit sensitivity. These methods may overlook potential producing strains, therefore screening of β-lactam producers using molecular methods such as the PCR and Southern blotting may improve sensitivity. PCR-based methods were examined as a a more sensitive and rapid screening system than conventional methods. The X-Shell depot strains designated as the "wild" group were tested for the production of antimicrobial substances using conventional methods and were also screened using PCR based methods (Chapter 3). A control group of organisms obtained from established culture collections was used to verify result obtained with genetic screening. PCR can amplify a single gene that is common to a varied group of organisms and therefore dependance on optimum culturing conditions becomes less important.

CHAPTER 3

DETECTION OF THE *pcbC* GENE USING THE POLYMERASE CHAIN REACTION

Primers that amplify the *pcbC* gene, encoding isopenicillin N synthase were used. Essentially, it was the primers that governed the success of the PCR reaction. Incorporation of a GC-clamp and restriction enzyme sites in the primers was a simple way of introducing useful sequences into the amplification product. Due to the high GC content of streptomycetes (68-72%) amplification often did not respond to usual PCR conditions. The addition of 3% (w/v) acetamide to the reaction enhanced the specificity of the reaction. Amplification of the IPNS gene in *S. clavuligerus, S. tanashiensis, S. griseus, S. olivaceus, S. lipmanii, S. chartreusis* and other *Streptomyces* spp. was successful. Amplification of the IPNS gene can therefore be used for the detection of species containing the β-lactam gene.

3.1 INTRODUCTION

A unique method of amplifying DNA, which has found application in virtually all fields of molecular biology, is the polymerase chain reaction (PCR) (Erlich, 1989; Erlich *et al.*, 1988; Innis *et al.*, 1990; Saiki *et al.*, 1988; Tower and Cockayne, 1993). The specificity of the PCR reaction can be evaluated by the production of the target fragment relative to other products by gel electrophoresis. Enzymatic amplification of target genomic sequences has been used as a rapid diagnostic tool (Border *et al.*, 1990; Brisson-Nöel *et al.*, 1989). Its utility has been extended for the detection and isolation of antibiotic coding genes which could lead to a more rapid and specific antibiotic screening system.

Various factors can influence the specificity of the PCR reaction such as the concentration of the target sequence, reaction components, and the temperature profile (Innis and Gelfand, 1990). However, it is the primers that chiefly govern the success or failure of the PCR reaction.

3.1.1 Primer selection

Primers should preferably be between 20 and 30 bases in length; have a random base

distribution; and extended sequences of polypurines, polypyrimidines, or unusual sequences should be avoided as they can pair outside the desired sequence or complementary sequences within the same primer (Saiki, 1989). Extended sequences (three or more) of G's and C's at the 3' end of the primers should be avoided as it may promote mispriming at G+C-rich sequences. Palindromic base sequences can cause the primer to fold and pair with itself. It has been shown that the match at the 3' end of the primer is essential for the success of PCR because T*aq* polymerase lacks 3'-5' exonuclease activity (Hewitt *et al.,* 1990). Primer with complementary 3' ends can pair with themselves and amplify to form primer dimers which compete for enzyme and nucleotides at the expense of the target fragment (Saiki, 1989).

Scharf *et al.* (1986) first showed that it is quite simple to add restriction sites to the 5' end of the primer with no affect on the PCR reaction. In the event of a novel β-lactam antibiotic being discovered, the incorporation of restriction sites, regulatory elements (*eg.* promoters) and GC-clamps on the 5' end of the primer, which become included at the ends of the double-stranded PCR product, may facilitate further genetic manipulation of the gene product (Higuchi, 1989). Restriction sites can also be incorporated internally in the primer near the 5' end of the primer so that the 3' end matches the target sequence and is not mispaired. The attachment of a 40-45 bp G+C- rich sequence, called a GC-clamp, to one end of the amplified fragment can enhance the resolution on denaturation gradient gel electrophoresis (DGGE) of fragments that differ by as little as a single base (Fischer and Lerman, 1983; Myers *et al.,* 1989; Sheffield *et al.,* 1989). Amplification of the IPNS fragment followed by DGGE analysis can therefore be used to discriminate between closely related sequences without the need to sequence. The PCR reaction offers a rapid and simple means of detecting and modifying antibiotic genes which can have great implications in an antibiotic screening program.

3.2 METHODS

3.2.1 Standard buffers and media

All the standard media and buffers used are listed in Appendix A and B, respectively.

3.2.2 Culture conditions

3.2.2.i Growth of *Streptomyces* for the isolation of total DNA

Mycelia for DNA extractions of *Steptomyces* were obtained by inoculating 50 ml of M_3 broth (Kirby and Lewis, 1981) with 100 μl of *Streptomyces* spore suspensions (prepared as in Chapter 2). Cultures in broth media were incubated at 28°C on orbital shakers at 200 rpm for 5 to 7 days.

3.2.2.ii Growth of fungi for the isolation of DNA

Fungal cultures were grown in the appropriate broths (refer to chapter 2) at 25°C on an orbital shaker (150 rpm) for 7-10 days. Mycelia were harvested by filtration through a Buchner funnel onto Whatman 3 MM filter paper. All fungal cultures used for DNA extractions were checked for contamination by inoculation onto appropriate agar plates.

3.2.3 DNA extractions

3.2.3.i Optimization of DNA extractions for the PCR reaction

Total genomic DNA was obtained using a mini-prep method that was adapted for *Streptomyces*, *E. coli* and *B. subtilus* samples. Mycelia were harvested by microfuging 1.5 ml of broth culture in Eppendorf tubes for 3 minutes and the pellet was washed twice with 1 ml of sterile TE (Appendix B). The pellet was then resuspended in 400 μl of TE buffer with 10 μl lysozyme (20 mg/ml), by vortexing, and was digested at 37°C for 1 hour. Lysis of the cells was achieved by the addition of 200 μl of CTAB buffer (Appendix B) with 10 μl Proteinase K (200 μg/ml) which was incubated at 65°C for 30 min. The homogenate was emulsified with an equal volume of chloroform:isoamyl alcohol (24:1), and centrifuged for 15 minutes at 10 000 rpm. The aqueous layer (containing the DNA) was transferred to a sterile 1.5 ml Eppendorf tube, using autopipette tips with widened orifices. This was achieved by removing the last 5 mm of the tip, in order to prevented the shearing of the DNA and mixing of the interphases. The chloroform:isoamyl alcohol extraction was

repeated only if the aqueous layer needed further clarification. The DNA was precipitated from the aqueous phase with the addition of 0.6-1 volume of isopropanol and 0.1 volume of 3 M sodium acetate and was incubated at room temperature for 10 minutes. The sample was centrifuged for 20 minutes, after which the DNA pellet was washed twice in 70% ethanol in order to remove salts, vacuum dried, and resuspend in 100-200 μl of TE buffer, depending on the size of the pellet. RNAase treatment was not found to be essential for DNA used for PCR, however untreated DNA gave higher DNA concentration readings which had to be accounted for when optimising the PCR reaction. RNAase treatment required the addition of 10 μl RNase A (5 mg/ml), followed by digestion for 30 minutes at 37°C. The RNase was inactivated by boiling the sample for 10 minutes. A phenol:chloroform extraction after RNase treatment was not required if the DNA was to be used for PCR. DNA solutions were kept sterile by the addition of 1% chloroform (Hopwood *et al.*, 1985), and were stored at 4°C.

3.2.3.ii Extraction of fungal DNA

Various DNA extraction methods were tried (Cenis, 1992; Cobb and Clarkson, 1993) of which the method by Möller *et al.* (1992) was found to be most efficient when the DNA was to be used for PCR.

3.2.4 Quantification of DNA

DNA was diluted to 10^{-2} in ddH_20, for spectrophotometric quantitation using a Shimadazu UV-VIS Spectrophotometer UV-160A. Spectrophotometric readings were converted to concentrations by the following equation:

$$1 \text{ OD} = 50 \ \mu\text{g/ml}$$

(Sambrook *et al.*, 1989).

Quantitation of genomic DNA was also performed using ethidium bromide fluorescence as in Sambrook *et al.*, (1989).

3.2.5 Estimation of oligonucleotide concentrations

The concentration of the oligonucleotides was determined by the Beer-Lambert Law:

$$\text{Absorbance} = \text{Extinction coefficient} \times \text{Concentration}$$

The extinction coefficient was determined by the base composition of each primer (Appendix

C). Primers were diluted in ddH_2O to give 50 pmol/μl as stock solutions.

3.2.6 Electrophoresis of PCR products

3.2.6.i Agarose gels

Agarose gels (SeaKem GTG, FMC, Rockland) were prepared as in Sambrook *et al.* (1989), using TBE buffer (Appendix B). The agarose gels were stained with a fluorescent dye (ethidium bromide) (Sambrook *et al.*, 1989) which was incorporated into the gel at a concentration of 0.5 μg/ml. After use, ethidium bromide solutions were decontaminated (Appendix B).

3.2.6.ii. Polyacrylamide gel electrophoresis (PAGE)

PAGE gels were used for analysis of PCR products as they are more sensitive than agarose and gave better resolution of picogram quantities of DNA when stained with silver nitrate. The buffer system used for SDS-polyacrylamide gel electrophoresis was a modification of the Laemmli system (Laemmli, 1970) (Appendix C) and was found to give sharper resolution and less distortion of DNA bands. PCR products were separated on the Tall Mighty Small Vertical slab unit (Hoefer Scientific Instruments, California). For analysis PCR products were run on 20 or 50 lane PAGE systems (OMEG). Approximately 3 μl specific PCR product were used for analysis on PAGE gels and 8 μl on agarose gels. All DNA samples were mixed with 0.25 volumes Loading Buffer (Appendix B) before electrophoresis.

The composition of the stock solutions is shown in Appendix C. Typically a resolving gel of 10% and stacking gel of 5% acrylamide was used to fractionate nucleic acids 10-1000 bp long. Analytical gels were electrophoresed at a constant voltage of 10 V/cm for 18 hours.

3.2.6.iii Silver staining

Silver staining of the polyacrylamide gels was an adaptation of the Qiagen protocol for silver staining of TGGE gels (Appendix C). Developed gels were stored at 4^0C in plastic bags.

3.2.6.iv Photography of gels

All gels were photographed immediately after electrophoresis for analysis using the UVP Gel Documentation system (UVP Inc., California).

3.2.6.v Size markers for gels

For accurate size estimation of the PCR products and other DNA fragments that were run on agarose and polyacrylamide gels, a range of DNA fragments of known size were run concurrently with the unknown fragments. Marker DNA was prepared by restriction enzyme digest of pBR322 with *Hinf* I and Lambda DNA with *Hind* III and *Eco* RI (Appendix C).

3.2.7 The Polymerase Chain Reaction (PCR)

3.2.7.i Design of primers for the IPNS gene

IPNS-A primers were originally designed based on sequence information of the IPNS gene from *S. clavuligerus* (Samson *et al.*, 1985) (Table 3.1). New primers were designed (IPNS-B) based on more recent sequence information of the IPNS gene from *Flavobacterium species*, *A. chrysogenum*, *S. griseus* (Shiffman *et al.*, 1988); *S. jumonjiensis*; *S. lipmanii* (Weigel *et al.*, 1988); *S. clavuligerus*, *C. acremonium* (Samson *et al.*, 1985), *P. chrysogenum* (Diez *et al.*, 1989), *A. nidulans* (García-Domíniguez *et al.*, 1991). Primers were designed based on areas showing the highest percent homology between all the IPNS sequences.

Table 3.1 : IPNS-A and IPNS-B primer sequences: IPNS-B primers were designed with incorporated restriction sites (highlighted) and GC clamp that is designated by the underlined sequence of the IPNS-B reverse (R) primer.

Primer	F/R*	Primer sequence		Fragment size
IPNS-A	F	3'-5'	TCGACATCTCGCCGCTGTTC	930 bp
	R	3'-5'	AGCAGTTCTTGCCGGTCTGG	
IPNS-B	F	3'- 5'	CTTGTCGCCGC**tctaga**GCT	
	R	3'- 5'	GCGCCTCGCCGACAGGGAC**cggccgc**GCCCCCG CCGCCCCGCCCGCGCCCCGCGCCGCGC	844 bp

* F and R pertains to the orientation of the primer, Forward and Reverse respectively. Primers were synthesized by Department of Biochemistry, UCT, Cape Town, SA.

3.2.7.ii Optimization conditions for PCR using IPNS-A primers

Optimizations for the amplification of a 930 bp fragment using the IPNS-A primers entailed testing within the following concentration ranges: 1-5 U T*aq* polymerase; 1-10 mM Mg^{++}concentrations; 0.05 μM-2 μM of each primer per reaction; 25-200 μM of each dNTP; 1-1000 ng template DNA. Amplifications were examined at annealing temperatures from 45-65°C with extension and denaturation at 72°C and 94°C respectively. Optimization reactions for the amplification of the IPNS fragment included: i) the addition of T*aq* polymerase after the initial denaturation step (hot-starts) (Wittwer and Garling, 1989); ii) genomic DNA digested with *Eco* RI before amplification (Sharma *et al.*, 1992); iii) inclusion of cosolvents such as 5-10% DMSO (Innis *et al.*, 1990); iv) and pre-boiling of the primers followed by rapid cooling on ice before they were used in the PCR reaction (Kureishi and Bryan, 1992).

3.2.7.iii Optimizations of the PCR reaction using IPNS-B primers

Optimization conditions were tested as for IPNS-A primers. The addition of 0-10% (w/v) acetamide to a PCR mixture was investigated following the findings of Reysenbach *et al.* (1992), where acetamide was successfully used as a denaturant in high GC archaebacterial DNA.

3.2.7.iv Optimized profile for the amplification of the IPNS fragment

An amplification profile was established based on the aforementioned optimizations, that successfully amplified a 844 bp fragment using the IPNS-B primers: Each 50 μl amplification reaction contained 60-100 ng of total DNA; 0.5 μM of each primer (IPNS-B); 100 μM of each of the deoxynucleotide triphosphates; 3% acetamide; 5 μl 10x reaction buffer (Advances Biotechnologies Buffer V: 500 mM KCl; 100 mM Tris-HCl pH 8.8; 1% Triton X-100; 15 mM $MgCl_2$) (Advanced Biotechnologies Ltd., London); 1.5 mM $MgCl_2$; and 1 U of T*aq* polymerase (Advanced Biotechnologies). All reactions were overlaid with 50 μl mineral oil. Amplification of the IPNS fragments was performed in a thermal cycler (Hybaid, OmniGene) at the following temperatures: An initial denaturation step at 94°C for 2 minutes was followed by 40 cycles of 94°C denaturation for 45 seconds; an annealing step at 55°C for 40 seconds; and an extension step at 72°C for 90 seconds; with the final extension step at 72°C for 5 minutes.

3.2.8 Mapping of amplification product

The 844 bp fragment was subjected to restriction digestions to confirm that the amplification fragment was part of the IPNS gene. Known sequence information of IPNS gene from *S. clavuligerus* was used for selection of restriction enzymes that would map the amplified fragment of *S. clavuligerus* (Samson *et al.*, 1985). The following enzymes were used: *Hinf*I; *Sma*I; *Bgl*II; *Msp*I; *Dra*I (Boehringer Mannheim). Restriction enzyme digests were performed on the amplified IPNS gene as follows:

2.5 μl Amplified DNA
1 μl Restriction enzyme
1 μl Restriction buffer
5.5 μl dH_20

The PCR product was digested at 37°C for 4 hours after which the whole digest was electrophoresed on a 12% polyacrylamide gel and silver stained for analysis. Fragment sizes were determined using the UVP Gel Documentation system (UVP Inc., California) which converts bands into molecular weight data with reference to markers on each gel.

3.3 RESULTS

3.3.1 Optimization of DNA extractions

DNA extractions using the modified CTAB protocol yielded approximately 2-10 μg of DNA according to spectrophotometric readings. A common problem with streptomycete chromosomal DNA is the high viscosity which makes sampling difficult (Hopwood *et al.*, 1985). The use of SDS after the cells had been digested with lysozyme was effective in the lysis of the cells yielding a clear lysate; however, the solutions were found to become viscous making further manipulations difficult. The procedure was modified by the addition of Sarkosyl in place of SDS in a CTAB buffer. The resulting lysate was not as viscous and the efficiency of lysis was not affected.

Fungal and streptomycete strains grew in pellet form in broth media making conventional lysis procedures ineffective and DNA yields low. Steps taken to favour dispersed growth of mycelia included the use of glass balls in the growth media; increasing the sucrose content

of the media; or incubation of flasks on an orbital shaker with vigorous agitation. For most cultures, incubation with shaking at 180-200 rpm was adequate to reduce large pellet formation. Amplification of RNase treated samples was found to be the same as untreated samples and this was supported by the findings of Jones *et al.* (1994).

3.3.2 Spectrophotometric readings

Genomic DNA was run on agarose gels (stained with ethidium bromide) and compared to known concentrations of DNA that had been loaded onto the same gel. DNA concentration readings by spectrophotometry were 5 to 10 times higher than those obtained on agarose gel. In the optimization of the DNA concentration for PCR, the DNA was diluted to different concentrations, according to spectrophotometric readings. The concentration that yielded reproducible bands with the least smearing was chosen. This concentration was compared with agarose estimates, and the amount of DNA used for PCR was determined. Approximately 60-100 ng DNA/50 μl PCR reaction was found to give the most reproducible results.

3.3.3 Gels used in the analysis of PCR products

A concentration of 10-12% of polyacrylamide was found to give the most effective resolution for PCR products.

3.3.4 Amplifications of IPNS gene by PCR

3.3.4.i Amplification using IPNS-A primers

Variations in the concentrations of the PCR components and amplification conditions were tested in an attempt to reproduce the IPNS fragment using IPNS-A primers. Modifications of the PCR protocol that were attempted to amplify the IPNS gene were not successful with the IPNS-A primers (Figure 3.1).

Although amplification products were obtained at DNA concentrations of 60 ng/50 μl reactions, the desired 930 bp product was not amplified. Instead IPNS-A primers yielded amplification products in the range of 220-280 bp. Different concentrations of PCR components were tried at temperatures ranging from 45°C to 65°C. Temperatures below 50°C produced smearing due to non-specific amplification, whereas temperatures above 60°C

failed to amplify (Figure 3.1). Two stage PCR reactions, where the first four rounds were at lower specificity followed by 30 cycles at high stringency (60°C), did not eliminate non-specific amplicons (Figure 3.2). Mg^{++} concentrations from 1-10 mM greatly influenced the specificity and the yield of the amplification. $MgCl_2$ concentrations were adjusted to 3.0 mM so as to reduce non-specific amplification without loss of yield. An oligonucleotide concentration of 1 μM primer was found to yield amplification products with the least background interference, *ie.* smearing and spurious amplifications. Different species were amplified using various optimization parameters but to no avail.

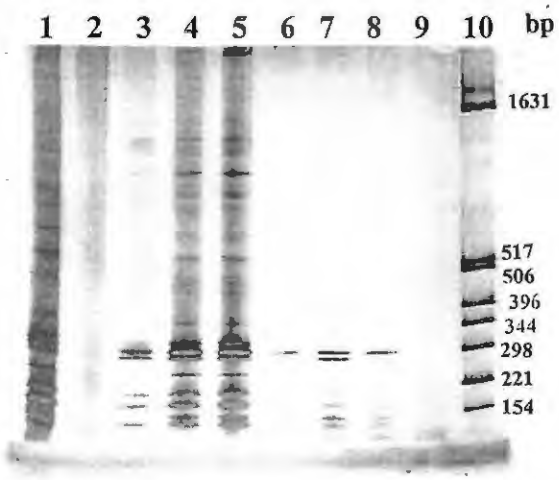


Figure 3.1: *S. clavuligerus* was amplified at 50°C, 55°C, and 60°C in order to eliminate non-specific amplification and select for the 930 bp IPNS fragment using the IPNS-A primer. Lane 1: Amplification of *S. clavuligerus* was conducted at 50°C; Lanes 2-5: Amplification at 55°C; and Lanes 6-9: 60°C; Different primer concentrations of primer IPNS-A were used for samples as follows: lanes 2 and 9: 1 pmol; 3 and 6: 25 pmol; lane 4 and 7: 50 pmol; lane 5 and 8: 100 pmol. 10: Marker DNA (pBR322/*Hinf*I).

Irrespective of conditions tested, amplification products were approximately 220-290 bp for various β-lactam producing species. Other conditions tested included the digestion of genomic DNA with *Eco*RI before being subjected to amplification (Figure 3.2) so as to make the target sequence more accessible to the primers (Sharma *et al.*, 1992). The amplification of 220-290 bp bands, even after amplification at higher temperatures, may be an indication that the oligonucleotides used were annealing to similar or identical sequences in the genome, possible to regions that represent low-, medium-, or high-copy repeats. Since all optimization attempts to amplify the 930 bp IPNS fragment failed, new oligonucleotides were synthesised that would amplify a shorter fragment than that initially chosen.

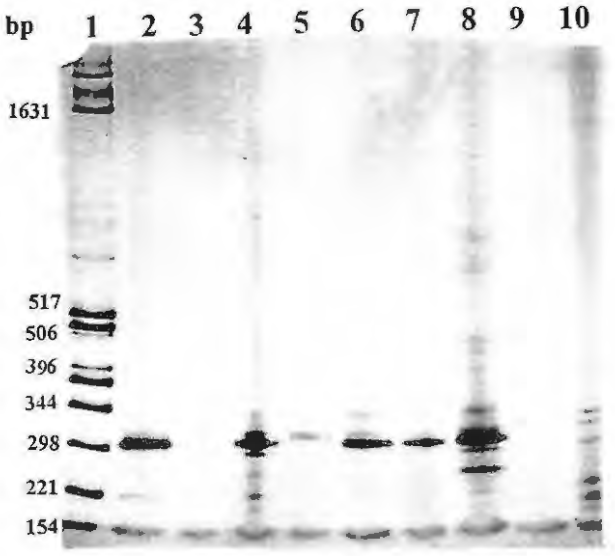


Figure 3.2: Amplification optimizations of various β-lactam species using IPNS-A primers. Lanes 1. Marker (pBR322/*Hinf*I); 2. *S. clavuligerus*; 3. *A. chrysogenum*; 4. *S. tanashiensis*; 5. *S. clavuligerus/Eco*R I (Lanes 2-5 were amplified at 45°C); 6. *S. clavuligerus*; 7. *A. chrysogenum*; 8. *S. tanashiensis*; 9. *S. clavuligerus/Eco* R I 10. *S. griseus* 10137; (Lanes 6-10 were amplified using a two stage PCR reaction with annealing temperatures of 42°C X 5 cycles followed by 60°C X 30 cycles).

3.3.4.ii Design of IPNS-B primers

Primer IPNS-B oligonucleotides were designed based on published DNA sequences of the IPNS gene of *S. griseus*, *S. lipmanii*, *A. nidulans*, *S. clavuligerus*, *Flavobacterium spp.*, *A. chrysogenum*, *P. chrysogenum*, *C. acremonium*, and *S. jumonjinensis*. The IPNS sequences from these species were aligned and primers were designed based on highly conserved areas of the *pcb*C genes. The first four bases of the 3' end of the primers were designed according to areas of the sequence that showed at least 95% homology between the above mentioned species. In the event of a novel β-lactam antibiotic being discovered, restriction sites and a 40 bp GC-clamp were included in the IPNS-B primers which would facilitate further genetic manipulations of the IPNS gene. The restriction sites included are the *Bgl*II site in IPNS-B/F primer and the *Not*I in IPNS-B/R primer. Both restriction sites are not present internally in the IPNS gene. The GC-clamp was added to primer IPNS-B/R (3'-5') that could allow for DGGE studies of the IPNS fragment.

3.3.4.iii Amplification optimization using IPNS-B primers

Irrespective of primers used non-specific amplification and/or very weak amplification of the target DNA was obtained. The streptomycete genome has a G+C ratio of 69-78% (Williams *et al.*, 1989), therefore requires higher temperatures for denaturation than most other organisms. "Hot starts" (Erlich *et al.*, 1991) and inclusion of cosolvents such as DMSO as a denaturant, did not lead to successful amplification.

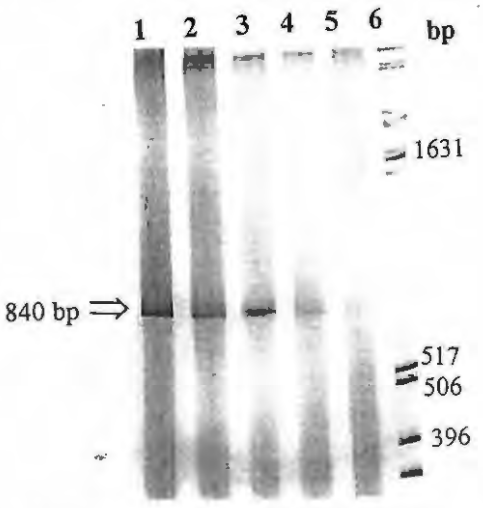


Figure 3.3: The effect of acetamide concentractions on PCR reaction specificity. Acetamide was used at the following concentrations in the PCR reaction for the amplification of *S. griseus* ATCC 10137 using the IPNS-B primers. Lanes: 1. 10%; 2. 5%; 3. 3%; 4. 1%; 5. 0%; 6. Marker (pBR322/*Hinf*I).

Acetamide was added to the PCR reaction at concentrations between 0-10%. The addition of acetamide allowed for the amplification of a single 844 bp fragment in *S. clavuligerus* (Figure 3.3). There was a linear relationship between the acetamide concentration and yield of the amplicon. At concentrations greater than 5% acetamide, smearing occurred due to non-specific amplification. A concentration of 3% acetamide was chosen as it yielded the highest concentration of the 844 bp fragment with the least background interference. The IPNS-A primers, when amplified with acetamide as a denaturant, failed to yield the 930 bp fragment. However, the IPNS-B primers at 55°C annealing successfully amplified an 844 bp fragment in streptomycete β-lactam producers:

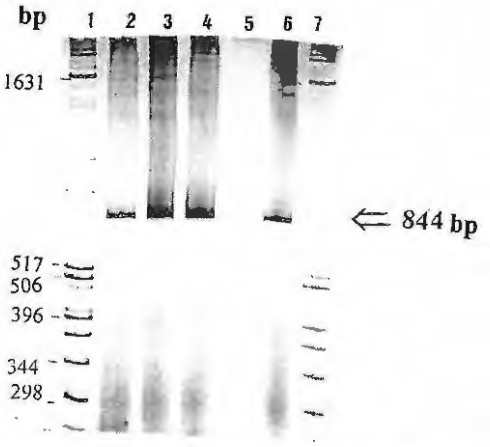


Figure 3.4: Amplification of an 844 bp fragment using IPNS-B primers from different β-lactam producer: Lanes 1 and 7. Marker (pBR322/*Hinf*I); 2. *S. tanashiensis*; 3. *S. griseus* ATCC 10137; 4. *S. clavuligerus*; 5. *S. olivaceus*; 6. *S. lipmanii*.

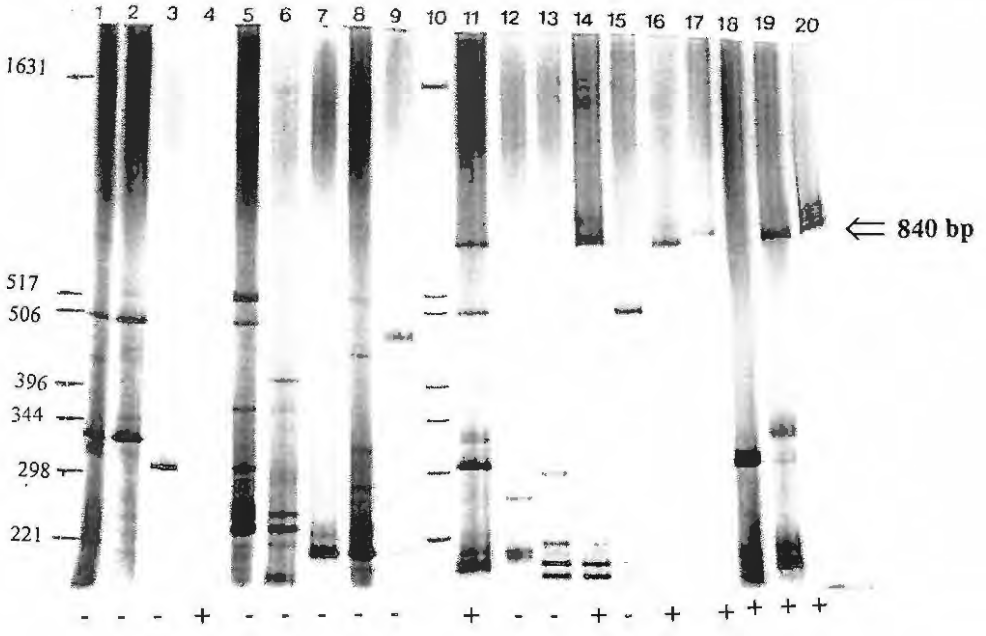


Figure 3.5: PCR amplification of β-lactam and non-β-lactam producers using the IPNS-B primers. 1. *S. venezuelae* ATCC 10595; 2. *S. venezuelae* ATCC 10712; 3. *S. rimosus*; 4. *S. tanashiensis*; 5. *S. antibioticus*; 6. *S. halstadii*; 7. *S. ambofaciens* ATCC 23877; 8. *S. ambofaciens* ATCC 15154; 9. *S. galbus*; 10. Marker (pBR322/*Hinf*I); 11. *S. argenteolus*; 12. *S. griseus* ATCC 13968; 13. *S. griseus* ATCC 31031; 14. *S. griseus* ATCC 10137; 15. *S. hygroscopicus* ATCC 21703; 16. *S. hygroscopicus* ATCC 31039; 17. *S. chartreusis*; 18. *S. olivaceus*; 19. *S. lipmanii*; 20. *S. clavuligerus*.
'+' indicates the β-lactam producers; '-' indicates the non-β-lactam producers.

An 844 bp fragment was amplified in *S. clavuligerus; S. lipmanii; S. tanashiensis; S. griseus* ATCC 10137 (Figure 3.4) whereas the other β-lactam producers such as *S. olivaceus* failed to amplify. Since highly stringency amplifications failed to produce amplicons for some of

the strains tested, lower stringency amplifications were tested. When amplification was conducted at 45°C annealing, *Streptomyces* species, bacterial and fungal samples produced a range of bands (Figure 3.5). An 844 bp band was obtained in the following strains: *S. clavuligerus, S. lipmanii, S. chartreusis, S. hygroscopicus* ATCC 31039, *S. griseus* ATCC 10137, *S. tanashiensis* and *S. argenteolus*. The additional amplification products may be length variants of the IPNS gene or IPNS-like sequences within the genome.

3.3.5 Confirmation of the identity of the amplification product

Apart from size determination of the amplified IPNS fragment using electrophoretic methods, the identity of the amplification fragment was confirmed by examination of RFLPs of the PCR product based on known sequence information of the *S. clavuligerus* IPNS gene (Samson *et al.*, 1985). The expected restriction enzyme map of the IPNS gene was constructed as follows:

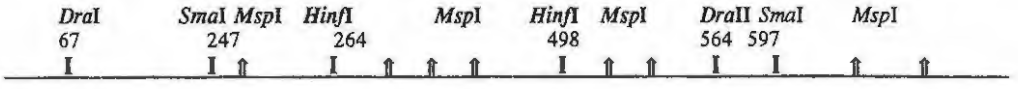


Figure 3.6: Theoretical restriction enzyme map of the IPNS fragment from *S. clavuligerus*.

Restriction enzymes which were used to map the *S. clavuligerus* 844 bp amplification product produced the following profiles:

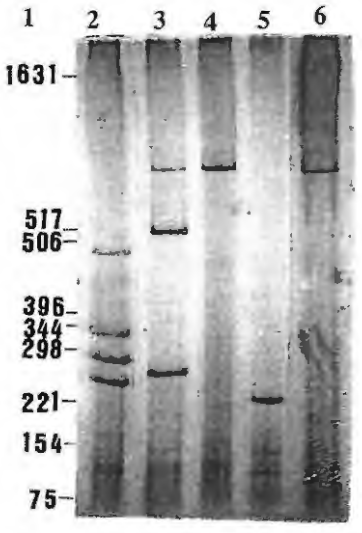


Figure 3.7: Restriction enzyme digests of the *S. clavuligerus* amplification product using various restriction enzymes: Lanes: 1. Marker (pBR322/*Hinf* I); 2. *Hinf* I; 3. *Sma* I; 4. *Bgl*II; 5. *Msp* I; 6. *Dra* I.

Fragment sizes greater than 500 bp do not show a linear relationship between distance migrated and size on a 10% acrylamide gel (Sealey and Southern: cited in Rickwood and Hames, 1982), thus, only approximate estimations could be taken. Although, partial digestion was evident with *Hinf*I and *Sma*I, the fragments were found to add to the expected size of the IPNS fragment (Table 3.2).

*Msp*I did not produce any conclusive results as it cut the IPNS gene at 10 sites of which the largest fragment produced was 240 bp. Most of the fragments produced by this enzymes were less than 70 bp therefore were not efficiently resolved on a 10% polyacrylamide gel (Sealey and Southern: cited in Rickwood and Hames, 1982).

Table 3.2: Approximate fragment sizes from Figure 3.7 are represented below as base pairs which have been added to confirm the original fragment size. All fragment sizes have been rounded off to the nearest decimal. The figure in brackets indicates the fragment that was expected but was not visualised on a 10% polyacrylamide gel due to its small size.

Restriction enzymes:	*Hinf*I (partial digest)	*Hinf*I	*Sma*I	*Bgl*I	*Dra*I
Fragment sizes in base pairs:	500*=260+240 340	340 260 240	840* 590 250	840 (4)	840
Total fragment size	840	840	840	840	840

* Products of partial or incomplete digest.

Fragments obtained using the enzymes *Dra*I, *Sma*I and *Msp*II produced fragments that are similar to the expected sizes. Presence of the 844 bp fragment after digestion with *Sma*I and the 510 bp fragment produced by *Hinf*I is due to partial digestion. Higher concentrations of polyacrylamide gels could be used to resolve smaller fragments that were produced by enzymatic digestion but would not efficiently resolve larger fragments. PCR has been show to have an error rate of $\sim 2 \times 10^{-4}$ nt/cycle (Erlich, 1989) and in such a case could produce slight variations in enzymatically cleaved fragments. However, the overall restriction pattern of the 844 bp fragment from *S. clavuligerus* suggests that the 844 bp fragment to be part of the IPNS fragment.

3.4 DISCUSSION

The most likely avenue for the chance introduction of a foreign DNA is during sample preparation. Conventional procedures for isolation of streptomycete DNA (Hopwood *et al.*, 1985) requires the harvesting of mycelia through a Buchner filter onto filter paper, using large working volumes, which makes extractions cumbersome, time-consuming and open to contamination. It was necessary to optimize the extraction of DNA from microorganisms for use in PCR reactions in order to eliminate potential avenues of contamination. CTAB precipitates acidic polysaccharides which cause the solutions to become viscous making extractions difficult. The modified CTAB extraction method proved to be effective for the extraction of DNA from as little as 1.5 ml of broth culture. Extraction of DNA from Eppendorf volumes reduced the risk of contamination and allowed for the simultaneous preparation of a large number of samples. Although spectrophotometric readings of DNA appeared to give an overestimation of DNA concentration, probably due to RNA contamination, results were rapid and gave relative estimates of DNA to be used in PCR reactions.

PCR amplification of the IPNS gene offers the possibility of a faster and easier high resolution screening method for β-lactam antibiotics. Failure to amplify the *pcbC* gene in all the β-lactam producers could suggest that the IPNS sequence between species is less conserved than originally speculated; or this could be an indication of complementary sequences found within the IPNS gene itself. Although *S. chartreusis* has not to date been identified as a β-lactam producer, an 844 bp fragment was amplified in this organism. The presence of the IPNS gene in a non-β-lactam producer may indicate the presence of a non-functional gene or the level of antibiotic produced may be at levels that escape detection. The IPNS gene was not amplified in *S. griseus* 31031 which is a known β-lactam producer. Antimicrobial activity was also not detected in this organism when conventional antibiotic screening methods were employed. The IPNS gene may have been defective or deleted due to long term storage. Therefore, PCR can be employed as a rapid screen for the presence of the IPNS gene. Smith *et al.* (1989: cited in Smith *et al.*, 1990a) has shown that the *pcbC* gene was present in repeated copies in a penicillin-producing strain of *P. chrysogenum*, which may account for the additional amplification products. However, Southern hybridization or sequence analysis would be required to determine the identity of these additional

amplification products.

3.5 FUTURE CONSIDERATIONS

Since the design of new primers failed to eliminate non-specific amplification in some of the β-lactam producing strains, the detection of the IPNS gene over the background of non-specific fragments can be performed by sequencing; by using a radioactive probe complementary to the sequence; or an asymmetric reamplification method may be used (Arbuthnot and Fitschen, 1993). In the last method, the amplified DNA is subjected to a second round of PCR using a single internal primer to amplify a shorter fragment, excluding contaminating or non-specific DNA as it does not have an effect on the extension of the internal annealing primer. A similar approach that has been used to increase the sensitivity of the PCR reaction is nested PCR (Leys *et al.*, 1994; Sellner *et al.*, 1994) where a first set of primers can be used to amplify sequences that are specific to all β-lactam producers. This is followed by a second and more stringent round of amplification with primers that are homologous to internal parts of the sequence produced in the first round of amplification. It has been reported that nested PCR reactions offer sensitivity that is greater than PCR followed by hybridizations with specific probes (Sellner *et al.*, 1994). The use of longer primers (25 to 30 versus 20 nt), may improve the specificity of the reaction and allow for the direct cycling between two temperatures in PCR (usually 70 to 72°C and 94°C), which may help eliminate spurious amplification. Alternatively, changing one of the two primers so that a shorter or longer fragment is amplified, may improve specificity.

3.6 CONCLUSION

The polymerase chain reaction was found to be a rapid and reliable way to amplify the *pcb*C gene without the need to clone in *S. clavuligerus; S. lipmanii; S. tanashiensis;* and *S. griseus* ATCC 10137. Although conceptually quite simple the successful amplification by PCR involved balancing a large number of variables, including the quality and quantity of the DNA, the hybridization temperatures and the concentration of the reaction components. Yet, it was the selection of primers more than anything else that governed the success of the reaction. The incorporation of a 'GC' clamp and restriction enzyme sites in the primers will enable further genetic manipulations of the amplified IPNS gene in the event of a novel antibiotic being discovered. PCR of the IPNS gene coupled with restriction enzyme digest

was used to confirm the identity of the amplification product. However, the presence of the IPNS gene could not be confirmed in all species by PCR and gel electrophoresis due to the presence of non-specific amplification products. Further verification of these amplification products was required in order to determine the presence of the IPNS gene, which will be discussed in the following chapter.

CHAPTER 4

DETECTION OF THE *pcbC* GENE USING SOUTHERN HYBRIDIZATION AND DOT BLOT ANALYSIS

The polymerase chain reaction was used to amplify the IPNS gene from *S. clavuligerus* which was used to probe analogous genes in *Streptomyces*, fungi and Gram⁻ bacteria using Southern hybridization and dot blot analysis. The IPNS gene was detected by high stringency hybridization in *S. chartreusis*, *S. clavuligerus*, *S. griseus* ATCC 10137, *S. cattleya*, *S. hygroscopicus* ATCC 31039, *S. lipmanii*, *S. olivaceus*, *S. tanashiensis*, and by low stringency hybridization in *A. persicinum* DSM 1073, *A. strictum* DSM 2399, *A. chrysogenum* DSM 880, *P. chrysogenum* and one *Streptomyces* isolated from the soil, sample No. 22. IPNS-like sequences were found to be present in two antibiotic non-producing strains, *S. hygroscopicus* ATCC 21703 and *S. venezuelae* ATCC 10712 by low stringency hybridization.

4.1 INTRODUCTION

4.1.1 Detection of *pcbC* sequences within Gram⁻, Gram⁺ and fungal DNA

The isolation of genes that are involved in the biosynthesis of β-lactam antibiotics is of considerable clinical and commercial interest. The application of molecular techniques such as Southern hybridization analysis and PCR may lead to new approaches for strain improvement (Skatrud *et al.*, 1989) and synthesis of novel β-lactam structures. The technique used to locate a sequence of interest within a larger fragment of genome was first described by Southern (1975), and although experimental methods have been modified and updated (Dybczynski and Puciennicza k, 1988; Hames and Higgins, 1985; Kessler, 1994; Nygaard and Hall, 1963; Sambrook *et al.*, 1989) the principle still remains essentially the same, where single stranded DNA will bind to a complementary strand by hydrogen bonding.

Southern hybridization analysis has found application in the isolation of antibiotic coding genes such as the *pcbC* (Carr *et al.*, 1986; Ramón *et al.*, 1987; Shiffman *et al.*, 1988), *cefD* and *cefE* (Coque *et al.*, 1993b); *lat* genes (Coque *et al.*, 1991) and *act*I and *act*III (Malpartida *et al.*, 1987). The alignment of IPNS sequences have shown that close homology exists between IPNS genes (Weigel *et al.*, 1988) to the extent that allows their cross hybridization (Shiffman *et al.*, 1988). The first requirement when making DNA probes is the identification of an antibiotic specific sequence. This has been obtained in a limited

number of situations by normal cloning procedures (García-Domíniguez *et al.*, 1991; Leskiw *et al.*, 1988; Peñalva *et al.*, 1990). There have been suggestions that the β-lactam gene is cryptic (Shiffman *et al.*, 1988), or may be in low copy number, thus making detection difficult. In addition, procedures that require cloning and the construction of genomic libraries make screening for such genes labour intensive and time consuming, thus only a limited number of species can be screened (Smith *et al.*, 1990a; Weigel *et al.*, 1988). The rapid production of labeled probe using limited sequence information is now possible by means of the PCR reaction (Barry *et al.*, 1990; Bugawan *et al.*, 1988; Kuczek *et al.*, 1994). Radioactive probe that is prepared by PCR yields the probe of interest at high specific activity that can be used for numerous hybridizations. The structure of ^{32}P containing nucleotide triphosphates is almost identical to that of the nonradioactive components which renders them non-inhibiting to the PCR reaction (Ausubel *et al.*, 1995). In order to confirm the presence of the IPNS gene in β-lactam producing and non-producing organism, a PCR-generated probe was used to isolate heterologous IPNS genes using dot blot and Southern hybridization techniques.

4.2 METHODS

4.2.1 Standard buffers and methods

Appendix D lists all the standard techniques and buffers used.

4.2.2 Bacteria and fungal strains

All bacterial and fungal strains (Table 2.1) used in this study were cultured and maintained as described previously in Chapter 2.

4.2.3 Preparation of DNA for Southern and dot blot analysis

Hybridization studies were performed using genomic and PCR amplified DNA of bacterial and fungal origin. Genomic DNA was prepared using a modified CTAB protocol as described in Chapter 3; PCR products for Southern transfer or for dot blots were obtained either by amplification of the IPNS fragment (Chapter 3) or by RAPD PCR of bacterial and fungal DNA.

4.2.4 PCR products for hybridization studies

4.2.4.i Amplification of the IPNS fragment

Streptomycete and fungal cultures were amplified using the IPNS-A and IPNS-B primers as described previously (Chapter 3). The resulting amplification products (Figure 3.5) were immobilised onto Hybond-N^+ membranes (Amersham Inc., Amersham, United Kingdom) that were to be used in Southern hybridization and for dot blot analysis.

4.2.4.ii RAPD PCR for hybridization studies

RAPD PCR for streptomycetes, was optimised as follows: PCR amplifications were carried out in 50 μl amplification reaction volumes containing 60-100 ng of total DNA. A master mix was prepared comprising 0.25 mM of the 10-mer oligonucleotide primer (OP2: 5'- 3' AGCCGTTCAG; OP3: 5'- 3'GGGTCCAAAG) which were purchased from Operon Technologies; 100 μM of each of the deoxynucleotide triphosphates (Promega); 1% acetamide; 5 μl 10x reaction buffer (Buffer IV from Advanced Biotechnologies Ltd., London) comprising: 50 mM KCl; 10 mM Tris-HCl pH 8.8; 1% Triton X-100; 15 mM $MgCl_2$; an additional 1.5 mM $MgCl_2$ (Advanced Biotechnologies Ltd.) was added; and 1 U of T*aq* polymerase (Advanced Biotechnologies). All reactions were overlaid with 50 μl sterile mineral oil. Amplification were performed in a high performance temperature profiler (JDI Model 8021- James Duncan Instruments), at the following temperatures: an initial denaturation step at 94°C for 2 minutes followed by 40 cycles of 94°C denaturation for 45 seconds; 72°C extension for 90 sec; annealing at 37°C for 40 sec; and the final extension step was at 72°C for 5 minutes. Negative controls were prepared using water instead of template DNA to reveal possible non-specific DNA amplification. Reproducibility between amplifications was confirmed for all primers when performed with 40-100 ng template DNA. All PCR products were detected and analysed on silver stained 10% polyacrylamide gels (Appendix C) that were cast on a Tall Mighty Small 11 cm Vertical Slab Unit (Hoefer Scientific Instruments, California).

4.2.5 Preparation of radiolabled probe by PCR

PCR was used to generate a radioactively labelled IPNS fragment as a hybridization probe. The PCR reaction was performed as in Chapter 3 with the following modifications: 200 ng *S. clavuligerus* genomic DNA was used and the reaction was spiked with 50 μCi of α^{32}P

dCTP (≈3000 mCi/mmol) (Amersham), in addition to the cold nucleotides already present. After amplification, 15 μl of the PCR products were electrophoresed at 7 V/cm on 0.9% low melting agarose (SeaKem GTG agarose gel FMC, Rockland, MA) in TAE buffer, that was stained with ethidium bromide (0.4 μg/ml). Visualisation of the amplification products by UV was kept to a minimum as prolonged exposure leads to the cross-linking and fragmentation of the nucleic acids. The 844 bp band was isolated from the low melting agarose (SeaKem) using conventional procedures (Appendix D). The final DNA pellet was resuspended in 150 μl TE buffer, of which 3 μl was loaded onto a 10% polyacrylamide gel Tall Mighty Small System (Hoefer), in order to confirm the identity of the isolated fragments. The PCR mixture was diluted 1:5 in ddH_20 and 1 μl was used to run concurrently with the probes. The polyacrylamide gel was electrophoresed in TBE buffer at (7 V/cm), using Blue Loading Buffer (Appendix B) as the tracking dye. After electrophoresis the gel was wrapped in cling-wrap, and was exposed to X-ray film (Amersham) for 24 hours at room temperature (Figure 4.1). Development of the autoradiograph was as in Appendix D. After agarose gel purification, the probe was stored at -20°C.

4.2.6 Measurement of the specific activity of the IPNS probe

The specific activity of each probe was calculated by the incorporation assay in Sambrook *et al.* (1989) (Appendix D).

4.2.7 Dot blot using a manifold

The nylon membrane was soaked in water before being transferred to 15 X SSC buffer. The wet filter was placed in position on the upper half of the manifold, avoiding air bubbles. The two halves were assembled and clamped together. Approximately 1 μg of genomic DNA or 30 μl of PCR product were used to spot onto the membrane. The nucleic acid samples were prepared by diluting to 50 μl in TE buffer, to which one volume of 20 x SSC was added. The samples were denatured by heating to 95°C for 5 minutes, then chilled on ice before they were applied to the wells in the manifold. The water flow rate was set to produce a gentle suction so that the sample would require at least five minutes to flow through the filter. Each well was then washed twice with 6 X SSC buffer. After the manifold was dismantled, the membrane with DNA side face up, was placed on Whatman paper soaked in denaturation

solution for five minutes. The membrane was then transferred onto a filter paper soaked in neutralisation buffer for 1 minute. The blot was fixed onto the filter using alkali transfer (Appendix D).

4.2.8 Southern transfer of DNA

The following microorganisms were amplified by the PCR reaction using the IPNS-B primers (Chapter 3): *S. chartreusis*, *S. clavuligerus* ATCC 27064, *S. lipmanii* ATCC 27357, *S. griseus* ATCC 10137, *S. tanashiensis* ATCC 33159, *S. hygroscopicus* ATCC 21703, *S. griseus* ATCC 31031, *S. olivaceus*, *S. hygroscopicus* ATCC 31039, *S. halstedii*, *P. chrysogenum* DSM 895, *S. fulvoviridis* ATCC 21954, *Flexibacter sp.* DSM 3098, *S. ambofaciens* ATCC 23877, and *A. nidulans*. PCR products were electrophoretically separated on 1% agarose, and transferred to Hybond-N^+ membranes according to the manufacturers instructions (Amersham).

4.2.9 Filter prehybridizations and hybridizations

Procedures for filter hybridization were performed according to the Amersham protocol for Hybond-N+ membranes (Appendix D). Prehybridization and hybridization were performed in a solution containing 0.5 ml Sheared Herring DNA (1 mg/ml) as the blocking agent. Southern blots (Figures 4.4 and 4.5) and dot blot 1 (Figure 4.2) were performed at 70°C, whereas dot blot 2 (Figure 4.3) was performed at 65°C . The ^{32}P-labelled probe, synthesised by PCR, was boiled in a volume of 150 μl TE buffer for 10 min and added to 25 ml of hybridization solution (containing 5 X SSPE, with blocking DNA). Following hybridization, the Southern blot and dot blot filters were washed twice in 2 X SSPE, 0.1% [w/v] SDS at room temperature for 15 minutes and once with 1 X SSPE, 0.1% [w/v] SDS at 70°C for a further 15 minutes.

The high stringency wash was omitted as related and not identical sequences were being probed. The filter was removed from the solution and blotted between two dry pieces of Whatman 3 MM paper, then wrapped in cling-film without allowing the filter to dry out as this would cause irreversible binding of the probe to the filter. The hybridization solution was stored after hybridization at -20°C for re-use. DNA of *E. coli* K12 was used as the negative control and DNA from *S. clavuligerus* was used as the positive control.

4.2.10 Discriminating between closely related sequences

In order to discriminate between perfect and imperfect hybrids it was necessary to consider the various conditions that affect the hybrid stability. The melting temperature (T_m) (the temperature at which half the hybrids dissociate) is dependant on the base composition (%G+C), ionic strength (M in mol/Litre), the length of the shortest duplexed segment in the DNA (n) and the concentration of any agents that cause helix instability (*eg.* formamide). From these combined results the melting temperature (T_m) of a given DNA hybrid can be shown as:

$T_m = 81.5^0C + 16.6 (\log M) + 0.41 (\%G+C) - 0.61 (\%\text{formamide}) - (500/l)$

l= the length of the hybrid in base pairs (Hopwood *et al.*, 1985).

This equation is valid for concentrations of Na^+ in the range of 0.01 M to 0.4 M and a G+C content that is between 30%-75% for accurate estimations (Sambrook *et al.*, 1989).

4.2.11 Removal of probe and re-use of blots

The probe was stripped from the filter by immersing the filter into boiling 0.5% (w/v) SDS, and allowing to cool to room temperature. Autoradiography was then performed to check that the probe had been removed (Reed and Mann, 1985).

4.3 RESULTS

4.3.1 Preparation of PCR products for hybridization studies

4.3.1.i Preparation of DNA for Southern and dot blotting hybridizations

The DNA used for Southern and dot blot hybridizations was prepared using PCR amplified IPNS genes (Chapter 3). The amplification of RAPD PCR products were screened for possible IPNS-marker bands using Southern hybridizations and dot blot analysis.

4.3.1.ii Synthesis of IPNS probe using PCR

The PCR reaction was used to produce an 844 bp radiolabelled probe in one step by incorporation of radiolabelled dCTP in the reaction mixture. This procedure produced probes with specific activity of approximately 6 X 10^8 d.p.m. (Appendix D.12). The size of the probe was confirmed as the 844 bp fragment by running it on a 10% acrylamide gel

that was subsequently exposed to an X-ray and autoradiographed. Refer to Figure 4.1:

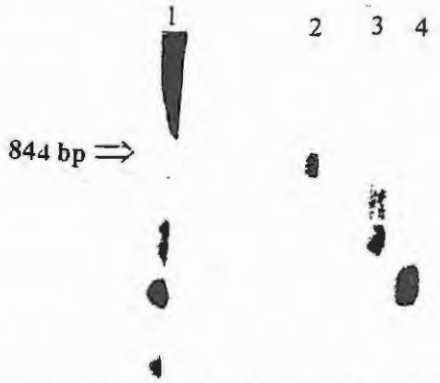


Figure 4.1: Autoradiograph of polyacrylamide gel with radiolabelled amplification products: Lane 1: radiolabeled PCR products; Lane 2-4: DNA fragments that were isolated from agarose and radiolabelled; fragment in lane 2 is the 844 bp fragment that was subsequently used as a probe.

4.3.2 Dot blot analysis

Genomic DNA, PCR-amplified DNA using the IPNS-B primers and RAPD PCR products were transferred onto the nylon membrane using a dot blot manifold. The IPNS probe from *S. clavuligerus* was used to probe for IPNS-like sequences in the organisms listed in Tables 4.1 and 4.2 at moderately high stringency and low stringency, respectively (calculated using the equation in Chapter 4.2.10). The intensity of the signal of the dots could be interpreted in terms of the degree of relatedness of the different sequences.

The *pcbC* gene from genomic DNA samples was detected in *S. clavuligerus* and *S. chartreusis*. The use of PCR amplified DNA samples for hybridization analysis increased the sensitivity of the screening procedure, with the result that positive hybridization signals were obtained at low and high stringency in the DNAs of *S. clavuligerus*, *S. chartreusis, S. tanashiensis, S. griseus* ATCC 10137, *S. hygroscopicus* ATCC 21703, *S. lipmanii* and *S. cattleya*. In addition, at low stringency hybridization a strong positive signal was detected for *S. argenteolus*, and weaker but positive signals were obtained for *S. hygroscopicus* ATCC 31039, *S. olivaceus*, *S. venezuelae* ATCC 10712, *A. strictum* DSM 2399, *A. chrysogenum* DSM 880, *A. persicinum* DSM 1073 and *P. chrysogenum*. Only one *Streptomyces* isolated from the soil, sample No. 22, hybridized weakly to the IPNS probe (Figure 4.3).

Table 4.1: PCR products were blotted according to the following table. Organisms that produced positive signals with the IPNS probe are indicated with the + sign. Refer to Figure 4.2 for dot blot. Rows A to G consist of genomic DNA samples. Rows H to L contain PCR amplified DNA that were amplified with primers IPNS-B.

Rows	LANE		
	A -DNA samples	**B -DNA samples**	**C -DNA samples**
1	*S. hygroscopicus* ATCC 31039	*S. tanashiensis*	*S. griseus* No.11
2	*S. argenteolus*	*S. hygroscopicus* ATCC 21703	*S. antibioticus*
3	*S. clavuligerus* +	*S. griseus* ATCC 13968	*S. ambofaciens* ATCC 23877
4	*S. argenteolus*	*S. flavogriseus*	*S. venezuelae* ATCC 10712
5	*S. lipmanii*	*S. olivaceus*	*S. ambofaciens* ATCC 15154
6	*S. chartreusis* +	*S. griseus* No.11	*S. antibioticus*
7	*S. griseus* ATCC 10137	*S. fulvoviridis*	*S. cattleya*
8	*E. coli* HB101	*S. griseus* ATCC 31031	*S. halstedii*
	D -DNA samples	**E -DNA samples**	**F -DNA samples**
1	*Flexibacter sp.*DSM 3589	3	15
2	*P. chrysogenum* DSM 895	2	12
3	*S. venezuelae* ATCC 10712	*A. chrysogenum* DSM 880	11
4	*S. venezuelae* ATCC 10595	*Flexibacter sp.* DSM 3098	10
5	*S. aureofaciens* ATCC 10762	*A. persicinum* DSM 1073	7
6	*S. galbus*	*A. strictum* DSM 2399	9
7	*S. fradiae*	*A. persicinum* DSM 1052	5
8	*S. rimosus*	*A. nidulans*	4
	G -DNA samples	**H -PCR samples**	**I -PCR samples**
1	25	13	*P. chrysogenum*
2	24	27a	*S. aureofaciens* ATCC 10762
3	23	$27a^2$	*P. chrysogenum*
4	17	*E. coli* K12	*S. olivaceus*
5	22	*S. clavuligerus* (+ Control)	*S. halstedii*
6	16	*S. tanashiensis* +	*A. nidulans*
7	14	*S. griseus* ATCC 10137 +	*S. venezuelae* ATCC 10712
8	26	*S. lipmanii*	*S. griseus* ATCC 10137 +
	J- PCR samples	**K- PCR samples**	**L-PCR samples**
1	*S. hygroscopicus* ATCC 31039 +	*S. flavogriseus*	-
2	*A. persicinum* DSM 1052	*A. chrysogenum* DSM 880	*S. halstedii*
3	*S. fulvoviridis*	*S. galbus*	*S. chartreusis* +
4	*A. persicinum* DSM 1073	*S. antibioticus*	*S. lipmanii* +
5	*S. venezuelae* ATCC 10595	*S. cattleya* +	*S. fulvoviridis*
6	*S. venezuelae* ATCC 10712	*Flexibacter sp.* DSM 3098	*S. griseus* ATCC 31031
7	*S. griseus* ATCC 13968	*S. fulvoviridis*	*S. fradiae*
8	*S. hygroscopicus* ATCC 21703	*S. ambofaciens* ATCC 23877	*S. rimosus*

Table 4.2: PCR amplified samples were used for dot blot analysis (Figure 4.3). Organisms that hybridized with the IPNS probe are indicated by the + sign. The samples that were amplified with IPNS-A primers and IPNS-B primers are indicated by the letters A, and B, respectively. C2 and C3 represent the amplification products that were obtained by RAPD PCR using the 10-mer primers OP2: 5'- 3' AGCCGTTCAG, and OP3: 5'- 3' GGGTCCAAAG, respectively.

Rows	LANES		
	A	**B**	**C**
1	*Flexibacter sp.* DSM 3098 ^{B}	*S. griseus* ATCC 13968^{B}	*A. persicinum* DSM 1073^{B} +
2	*S. olivaceus*^{B} +	*S. griseus* ATCC 31031^{B}	*A. strictum* DSM 2399^{B} +
3	*S. griseus* ATCC 10137^{B} +	*S. halstedii*^{B}	*A. chrysogenum* DSM 880^{B} +
4	*S. argenteolus*^{B}	*S. halstedii*^{B}	*S. flavogriseus*^{B}
5	*S. lipmanii*^{B} +	*S. cattleya*^{B} +	*P. chrysogenum*^{B} +
6	*S. tanashiensis*^{B}	*S. cattleya*^{A}	*E. coli* K12^{B}
7	*S. clavuligerus*^{B}+	*S. chartreusis*^{B} +	*S. clavuligerus*^{B} +
8	*S. chartreusis*^{A}	*S. tanashiensis*^{C3}	*S. tanashiensis* ^{C3}
	D	**E**	**F**
1	*S. fradiae*^{B}	*S. griseus* ATCC 31031^{B}	9^{B}
2	*S. hygroscopicus* ATCC 21703^{B}+	*S. griseus* No.11^{B}	26^{B}
3	*S. venezuelae* ATCC 10712^{B} +	*S. antibioticus* ATCC 11891^{B}	24^{B}
4	*S. ambofaciens* ATCC 15154^{B}	*A. nidulans*^{B}	11^{B}
5	*S. argenteolus*^{B} +	*S. aureofaciens* ATCC 10762^{B}	27a^{B}
6	*S. fulvoviridis*^{B} +	*S. galbus*^{B}	22^{B} +
7	*S. olivaceus*^{B}	*S. ambofaciens* 23877^{B}	17^{B}
8	*S. clavuligerus*^{A}	*S. clavuligerus*^{A}	*S. griseus* ATCC 10137^{B} +
	G	**H**	**I**
1	14^{B}	*P. chrysogenum*^{B}	*S. tanashiensis*^{C3}
2	10^{B}	*P. chrysogenum*^{B}	*A. nidulans*^{A}
3	12^{B}	*Flexibacter sp.* DSM 3589^{A}	*A. strictum* DSM 2399^{A}
4	16^{B}	*S. clavuligerus*^{C2}	*Flexibacter sp.* DSM 3589^{A}
5	17^{B}	*S. hygroscopicus* ATCC 31039^{C2}	*S. hygroscopicus* ATCC 31039^{A}
6	13^{B}	*P. chrysogenum*^{A}	*Flexibacter sp.* DSM 3098^{A}
7	15^{B}	*Flexibacter sp.* DSM 3098^{A}	*A. persicinum* DSM 1052^{A}
8	*S. clavuligerus*^{B} +	*S. clavuligerus*^{A}	*S. venezuelae* ATCC 10712^{A}
	J	**K**	**L**
1	*S. griseus* ATCC 10137^{A}	*S. galbus*^{C3}	*S. lipmanii*^{B} +
2	*S. argenteolus*^{C3}	*S. clavuligerus*^{C3}	*S. hygroscopicus* 21703^{A}
3	*S. hygroscopicus* ATCC 21703^{C3}	*S. griseus* ATCC 31031^{C3}	*S. hygroscopicus* ATCC 21703^{C2}
4	*S. halstedii*^{C3}	*S. griseus* ATCC 10137^{C3}	*S. halstedii*^{C2}
5	*S. argenteolus*^{C3}	*S. halstedii*^{C3}	*S. olivaceus*^{C2}
6	*S. clavuligerus*^{C2}	*S. olivaceus*^{C3}	*S. hygroscopicus* ATCC 31039^{C3}
7	*S. lipmanii*^{C2}	*S. clavuligerus*^{C3}	*S. hygroscopicus* ATCC31039^{B}+
8	*E. coli* K12^{C2}	*S. griseus* ATCC 10137^{C3}	*S. chartreusis*^{B} +

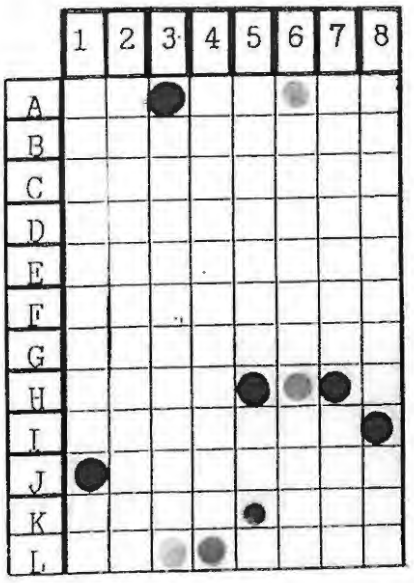


Figure 4.2: Dot blot analysis of samples from Table 4.1. The IPNS probe hybridized to genomic and PCR amplified DNA at high stringency. Positive signals that were detected are listed in Table 4.3.

Table 4.3: Organisms that correspond with positive signals in Figure 4.2 are listed below with their corresponding column/row numbers:

A3.	*S. clavuligerus*
A6.	*S.chartreusis*
H5.	*S. clavuligerus*
H6.	*S. tanashiensis*
H7.	*S. griseus* ATCC 10137
I8.	*S. griseus* ATCC 10137
J1.	*S. hygroscopicus* ATCC 21703
K5.	*S. cattleya*
L3.	*S.chartreusis*
L4.	*S. lipmanii*

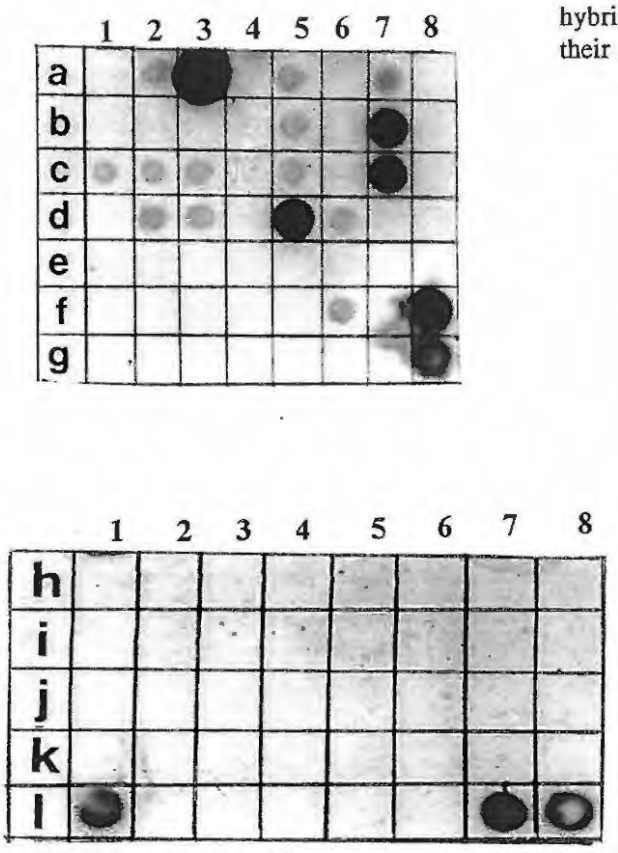


Figure 4.3: Dot blot analysis of 108 DNA samples that were enzymatically amplified and screened for the presence of the IPNS gene. This dot blot was produced at low stingency and corresponds to table 4.2. Table 4.4 lists all the organisms that produced a positive signal.

Table 4.4: Organisms that produced positive hybridization signals in Figure 4.3 are listed below with their corresponding row/column numbers:

A2.	*S. olivaceus*
A3.	*S. griseus* ATCC 10137
A5.	*S. lipmanii*
A7.	*S. clavuligerus*
B5.	*S. cattleya*
B7.	*S.chartreusis*
C1.	*A. persicinum* DSM 1073
C2.	*A. strictum* DSM 2399
C3.	*A. chrysogenum* DSM 880
C5.	*P. chrysogenum* DSM 895
C7.	*S. clavuligerus*
D2.	*S. hygroscopicus* ATCC 21703
D3.	*S. venezuelae* ATCC 10712
D5.	*S. argenteolus*
D6.	*S. fulvoviridis*
F6.	sample No. 22 from the soil isolates
F8.	*S. griseus* ATCC 10137
G8.	*S. clavuligerus*
L1.	*S.chartreusis*
L2.	*S. hygroscopicus* ATCC 21703
L8.	*S. lipmanii*

Southern blots were prepared in order to determine whether the positive signals obtained by the dot blot method were in fact the IPNS gene or IPNS-like sequences. DNA from β-lactam producers and non-producers which amplified using the IPNS-B primers were transferred onto nylon membranes. The IPNS gene was then used to probe DNA that had been blotted onto the nylon membrane at high stringency:

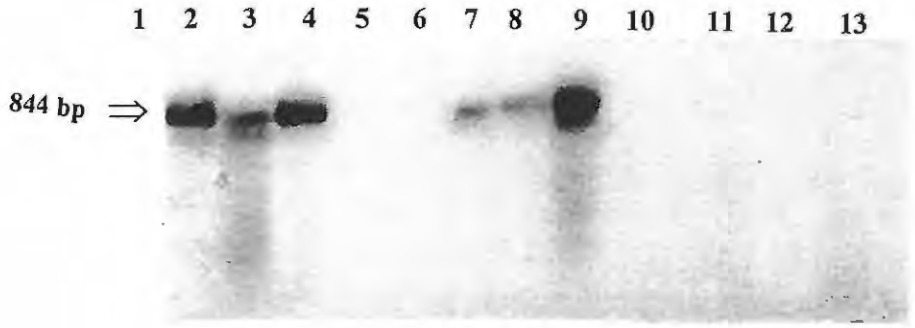


Figure 4.4: Southern hybridization of the *S. clavuligerus* IPNS gene with PCR amplified DNA using the IPNS-B primers. Lanes: 1. Size of DNA band in base pairs; 2. *S. clavuligerus*; 3. *S. lipmanii*; 4. *S. griseus* ATCC 10137; 5. *S. olivaceus*; 6. *S. tanashiensis;* 7. *S. griseus* ATCC 10137; 8. *S. chartreusis*; 9. *S. hygroscopicus* ATCC 31039; 10. *S. hygroscopicus* ATCC 21703; 11. *P. chrysogenum* DSM 895; 12. *Flexibacter sp.* DSM 3098; 13. *A. nidulans* ATCC 32353.

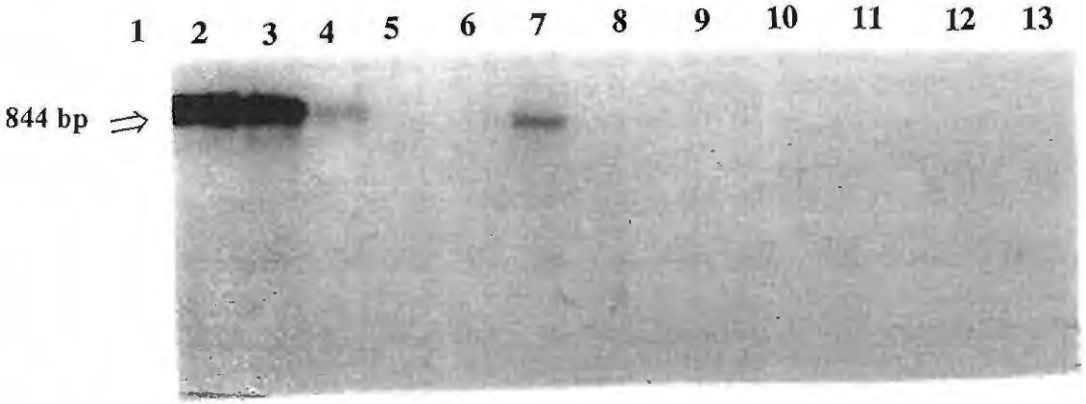


Figure 4.5: Southern hybridization of PCR amplified DNA of several streptomycetes with the 844 bp probe carrying the *pcbC* gene from *S. clavuligerus*. Lanes: 1. Size of DNA band in base pairs; 2. *S. clavuligerus*; 3. *S. chartreusis*; 4. *S. lipmanii*; 5. *S. argenteolus*; 6. *S. halstedii*; 7. *S. tanashiensis*; 8. *S. hygroscopicus* ATCC 31039; 9. *S. griseus* ATCC 31031; 10. *S. halstedii*; 11. *Flexibacter sp.* DSM 3098; 12. *S. ambofaciens* ATCC 23877; 13. *B. subtilus*.

Table 4.5: A summary of the results from Southern hybridization and dot blot analyses.

Organisms that hybridized at low and high* stringency with IPNS probe from *S. clavuligerus*	**Non β-lactam producers that hybridized to the IPNS probe**	**Organisms that did not produce any positive signals with the IPNS probe. Known β-lactam producers are indicated with the ‡ symbol.**
A. chrysogenum DSM 880	*S. venezuelae* ATCC 10712	*A. nidulans*‡
A. persicinum DSM 1073	*S. hygroscopicus* ATCC	*B. subtilis*
A. strictum DSM 2399	21703	*E. coli*
P. chrysogenum		*Flexibacter* spp.‡
S. argenteolus		*S. ambofaciens* ATCC 15154
*S. cattleya**		*S. ambofaciens* ATCC 23877
*S. chartreusis**		*S. antibioticus*
*S. clavuligerus**		*S. aureofaciens*
S. fulvoviridis		*S. flavogriseus*
S. griseus ATCC 10137*		*S. galbus*
S. hygroscopicus ATCC 21703*		*S. griseus* ATCC 13968
S. hygroscopicus ATCC 31039		*S. griseus* ATCC 31031‡
*S. lipmanii**		*S. griseus* No.11
S. olivaceus		*S. halstadii*
*S. tanashiensis**		*S. rimosus*
S. venezuelae ATCC 10712		*S. venezuelae* ATCC 10595
Soil sample No.22		

4.4 DISCUSSION

In order to determine the prevalence of the IPNS gene in nature, an IPNS probe was produced using PCR, and was used to isolate heterologous *pcbC* genes in *Streptomyces*, fungi and Gram⁻ bacteria. These strains included known producers of antibiotic, non-producers and soil isolates of unknown etiology. The hybridization probe derived from *S. clavuligerus* IPNS gene by PCR was used to screen for analogous IPNS genes using Southern blotting and dot blot analysis. A positive response was taken to mean the presence of IPNS-like sequences present in the genome.

4.4.1 PCR-generated probe

The PCR reaction was successfully used to produced an 844 bp ^{32}P-labelled probe which has a number of advantages over the conventional procedures: i) incorporation of the label was not disruptive to the DNA template (Schofield *et al.*, 1991); ii) three probes per PCR reaction were produced; iii) PCR-generated probes that were hybridized overnight did not produce any background noise; iv) the method was rapid, specific and it was a short single step process for obtaining a high concentration of the target sequence; v) longer sequences

or sequences that have a low repeat number, do not require cloning strategies in order to obtain sufficient probe to give a detectable signal in a hybridization reaction; vi) and one set of PCR-primers can be used to produce probes from different organisms by altering the stringency of the PCR reaction. Radioactive labels originally had some of the highest sensitivities amongst labelling methods with the capacity of detecting ≤ 1 pg of target DNA (Tower and Cockayne, 1993). One of the major disadvantages of the method is that radioactive probes are not suitable on a routine basis due to the possible health and safety hazards associated with the use and disposal of radioactive waste.

4.4.2 Dot blot and DNA-DNA hybridizations

Conventional microbial screening of soil isolate No. 22 and of known β-lactam producers *viz.* *S. chartreusis* and *S. tanashiensis* (Kuczek *et al.*, 1994; Williams *et al.*, 1989), did not display any antimicrobial activity against *E. coli* DH5α, *B. subtilis*, *S. aeruginosa*, or *S. aureus* (Chapter 2). However, these species hybridized to the IPNS probe signifying the presence the antibiotic coding gene. Sample No. 22 may be a potential β-lactam producer that would have been overlooked in a conventional screening program. Various factors could have contributed to these organisms being overlooked as potential β-lactam producers when using conventional procedures: i) these species may have lost their ability to produce antibiotic due to subculturing; ii) indicator strains used in microbial screening may not have been sensitive enough to detect the β-lactam produced; iii) or culture conditions were not optimal for antibiotic production. Results obtained are consistent with previous studies where the IPNS gene from *S. clavuligerus* has been shown to hybridize to *P. chrysogenum* (Smith *et al.*, 1990a), *Cephalosporium chrysogenum* (syn. *Acremonium chrysogenum*) (Leskiw *et al.*, 1988), *S. lipmanii*, *S. griseus* ATCC 10137 and *S. cattleya* (Shiffman *et al.*, 1988). Such studies required the construction of genomic libraries and cloning which can be time consuming and laborious.

Beta-lactam producers that failed to hybridize with the *S. clavuligerus* IPNS probe at low and high stringecy included *S. griseus* ATCC 31031, and the Gram$^-$ bacteria, *viz. Flexibacter* spp. DSM 3098/3589. *S. griseus* ATCC 31031 did not show any antimicrobial production (Chapter 2), therefore failure to hybridize to the IPNS probe may indicate an absent or defective IPNS gene, but more likely low homology with the *S. clavuligerus* IPNS gene. The difference in the %G+C content between the streptomycetes and the Gram$^-$ bacteria

could explain the low homolgy between the probe and the *Flexibacter* spp. DSM 3098/3589.

Unexpectedly, two non-producing strains, *S. hygroscopicus* ATCC 21703 and *S. venezuelae* ATCC 10712, exhibited distinct signals with the IPNS-probe. Although a positive signal does not signify the existence of a functional IPNS gene, it may suggest the presence of homologous sequences that have been conserved in many actinomycetes. It is plausible that these strains contain defective, cryptic, IPNS genes that are either poorly expressed or not expressed at all. Alternatively, it could be an ancestral gene or related gene with another function. Such microorganisms could harbour 'extinct' β-lactams that could be mutated to produce novel antibiotics.

It has been previously reported that non-penicillin producing strains produced weak but positive responses using the *pcbC* gene from *S. clavuligerus* (Shiffman *et al.,* 1988). Results from previous studies were unexpected as some non-producing species responded to one or more of the IPNS probes, for example, two *S. griseus* isolates that had been characterized as penicillin and cephalosporin non-producers hybridized strongly to two of the IPNS probes. Similar studies were conducted using PCR amplified probes of the deoxyerythronolide B synthase that was used to screen for genes involved in polyketide biosynthesis genes in *Streptomyces* (Kuczek *et al.,* 1994). Polyketide and some non-polyketide producers were found to hybridize with the probe. The wide distribution of sequences similar to antibiotic coding genes in producing and non-producing strains among the streptomycetes appears to be more widespread than predicted.

4.4.3 Southern blotting and DNA-DNA hybridization

From the Southern blots in Figure 4.5 it appears that the IPNS probe is hybridizing to the 844 bp IPNS gene in *S. clavuligerus*, *S. chartreusis*, *S. lipmanii* and as well as IPNS-like sequences in *Flexibacter* sp. DSM 3098. However, it was not determined whether these IPNS-like sequences are part of the IPNS gene.

The IPNS probe proved to be useful in dot blots with the additional advantage that a large number of samples could be processed at the same time. However, if dot blots are to be used as a screening process, further verification of positive signals is essential to check for cross-hybridization with IPNS-like sequences that may be present. Southern hybridization

analysis or sequencing of positive signals could be used to confirm the presence of IPNS gene.

4.4.4 Screening of RAPD PCR profiles for IPNS-marker bands

A RAPD profile that contains an IPNS-marker band within the profile could be used to simultaneously screen and identify β-lactam producers. Microorganisms that were amplified with the RAPD primers OP2 and OP3 were screened for the presence of possible IPNS marker bands within the RAPD profiles (Figure 4.3) however, no positive signals were obtained. Extensive screening of RAPD profiles that are amplified by different primers would be required before a primer that amplifies IPNS marker bands can be identified.

4.4.5 Evolutionary implications

It is interesting to note that the *S. clavuligerus* IPNS probe hybridized to the fungal DNA at low stringency, but failed to produce a positive signal with any of the Gram$^-$ β-lactam producers. This may indicate that the streptomycete IPNS gene is more closely related to the fungal gene than to that of the Gram$^-$ bacteria. Such a finding supports the theory that the IPNS gene was transferred to the fungi some 370 million years ago (Weigel *et al.*, 1988) and not 1.0-1.5 billion years ago as postulated by Cohen *et al.* (1990). The IPNS gene of the *Streptomycetes* and the fungi would have evolved separately over 370 million years whereas IPNS gene of the Gram$^-$ bacteria and the *Streptomyces* would have evolved over a greater length of time, which would correspond to the split between the Gram$^-$ and Gram$^+$ bacteria. Another, and perhaps more plausible hypothesis is that multiple transfer events occurred (Vining, 1992).

4.5 FUTURE CONSIDERATIONS

These results suggest that the IPNS gene can be used as a specific probe for the screening of β-lactam producing microorganisms. Genes that are found early in the biosynthetic pathway and show a high degree of homology between different strains may be used in similar studies. Such genes include: the *lat* gene encoding lysine 6-aminotransferase (Coque *et al.*, 1991; Tobin *et al.*, 1991); the *cefD* and *cefE* genes (Coque *et al.*, 1993b; Kovacevic and Miller, 1991); and the genes encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) from the first step of the biosynthetic pathway (Smith *et al.*, 1990a). Southern blotting in conjunction with the PCR reaction may therefore facilitate the isolation

of genes that are involved in the biosynthesis of novel antibiotics.

Although ^{32}P-labelled sequence specific probes represent an informative and general approach to genetic analysis, their use in screening for β-lactam producing organisms on a routine basis will be restricted due to the structural instability and biohazards associated with radioactive probes. The advantages of using PCR-generated nonradioactive genetic probes (Hewitt *et al.*, 1990; Higuchi, 1989; Preus and Russell, 1994) are that the hybridization solutions can be stored for long periods of time until they can be used again, and radioactive hazards are avoided. The use of non-radioactive hybridization using covalently immobilised oligonucleotide on a microtitre plate (Chevrier *et al.*, 1995) for the screening of the IPNS gene may be automated thus allowing for the rapid screening of a large number of samples.

4.6 CONCLUSION

Fluctuations in growth conditions can directly lead to potential antibiotic producers being overlooked when using conventional screening programs. Such limitations can be overcome when antibiotic screening is focused on detecting the genetic potential of the organism as an antibiotic producer.

PCR has been found to be a quick and very efficient way to label a large amount of probe with high specific activity. The PCR reaction was also found to improve the sensitivity of the screening process as samples that had been previously amplified, hybridized with the probe more readily than genomic DNA samples. This method can be used to screen other β-lactam synthesis genes or can be extended to the screening of other antibiotics using limited sequence information. More significantly, however, are the cumulative savings of time and effort during the several rounds of screening that are required to isolate potential β-lactam organisms.

CHAPTER 5

THE EVALUATION OF NUMERICAL ANALYSIS OF RAPD PCR AND SOLUBLE PROTEIN PATTERNS FOR THE DIFFERENTIATION OF *STREPTOMYCES*

'Fingerprinting' techniques such as RAPD PCR and soluble proteins patterns were employed to investigate their utility as identification and classification tools for *Streptomyces*. In RAPD PCR primers of arbitrary sequence were used to produce profiles that were strain specific. RAPD profiles could therefore be used to identify duplicate samples, *viz.* a sample labelled *UNK* was identified as *S. argenteolus* and soil isolated *Streptomyces* S7 and S15 were found to be duplicate strains. The RAPD PCR technique was used to examine phylogenetic relationships between *Streptomyces* using strains of established identity for comparative purposes. Results are discussed in the context of current work in molecular biosystematics. For comparison, SDS-PAGE analysis of whole cell extracts was performed to obtain protein band profiles of nine streptomycetes. These strains shared 50-78% similarity between proteins profiles produced by SDS-PAGE and although subspecies clustered together they showed low similarity. Analysis of protein and RAPD profiles reflect the streptomycetes as genetically diverse organisms.

5.1 INTRODUCTION

5.1.1 Random Amplified Polymorphic DNA

Microbial screening is often restricted due to lengthy identification and classification schemes and repetitive encounters with possible duplicate strains. To improve the elimination of duplicate strains, random amplified polymorphic DNA (RAPD) analysis (Caetano-Anollés, 1993; Williams *et al.*, 1990) has been successfully used with *Streptomyces* (Anzai *et al.*, 1994), bacteria (Tanaka *et al.*, 1994), and fungi (Fujimori and Okuda, 1994); however, its contribution to *Streptomyces* phylogenetic relationships has not been investigated. Since DNA is the core basis of evolutionary history (Zuckerkandl and Pauling, 1965), RAPD data should theoretically contain information about ancestry and thus may be used to elucidate evolutionary pathways (Kambhampati *et al.*, 1992). Although the nature and variation of RAPD data have been investigated (N'Goran *et al.*, 1994; Smith *et al.*, 1994; Stammers *et al.*, 1995; Weeden *et al.*, 1992), their contribution to phylogenetic analyses remains a subject of debate.

There are some clear advantages to RAPDs (Newbury and Ford-Lloyd, 1993; Wilde *et al.*, 1992; Welsh and McClelland 1990): i) many loci are examined from diverse regions of the genome at the same time, thus giving data on the divergence of the whole genome; ii) RAPDs allow for rapid groupings of organisms; iii) it can cater for a large number of organisms; iv) an unknown organism can be allocated to a group for possible identification; v) a large number of patterns can be stored in data banks for reference; vi) and it can be used as a quick decision on whether two colony types in a culture are due to contamination or due to variation. Unlike protein fingerprint data, DNA fingerprinting is unaffected by fluctuations in growth conditions. In this report, various primers were examined as to their applicability in establishing RAPD assays as a rapid identification system for various streptomycete strains.

5.1.2 Analysis of proteins

Comparison of gene products have been used to portray taxonomic relationships in microorganisms (Andersen *et al.*, 1987; Costas *et al.*, 1989; Jackman and Pelczynska, 1986; Moore *et al.*, 1980). Soluble cytoplasmic proteins, or proteins solubilised by a denaturant such as SDS, that are subjected to polyacrylamide gel electrophoresis are separated on the basis of the size of the protein alone (Johnstone and Thorpe, 1982; Weber and Osborn, 1975). Since the molecular weight of homologous proteins is more conserved than the net charge, SDS electrophoretic patterns should detect relationships of a broad range (Vauterin *et al.*, 1993). The resulting fingerprints are characteristic of the bacterial strain (Kersters and Ley, 1975; Manchester *et al.*, 1990; Swing *et al.*, 1976; Van den Mooter *et al.*, 1987) and have been used to classify *Streptomyces* and *Streptovericillium* species (Manchester *et al.*, 1990). RAPD PCR has been shown to be more sensitive than multilocus enzyme electrophoresis (MLEE) (Wang *et al.*, 1993). MLEE is a protein-based typing method where enzymes that are coded by specific alleles are scored by their electrophoretic mobilities. The difference in the charge of the protein is reflected by differences in the mobility of the enzyme, hence single base differences in the genes encoding them. Studies of protein pattern similarity have shown good correlation to DNA-DNA homology (Kersters and De Ley, 1975; Owen and Jackman, 1982; Van den Mooter *et al.*, 1987). The correlation between protein and RAPD pattern analysis of *Streptomyces* was examined and compared to an established numerical classification system for *Streptomyces* (Williams *et al.*, 1983a).

5.1.3 Electrophoregram analysis of protein and RAPD patterns

Computer aided numerical analysis (Austin and Priest, 1986; Sacking and Jones, 1993) of 'fingerprint' patterns has great potential in strain identification (Kesters and De Ley, 1975; Vauterin *et al.*, 1993). Numerical analysis is a relatively rapid, inexpensive and objective method of processing a large number of organisms. A computerised data bank can be established that can be used for identification of unknown isolates, which is of value in any screening program.

Although RAPDs have been used to determine relationships between organisms, methods of analysis have not been investigated and standardised. When analysis of RAPD and protein profiles was considered, the method that would extract the most useful phylogenetic information was investigated. A search for patterns in RAPD and protein data was conducted using various cluster analyses programs such as *GelManager*, PRIMER, RAPDPLOT and PHYLIP, and compared to other methods of streptomycete classification.

5.2 METHODS

5.2.1 Buffers and solutions

Standard buffers and solutions used in this section are described in Appendices B, C and D.

5.2.2 Organisms and growth conditions

Strains used in this section are listed in Tables 2.1 and 2.2 (Chapter 2). Streptomycetes that had been isolated from the soil (Chapter 2) were also used for RAPD analysis. In addition, a culture that was labelled *UNK* was analysed in order to determine its identity.

The strains that were subjected to protein analysis were: *S. hygroscopicus* ATCC 31039; *S. griseus* ATCC 31031; *S. griseus* ATCC 10137; *S. aureofaciens*; *S. fulvoviridis*; *S. venezuelae* ATCC 10595; *S. venezuelae* ATCC 10712; *S. ambofaciens* ATCC 23877; *S. ambofaciens* ATCC 15154; and *E. coli* HB101.

All *Streptomyces* cultures that were used in protein and RAPD analysis were grown in 50 ml M3 broth at 25°C, on a rotary shaker at 170 rpm, for 5 to 7 days.

5.2.3 RAPD PCR of *Streptomyces*

5.2.3.i DNA isolation for RAPD PCR

Procedures involving isolation of DNA are described in Chapter 3.

5.2.3.ii Generation of RAPD profiles

All RAPD PCR amplifications were performed as in Chapter 4 using RAPD primers that are listed in Table 5.1. OP1, OP2, OP3, OP13 and OP18 were purchased from Operon Technologies Ltd; primers 1, 7 and 12 were synthesised by the Department of Biochemistry, University of Cape Town, Cape Town, RSA.

Table 5.1: Primers used for RAPD PCR amplifications and their respective sequences and CG ratios.

Primer No.	Primer sequence	GC ratio
OP1	5'- 3' GGCATCGGCT	70
OP2	5'- 3' AGCCGTTCAG	60
OP3	5'- 3' GGGTCCAAAG	60
OP13	5'- 3' ACGCTGCGAC	70
OP18	5'- 3' TCGCGGAACC	70
IPNS-B/F	5'-3' CTTGTCGCCGCTCTAGAGCT	60
1	5'-3' ACGGTACACT	50
7	5'-3' TCACGATGCA	50
12	5'-3' ATTGCGTCCA	50

5.2.3.iii Electrophoresis of RAPD PCR products

Ten microlitres of all RAPD PCR products were separated on 10% polyacrylamide slab systems (Appendix C) using the Tall Mighty Small Vertical Slab Unit (Hoefer Scientific Instruments, California), and Owl Systems (Omeg Instruments, UK). DNA standard markers that were used on all gels were pBR322/*Hinf*I or λ/*Eco*RI/*Hind*III (Appendix C). DNA migrated at constant voltage (150V) for approximately 10 hours or until the loading buffer was at the bottom edge of the gel. DNA was visualized by the silver staining procedure (Appendix C).

5.2.4 Total soluble protein analysis of *Streptomyces*

5.2.4.i Preparation of cell-free extracts

Protein extraction was conducted by pelleting 30 ml of the bacterial broth culture at 5000 rpm for 10 minutes. Pellets were washed once in 0.1 M phosphate buffer, pH 8.0 before they were resuspended in 3 to 5 ml dissociation buffer which contained SDS and 2-mercaptoethanol (Appendix E). The cultures were sonicated at 80% amplitude for 3 minutes at 30 seconds bursts. All sonication was carried out on ice to prevent heat build up. Intact bacteria and cell debris were removed by centrifugation (12 000 rpm for 10 minutes) at 4°C. The supernatant was then aliqoted into 1.5 ml Eppendorfs and was stored at -20°C until it was used for electrophoresis.

Protein concentration of the clear supernatant was determined by the standard Bradford method (Ausubel *et al.*, 1988). Coomassie Brilliant Blue (R250; Fluka Chemica, Switzerland) was used as the protein binding dye which was then quantitated spectrophotometrically (A_{595}).

5.2.4.ii Electrophoresis of streptomycete proteins

Discontinuous PAGE in the presence of SDS (SDS-PAGE) was applied as described by Laemmli (1970) with a stacking gel of 5% and resolving gel of 12%. Approximately 25-100 μg of the whole-cell lysate was added per lane. The protein standards markers were alpha$_2$-macroglobulin 170 kD; beta-galactosidase 116 kD; fructose 6-phosphate kinase 85.2 kD; glutamate dehydrogenase 55.6 kD; aldolase 39.2 kD; triosephosphate isomerase 26.6 kD; trypsin inhibitor 20.1 kD; lysozyme 14.3 kD (Boehringer Mannheim Combithek ^{R}, Calibration Proteins). The gels were electrophoresed at 40 V for 16 hours at room temperature. Staining of protein profiles of polyacrylamide gels was performed as by Nxomani *et al.* (1994).

5.2.5 Analysis of protein and RAPD profiles

All protein and RAPD gel images were captured on black and white photographic prints from which numerical analysis was conducted. Gels were recorded using the UVP Gel Documentation System (UVP Inc., California). UVP was used to convert the RAPD profile into molecular weight data with reference to markers on each gel. Analysis was therefore

reliant on the quality of the image capture of the RAPD profile from the polyacrylamide gels and the efficiency with which the profile was corrected between gels. RAPD profiles produced by primer 13 were used to optimize analysis by using the following scoring methods:

i) Weighted analysis of the RAPD profile was conducted by assigning the bands equal weight. The UVP System was used to obtain band sizes which were then converted to a data matrix of 1 for presence and 0 for absence of a band. The resulting matrices were used as input into various clustering packages such as PHYLIP (Felsenstein, 1989); *GelManager* (Version 1.01); PRIMER's (Version 3.1b) Hierarchical Agglomerative Clustering using various statistical combinations and by Non-Metric Multidimensional Scaling (MDS); and RAPDPLOT (Version 2.3).

ii) Unweighted analyses of RAPD and protein profiles were performed by *GelManager* (Version 1.01). This package allowed for the conversion of RAPD and protein profiles into absorbance values thus taking into account band breadth, with broader bands carrying more weight than finer bands. Once the data had been calculated into a similarity matrix they were grouped into clusters using arithmetic averaging of pair groups (UPGMA) (Sneath and Sokal, 1973), which were then displayed as dendrograms. The intercluster distance is described as the average pairwise distance between members of two clusters (Li and Graur, 1991). Nei *et al.* (1983) performed simulation studies and found that the UPGMA performs well when the mutation rates were the same along all branches of the tree. Cluster analysis that was performed using UPGMA was conducted by PHYLIP's NEIGHBOUR program (DRAWTREE and DRAWGRAM) and *GelManager*.

5.2.5.i *GelManager*

[Jackman, P.J.H. 1992. (Copyright). GelManager* Biosystematica. Prague, Czechoslovakia]. Unweighted analysis of RAPD and protein profiles were performed by *GelManager* which were recorded from photographic prints as 256 grey level TIFF files (Tag Image File Format). *GelManager* converted fingerprint profiles to absorbance values that were recorded

as integer values in a database. Markers were used on all gels to compensate for gel-to-gel variation. The patterns were compared by the Pearson product moment coefficient, (r) between absorbance profiles (Equation 5.1.A), and were placed in a similarity matrix. The profiles showed correlation values that are between 0 and +1. Absence of a band was taken to show a similarity when r was applied.

5.2.5.ii RAPDPLOT

[Black B., (1994), RAPDPLOT version 2.3. Distributed by author. Department of Microbiology, Colorado State University, Fort Collins]. RAPDPLOT was used to analyze genetic relationships between individuals using RAPD PCR markers. The RAPD profiles from each individual were scored as 0 for absence and 1 for presence of a band. Two genetic distance matrices were produced by RAPDPLOT: Nei and Li (1985) similarity matrix (Equation 5.2.B), and a joint presence/absence matrix (M) (Equation 5.2.C).

5.2.5.iii PHYLIP

[Felsenstein, J., (1993), PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle]. Various packages found in PHYLIP were used for analysis including NEIGHBOR, MIX, PENNY, and DRAWGRAM. The UPGMA option in NEIGHBOR was used to constructs trees by successive (agglomerative) clustering. PENNY and MIX are both parsimony programs. The latter carried out the Wagner and Camin-Sokal parsimony method.

5.2.5.iv PRIMER

PRIMER was a program used to examine RAPD profiles of streptomycete species using the Bray-Curtis Coefficient (Bray and Curtis, 1957) and Euclidean distance with UPGMA analysis. Since data were entered as 0,1 characters, the Bray-Curtis was reduced to the Dice coefficient of Sneath and Sokal (1973) (Equation 5.2.A). When using the Bray-Curtis Coefficient 'joint absences' have no effect on similarity (S). PRIMER converted the 0,1 matrix into Euclidean distance which takes joint absences into account (Sacking and Jones, 1993).

5.2.5.v Multidimensional Scaling

Another method of representing the matrix data was by ordination. Multidimensional scaling was performed by the MDS option in the PRIMER program. When transposing a cluster into a MDS plot the rank order of the samples had to be preserved exactly. This was not always possible, resulting in some distortion or stress between dissimilarities and the corresponding distances in the plot (Field *et al.*, 1982). A 'Stress' value of zero indicates that the rank order of the dissimilarity (δ) (Equation 5.1.C) is preserved. Stress values of < 0.1 are good; $0.1 < 0.2$ are useful; and anything > 0.3 represents random points. When stress is low (< 0.1), then MDS is a better representation of data than cluster analysis.

A.

$$r = \frac{\sum_{i=1}^{n} (X_{ij} - \bar{X}_j)(X_{ij} - \bar{X}_k)}{\sqrt{\sum_{i=1}^{n} (X_{ij} - \bar{X}_j)^2 \sum_{i=1}^{n} (X_{ij} - \bar{X}_k)^2}}$$

B.

$$d_{jk} = \sqrt{[\sum_{i=1} (y_{ij} - y_{ik})^2]}$$

C.

$$\text{Stress} = \Sigma_j \Sigma_k (d_{jk} - d_{jk})^2 / \Sigma_j \Sigma_k d^2_{jk}$$

Equation 5.1: **A)** Equation for the Pearson product-moment correlation coefficient (r) used by the *GelManager* program in the comparison algorithms. Resemblance is calculated between j and k samples.
B) Dissimilarity Coefficients are turned to distance (d) between pairs of samples. **C)** Regression used to measure the amount of stress (or goodness-of-fit) of a cluster that has been transposed to an MDS plot
d_{jkm} = the distance for the fitted regression line for the dissimilarity (δ_{jk}).

A.

$$S_{jk} = 100 \left(\frac{2a}{2a+b+c}\right)$$

B.

$$S = \frac{2NAB}{(NA + NB)}$$

C.

$$M = \frac{NAB}{NT}$$

Equation 5.2: **A)** Dice coefficient of Sneath and Sokal (1973): a = number of bands present in the both samples; b + c = number of bands present in the one sample and not the other. **B)** Nei and Li (1985) similarity matrix (S). **C)** Nei and Li (1985) joint presence/absence matrix (M). NA: the number of bands in sample A; NB: the number of bands in sample B; NAB: the total number of matches in samples A and B; NT: the total number of loci that are scored in using a specific primer.

5.3 RESULTS

5.3.1 Proteins

Cell-free protein extracts from different streptomycete species were used to generate protein fingerprint profiles (Figure 5.1). Due to high proteolytic activity of certain streptomycetes only a small number of strains could be used to generate reproducible protein profiles. Even after the cell-free extracts were heated to 100^{0}C in order to inactivate proteases, proteins

were found to degrade rapidly. Certain streptomycete species were found to be more susceptible to protein degradation than other species.

5.3.2 Numerical analysis of streptomycete protein profiles

The dendrogram (Figure 5.2) was obtained by the Pearson product moment correlation coefficient and UPGMA cluster analysis as it has been reported to be the most satisfactory method for comparing protein electrophoregrams (Vauterin *et al.*, 1993). Bands between 26.6 and 116 kD were considered for analysis. *E. coli* HB101 was used as an outgroup. Two streptomycete clusters could be distinguish at $r = 0.50$, with subspecies differentiating as follows: *S. griseus* ATCC 10137 and ATCC 31031 grouped at $r = 0.75$; *S. venezuelae* ATCC 10712 and ATCC 10595 showed a $r = 0.78$; and *S. ambofaciens* ATCC 23877 and ATCC 15154 clustered at $r = 0.67$.

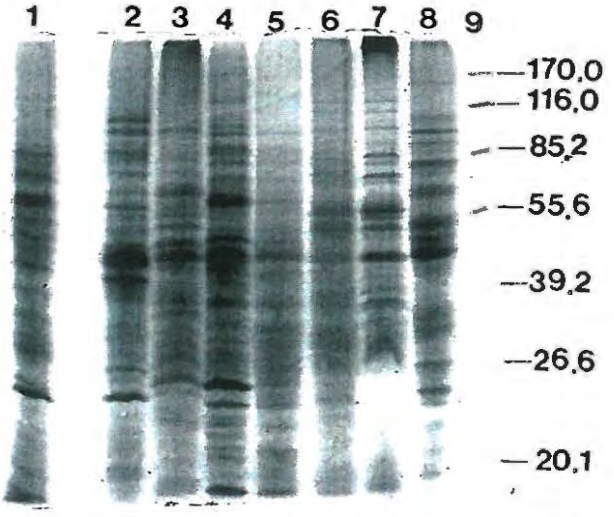


Figure 5.1: Electrophoretic SDS whole-cell protein patterns of eight streptomycetes. Lanes 1. *S. aureofaciens*; 2. *S. chartreusis*; 3. *S. fulvoviridis*; 4. *S. venezuelae* ATCC 10595; 5. *S. venezuelae* ATCC 10712; 6. *S. ambofaciens* ATCC 23877; 7. *S. ambofaciens* ATCC 15154; 8. *E. coli* HB101; 9. Protein molecular weight markers in KDa.

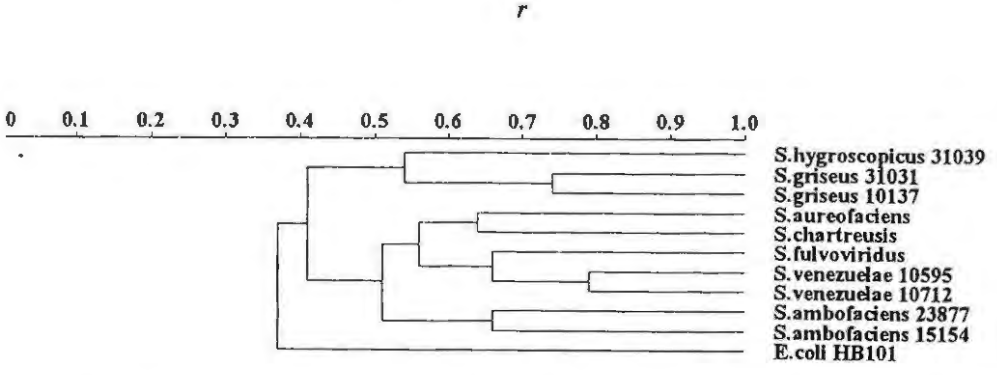


Figure 5.2: Dendrogram of *Streptomyces* based on whole-protein patterns using the Pearson product moment correlation coefficient and UPGMA clustering.

5.3.3 Optimization of RAPD PCR for streptomycetes

In order to determine the suitability of RAPD PCR for streptomycete taxonomy, 5 different 10-mer primers of arbitrary sequence and one 20-mer primer were used. Differences in the number and nature of bands of RAPD profiles were not affected by small variations in temperature, but were attributed to DNA quality, primer, magnesium, and dNTP concentrations (Caetano-Anollés, 1993; Rasmussen *et al.*, 1994; Weeden *et al.*, 1992). DNA and all the PCR reaction component concentrations had to be kept within a narrow range in order to obtain reproducibility. The addition of acetamide as a denaturant aided RAPD PCR of streptomycete stains by reducing their denaturation temperature (T_m) (Reysenbach *et al.*, 1992) as streptomycetes have a high G+C content (Wright and Bibb, 1992). The reaction components were prepared as a master mix which had numerous advantages: this procedure allowed for standardization between samples with the only variable being the DNA; it reduced possible contamination due to handling; and aided in the preparation of a large number of samples.

The time taken for temperatures in the PCR profile to increase from annealing to extension temperature and to decrease from denaturation to annealing temperature (ramp time) was found to affect the overall success of the PCR reaction. Shorter ramp times produced clearer RAPD profiles with less background smearing. Optimization of ramp times has previously been reported to improve RAPD profiles (Martin-Kearley *et al.*, 1994).

One-dimensional PAGE electrophoretic gel patterns of RAPD PCR profiles (Figures 5.3-5.7) were visualised by silver staining procedures, as this method has been reported to produce high resolution profiles (Bassam *et al.*, 1991; Caetano-Anollés, 1992). Reproducibility was also determined by the quantity of DNA that was used for the PCR reaction. A concentration of 60-100 ng template DNA produced RAPD profiles with major bands that were reproducible.

5.3.4 *Streptomyces* RAPD profiles

All the RAPD primers that were tested produced different profiles (Keil and Griffin, 1994) that were strain specific. *S. argenteolus* and an unidentified strain (*UNK*) produced similar banding patterns with primers OP1, OP13 and OP18 (Figures 5.5, 5.4, and 5.7,

respectively), and the soil isolates S15 and S7 were found to have the same RAPD profiles (Figure 5.6). Although major bands in RAPD profiles were reproducible, fingerprint patterns were complex and difficult to interpret. An average of 26 bands per profile were obtained with the 10-mer primers. RAPD PCR profiles showed high variability between species and even sub-species, for example *S. griseus* ATCC 10137/31031/13968/No11 and *S. hygroscopicus* ATCC 21703/31039. In contrast, the ecosemer primer (IPNS/B) produced profiles that were more conserved between subspecies, with an average of 12 bands per profile; however, certain samples failed to amplify with this primer. Representative samples amplified with the 10-mer primers are depicted in Figures 5.3-5.7:

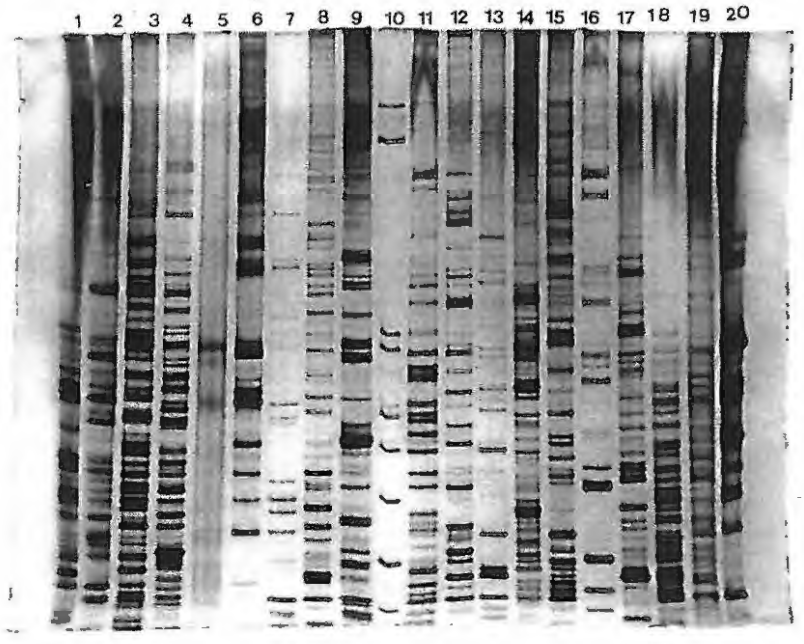


Figure 5.3: RAPD profile using primer OP3: Lanes: 1. *B. subtilus*; 2. *E. coli* HB101; 3. *S. flavogriseus*; 4. *S. antibioticus*; 5. *S. halstedii*; 6. *S. fradiae*; 7. *S. hygroscopicus* ATCC 31039; 8. *S. griseus* No. 11; 9. *S. ambofaciens* ATCC 15154; 10. *Flexibacter sp.* DSM 3098; 11. Marker (pBR322/*Hinfl*); 12. *S. fulvoviridis*; 13. *S. flavogriseus* ; 14. *E. coli* K12; 15. *S. lipmanii*; 16. *S. rimosus*; 17. *S. cattleya*; 18. *S. hygroscopicus* ATCC 31039; 19. *S. hygroscopicus* ATCC 21703; 20. *S. fulvovoridis*.

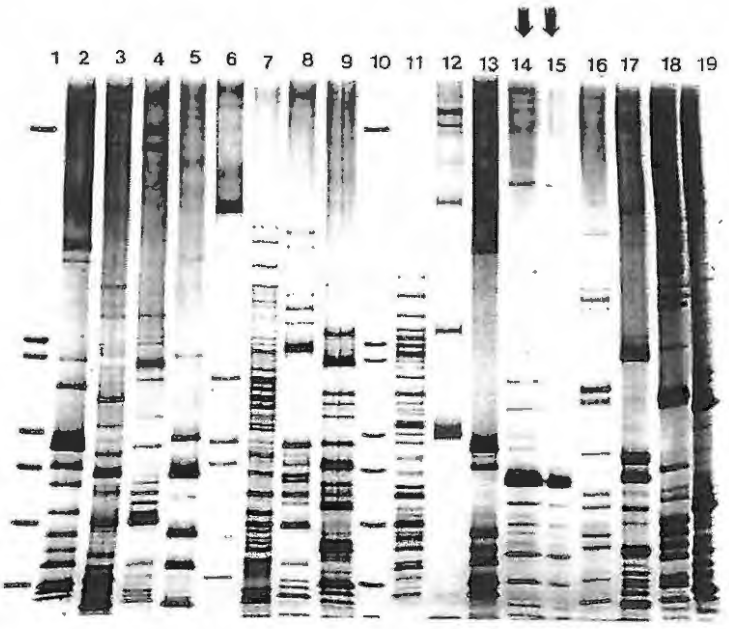


Figure 5.4: RAPD profile obtained with primer OP13: Lanes: 1 and 10. Marker (pBR322/*HinfI*); 2. *S. hygroscopicus* ATCC 31039; 3. *S. aureofaciens*; 4. *S. chartreusis*; 5. *S. clavuligerus*; 6. *S. fulvoviridis*; 7. *S. tanashiensis*; 8.*S. venezuelae* ATCC 10712; 9. *S. rimosus*; 11. *E. coli* HB101; 12. *B. subtilus*; 13. *S. hygroscopicus* ATCC 21703; 14. *S. argenteolus*; 15. *UNK*; 16. *S. griseus* ATCC 31031; 17. *S. griseus* ATCC 13968; 18. *S. griseus* ATCC 10137; 19. *S. griseus* No.11. (Note the banding similarity between *S. argenteolus* and the *UNK* as indicated by the arrows).

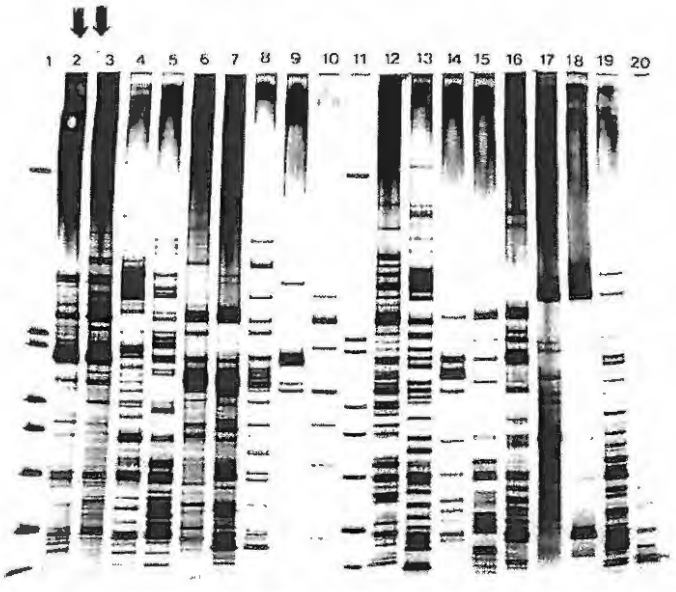


Figure 5.5: RAPD amplification profile using primer OP1: Lanes: 1 and 11. Marker (pBR322/*HinfI*); 2. *S. argenteolus*; 3. *UNK*; 4. *S. aureofaciens*; 5. *S. ambofaciens* ATCC 23877; 6. *S. chartreusis*; 7. *S. fulvoviridis*; 8. *S. hygroscopicus* ATCC 31039; 9. *S. hygroscopicus* ATCC 21703; 10. *S. olivaceus*; 12. *S. rimosus*; 13. *S. griseus* No. 11; 14. *S. griseus* ATCC 10137; 15. *S. griseus* ATCC 13968; 16. *S. griseus* ATCC 31031; 17 and 18. *B. subtilus*; 19 and 20. *E. coli* HB101. (Note the banding similarity between *S. argenteolus* and the *UNK* as indicated by the arrows).

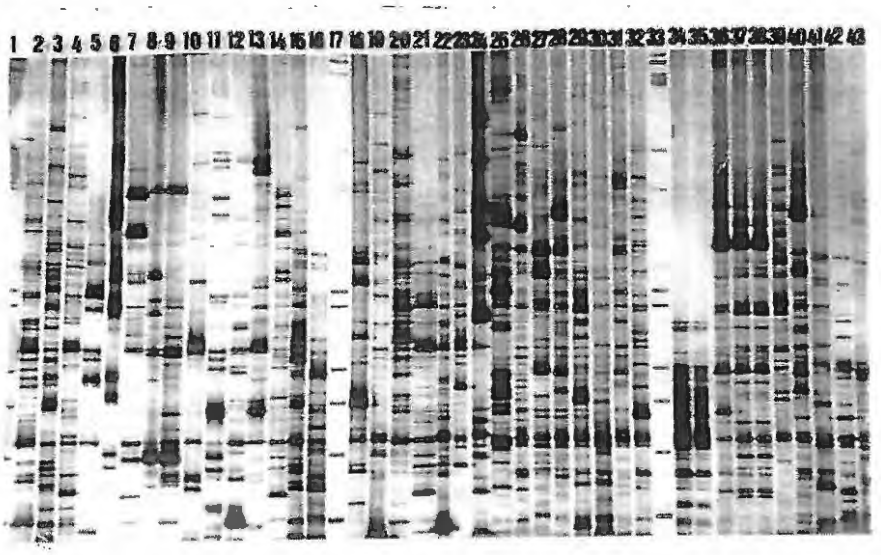


Figure 5.6: RAPD profile obtained with primer OP2: Lanes: 1, 17 and 33. Marker (pPBR322/*HinfI*); 2. *S. aureofaciens*; 3. *S. rimosus*; 4. *S. fradiae*; 5. *S. clavuligerus*; 6. *E. coli* K12; 7. *S. argenteolus* 11009; 8. *S. venezuelae* ATCC 10712; 9. *S. venezuelae* ATCC 10595; 10. *S. griseus* ATCC 10137; 11. *S. halstedii*; 12. *S. ambofaciens* ATCC 15154; 13. *S. hygroscopicus* ATCC 31039; 14. *S. hygroscopicus* ATCC 21703; 15. *S. olivaceus*; 16. *S. fulvoviridis*; 18. *S. chartreusis*; 19. *S. galbus*; 20. *Flexibacter sp.* DSM 3098; 21. *S. lipmanii*; 22. *S. griseus* ATCC 13968; 23. *S. antibioticus*; 24. *A. nidulans*; 25. *E. coli* HB101; soil isolates (S): 26. S2; 27. S11; 28. S15; 29. S13; 30. S9; 31. S27a; 32. S24; 34. S10; 35. S10; 36. S7; 37. S15; 38. S14; 39. S16; 40. S17; 41. S22; 42. S4; 43. S12.

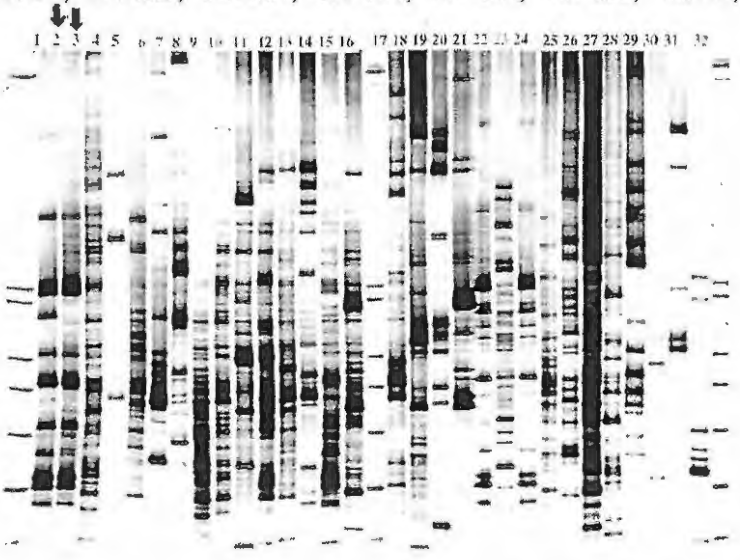


Figure 5.7: RAPD profile using primer OP18: Lanes: 1, 17, 33, 48: Marker (pBR322/*HinfI*); 2. *UNK* 3. *S. argenteolus* ATCC 11009; 4. *S. ambofaciens* ATCC 23877; 5. *S. ambofaciens* ATCC 15154; 6. *S. aureofaciens* ATCC 10762; 7. *S. chartreusis*; 8. *S. clavuligerus*; 9. *S. fulvoviridis*; 10. *S. flavogriseus*; 11. *S. griseus* ATCC 31031; 12. *S. griseus* ATCC 13968; 13. *S. griseus* ATCC 10137; 14. *S. galbus*; 15. *S. lipmanii* 27357; 16. *S. olivaceus*; 18. *S. tanashiensis*; 19. *S. venezuelae* ATCC 10595; 20. *S. venezuelae* ATCC 10712; 21. *S. rimosus*; 22. *S. hygroscopicus* ATCC 31039; 23. *S. hygroscopicus* ATCC 21703; 24. S. *hygroscopicus* ATCC 31039; 25. *Flexibacter sp.* DSM 3098; 26. *A. nidulans*; 27. *E. coli HB101*; 28. *P. chrysogenum*; 29. *E. coli K12*; 30. *S. antibioticus*; 31. *S. clavuligerus*; 32. *S. fradiae*. (Note the banding similarity between *S. argenteolus* and the *UNK* as indicated by the arrows).

5.3.5 Optimization of RAPD analysis

The use of different coefficients was used to optimize analysis and confirm the validity of clusters obtained with Primer 13. Amplification products from 279 to 1600 base pairs were found to be reproducible and were used in RAPD analysis. RAPD PCR bands were translated into a (0,1) matrix (Table 5.2). The deviation between gels was minimized by using black and white photographic images of gels for analysis and the scoring of bands from molecular weight data.

Table 5.2: RAPD PCR profile of *Streptomyces* using primer OP13, scored as presence (1) and absence (0) of bands. Numbers following species names are ATCC numbers used to differentiate between subspecies.

RAPD marker sizes in base pairs:	270 ▼	400 ▼	600 ▼	800 ▼	1000 ▼	1600 ▼
S. tanashiensis	0111011101010111001110110111011110011001111001000000000000000					
S. argenteolus	0110010101000100000010000100000000001000101000000000000000000					
S. aureofaciens	1011001001010101000110111010100101010000000000000000000000000					
S. chartreusis	0000111101100110100100110011111000000000000000000000000000001010					
S. clavuligerus	0010001001000100000111000011110100000000000000000000010000100000					
S. fulvoviridis	0010010000100100100111000110000000000000000000000100000000000000					
S. venezuelae 10712	0001011101110101001010100010100011010000001010000000000000000					
S. rimosus	0111011100100100100110010001010000100011100100000000000000000					
S. hygroscopicus 21703	0110000000100100000010000100111010000000011000010100000001000 1					
S. hygroscopicus 31039	0111111001000110001010100110100001010010110011010110001000000					
S. griseus 31031	0111011001010000000110000110001111000001000100000000000000000					
S. griseus 13968	0101011001010000000000001001001000000000001000011000000000000					
S. griseus 10137	0111011100111000000000000100000011110010000000000000000000000					
S. griseus No11	0110010100101011000011010100100001000010000000000000000000010					
S. venezuelae 10712	0101001101101000100001000100101001000000011000000000000000000					
S. venezuelae 10595	0111001101011101101010100111001010100000000000000000000000000					
S. lipmanii	0101010000010100000010100100100011001000000100000000000000000					
S. ambofaciens 23877	0100011101010001000000010100001000000000000000000000000000000					
S. halstadii	0011010101111010101010111011010100000001000001001000000000000					
S. olivaceus	0110011001010010100001100110010011000100100000000000000000000					
S. flavogriseus	0011001001000110011010110110100000001010000000000000000000000					
S. galbus	0101011001000001100100010101000000000100000010000000000001000000					
S. ambofaciens 15154	0011001100000100100000100100100010000100000010000000000000000					
S. antibioticus	0001011101111000001100010010101000001000100000000000000000000					
S. argenteolus	1011001001001000100010100000000000000000001001101000000000000					
S. cattleya	0111001101000110001011110110010010001001001000100100100001000					
S. fradiae	0011011110010100101010100100101001001010001000110001001010100					

Dendrograms obtained using the 0,1 matrix which was used as input into RAPDPLOT, PRIMER, and PHYLIP's PENNY and MIX programs, are represented in Figure 5.8.

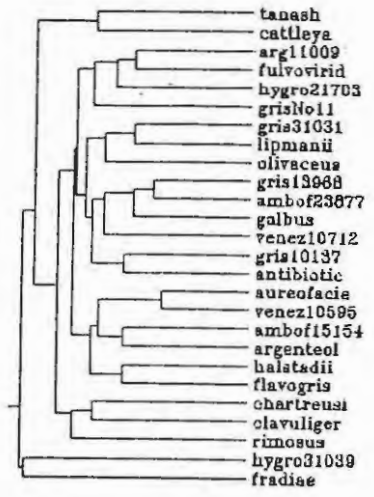
Coefficients can be divided into those that are based on band presence only and those that take joint presence and absence values into consideration. Jaccards, Nei and Li (1985) coefficient of similarity, and Dice coefficients accounted for band presence only. The Pearson correlation coefficient (r), Euclidean distance and Simple Match (S_{SM}) coefficients

take joint absences into account. The most commonly used coefficient for electropherograms is the Pearson product-moment correlation coefficient (r) which is based on deviations from the mean score (Weir, 1990) and takes into account joint absences; therefore, S increases with more joint absences. The major disadvantage when using this method is its tendency to weight towards larger features such as large peaks or gaps (Weir, 1990). The Dice measure, on the other hand does not take joint absences into account.

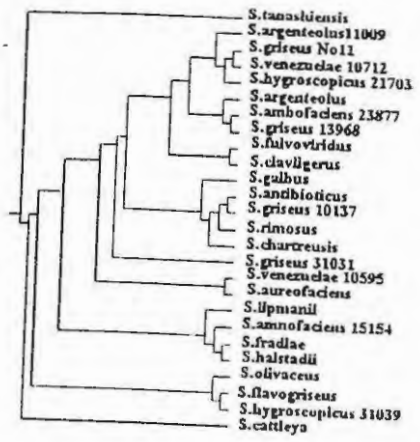
PENNY and MIX are parsimony programs. The most parsimonious tree is found to be the one that requires the least number of changes to transverse the whole tree. The parsimony method minimizes the number of evolutionary events. If the amount of evolutionary changes is small then the parsimony method applies; however, for large amount of evolutionary changes this method is more likely to give the wrong tree (Felsenstein, 1978).

The parsimony methods (MIX and PENNY), ordination (MDS), Nei and Li similarity matrix (PRIMER), the Dice and Euclidean distance algorithms (PHYLIP) that were examined showed different relationships between the *Streptomyces*. Only the algorithems that showed some congruency between different dendrograms and MDS are represented in Figure 5.8.

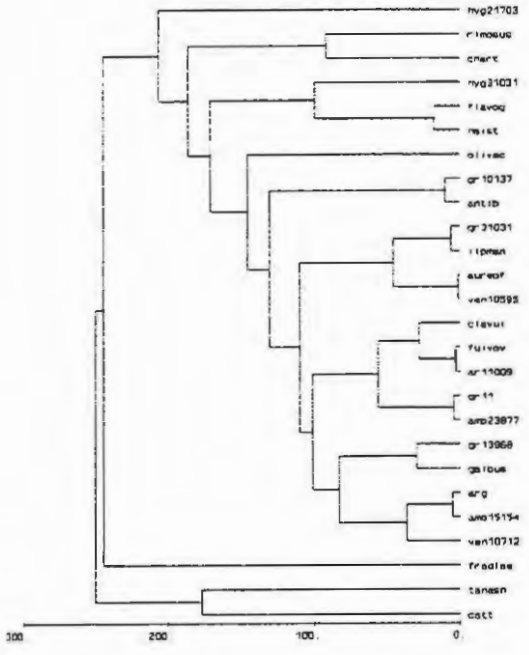
The different clustering algorithms used differentiated between the major groupings, *viz. S. tanashiensis, S. fradiae,* and *S. cattleya* but did not portray consistent classification in inter-cluster relationships (Figure 5.8). Closely related strains were not found to cluster together even though visual assessment of RAPD electrophoregrams showed profiles to contain identical or highly related patterns between the strain. This suggests that useful information was lost when RAPD bands were reduced into a 0,1 matrix. Broader bands which were found to be reproducible, carried the same weight as the finer bands which were spurious amplification products. Exclusion of finer bands in the analysis of polyacrylamide electrophoretic patters was not possible without introducing a degree of subjectivity, due to the complexity of the fingerprints obtained.



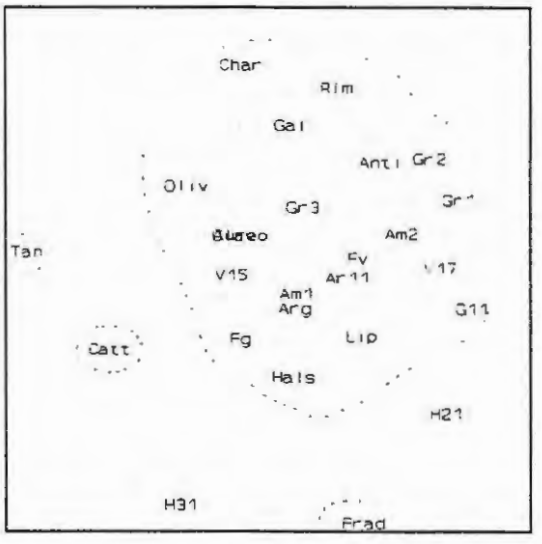
A.



B.



C



D.

Figure 5.8: The effect of different clustering algorithms on a 0,1 data matrix for *Streptomyces*, using primer 13: **A)** RAPDPLOT; **B)** PENNY; **C)** PRIMER: Euclidean distance with UPGMA analysis; **D)** Two dimensional configurations of MDS ordination of streptomycete strains that had been amplified with Primer 13. Stress value was 2.7; the strain designations in the dendrograms/MDS plot indicate the species names as follows: amb15154/Am1: *S. ambofaciens* ATCC 15154; amb23877/Am2: *S. ambofaciens* ATCC 23877; antib/anti: *S. antibioticus*; ar11009/Ar11: *S. argenteolus* ATCC 11009; arg: Unknown; aureof: *S. aureofaciens*; catt: *S. cattleya*; char: *S. chartreusis*; clavul/Clav: *S. clavuligerus*; flavog/Fg: *S. falvogriseus*; fradiae/Frad: *S. fradiae*; fulvov/Fv: *S. fulvovoridus*; galbus/Gal: *S. galbus*; gr10137/Gr1: *S. griseus* ATCC10137; gr11/G11: *S. griseus* No.11; gr13968/G2: *S. griseus* ATCC 13968; gr31031/Gr3: *S. griseus* ATCC 31031; Hals: *S. hastadii*; hyg21703/H21: *S. hygroscopicus* ATCC 21703; hyg31039/H31: *S. hygroscopicus* ATCC 31039; lipman/Lip: *S. lipmanii*; olivac/oliv: *S. olivaceus*; rimosus/rim:*S. rimosus*; tanash/Tan: *S. tanashiensis*; van10595/V15: *S. venezuelae* ATCC 10595; ven10712/V17: *S. venezuelae* ATCC 10712.

Weighted analysis was conducted using the *GelManager* program which scored the bands according to band breadth. Absence of a band was also considered a character state. Finer bands carried less weight, thus analysis was more reproducible than that conducted using 0,1 discreet data.

5.3.6 Ordination

Dendrograms are not always the best means of representing relationships between organisms as two adjacent samples are not necessarily the most similar. An alternative method of recording RAPD data, which allowed the differences and similarities between genotypes to be simultaneously presented as distances, is shown in Figure 5.8.D. The MDS retains the original RAPD information, highlighting similarities and differences as distances in ordinary Euclidean space. MDS plot is a better representation of clusters than dendrograms when the stress level is <0.1. The MDS plot illustrated the grouping out of *S. cattleya*, *S. rimosus*, and *S. fradiae* which is in agreement with dendrograms obtained with the Williams *et al.*, (1983a) classification. The *Streptomyces* belonging to cluster Group A (Figure 1.4) were found dispersed in an almost random set of points which is reflected in the groupings observed in the MDS plot (Figure 5.8.D). The 'goodness of fit' was measured using equation C in Equation 5.1C, known as the stress formula. The stress for the present ordination was large, 0.27, which indicates that the sample relationships were not represented well in the specified dimensionality. Stress can also be viewed as the distortion involved in 'compressing' the data to a small number of points.

Individual primers differed significantly in the amount of inter-population variation detected especially within the group A organisms (Figure 5.9). Unknown soil samples amplified with Primer OP2, were found to group in clusters with the streptomycetes of known identity. Samples S7 and S15 showed 100% similarity, therefore may be assumed to be the same strain.

Most of the streptomycetes failed to amplify with primers No. 1, 7 and 12 whereas primers with a CG ratio of $>60\%$ produced RAPD patterns. The 20-mer IPNS/B primer produced RAPD profiles with higher similarity between major clusters than with the 10-mer primers. *S. ambofaciens* ATCC 15154 and 23877 clustered at 90% similarity with the IPNS/B primer;

at 50% similarity with primer OP13; and at 65% similarity with protein analysis. *S. hygroscopicus* ATCC 31039 and 21703 were not found to cluster together with any of the RAPD primers that were tested or with protein analysis. *S. venezuelae* ATCC 10172 and 10595 clustered together with the 10-mer primers at 55% similarity with primers OP1 and OP18, at 40% similarity with primer OP2 and showed 78% similarity with protein profiles.

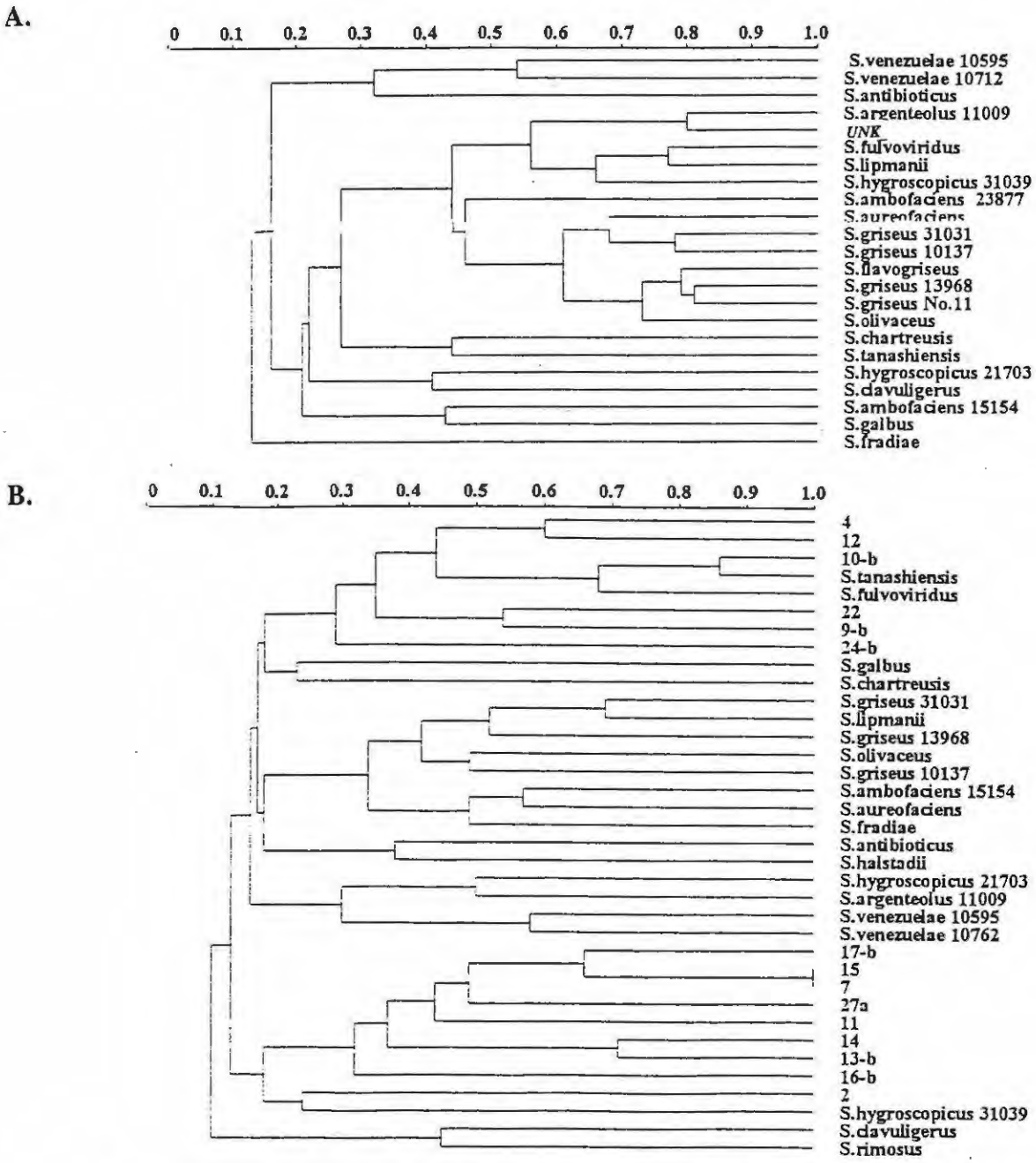
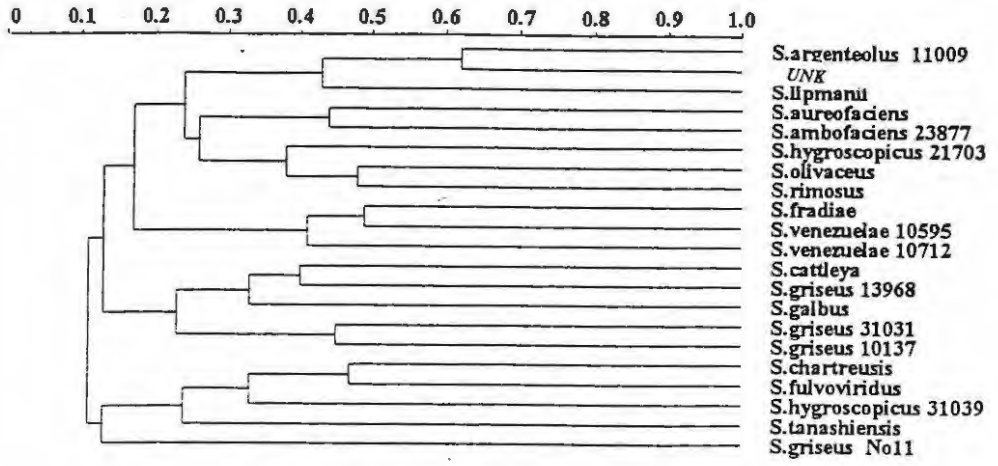
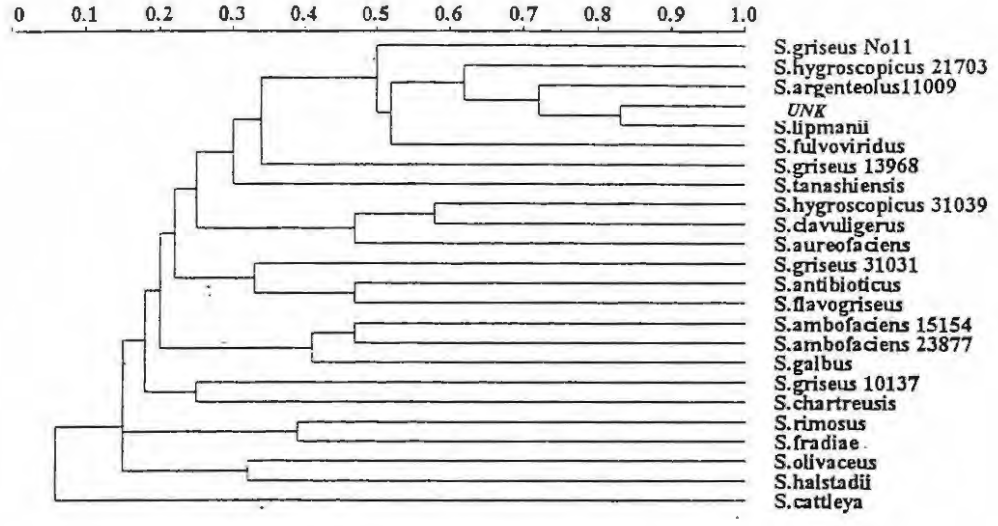


Figure 5.9 a: Dendrogram obtained using the Pearson product-moment correlation coefficient (*r*) with UPGMA clustering which was done by *GelManager*: **A.** Primer OP18; **B.** Primer OP2.

C.



D.



E.

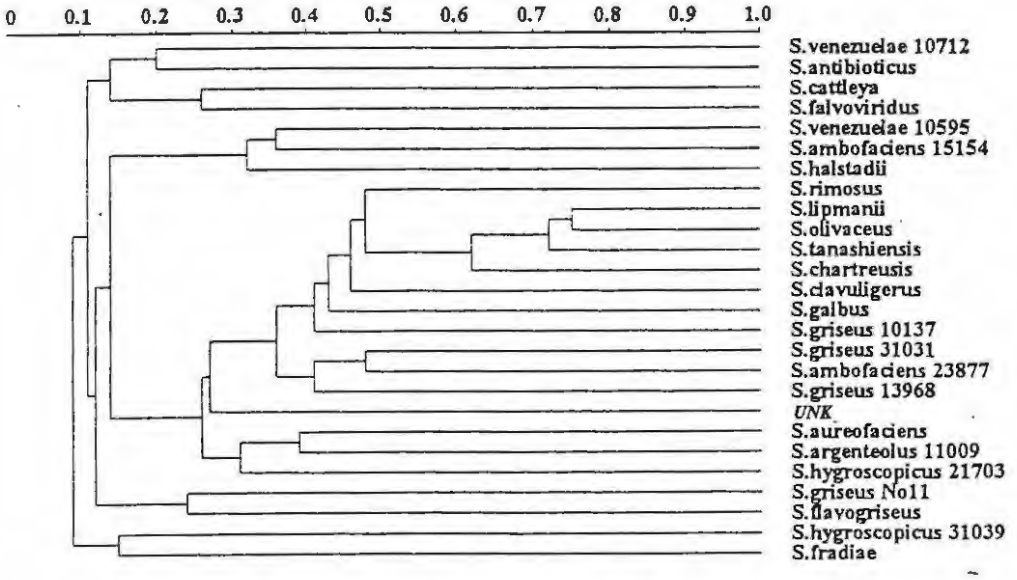


Figure 5.9 b: Dendrograms obtained using *GelManager*: **C.** Primer OP1; **D.** Primer OP13; **E.** Primer OP3.

F.

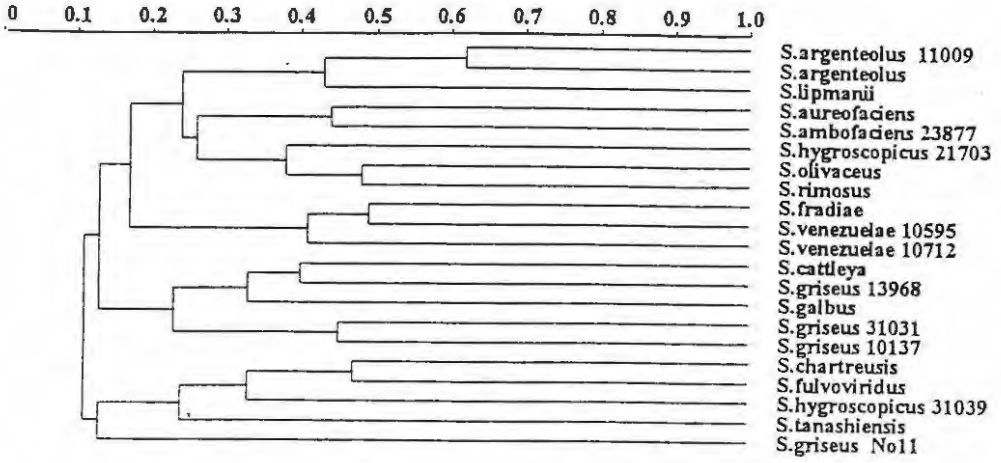


Figure 5.9 c: Dendrogram obtained using *GelManager*: **F.** 20mer IPNS-B primer.

5.4 DISCUSSION

The computer-assisted classification methods available for the classification of streptomycetes involves the use of large number of tests which become time consuming and difficult to interpret (Williams *et al.*, 1983a). Protein and RAPD PCR fingerprinting techniques were tested as potential identification and classification methods that would be required to accommodate the high throughput of samples in an antibiotic screening program. Morphological and biochemical diversity in microorganisms is paralleled by significant variations in the physical properties of the proteins, therefore protein profiles should portray these differences. Since RAPDs have been reported to provide a rapid and representative profile of the genome (Wang *et al.*, 1993; van Belkum *et al.*, 1994), their utility as a taxonomic tool in differentiating between species, identifying unknown samples, and in the reconstruction of phylogeny of *Streptomyces* was evaluated. Numerous factors need to be considered before RAPD data can be used to illustrate relationships between streptomycete species. Reproducibility of RAPD profiles, the nature of the amplicons, method of analysis employed, agarose versus PAGE electrophoresis, scoring methodologies and algorithms used, were found to have significant influence on the results obtained.

5.4.1 Proteins

The bacterial genome is largely devoted to the production of some 200 proteins with either

enzymatic or structural functions (Austin and Priest, 1986). In contrast to DNA composition, the protein content of a microorganism can be influenced by its environment (Kesters and De Ley, 1975). Therefore, all growth regimes (medium, temperature, and growth time) were kept constant.

Extensive protein degradation was observed even after samples were treated with 1% SDS and 1% 2-mercaptoethanol at 100°C to inactivate the proteases. Streptomycetes therefore require additional treatment against proteolytic degradation such as phenylmethylsulphonyl fluroride (PMSF) or guanidine-HCl at 100°C which may be added to the buffer used to resuspend the cells before lysis (Weber and Osborn, 1975). The Williams *et al.* (1983a) classification which is based on morphological and biochemical data define the nine strains used in this study into a major cluster A at a 77.5% similarity level (Figure 1.4), using the simple matching coefficient (S_{SM}) with UPGMA analysis (Sokal and Michener, 1958). Although the streptomycetes formed 2 clusters at 50% similarity and subspecies were found to cluster at levels of similarity above 67% but less than 80%, meaningful taxonomic conclusions could not be drawn with levels of similarity less than 85% (Kesters and De Ley, 1985).

5.4.2 Classification of *Streptomyces* using RAPD profiles

Streptomycetes subject to RAPD analysis (Table 2.1) were representatives of the phenetic cluster groups A, B, C, G and J, which together represent 80% of the 475 strains tested by the numerical phenetic study of Williams *et al.* (1983a) (Table 5.1). According to the Williams classification: *S. fradiae* belongs to cluster group G, *S. cattleya* and *S. galbus* are from cluster C, *S. rimosus* is from cluster B and *S. clavuligerus* from J and the rest of the streptomycetes tested in this study belong to cluster A (refer to Figure 1.4). The various primers tested reflected different aspects of the Williams *et al.*, (1983a) classification, for example, *S. fradiae* (primers OP3, OP13 and OP18), *S. rimosus* (primer OP2), *S. clavuligerus* (primers IPNS-B and OP2), *S. cattleya* (primers OP13 and OP3), *S. tanashiensis* (primers OP13, OP1) and *S. galbus* (primer OP18) differentiated as distantly related strains. RAPD profiles with all the primers tested did not show any similarity between the *S. hygroscopicus* ATCC 31039/21703 subspecies whereas *S. ambofaciens* ATCC 15154/23877 and *S. venezuelae* ATCC 10172/10595 were grouped together with various primers (Figure

5.8) as were *S. ambofaciens* and *S. aureofaciens*. The *S. griseus* β-lactam producers grouped together when amplified with the OP18, OP2, OP1, OP3 and the IPNS-B primers.

The average similarity between the major clusters was 10% which suggests long evolutionary separation between the species. There was no correlation between inter-cluster grouping of Group A organisms with the 10-mer primers. This implies that RAPD primers differentiated between the major taxonomic groups but differed significantly at the inter-cluster level. Within Group A organisms there were bands that were common between the species, however, spurious amplification products detected on PAGE gels stained with silver, may have resulted in incorrect inferences.

5.4.3 Elimination of duplicate strains

A sample labelled *UNK* showed high similarity to *S. argenteolus* ATCC 11009, and soil samples S7 and S15 were identified as duplicate strains. The results obtained in this study provide some evidence that independently isolated strains can be identical in their RAPD patterns thus may be used to reducing the number of closely related stains in a screening program.

5.4.4 Reproducibility of RAPD PCR

Many researchers have examined the reproducibility of the RAPD technique and its ability to discriminate between individuals (Keil and Griffin, 1994; Caetano-Anollés *et al.*, 1991). The success of RAPD reproducibility was found to be associated with the quality of the DNA (Keil and Griffin, 1994), especially when analysis was conducted from PAGE gels stained with silver. Although silver staining of amplification products allowed better resolution and visualization of fragments (Caetano-Anollés *et al.*, 1991), facilitating detection of 1 pg DNA/mm^2 band cross-section (Bassam *et al.*, 1991), spurious amplification products were detected which were not reproducible. Hence, RAPD analysis from agarose gels stained with ethidium bromide may simplify scoring of profiles as it allow for detection of a few major fragments (Welsh and McClelland, 1990; Martin-Kearley *et al.*, 1994). However, fragments amplified in all samples are sometimes not detected when analysis is conducted from agarose gels (Smith *et al.*, 1994).

5.4.5 RAPD Primers

Different primers showed different relationships between the species (Anzai *et al.*, 1994) (Figure 5.9). Kambhampati *et al.* (1992) found that the percentage of correctly classified presence/absence data varied according to the primer that was used. When data from two primers were combined, 100% of the individuals were classified correctly. This suggests that different primers produce different levels of information in a population (Dweikat *et al.*, 1993). Each RAPD primer appears to offer information from different regions of the genome. Therefore, a better representation of the streptomycete genome may be obtained by pooling RAPD data using an large number of RAPD primers.

The 10-mer primers formed clusters which were more diffuse, with operational taxonomic units (OTUs) joining together at much lower levels of similarity than with the 20-mer primers and the proteins. The IPNS/B grouped the *S. ambofaciens* ATCC 23877/15154 subspecies at 90% similarity which either suggests that it is the primer sequence or the increased length of the primer that determines the fingerprint profile. Studies have shown that the fingerprint patterns obtained were not related to the length of the primer (Caestro-Annolés, 1993) but the particular sequence that was chosen, especially at the 3'end of the primer (Klein-Lankhorst *et al.*, 1991; Mehling *et al.*, 1995). If RAPDs are to be used in a screening program a large number of primers would have to be screened to determine sequences that show conserved patterns between the species.

5.4.6 Nature of amplicons

Primer-sequence homology may determine a specific profile, but it is other factors such as enzymatic anchoring or reaction conditions and components which may affect primer annealing either by enhancing or restricting amplification of a fragment (Caetano-Anollés *et al.*, 1992). The result is a variation in band breadth (Caetano-Anollés *et al.*, 1991). The nature of the RAPD bands has been investigated as it may have direct consequences on the type of results obtained (Smith *et al.*, 1994). A common characteristic found in RAPD profiles was the intensity of various bands in a RAPD profile (Caestro-Anollés *et al.*, 1991; Weeden *et al.*, 1992). Relatively intense bands were found to be highly reproducible (Wang *et al.*, 1993), 'intermediate' bands were found to have an apparent error rate of 2% and the faint bands appeared at an error rate of 7% (Weeden *et al.*, 1992). This error rate can be

as high as 10% when spurious bands are not reproduced. Overall, an error rate less then 4% error could be attributed to RAPDs if PCR components, with high quality DNA, amplification and electrophoresis were kept constant. However, a 4% error rate can have serious implications in analysis and may result in incorrect inferences especially when bands are scored on presence and absence alone.

Chachaty *et al.* (1994), found that by scoring bands as 1 for faint, 2 for medium, and 3 for broad before calculating the similarity coefficient produced results that were similar to those produced by PFGE and ribotyping methods. Scoring was conducted from agarose gels stained with ethidium bromide, whereas banding patterns on silver stained acrylamide gels are more complex, which makes this type of analysis almost impossible without subjectivity.

Phylogenetic analysis of a cladistic nature requires comparison of independent, homologous, and variable characters that are genetically based (Selander *et al.*, 1991). Southern blot analysis has revealed that amplification products consisted of both single copy and highly repeated sequences (N'Goran *et al.*, 1994; Smith *et al.*, 1994; Stammer *et al.*, 1995). Varying band intensities were also attributed to comigrating fragments of the same length but different sequences (Smith *et al.*, 1994). The use of RAPDs in cladistic analysis could lead to erroneous results as scoring of multiple related bands may lead to overestimation of between-strain relatedness and differences. Clark and Lanigan (1993) used statistical methods to estimate sequence divergence with RAPDs and made suggestions as to criteria that must be met before RAPDs can be used: i) all bands should be scored and there should be no bias in scoring monomorphic versus polymorphic bands; ii) primers should not be selected according to the ones that show the highest polymorphisms as this leads to a bias in results; iii) the homology between bands in different species should be determined, for example with Southern blot analysis; iv) and allelism of bands should also be determined with the use of Southern blotting or segregation analysis and the allelic bands should only be scored once.

Other issues that need to be considered before making phylogenetic inferences concerning RAPD data are: i) gel-to-gel variation; ii) co-migration of non-homologous amplification products; iii) loss of low molecular weight products may lead to biased estimates of percent

similarity; iv) and an estimate of 'noise' factor obtained when unrelated taxa share common band sizes (Chapco *et al.*, 1992).

5.4.7 Analysis of phylogenetic data

GelManager was used to analyse RAPD profiles with different primers as the program scores profiles based on band intensity unlike all the other programs tested. This program is based on the Product moment coefficients which records band absence as a similarity, thus various assumptions had to be made: fragments that comigrate are assumed to be from identical alleles; and the absence of a band is due to identical ancestral mutations. These assumptions may not always hold true for RAPD analysis as migration of bands of similar size may not always be from identical alleles (Stammers *et al.*, 1995) and the absence of a band may be due to point mutations at the primer annealing site that may prevent annealing. Therefore, similarity matrices that only take the shared presence of a band into account may be a better representation of RAPD PCR profiles, such as the S^{SM} (Sokal and Michener, 1958), and the Nei and Li (1985) similarity index. Although *GelManager* (Version 1.01) enabled analysis to be conducted according to band intensity, it does not allow for the pooling of RAPD data from different primers into a single databank. Inefficient handling of RAPD data appears to be the major limitation in RAPD analysis as established programs do not take the nature of the amplicons into account.

The 16S rRNA data showed over 96% homology between *S. coelicolor, S. lividans* and *S. ambofaciens* (Kim *et al.*, 1990), hence they may be useful in assessing genetic distance between diverse bacteria (Mehling *et al.*, 1995; Rainey *et al.*, 1994; Rogall *et al.*, 1990). In contrast, RAPD profiles show an average of 10% similarity between major clusters, which suggests that the streptomycete genome is less conserved than originally thought. Fatty acid methyl esters patterns of *Streptomyces* conducted by Garrit *et al.* (1993) shows streptomycetes to be a heterogenous population which is consistent with the RAPD data presented in this study.

5.5 FUTURE CONSIDERATIONS

Complex RFLP patterns were scored by Forbes *et al.* (1991) by sectioning the fingerprint

with the use of an internal DNA ladder. This method could be adapted for RAPD profiles as it was found to reduce scoring problems that arise due to fragments comigrating. RAPD analysis conducted using denaturing-gradient-gel electrophoresis (DGGE) (Dweikat *et al.*, 1993) or Single Stranded Conformation Polymorphisms (SSCP) (McClelland *et al.*, 1994) gels have been reported to produce superior fingerprints when compared to agarose-RAPD, or polyacrylamide-RAPD methods. The amplification of conserved regions of actinomycete genomes using RAPD PCR (Mehling *et al.*, 1995) followed by DGGE analysis (Fisher and Lerman, 1983) may produce information of phylogenetic value. Cells amplified directly from culture (Harrison *et al.*, 1992), would facilitate the rapid detection of duplicate strains isolated in a screening program.

5.6 CONCLUSIONS

RAPDs were useful in generating strain specific patterns, and could therefore be used to eliminate duplicate strains in an antibiotic screening program. However, streptomycetes are a genetically diverse group which is reflected in their fingerprint profiles, therefore the successful classification and identification of new species can not be based on protein and RAPD-fingerprinting data alone. In this study, there was a better congruency between the numerical phenetic classifications (Williams *et al.*, 1983a) and the protein profiles than with RAPD fingerprints. However, proteins were subject to proteolytic activity, which did not affect analysis by RAPD PCR methods. Improvements in RAPD PCR analysis will not come primarily from better theoretical treatments or better algorithms per se. Improvements will be the result of large RAPD data banks which will provide a more complete picture of the genome.

CHAPTER 6

FINAL DISCUSSION

The PCR reaction has been shown to be a dynamic molecular tool with wide application; however, it has not been tested in β-lactam antibiotic screening programs. The application of PCR-based methodologies were examined in the differentiation of *Streptomyces* species; the reconstruction of phylogeny; identification of unknown samples; and detection of potential antibiotic producers.

Conventional antibiotic screening procedures were found to be reliant on the method of isolation, maintenance and preservation of cultures. Since DNA based methodologies are not dependent on culture conditions, the screening of organisms for the presence of antibiotic coding genes could therefore improve the sensitivity of the screening process. The objectives of this study which included conventional and molecular antibiotic screening procedures for detection of β-lactam producing organisms were fulfilled as follows:

i) The successful amplification of the IPNS gene for the screening of potential producing organisms required the optimization of the PCR reaction for high GC organisms such as the streptomycetes. PCR primers were designed based on consensus sequences of the *pcbC* gene from various β-lactam producers. Although the 844 bp IPNS fragment was amplified in *S. clavuligerus, S. tanashiensis, S. griseus, S. olivaceus, S. lipmanii, S. chartreusis*, the IPNS primers did not successfully amplify the IPNS gene from all the β-lactam producers. Southern and dot blot analysis improved the sensitivity of the antibiotic screening process and allowed the investigation of evolutionary relationships amongst the genes.

ii) PCR coupled with Southern and dot blot analysis was used to screen isolates from know culture collections and streptomycetes isolated from the soil for the presence of the *pcbC* gene. Cultures that tested positive included: *S. chartreusis, S. clavuligerus*, *S. griseus* ATCC 10137,

S. cattleya, *S. hygroscopicus* ATCC 31039, *S. lipmanii*, *S. olivaceus*, *S. tanashiensis*, *S. argenteolus*, *S. fulvoviridis* *A. persicinum* DSM 1073, *A. strictum* DSM 2399, *A. chrysogenum* DSM 880, *P.chrysogenum*, *S. hygroscopicus* ATCC 21703, *S. venezuelae* ATCC 10712, and one *Streptomyces* isolated from the soil, sample No. 22. Genetic screening methods proved to be more sensitive and specific than conventional screening procedures as β-lactam producers that had lost their antibiotic producing ability due to frequent subculturing were detected using PCR and Southern blot analysis. This may have great implications in a conventional antibiotic screening program as potential antibiotic producers may be overlooked.

iii) This study was a preliminary investigation into the potential use of RAPDs as a tool for solving discrepancies in *Streptomycete* identification, especially among "novel" and sub- species. RAPD PCR showed potential as a rapid detection tool of duplicate strains in an antibiotic screening program.

iv) It is becoming increasingly important to derive and integrate consensus classifications from chemical, phenetic and molecular data. The contribution of RAPD and protein-fingerprinting to streptomycete systematics with reference to numerical classification systems was examined. The application of RAPDs in phylogenetic analysis was found to be dependant on the nature of the amplicons and the type of analysis employed. When RAPD bands were reduced to binary scores (0,1 matrices), useful information was lost; whereas, RAPD profiles that were scored based on band breadth resulted in more reproducible dentrograms. Different RAPD primers differentiated between the major cluster groups as defined by William *et al.* (1983a); however, phylogenetic clustering of *Streptomyces* strains did not reflect antibiotic production, therefore RAPDs can not be relied upon to assist in selecting potential producers. The streptomycetes have been reported to be genetically diverse organisms as was reflected in their RAPD profiles.

v) The suitability of various phylogenetic packages to RAPD data was investigated. Such packages which included *GelManager*, PRIMER, RAPDPLOT and PHYLIP were not found to

be satisfactory analyses systems for RAPD data. The successful analysis of RAPD data will require that various factors be taken into account such as the nature of the amplicons which includes the band breadth; determination of the presence absence of a band if it is due to mutation or PCR artifact; the comigration of bands; whether bands are length variants of the same amplicon or whether bands are from various parts of the chromosome; analysis from PAGE versus agarose gels; gel-to-gel variation; pooling of RAPD data into large data banks. RAPDs do not appear to be suitable for genetically diverse organisms such as the *Streptomyces*, but their application should be limited to the elimination of duplicate strains until more is understood about the nature of RAPD fingerprints. Protein fingerprinting successfully grouped subspecies into clusters therefore may provide useful phylogenetic information.

CONCLUSION

PCR was found to have numerous applications in an antibiotic screening program: PCR was utilized to amplify the IPNS gene; incorporate restriction sites and a GC clamp into the amplified IPNS fragments which may be used for future genetic manipulations; a radioactive-probe was produced using PCR that could be used to screen for novel genes using Southern hybridization and dot blot analysis; and RAPD PCR produced strain specific fingerprints that could be used for the elimination of duplicate strains. Protein fingerprinting of *Streptomyces* may provide insight to discrepancies in taxonomy; however, it can not be used alone in the classification of the genus. Therefore, nucleic acid hybridization probes and polymerase chain reaction methodologies have shown potential in the detection of β-lactam producers as specific screening tools that may result in the discovery of β-lactam genes with new and useful properties.

APPENDICES

APPENDIX A

APPENDIX B

APPENDIX C

APPENDIX D

APPENDIX E

APPENDIX A

CULTURE MEDIA

All media was autoclaved at 120°C for 20 minutes unless otherwise stated.

A.1 Aspergillus Media

Yeast extract	1 g
Peptone	1 g
$NaNO_3$	6 g
Casamino acids (Difco 0230)	1 g
Agar	15 g
Adenine	0.15 g
Vitamin solution	10 ml
Distilled water	1000 ml

pH was adjusted to 6.0

The media was sterilized at 121°C for 10 minutes.

Vitamin solution:

Biotin	0.01 g
Pyridoxin HCl	0.01 g
Thiamine HCl	0.01 g
Riboflavin	0.01 g
p-Aminobenzoic acid	0.01 g
Nicotinic acid	0.01 g
Distilled water	100 ml

Solution was sterilized for 10 minutes at 121°C

A.2 CY-Agar

Casitone	3 g
$CaCl_2.2H_20$	1.36 g
Yeast extract	1 g
Agar	15 g
Distilled water	1000 ml

pH was adjusted to 7.2

A.3 GFY Medium

Glucose	5 g
Fructose	5 g
Yeast extract	5 g
$CaCO_3$	3 g
Agar	15 g

Distilled water	1000 ml
After autoclaving Vitamin B_{12} was added:	
Vitamin B_{12}	0.002 g

A.4 M_3 Media

Broth:	
Malt extract (Difco)	24 g
Yeast extract (Oxoid)	5 g
Distilled water	1000 ml
The pH was adjusted to 7.4.	
Plates:	
Agar (Oxoid No.1)	15 g

A.5 Nutrient Agar Plus Crystal Violet

Soil or other extract	100 ml
Tap water	900 ml
Beef extract	5.0 g
Yeast extract	5.0 g
Agar	15.0 g

Ingredients were mixed together and autoclaved for 3 minutes at 121°C. 1.0 ml of filter-sterilized 0.1% solution of crystal violet was added.

A.6 Nutrient agar

Peptone	5 g
Meat extract	3 g
Agar	15 g
Distilled water	1000 ml

A.7 Potato Dextrose Agar

Potato extract	4 g
Dextrose	20 g
Agar	15 g
Distilled water	1000 ml
(pH 5.6)	

A.8 Soil infusion agar

Distilled water	600 ml
*Soil infusion	400 ml
Yeast extract	3.0 g
Glucose	7.5 g
K_2HPO_4	0.5 g

KH_2PO_4	0.5 g
$MgSO_4 7H_20$	0.5 g
Agar	16.0 g

Ingredients were mixed and autoclaved at 121°C for 15 min.

*Soil infusion
Fresh Super soil (any good loam potting soil) 500 g
Distilled water 1000 ml
Soil and water were mixed and heated for 15 min. without boiling. The water was strained through a double-layered cheese cloth and filter sterilized. The soil infusion was stored at 4°C until needed.

A.9 Tryptose Soya Agar (TSA)

Tryptone	15 g
Soy/Peptone	5 g
NaCl	5 g
Agar	13 g
dH_2O	1000 ml

The pH was adjusted to 7.3 (±1)

A.10 TSP Broth

Tryptone	17 g
Soy Peptone	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Dextrose	2.5 g

pH was adjusted to 7.3 (±1)

A.11 Yeast Glucose broth (YG)

Yeast extract	0.5%
Glucose	2%

A.12 YpSs Media

Yeast extract	4.0 g
Soluble starch	15 g
K_2HPO_4	1 g
$MgSO_4X\ 7H_2O$	0.5 g
Agar	15 g
Distilled water	1000 ml

APPENDIX B

STANDARD BUFFERS AND SOLUTIONS

B.1 Acetamide (10X solution)

Acetamide	5 g
ddH_2O	10 ml

After the solution was sterilized by autoclaving at 121°C for 20 minutes, it was passed through a 0.22 μm filter.

B.2 Blue Loading Buffer

EDTA	0.1 M
Bromophenol Blue	0.25% (w/v)
Sucrose	40% (w/v)

B.3 CTAB Buffer (2X)

CTAB	2 % (w/v)
NaCl	1.4 M
EDTA	25 mM
Sarkosyl	1%
Tris/HCl (pH 9.5)	100 mM
Proteinase K	100 mg/ml
Sarkosyl	30% N-Lauroyl-Sarcosine

B.4 Ethidium Bromide

Ethidium Bromide	10 mg
ddH_2O	10 ml

(Was stored at 4°C in the dark)

B.4.i Decontamination of Ethidium Bromide Solutions (>0.5 mg/ml)

Sufficient water was added to reduce the concentration of the ethidium bromide to <0.5 mg/ml. One volume of 0.5 M $KMnO_4$ was mixed into the ethidium bromide solution, followed by the addition of one volume of 2.5 N HCl. Finally, one volume of 2.5 N NaOH was mixed before the solution was discarded (Sambrook *et al.*, 1989)

B.5 Lysis Buffer

SDS	2%
EDTA	0.1 M
Tris/HCl	10 mM
Proteinase K	100 mg/ml

B.6 Phenol:Chloroform:Isoamyl alcohol

A mixture that containing phenol:chloroform:isoamyl alcohol in the a 25:24:1 ratio was used to remove proteins. The mixture was prepared as in Sambrook *et al.* (1989), and was stored under 100 mM Tris/HCl (pH8.0) in a dark bottle at 4°C.

B.7 Proteinase K

Stock solution	20 mg/ml in dH_2O
Concentration in reaction	50-100 mg/ml
Temperature of activity	37-56°C
Storage temperature	-20°C

B.8 Ribonuclease A (RNase A)

A stock solution of 20 mg/ml pancreatic RNAse A was made in 10 mM Tris/HCl (pH 7.5) and 15 mM NaCl. The solution was then heated at 100°C for 15 minutes and then slowly cooled down to room temperature. RNase A Stock solution was aliquoted and stored at -20°C.

B.9 Saline

NaCl	8.7 g
dH_2O	1000 ml

Saline water was aliquoted and autoclaved.

B.10 Sodium Acetate 3 M (pH 5.5)

Sodium acetate.$3H_2O$	408.1 g
dH_2O	800 ml

The pH was adjusted with glacial acetic acid. The volume was then adjusted to 1000 ml with dH_2O. The solution was dispensed in aliquotes and sterilized by autoclaving.

B.11 TAE (50 X)

Tris base	242 g
Glacial acetic acid	57.1 ml
$Na_2EDTA.2H_20$	37.2 g
pH 8.5	

B.12 $T_{10}E_1$ Buffer

Tris/HCl (pH8.0)	10 M
Na_2EDTA	1 mM

B.13 TBE 10X Buffer

Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 ml

B.14 Tris/SDS/EDTA Solution

Tris/HCl (pH8.0)	10 mM
SDS	2% (w/v)
Na_2EDTA	1 mM

B.15 Tris-Sucrose Solution

Tris/HCl (pH 8.0)	1000 mM
Sucrose	25% (w/v)

APPENDIX C

PAGE GELS FOR DNA: REAGENTS AND STANDARD PROCEDURES

C.1 Polyacrylamide Gels

Acrylamide (BDH) 30%
NN-methylene-bis-acrylamide 0.5%
Solution was filtered and then stored in the dark at 4°C.

Table C1 : An adaptation of the Laemmli system by Carlos Bezuidenhout (Personal communications) for DNA preparations.

Stock Solutions	Final polyacrylamide gel stock concentrations		
	5% Stacking gel	10% Resolving gel	12% Resolving gel
Acrylamide stock (ml)	3.0	12.0	15.0
0.5 M Tris/HCl pH6.8/ 0.4% SDS (ml)	4.5	-	-
1.5 M Tris-HCl pH 8.8/ 0.4% SDS (ml)	-	9.0	9.0
ddH_20 (ml)	9.8	15.0	12.0
TEMED (μl)	10	20	20
10% Ammonium Persulphate (ml)	150	300	300
Final volume (ml)	18.0	36.0	36.0
Running buffer	TBE (Appendix B)		

C.2 Estimation of Oligonucleotide Concentration

Oligonucleotide were diluted $^1/_{100}$ in ddH_20 and the absorbances (OD) were read at 260 nm. The concentration of the oligonucleotide could then be calculated using the following extinction coefficients:

Table C2: Extinction coefficients (E) values for nucleotides

Nucleotides	E (ml/mmole)
dGTP	11.7
dCTP	7.3
dATP	15.4
dTTP	8.8

(Sambrook *et al.*, 1989)

The concentration of each nucleotide could then be calculated by multiplying the number of times each base appeared by its extinction coefficient (E). Having obtained an absorbance value ($OD_{260\ nm}$), the concentration (c) could then be calculated using the Beer-Lambert equation, (for 1 cm path-length):

$$\text{Absorbance} = E \times c$$

An OD_{260} of 1 is equivalent to 50 mg/ml of double stranded DNA (Ausubel *et al.*, 1995).

C.3 DNA Size Markers

DNA size markers were prepared by the enzymatic digestion of 10 μl DNA (PBR322), 1 μl restriction enzyme (Boehringer Mannheim, Germany), 10 μl restriction enzyme 10 X buffer and 79 μl ddH_20. Digestion was carried out at 37°C for 2-5 hours. The reaction was stopped by the addition of 25 μl of Blue Stop Buffer (see Appendix B). Approximately 7 μl of the DNA size marker was loaded onto 1% agarose gels and 3 μl was loaded onto 10-12% polyacrylamide gels for analysis.

Table C3: Sizes of restriction fragments in base pairs

DNA PBR322[a] *HinfI* (bp)	*EcoRI*[b] (bp)	*HindIII*[b] (bp)
1631	21240	23150
517	7420	9420
506	5810	6560
396	5650	4380
344	4880	2320
298	3540	2020
221		560
220		125
154		
75		

[a]Fragment sizes as by Stephan Minter and Paul Sealey: cited in Rickwood *et al.* (1984).
[b]Fragment sizes of double digest

C.4. Silver Staining

C.4.i Buffer A

Acetic Acid	0.5%
Ethanol	10%

C.4.ii Buffer B

$AgNO_3$	0.1%

C.4.iii Buffer C

NaOH	1.5%
$NaBH_4$	0.01%
Formaldehyde	0.15%

Buffer C was made fresh every time.

C.4.iv Silver Staining of PAGE Gels

Polyacrylamide gels were soaked in 200 ml Buffer A for 2 X 3 minutes; followed by 10 minutes in 200 ml Buffer B; the gels were washed twice in distilled water; and finally, the gels were developed in 200 ml Buffer C. Buffer B was stored in the dark and was reusable.

APPENDIX D

STANDARD SOLUTIONS AND PROCEDURES USED FOR SOUTHERN BLOTTING AND HYBRIDIZATIONS

D.1 Alkali Fixation Buffer

NaOH	0.4 M

D.2 Alkali Transfer Buffer

NaOH	0.4 M

D.3 Denaturing Solution

NaCl	1.5 M
NaOH	0.5 M

D.4 Neutralizing Solution

NaCl	1.5 M
Tris-HCl pH 7.2	0.5 M
EDTA	0.001 M

D.5 20 X SSC (pH7)

NaCl	3 M
Na_3citrate	0.3 M

D.6 20 X SSPE

NaCl	3.6 M
Sodium phosphate	0.2 M
EDTA pH 7.7	0.002 M

D.7 Filter Hybridization Solutions

D.7.i 100 X Denhardt's Solution

BSA (Fraction V)	2% (w/v)
Ficoll™	2% (w/v)
PVP (polyvinylpyrolidone)	2% (w/v)

D.7.ii 10 mg/ml Non-Homologous DNA

Herring DNA was sheared by repeated pipetting of the DNA in an eppendorf; the DNA was then denatured by boiling at 100°C for 10 minutes, and rapidly cooled on ice just before it was added to the pre-hybridization and hybridization mixture.

D.7.iii Pre-hybridization Solution (25 ml)

5 x SSPE [6.25 ml, 20 x SSPE]
5 x Denhardt's solution [1.25 ml, 100 x Denhardt's solution]
0.5% (w/v) SDS [1.25 ml, 10% (w/v) SDS]
Made to 25 ml with sterile water

D.7.iv Hybridization Wash I:

2 x SSPE
0.1% (w/v) SDS

D.7.v Hybridization Wash II:

1 x SSPE
0.1% (w/v) SDS

D.8 Procedures of Southern Blotting

(Acid treatment of the samples was omitted as the fragments that were to be analyzed were all under 10 kb in size). The gels were stained in ethidium bromide solution, not exceeding 10 mg/ml and the bands visualized by UV and were photographed. The gels were washed in distilled water, after which the DNA was denatured by soaking the gel in denaturation buffer for 30 minutes at room temperature with shaking. The gels were then washed in distilled water, followed by a neutralization step where the gels were placed in neutralization buffer for 15 minutes at RT with shaking. Neutralization was repeated for another 15 minutes. The capillary blot was set up as follows:

D.8.i Southern Capillary Electrophoresis Blotting Procedure

A glass dish was filled with blotting buffer (20 x SSC or 20 x SSPE). A glass platform was placed in the centre of the dish and covered with a wick that dipped into the 20 x SSC on all four sides of the plate. Two sheets of Whatman 3 MM filter paper were cut slightly smaller than the glass plate and were placed ontop, saturated with blotting buffer. Excess SSC and air bubbles were smoothed out using a glass rod. The gel was placed onto a piece of glass and all unnecessary pieces were cut off before the gel was measured. The Hybond-N^+ was cut to size (Hybond-N^+ was handled with forceps at all times or between two pieces of protective paper as the membrane should never be touched) and floated on distilled water making sure that both side were thoroughly wet before transferring to 2 x SSC buffer. The gel was placed on the wick making sure that there were no bubbles trapped below the gel. Cling film was placed around the gel so as to prevent the blotting buffer being absorbed directly into the paper towels above, thus short circuiting the blot. Hybond-N^+ was positioned on top of the gel making sure that all air bubbles were eliminate. Once the membrane had made contact with the gel it could not be moved or removed as DNA transfer started immediately on contact. Three sheets of Whatman (1)3 MM paper were cut to size and were wetted with blotting buffer before being placed on top of the Hybond-N^+ membrane. Absorbent paper towels were stacked on top of the 3 MM, cut to size and approximately 5 cm high. A glass plate with a 0.5-1 kg weight were placed on top of the

paper towels. The system was secured with cling-film which also helped reduce evaporation. The transfer was allowed to take place for 24 hours at room temperature. After blotting the system was carefully dismantled and the gel was marked with a soft pencil to position the tracks. The top right hand corner was cut to allow for orientation. The DNA was then fixed onto the membrane using alkali fixation.

D.8.ii Alkali Fixation of Southern and Dot Blot

The membrane was placed onto a pad of filter paper (Whatman No.3) that had been pre-soaked with 0.4 M NaOH, and left for 30 minutes at room temperature for fixation of the DNA onto the nylon membrane. The final wash was in 5 x SSPE with gentle agitation and no more than one minute. The membrane was wrapped in cling film and stored at 4°C.

D.9 Procedures for Filter Hybridization

Sheared Herring DNA (0.5 mg) that had been heated to 100°C and rapidly cooled on ice was added to the prehybridisation solution. The membrane was pre-hybridized in a shaking water bath at 70°C for at least 1 hour. The probe was denatured by heating to 100°C for 5 minutes, and immediately after was incubated on wet ice for 5 minutes. A corner of the plastic bag (containing the filter in the pre-hybridization solution) was cut and the probe was added to the hybridisation mix (the probe concentration was less than 20 ng/ml and $\pm 10^6$ cpm). The bag was resealed and hybridisation was performed for more than 12 hours at 70°C. After hybridisation the filter was removed from the plastic bag and placed in a plastic box for washes. The filter was washed in relatively large volume of Hybridisation Wash buffer ($\pm$100 ml) at 70°C with gentle agitation. The first two washes were at low stingency using Wash solution I for 30 minutes each wash. Two more 15 minute washes at Moderate stringency with wash solution II were performed. The filter was then exposed to an X-Ray (Amersham) for autoradiography.

D.10 Isolation of DNA Fragments from Low Melting Agarose

The agarose was melted at 68°C to which 200 μl TE was added. The probe was purified by phenol:chloroform extraction followed by chloroform:isoamyl alcohol extraction (Appendix B). DNA was precipitated by the addition of 2 volumes of 95% ethanol and 0.2 volumes 10 M Ammonium Acetate at 4°C. The final DNA pellet was resuspended in 150 μl of TE buffer (Appendix B).

D.11 Autoradiography

Autoradiography was performed by exposing the nylon membranes to an X-Ray film (Amersham), in an autoradiograph cassette on an intensifying screen. The cassette was placed at -70°C for more than 24 hours. The X-ray film was developed for 150 sec. in developer (Cronex); 0.5 minutes in 2% acetic acid solution and 5-6 minutes in Fixer solution (Cronex Premix Fixer).

D.12 Measurement of the Specific Activity

Three microlitres of the probe sample was spotted onto glass fibre discs that was pinned onto a foil-covered polystyrene slab and were left to air dry. This filter represented the total number of counts per minute (cpm) in the reaction. An equal volume of the same probe sample was added to a tube containing 100 μl of 1 mg/ml Herring sperm DNA (Boehringer Mannheim Biochemica, Germany). After the sample was mixed, 0.5 ml of 5% trichloroacetic acid (TCA) was added and incubated on ice for 30 minutes. This sample was then filtered through glass fibre discs, washed three times with 5% cold TCA, rinsed with ethanol and left to dry and represented the incorporated radiolabel. When the filters were dry they were placed in scintillation vials containing 5 ml Packard Emulsifier Scintillation 299 scintillation fluid. Counts per minute were read by the Beckman LS 5801 scintillation spectrometer.

The incorporation of radioactivity into the nucleic acid was calculated as follows:

$$\text{cpm incorporation} = \frac{\text{incorporated cpm}}{\text{total cpm}}$$

total amount of the product = proportion incorporated X total amount of the product

$$\text{specific activity} = \frac{\text{cpm incorporated}}{\text{total amount of the product}}$$

(Sambrook *et al.*, 1989)

APPENDIX E

STANDARD BUFFERS AND REAGENTS USED FOR PROTEINS

E.1 Bath Buffer

Tris	30.3 g
Glycine	144.1 g
SDS	10.0 g
dH_2O	1000 ml

E.2 Bradfords Reagent

Coomasie Brilliant Blue	100 mg
Ethanol (95)%	50 ml
Phosphoric Acid (85%)	100 ml
dH_20	1000 ml

E.3 Destaining Solution

Methanol	450 ml
Glacial Acetic Acid	70 ml
dH_2O	480 ml

E.4 Dissociation Buffer (1X)

Mercaptoethanol	5 ml
SDS	5 g
Glycerol	7.5 ml
Bromophenol Blue (0.2%)	2.5 ml
1 M Tris/HCl pH 6.8	6.3 ml
dH_2O	28.7 ml

E.5 Sodium Phosphate Buffer pH 7.2

Solution B: 14.2 g/L Na_2HPO_4	72 ml
Solution A: 13.8 g/L $NaH_2PO_4.H_2O$	28 ml

E.6 Staining Solution

Methanol	450 ml
Glacial acetic acid	100 ml
Coomassie Blue	2 g
dH_2O	450 ml

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