ISOLATION AND CHARACTERISATION OF ANTIBIOTIC-PRODUCING MARINE ACTINOMYCETES

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ABSTRACT

Resistance to antibiotics poses a serious threat to healthcare and new drugs are needed. This is especially true for tuberculosis (TB), which is at epidemic levels in South Africa. Multidrug-resistance in *Mycobacterium tuberculosis* makes TB more difficult and expensive to treat and increases mortality rates.

The surfaces of 12 seaweed species found in South African coastal waters were screened for the presence of antibiotic-producing actinomycetes. Of the 67 strains isolated, 26 exhibited antibacterial activity against *Mycobacterium aurum* A+ and/or *Enterococcus faecium* VanA. These actinomycete strains were physiologically characterised. Three strains showing very strong antibacterial activity were further characterised by the use of chemical taxonomy, DNA sequencing and scanning electron microscopy and were shown to belong to the genus *Streptomyces*. A strain not showing activity was shown by the same methods to belong to the genus *Micromonospora*. Partial purification of the active compounds was carried out on the three strains exhibiting strong antibacterial activity. All were shown to produce moderately to highly polar compounds.

ABBREVIATIONS

A	Adenine
bp	Base Pairs
C	Cytosine
°C	Degrees Celsius
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra-acetic Acid
g	Grams
G	Guanine
h	Hours
I.U.	International Units
M	Molar
mM	Millimolar
ml	Millilitres
nm	Nanometres
%	Percentage
PCR	Polymerase Chain Reaction
psi	Pounds per Square Inch
rpm	Revolutions per Minute
s	Seconds
sp.	Species
T	Thymine
TAE	Tris-acetate-EDTA Buffer
TE	Tris-EDTA Buffer
µl	Microlitres
v/v	Volume per Unit Volume
w/v	Weight per Unit Volume

CHAPTER 1:
GENERAL INTRODUCTION

1.1 Antibiotic Resistance

1.1.1 Miracle Cures?

One of the greatest triumphs of modern medicine has been the ability to routinely treat and control the spread of infectious diseases that would once have led to certain death. The advances made in antimicrobial chemotherapy since the discovery of penicillin have led (in first world countries at least) to longer life expectancy and reduced fear that infectious disease may result in death (Cohen, 1992; Levy, 1998; World Health Organisation, 2002a).

Because of ignorance and misplaced confidence on the side of the public and complacency on the side of the medical profession, resistance to previously very useful drugs has increased at an alarming rate (Levy, 1998). It is now commonplace for the most widespread bacterial pathogens to be resistant to the first-line antibiotics: those that are cheapest and have the fewest side effects (World Health Organisation, 2002a). If resistance is encountered, it becomes necessary to treat the infection with more expensive drugs. The more widely these are employed, the more widespread resistance to these drugs becomes and a yet more expensive drug becomes necessary. The cost soon becomes prohibitive, especially in poorer communities and developing countries (Gibbons, 1992).

Worse still, some bacterial infections are now resistant to nearly all available antibiotics (Levy, 1998). These include strains of potentially lethal pathogens such as *Staphylococcus aureus* and members of the genus *Streptococcus*. Every year the list of untreatable infections grows as resistance spreads. This means higher morbidity and mortality rates associated with disease (Cohen, 1992). Treatment has to be delayed until an effective drug

has been found, decreasing the patient's possibility of recovery and increasing the possibility of resistance spread (Palumbi, 2001). If an antibiotic to which the pathogen is already resistant is administered to a patient not yet showing symptoms, the killing off of antibiotic-sensitive bacterial competitors will enable the resistant bacterium to spread, hastening the onset of disease (Levy, 1998). Apart from the cost of the drug, the cost of hospitalisation and laboratory work must also be considered (Palumbi, 2001).

1.1.2 Contributory Factors

As a result of the great success of antibiotics, the general public see them as cure-alls: take an antibiotic and the "germ" will be killed. So much so, that patients will often demand that their doctor prescribe a course of antibiotics for even the most minor of complaints (Gibbons, 1992). Prof. Stuart Levy, president of the Alliance for the Prudent Use of Antibiotics, conducted a seminar at which "more than 80 percent of the physicians present admitted to having written antibiotic prescriptions on demand against their better judgment" (Levy, 1998) This means prescribing antibiotics to treat an illness actually caused by a virus - against which antibiotics have no effect - or prescribing as a precautionary measure (World Health Organisation, 2002a). Sometimes broad-spectrum drugs are administered in place of a more appropriate narrow spectrum drug.

Even if antibiotics are prescribed for a valid reason, patients may misuse the drugs. Failure to finish the full course of treatment will result in the infective agent not being eliminated from the human body. Patients may think that because they are feeling better, they can cease treatment (Henry, 2000; Levy, 1998; World Health Organisation, 2002a). The remaining drugs are often kept for self-medication at a later stage to treat ailments against which they have no effect. Also, the drug dose used for imprudent self-medication may be too low to eliminate pathogens and may even promote disease.

A staggering 40-50% of antibiotics produced are fed to food-producing animals to promote growth or as a prophylactic measure (Henry, 2000; World Health Organisation,

2002a). The drugs are administered at subtherapeutic doses, supplying selective pressure that will promote the growth of resistant bacteria. These resistant strains can be passed to humans either through consumption of undercooked meat or environmental contact (Ferber, 2000; Levy, 1998). In addition, antibiotics are sprayed onto fruit trees to prevent bacterial infection. Apart from the consumption of fruit that may carry antibiotic-resistant strains, this also results in the development of a vast pool of resistance genes in the soil (Levy, 1998; Palumbi, 2001).

1.1.3 *Staphylococcus aureus*: A Case Study

In 1946, only three years after penicillin (more precisely, penicillin G) was made commercially available, a strain of the nosocomial pathogen *Staphylococcus aureus* was found to produce an enzyme (β-lactamase) which could cleave the β-lactam ring of the molecule, thereby destroying its antibiotic activity (Neu, 1992; Palumbi, 2001). Methicillin, a chemically modified penicillin resistant to penicillinase, was introduced in 1960. The first report of resistance (caused by altered penicillin-binding proteins) was in the following year (Spratt, 1994; Palumbi, 2001).

Today, most clinical isolates of *S. aureus* are resistant to penicillin G and the occurrence of methicillin-resistant *S. aureus* (MRSA) can approach 50% (Palumbi, 2001). These strains frequently possess resistance to other classes of drugs as well. In the 1980s, new fluoroquinolones such as ciprofloxacin were used successfully against MRSA. Now, however, fluoroquinolone resistance is widespread (Neu, 1992; Spratt, 1994). Another effective compound is mupirocin, which is applied nasally. However, staphylococci present on the skin inevitably find their way to the nose and are spread between people. Given this fact, it was inevitable that a resistance gene would be found on a plasmid (Neu, 1992).

There are now strains that are resistant to all antibiotics except vancomycin, a "drug of last resort" (Levy 1998). In 1996, glycopeptide intermediate *S. aureus* (GISA) strains

were first discovered in three patients (Henry, 2000). GISA strains have reduced susceptibility to vancomycin. Worse was to come. The first case of vancomycin-resistant *S. aureus* (VRSA) was reported in 2002. The strain isolated was able to survive a dose of vancomycin 30 times that which would be toxic to humans (Pearson, 2002). It is thought that vancomycin resistance may be related to the use of the antibiotic avoparcin (since 1974) as a growth supplement in livestock (Farber, 2000). Avoparcin-resistant enterococci are also resistant to vancomycin and may be the source of resistance in *S. aureus.*

The patients infected with VRSA were treated with linezolid (Zyvox®), the first of a new class of antibiotics, the oxazolidinones (Palumbi, 2001; Prescott *et al.*, 2002; Pearson, 2002). However, at the time of treatment some *S. aureus* strains had already shown signs of resistance to this new drug. In fact, resistance to linezolid had been reported even before the Food and Drug Administration of the United States had approved it for medicinal use (Henry, 2000). VRSA have also been treated with Synercid®, a combination of the streptogramin antibiotics quinupristin and dalfopristin (Palumbi, 2001). Yet again, resistance to these drugs has already been found in strains of *S. aureus* (Pearson, 2002)

1.2 The Spread of Resistance Genes

1.2.1 The Origin of Resistance

Resistance genes may originate in one of three ways. Firstly, antibiotic producers must protect themselves from the very compounds they produce. Indeed, resistance genes are well documented in those strains producing the particular drug (Davies, 1994). Secondly, random mutations in key genes may alter the drug target in otherwise susceptible strains, making them resistant (Davies, 1994; Levy, 1998). Finally, enzymes acting on antibiotics, rendering them inactive may evolve from regular housekeeping genes in the cell. Together, these three factors create a pool of resistance in the environment.

1.2.2 Gene Transfer

The severity of the antibiotic resistance problem stems from the ability of bacteria to acquire genes from other bacteria whether they are of the same species or only distantly related (Levy, 1998). Bacteria have several processes available to them to acquire resistance genes, such as conjugation with other bacteria (especially common), transduction by bacteriophages and transformation with naked DNA (Davies, 1994).

Resistance genes will often find their way onto plasmids in the cell. This eases the transmission of resistance through the bacterial population (Levy, 1998). Resistance (R) plasmids often carry resistance genes to several antibiotics, so that selection pressure from one drug can result in acquisition of resistance to several antibiotics. More often than not, resistance genes will be associated with transposable elements, which will promote the replication of resistance genes onto plasmids or integration of plasmids into the chromosome. Specialised transposons, called integrons, have a specific structure that enables them to easily acquire new resistance genes (Davies, 1994; Levy, 1998)

1.3. Mechanisms of Resistance

1.3.1 Reduced Intracellular Drug Concentration

To achieve its desired effect, a drug needs to be present at a sufficiently high level in the bacterial cell, which can prevent this by limiting entry of the drug or pumping the drug out of the cell (Pratt, 1990).

The thick layer of mycolic acids surrounding mycobacteria confers a high degree of resistance to the cell by preventing entry of many drugs. The outer membrane of Gram-negative bacteria has the same effect, hence their resistance to penicillin (Nikaido, 1994).

However, porins may result in the active uptake of some drugs. Tetracycline resistance in strains of *E. coli* can be caused by a defiency in porin IA (Pratt, 1990).

Efflux pumps may confer multi-drug resistance due to their non-specificity. Such pumps may be present in sensitive strains, but are overproduced in resistant strains. Uptake of the drug remains normal. Such pumps are widely distributed amongst both Gram-positive and Gram-negative bacteria (Nikaido, 1994).

1.3.2 Inactivation of the Drug

Penicillins and cephalosporins may be inactivated by β-lactamase enzymes, which cleave the β-lactam rings of the compounds. Gram-positive bacteria tend to produce penicillin-specific enzymes, while those of Gram-negatives fall into one of five groups, depending on their specificity (Neu, 1992; Pratt, 1990).

Aminoglycosides and chloramphenicol can be rendered inactive by the addition of acetyl groups to the antibiotic structure. Aminoglycosides can also be inactivated by phosphorylation and adenylation (Pratt, 1990). This prevents these drugs from binding to the ribosome and inhibiting protein synthesis (Pratt, 1990; Neu, 1992).

1.3.3 Increased Metabolite Concentration

Sulfonamides (and the related drug trimethoprim) act as antimetabolites in that they are structural analogues of folic acid (Pratt, 1990; Neu, 1992). An overproduction of para-aminobenzoic acid in resistant strains may be due to a gene mutation in the metabolic pathway, allowing the sulfonamide to be outcompeted. Alternatively, folic acid can be taken up from the surroundings instead of producing it in the cell. In this way the action of the antimetabolite is effectively bypassed (Pratt, 1990; Neu, 1992).

Table 1. Drugs used to treat TB and to which resistance genes have been found (adapted from Telenti and Iseman, 2000)

Drug	Mechanism of action	Genes involved in resistance
Isoniazid	Inhibition of mycolic acid biosynthesis	*katG, inhA, kasA, ahpC*
Ethionamide	Inhibition of mycolic acid biosynthesis	*inhA*
Rifampicin	Inhibition of transcription	*rpoB*
Pyrazinamide	Inhibition of fatty acid synthesis	*pcnA, fasA*
Ethambutol	Inhibition of arabinogalactan synthesis	*embCAB*
Streptomycin	Inhibition of protein synthesis	*rpsL, rrs*
Amikacin	Inhibition of protein synthesis	*rrs* (16S rRNA)
Kanamycin	Inhibition of protein synthesis	*rrs* (16S rRNA)
Fluoroquinolones	Inhibition of DNA gyrase	*gyrA, gyrB, lfrA*
Clarithromycin	Inhibition of peptidyltransfer function of ribosome	*rrs* (23S rRNA)
Cycloserine	Inhibition of peptidoglycan synthesis	*alrA*

Drugs used to treat TB are listed in Table 1 (Telenti and Iseman, 2000). In each case, resistance derives from a chromosomal mutation event. For example, isoniazid is administered as a prodrug and is activated by a mycobacterial catalase-peroxidase, KatG. Deletion or mutation of this gene will render the organism catalase negative and will confer isoniazid resistance (Petrini and Hoffner, 1999).

Multidrug resistance in *M. tuberculosis* is defined as resistance to at least isoniazid and rifampicin - the most effective anti-TB drugs (Petrini and Hoffner, 1999; Telenti and Iseman, 2000). As discussed in section 1.1.2, resistance is promoted by failure of patients to complete a treatment regimen (Bloom and Murray, 1992). Not only do such patients remain infected with *M. tuberculosis*, they are also applying a selective pressure to the TB bacilli in their lungs to acquire resistance. As a result, treatment is prolonged and more expensive. Whereas SCC lasts six months, treatment of multidrug-resistant TB may last up

to two years and be over 100 times more expensive (World Health Organisation, 2000). In addition, the drugs used are more toxic to patients. Cycloserine, for instance, has serious psychiatric side effects (Petrini and Hoffner, 1999). Cure rates among patients with multi-drug resistant TB may drop to 56%, depending on the number of antibiotics to which the strain is resistant. In severe cases, removal of the diseased lung must be considered (Telenti and Iseman, 2000).

DOTS (Directly Observed Treatment, Short-course) is a TB treatment strategy in which patients take their medication under the supervision of a health worker or trained volunteer (Petrini and Hoffner, 1999; World Health Organisation, 2000). This lowers costs, increases cure rates and helps prevent multi-drug resistance. In addition, the World Health Organisation has initiated the DOTS-Plus, whereby patients from low and middle income countries who are infected with MDR-TB are treated with second-line drugs according to the DOTS strategy (World Health Organisation, 2002b).

1.4.3 Tuberculosis in South Africa

In South Africa, more than 80% of all communicable diseases notified are cases of TB (Weyer *et. al.*, 1995). The Medical Research Council of South Africa estimated in 1997 that more than 180 000 South Africans were suffering from TB, an incidence of 419 cases per 100 000 people (Fourie, 1999). These figures make the epidemic in South Africa one of the worst in the world.

The TB epidemic is particularly serious in the Western Cape province, where the incidence of TB is the highest in the world (Donald, 1998). Amongst the Coloured population, infection rates of 700 cases per 100 000 people are commonplace (Weyer *et. al.*, 1995) and in the Cape Town suburbs of Guguletu and Ravensmead, infection rates of 1000 cases per 100 000 people have been reported (Donald, 1998). Fortunately, prevalence of multi-drug resistance in the Western Cape is relatively low (1.1-4.0%) compared to other developing countries (Weyer *et. al.*, 1995).

The spread of HIV/AIDS has excerbated the TB epidemic in southern Africa (Bloom and Murray, 1992; Kochi, 1991), where 69% of TB cases are attributable to HIV/AIDS (Fourie, 1999). In KwaZulu-Natal, Mpumalanga and Gauteng provinces of South Africa, TB infection rates have risen sharply due to rising HIV infection rates. Cure rates for TB can only be increased if HIV infection rates are significantly lowered – a situation that can only be remedied through a concerted attack against both epidemics.

1.5 Actinomycetes as Antibiotic Producers

Actinomycetes are important components of aerobic soils and sediments, where production of secondary metabolites such as antibiotics provides them with an advantage over competing microorganisms, especially during a period of nutrient deprivation. (Maplestone *et al*, 1992). In addition, antibiotic production is closely associated with aerial mycelium formation, such that species continuation is coupled with killing of competitors.

In 1939, Selman Waksman started a systematic study of the ability of the actinomycetes to produce antibiotics (Prescott *et al.*, 2002). Streptomycin, produced by *Streptomyces griseus*, was discovered in 1944. Antibiotic discovery reached a peak during the early 1970s, when close to 120 antibiotics were discovered each year. By 1992, 6000 antibiotics had been discovered, of which about two thirds originated from actinomycetes (Bull *et al.*, 1992; Takizawa, 1993; Watve *et al*, 2001).

However, in recent years the discovery rate of new antibiotics has dropped off severely. This is due to several factors, chief of which is the reduced emphasis placed on antibiotic screening (Watve *et al.*, 2001). This may be due to the false perception that new antibiotics are not needed. However, the increase in the occurrence of antibiotic resistance means that new drugs are needed now more than ever. Another factor is the rediscovery of the same drugs over and over again (Bull *et al.*, 1992; Takizawa, 1993; Watve *et al*, 2001), a consequence of the screening methods involved.

The genus *Streptomyces* accounts for the majority of new actinomycete isolates (Lacey, 1973; Elander, 1987) and streptomycetes are the producers of most of the antibiotics discovered (Kutzner, 1992; Locci, 1989; Watve *et al.*, 2001). A common screening strategy in recent years has been to select for the less common genera of actinomycetes, the rationale being that they are more likely to produce novel compounds (Lazzarini, *et al.*, 2000). It is also important to select against common species such as *S. albidoflavus* (Bull *et al.*, 1992). Due to the exploitation of terrestrial habitats, screening programs covering marine environments are now receiving increased attention.

1.6 Aims of this Study

TB has reached epidemic proportions in South Africa and new treatments are needed to bolster the fight against this disease. To this end, this study aimed: i) to isolate actinomycete strains from the marine environment; ii) to screen those strains for the presence of antimycobacterial activity; iii) to characterise physiologically those strains that show antibiotic activity; iv) to further characterise those strains with the greatest activity for the purposes of genus and species identification and v) to partially purify the active compounds produced by those strains.

CHAPTER 2:
ISOLATION OF ANTIBIOTIC-PRODUCING ACTINOMYCETES

2.1 Introduction

2.1.1 Actinomycetes as Marine Organisms

The broad metabolic capabilities of actinomycetes and their ability to degrade complex biopolymers such as pectin and chitin make them important chemoheterotrophic components of soil (Kutzner, 1992). In addition, their growth cycle, including the production of spores under unfavourable conditions, make them highly successful competitors in terrestrial environments.

For the above reasons, one would think that actinomycetes would form an equally important component of marine environments. Indeed, the isolation of actinomycetes from marine sediments is well documented, albeit at lower abundance than in terrestrial soils (Bernan, 1997; Bull *et al.*, 2000; Jensen *et al.*, 1991). However, the possibility of spores and/or mycelial fragments being washed in from terrestrial soils means there has long been doubt about the status of 'marine' actinomycetes (Goodfellow and Williams, 1983; Moran *et al.*, 1995).

One could postulate that 'marine' strains should show higher salt tolerance than strictly terrestrial strains. However, actinomycetes in general (including terrestrial forms) often show high salt tolerance and 'marine' strains often grow equally well on conventional media as on media with a higher salt content (Jensen *et al.*, 1991; Jensen and Fenical, 1994). Although salt tolerance in marine strains may be higher than for terrestrial strains, this can be explained by selection for salt tolerance and by adaptation to the higher salt content, which has been shown in *in vitro* studies. Alternatively, one would expect that the ability to degrade marine biopolymers such as alginate and laminarin would be restricted to

marine strains (Kutzner, 1992). As these are not standard inclusions in carbon utilisation tests, data for terrestrial strains is lacking.

The subject of 'marine' strains becomes clearer when considering the distribution of the various actinomycete groups. The distribution of the streptomycetes suggests that they are mainly of terrestrial origin, but nevertheless grow well under marine conditions (Kutzner, 1992). Streptomycetes occur only in shallow sea (near shore) sediments, with numbers dropping as distance from land increases, implying that they are facultatively marine (Jensen *et al.*, 1991). However, the results from one study suggest that the near-shore streptomycete population did not originate from the wash-in of terrestrial streptomycete spores (Moran *et al.*, 1995).

The two actinomycete groups most likely to contain *bona fide* marine strains are the actinoplanetes and nocardioforms. The actinoplanetes, especially members of the genus *Micromonospora*, have been found to increase in number as distance from shore and water depth increased (Jensen *et al.*, 1991). Isolates from deep-sea sediments appear to have an obligate requirement of seawater, as opposed to merely a tolerance of it. Deep-sea sediments from the Pacific Ocean have yielded large numbers of nocardioforms, members of which were among the first to be regarded as indigenous in the marine environment (Bull *et al.*, 2000).

2.1.2 Why the Marine Environment?

As researchers cast their net wider in search of new natural products, it is inevitable that the sea should receive increased attention (Bernan *et al.*, 1997; Takizawa *et al.*, 1993). Terrestrial sources of new compounds are generally more accessible than marine sources, leading to their being more widely studied and therefore more heavily exploited. As the technology to study the sea became more sophisticated, it became easier to study marine environments and exploit its natural products (Jensen and Fenical, 1994).

The potential of the sea as a natural resource should be apparent when considering that its area is twice that of the land on Earth and it also continues to a great depth (Bull *et al.*, 2000). It contains a diverse range of environments subject to extremes of temperature, pressure and nutrient availability (Bernan *et al.*, 1997; Bull *et al.*, 2000; Jensen and Fenical, 1994). Also, halogenation of compounds is more commonly found amongst marine organisms. Any compound produced under specifically marine conditions is not likely to be made by a terrestrial organism in its natural habitat. In addition, the metabolism and physiology of marine organisms must be adapted to suit its environment. This interplay of marine-adapted metabolism and marine conditions creates a great potential for unique products.

Actinomycetes have historically been regarded as primarily terrestrial soil organisms. However, screening of terrestrial strains often results in known compounds being "rediscovered" (Takizawa *et al.*, 1993). With the increased interest in marine research, it is natural that the actinomycetes, as the leading microbial source of bioactive compounds (especially antibiotics), should receive renewed attention.

2.1.3 This Study

The surfaces of marine algae are environments where space and access to nutrients are limited. Therefore, epiphytic bacteria living in such environments would be expected to possess metabolic capabilities, such as production of secondary metabolites that would enable them to compete successfully. Given their metabolic flexibility, actinomycetes in these environments must be considered as potential sources of novel antibiotics (Mearns-Spragg *et al.*, 1998; Zheng *et al.*, 2000).

In this study, actinomycetes were isolated from 12 seaweed species found in South African coastal waters and from two sediment samples taken from an adjacent location. Actinomycete strains were screened for antibacterial activity against three strains: *Mycobacterium aurum* A+ (acid-fast), *Escherichia coli* ATCC 25922 (Gram-negative)

and *Enterococcus faecium* VanA (Gram-positive). *M. aurum*, a fast-growing and non-pathogenic relative of *M. tuberculosis* has a similar antibiotic-resistance profile and was used as a suitable substitute. *E. faecium* VanA is a vancomycin-resistant clinical isolate. *E. coli* ATCC25922 is a standard antibiotic-susceptibility test strain.

2.2 Materials and Methods

2.2.1 Isolation of Actinomycete Strains

Seaweed samples were collected from Kommetjie and St. James (both Cape Town, South Africa) over the period April 2000 to March 2001. Species collected were *Gracilaria gracilis*, *Ecklonia maxima*, *Porphyra capensis*, *Ulva capensis*, *Cladophora capensis*, *Aodes orbitosa*, *Neuroglossum binderianum*, *Hymenena venosa*, *Grateloupia filicina*, *Gigartina radula*, *Codium* sp. and *Bifurcaria brassicaeformis*. Sea sand samples were collected from Sunny Cove (Cape Town, South Africa) on 17 June 2001.

Seaweed and sand samples (1g) were added to 5ml of sterile 2% NaCl solution and vigorously vortexed for 1 minute. Serial 10-fold dilutions (sterile 2% NaCl solution as diluent) were carried out to 10^{-4} and 100µl aliquots were plated on starch-casein (SC) (Vobis, 1989), seawater salt (SWS) (Pathirana *et al.*, 1991) and yeast extract-malt extract (YEME) (Shirling and Gottlieb, 1966) agars (Appendix A). SC and YEME agars were modified by the incorporation of 2% NaCl. Plates were incubated at 28°C for 14 days. Discreet colonies looking dissimilar to others on the same plate were picked and subcultured onto ISP Medium 4, on which the aerial and substrate mycelium colours were determined.

2.2.2 Determination of Antimicrobial Activity

Each actinomycete strain was stabbed into plates of each of the three isolation agars and incubated for 10 days at 28°C. Plates were then overlaid with 6 ml 0.7% Luria agar (Sambrook *et al.*, 1989) inoculated with one of the test bacteria and allowed to set. Plates were incubated at 37°C for 14-18 h (*E. coli* ATCC 25922 and *E. faecalis* VanA) or 40-45 h (*M. aurum* A+). The area of the zones of inhibition of bacterial growth were calculated from the diameter of the zones less the area of the actinomycete colony.

The test bacteria were grown as overnight cultures in 2YT broth (Ausabel *et al.*, 1989) at 37°C with agitation. Before inoculation into the agar overlays, the cultures were tested by Gram stain to check for contamination. In order to ensure similar bacterial cell counts, the volume of culture to be added to each sloppy agar overlay was determined such that the product of the volume in microlitres and the optical density of the culture at 600nm equaled 4 (*E. coli*) or 160 (*E. faecium* and *M. aurum*).

2.3 Results and Discussion

2.3.1 Actinomycete Strains Isolated

The actinomycete strains isolated are listed in Tables 2 and 3.

The number of strains isolated from each of the isolation media was not significantly different from the others, thus it cannot be said that any of the media was more favourable for the isolation of actinomycetes than the others.

With the exception of $R_2\mu$, the appearance of all strains was consistent with that of a streptomycete phenotype. Also, most strains displayed a yellow-brown substrate mycelium and grey aerial mycelium. While most streptomycetes have these characteristics (Locci, 1989), it is disappointing that a greater variety of actinomycetes were not isolated. While

the surfaces of intertidal seaweeds may be worthwhile sources of novel actinomycetes (Bernan *et al.*, 1997), further characterisation of these isolates will be necessary to demonstrate this.

Actinoplanetes and nocardioforms are often isolated in large numbers from marine environments (Bernan *et al.*, 1997; Jensen *et al.*, 1991). While the possibility that some of the isolates belong to one of these or other less common actinomycete groups cannot be ruled out without further characterisation, preliminary characterisation suggested that they are streptomycetes.

Due to its orange substrate mycelium, lack of aerial mycelium and production of a black mucous covering of spores, strain $R_2\mu$ was recognised as potentially being a member of the genus *Micromonospora* (Vobis, 1989). Although it was later shown not to exhibit any antimicrobial activity against the chosen test bacteria, as a non-streptomycete, it was chosen for further study.

In future studies, isolation procedures should be elaborated so as to select specifically for less common genera of actinomycetes, commented on in section 1.5. This is easily achieved through inclusion of inhibitory substances to which the genus is known to be resistant (eg. inclusion of potassium tellurite for isolation of *Nocardia* strains (Lechevalier, 1989)) or use of carbon sources used only by the genus being selected for (eg. inclusion of humic acid for isolation of streptosporangia and microbisporae (Goodfellow, 1989)). Pretreatment with inhibitory substances or raised temperature has also been utilised to good effect (Bull, *et al.*, 1992).

Table 2. Actinomycete strains isolated from seaweed samples

Strain name	Isolation Medium	Isolation Source	Spore Mass Colour[1]	Substrate Mycelium Colour[1]
HCBα	YEME	*Gracilaria*	Grey	Yellow-Brown
E_2a	SC	*Ecklonia*	White	Yellow-Brown
E_3b	SWS	*Ecklonia*	Grey	Yellow-Brown
G_2c	YEME	*Gracilaria*	Grey	Yellow-Brown
G_3d	YEME	*Gracilaria*	Grey	Yellow-Brown
E_1e	SC	*Ecklonia*	White	Yellow-Brown
P_2f	YEME	*Porphyra*	Green	Yellow-Brown
P_1g	SC	*Porphyra*	Grey	Yellow-Brown
P_1h	SC	*Porphyra*	White	Yellow-Brown
G_3i	SC	*Gracilaria*	Green	Yellow-Brown
E_4j	SC	*Ecklonia*	Green	Yellow-Brown
P_2k	YEME	*Porphyra*	Grey	Yellow-Brown
C_1l	SC	*Cladophora*	Green	Yellow-Brown
P_1m	YEME	*Porphyra*	Grey	Yellow-Brown
P_2n	SC	*Porphyra*	Grey	Yellow-Brown
U_1o	YEME	*Ulva*	Grey	Yellow-Brown
P_3p	SC	*Porphyra*	Green	Yellow-Brown
N_1q	SWS	*Neuroglossum*	Green	Yellow-Brown
A_1r	SC	*Aodes*	Grey	Yellow-Brown
U_2s	SWS	*Ulva*	Green	Yellow-Brown
U_2t	SWS	*Ulva*	Green	Yellow-Brown
E_3u	SWS	*Ecklonia*	Green	Yellow-Brown
R_1v	YEME	*Grateloupia*	Green	Yellow-Brown
U_2w	SWS	*Ulva*	Grey	Yellow-Brown

[1] Colour assignment based on Locci (1989) as applied to *Streptomyces*

Table 2. (cont.)

Strain name	Isolation Medium	Isolation Source	Spore Mass Colour[1]	Substrate Mycelium Colour[1]
G_3x	SC	*Gracilaria*	Grey	Yellow-Brown
U_2y	SWS	*Ulva*	White	Yellow-Brown
U_2z	YEME	*Ulva*	Green	Yellow-Brown
$R_1\beta$	SWS	*Grateloupia*	Green	Yellow-Brown
$C_2\gamma$	SWS	*Cladophora*	Green	Yellow-Brown
$A_1\delta$	YEME	*Aodes*	Green	Yellow-Brown
$H_1\varepsilon$	YEME	*Hymenena*	Grey	Yellow-Brown
$R_1\zeta$	SWS	*Grateloupia*	Green	Yellow-Brown
$H_2\eta$	SWS	*Hymenena*	Grey	Yellow-Brown
$R_2\theta$	SWS	*Grateloupia*	White	Yellow-Brown
$O_1\iota$	SWS	*Codium*	White	Yellow-Brown
$I_1\kappa$	SC	*Gigartina*	Green	Yellow-Brown
$I_1\lambda$	YEME	*Gigartina*	Green	Yellow-Brown
$R_2\mu$	YEME	*Grateloupia*	NA[2]	Orange
$B_1\nu$	SWS	*Bifurcaria*	Grey	Yellow-Brown
$B_1\xi$	YEME	*Bifurcaria*	White	Yellow-Brown
O_1o	YEME	*Codium*	White	Yellow-Brown
$O_2\pi$	SC	*Codium*	Grey	Yellow-Brown
$U_3\rho$	SC	*Ulva*	Grey	Yellow-Brown
$U_3\sigma$	SWS	*Ulva*	White	Yellow-Brown
$N_3\tau$	SC	*Neuroglossum*	Green	Yellow-Brown
$I_2\upsilon$	SC	*Gigartina*	Grey	Yellow-Brown
$I_2\varphi$	SWS	*Gigartina*	Grey	Yellow-Brown

[1] Colour assignment based on Locci (1989) as applied to *Streptomyces*

[2] NA, Not applicable: no aerial mycelium

Table 3. Actinomycetes isolated from sea sand

Strain Name	Isolation Medium	Spore Mass Colour[1]	Substrate Mycelium Colour[1]
SS1	SC	Grey	Yellow-Brown
SS2	SC	Grey	Yellow-Brown
SS3	SC	White	Yellow-Brown
SS4	YEME	White	Yellow-Brown
SS5	SWS	Grey	Yellow-Brown
SS6	YEME	Grey	Yellow-Brown
SS7	SWS	White	Yellow-Brown
SS8	SC	White	Yellow-Brown
SS9	YEME	Grey	Yellow-Brown
SS10	YEME	White	Yellow-Brown
SS11	YEME	Grey	Yellow-Brown
SS12	SWS	Green	Yellow-Brown
SS13	SC	Grey	Yellow-Brown
SS14	SC	Green	Yellow-Brown
SS15	SWS	White	Yellow-Brown
SS16	YEME	Grey	Yellow-Brown
SS17	SWS	White	Yellow-Brown
SS18	SC	White	Yellow-Brown
SS19	SC	Red	Yellow-Brown
SS20	SWS	Grey	Yellow-Brown

[1] Colour assignment based on Locci (1989) as applied to *Streptomyces*

2.3.2 Antibacterial Activity

The antibacterial activity shown by isolates against the test organisms are listed in tables 4 and 5. No antibacterial activity against *E. coli* was shown by any of the isolates.

Table 4. Strength of antibacterial activity shown by isolates against *Mycobacterium aurum* A+ on three different media.

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
HCBα	0	452	836
E_2a	626	1084	1084
E_3b	0	0	0
G_2c	0	0	0
G_3d	0	657	1206
E_1e	0	869	912
P_2f	1540	1583	1633
P_1g	204	552	0
P_1h	0	0	0
G_3i	741	979	1084
E_4j	657	1206	1807
P_2k	792	628	1084
C_1l	327	212	420
P_1m	5007	4398	5007
P_2n	0	0	0
U_1o	0	0	0
P_3p	0	776	1402
N_1q	0	1190	1270
A_1r	0	0	0
U_2s	0	0	0
U_2t	1583	1084	968

Table 4. (cont.)

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
E_3u	1070	1206	1540
R_1v	0	0	0
U_2w	0	0	0
G_3x	0	0	0
U_2y	0	0	0
U_2z	691	452	1084
$R_1\beta$	0	0	0
$C_2\gamma$	0	0	0
$A_1\delta$	1402	1583	968
$H_1\varepsilon$	0	0	0
$R_1\zeta$	0	0	0
$H_2\eta$	0	0	0
$R_2\theta$	0	0	0
$O_1\iota$	898	1540	1685
$I_1\kappa$	0	0	0
$I_1\lambda$	0	0	0
$R_2\mu$	0	0	0
$B_1\nu$	0	0	0
$B_1\xi$	754	1335	1347
O_1o	2124	707	2592
$O_2\pi$	0	0	0
$U_3\rho$	0	0	0
$U_3\sigma$	0	0	0

Table 4. (cont.)

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
$N_3\tau$	531	0	707
$I_2\upsilon$	520	0	720
$I_2\varphi$	804	0	520
SS1	0	0	490
SS2	0	0	0
SS3	0	0	0
SS4	0	0	0
SS5	0	0	0
SS6	0	0	0
SS7	0	0	0
SS8	0	0	0
SS9	0	0	0
SS10	0	0	0
SS11	0	0	0
SS12	720	3342	640
SS13	1490	0	0
SS14	0	0	0
SS15	0	0	0
SS16	0	0	0
SS17	0	0	0
SS18	0	0	0
SS19	0	0	0
SS20	0	0	0

Table 5. Strength of antibacterial activity shown by isolates against *Enterococcus faecium* VanA on three different media

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
HCBα	1206	226	852
E_2a	840	1084	660
E_3b	0	0	0
G_2c	0	0	0
G_3d	0	0	0
E_1e	467	823	235
P_2f	1034	1486	954
P_1g	452	247	1168
P_1h	0	0	0
G_3i	346	645	564
E_4j	423	534	643
P_2k	456	868	1362
C_1l	0	0	0
P_1m	2954	2388	3245
P_2n	0	0	0
U_1o	0	0	0
P_3p	1204	1642	1550
N_1q	726	430	620
A_1r	0	0	0
U_2s	0	0	0
U_2t	459	562	728
E_3u	230	638	480
R_1v	0	0	0
U_2w	0	0	0
G_3x	846	552	930

Table 5. (cont.)

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
U_2y	0	0	0
U_2z	552	872	930
$R_1\beta$	0	0	0
$C_2\gamma$	0	0	0
$A_1\delta$	1004	993	1208
$H_1\varepsilon$	0	0	0
$R_1\zeta$	0	0	0
$H_2\eta$	0	0	0
$R_2\theta$	0	0	0
$O_1\iota$	442	672	349
$I_1\kappa$	0	0	0
$I_1\lambda$	0	0	0
$R_2\mu$	0	0	0
$B_1\nu$	0	0	0
$B_1\xi$	0	0	0
O_1o	2531	1983	2738
$O_2\pi$	0	0	0
$U_3\rho$	0	0	0
$U_3\sigma$	0	0	0
$N_3\tau$	0	0	0
$I_2\upsilon$	0	0	0
$I_2\varphi$	0	0	0

Table 5. (cont.)

Strain Name	Inhibition Zone Area (mm^2)		
	YEME	SWS	SC
SS1	1080	770	1238
SS2	0	0	0
SS3	0	0	0
SS4	0	0	0
SS5	0	0	0
SS6	0	0	0
SS7	0	0	0
SS8	0	0	0
SS9	0	0	0
SS10	0	0	0
SS11	0	0	0
SS12	1972	2250	2390
SS13	340	774	927
SS14	0	0	0
SS15	0	0	0
SS16	0	0	0
SS17	0	0	0
SS18	0	0	0
SS19	0	0	0
SS20	0	0	0

All strains showing antibacterial activity against either *M. aurum* or *E. faecium* were selected for physiological characterisation. Three strains, P_1m, O_1o, and SS12, showed strong to very strong antibacterial activity (2000-3000 mm^2 inhibition zones) against *M. aurum* A+ and *E. faecium* (Table 6) and were thus chosen for further characterisation due to ease of isolation of their active compounds. It was disappointing that so few strains could be singled out in this way. Also, none of the strains isolated showed any antibacterial activity against *Escherichia coli* ATCC 25922, a Gram-negative bacterium. It was expected that strains showing activity against *E. coli* would be lower in number due to the inability of many antibiotics to penetrate the Gram-negative outer membrane (Pratt, 1990).

Table 6. Summary of antibacterial activity shown by P_1m, O_1o and SS12 against the three test bacteria.

Test Organism	**Growth Medium**	**Inhibition Zone Area (mm^2)**		
		P_1m	**O_1o**	**SS12**
E.coli	YEME	0	0	0
	SWS	0	0	0
	SC	0	0	0
E. faecium	YEME	2954	2531	1972
	SWS	2388	1983	2250
	SC	3245	2738	2390
M. aurum	YEME	5007	2124	720
	SWS	4398	707	3342
	SC	5007	2592	640

Only three of the 20 strains isolated from sea sand showed any antibacterial activity, which is surprising, as sediment is a well-known environment for actinomycetes (Bernan *et al.*, 1997). Also, five strains did not show any antibacterial activity against *M. aurum* on YEME, despite showing relatively strong activity on SWS and SC. For many strains, SC

was the medium on which greatest activity was shown, particularly against *M. aurum*. These differences in antibacterial activity for a given strain on different media suggest varying nutritional requirements for antibiotic production. For a given strain on a given medium, activity against *M. aurum* was generally greater than that demonstrated against *E. faecium*.

Six strains exhibited antibacterial activity against *M. aurum* only (Table7). As the focus of this study was on novel anti-TB drugs, it would have been interesting to continue investigation of these strains, but the level of activity shown was low in all cases. This would have hampered efforts to purify the active compounds.

Table 7. Actinomycete strains exhibiting antibacterial activity against *M. aurum* only.

Actinomycete strain	**Inhibition Zone Area (mm^2)**		
	YEME	**SWS**	**SC**
G_3d	0	657	1206
C_1l	327	212	420
$B_1\xi$	754	1335	1347
$N_3\tau$	531	0	707
$I_2\upsilon$	520	0	720
$I_2\varphi$	804	0	520

CHAPTER 3:
CHARACTERISATION AND IDENTIFICATION OF ACTINOMYCETE ISOLATES

3.1 Introduction: Actinomycete Taxonomy

3.1.1 Morphology

Although actinomycetes are traditionally defined as aerobic filamentous Gram-positive organisms (Lechevalier, 1989a), in many cases "traditional actinomycetes" are closely related to forms with simpler morphologies and lacking the complex actinomycete life-cycle (Goodfellow, 1989a; Embley and Stackebrandt, 1994). Thus "actinomycete" takes either its original meaning, or refers to the more inclusive group, the Gram-positive bacteria with a high guanine + cytosine DNA base composition. This confusion is circumvented by referring to the former by the previously tautological term "filamentous actinomycetes."

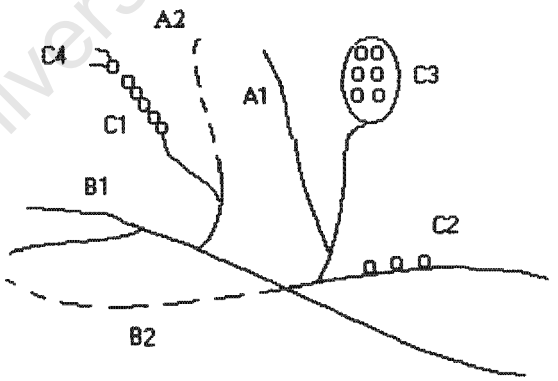


Fig.1 A schematic diagram showing morphological features of actinomycetes. The aerial mycelium (A) and substrate mycelium (B) may be stable (1) or fugacious (2). Spores may be found as conidia singly, in pairs or in chains on the aerial mycelium (C1) or the substrate mycelium (C2). Spores may also be borne in a sporangium (C3) and can be motile (C4) or immotile.

A schematic diagram of actinomycete morphological features is shown in Figure 1. The mycelial structure provides the most obvious characteristics. Often, only the substrate mycelium may be present and rarely, only the aerial mycelium. Those actinomycetes informally referred to as nocardioforms generally form a fugacious (unstable) mycelium (Lechevalier, 1989a).

Spores may be borne in various ways. Most common are various conformations of chains of conidia. Single conidia or pairs also occur and are characteristic of particular species. Conidia-bearing hyphae can form unique structures such as synnemata formed by *Actinosynnema* (Lechevalier, 1989a; McCarthy, 1989) or pseudosporangia formed by some species of *Actinomadura* (Goodfellow, 1989a). Sporangia can be sessile or occur on aerial hyphae. Many genera bear motile spores. These can originate from sporangia, conidia-bearing hyphae or fragmenting hyphae. Electron microscopy is needed to evaluate features such as ornamentation of spore surfaces (Lechevalier, 1989a).

3.1.2 Physiological Tests

Morphology aside, physiological characters provided taxonomists with the first useful method of classifying and identifying actinomycetes, particularly the genus *Streptomyces*, which accounts for a large proportion of the actinomycetes discovered thus far (Locci, 1989; Kutzner, 1992). Numerical taxonomic methods allowed *Streptomyces* strains to be grouped into broad clusters based on similarity of phenotypes. Clearly this method becomes more useful as the number of characteristics compared is increased (Langham *et al.*, 1989).

Although molecular and chemical methods are available to quickly identify any given isolate, probability matrices of physiological traits are invaluable in the identification of streptomycete strains, given the size of the genus and the variability shown (Bull *et al.*, 1992).

3.1.3 Chemical Taxonomy

When chemical methods became available, it was realised that classifications based on morphology alone were highly artificial (Embley and Stackebrandt, 1994; Goodfellow, 1989a). Most of the taxonomic clusters first defined by chemical methods, although in some cases amended with the advent of molecular methods, are still valid today (Goodfellow, 1989a). The suprageneric groups put forward in *Bergey's Manual of Systematic Bacteriology* (1989) were based on morpological, physiological and chemical characters (Figure 2).

Many properties of cell envelopes can be determined by chemical methods. Thin-layer chromatography (TLC) is used to determine which isomer of diaminopimelic acid (if present) is found in the cell wall peptidoglycan (Becker *et al.*, 1965). TLC is also used to determine the diagnostic sugar in the whole-cell hydrolysate of an isolate, as well as phospholipid type and presence of mycolic acids in the cell envelope (Lechevalier and Lechevalier, 1980). Among the other methods used in chemical taxonomy are the determination of the fatty acid profile of an isolate by gas-liquid chromatography of fatty acid methyl esters (Brian and Gardner, 1967) and the determination of the predominant menaquinone by high performance liquid chromatography (Kroppenstedt, 1985).

3.1.4 16S rDNA Sequencing

Analysis of the sequence of the 16S rRNA gene(s) has become the standard method of determining phylogenetic relationships and is the basis for modern classification systems (Stackebrandt *et al.*, 1997). As Embley and Stackebrandt (1994) remarked, actinomycete systematics can be approached in one of three ways: i) to view molecular methods as just another way to assess actinomycete relationships; ii) to view molecular methods as superior to the traditional methods or iii) to follow a polyphasic approach in which phenotypic and genotypic characters are reconciled in order to produce a classification.

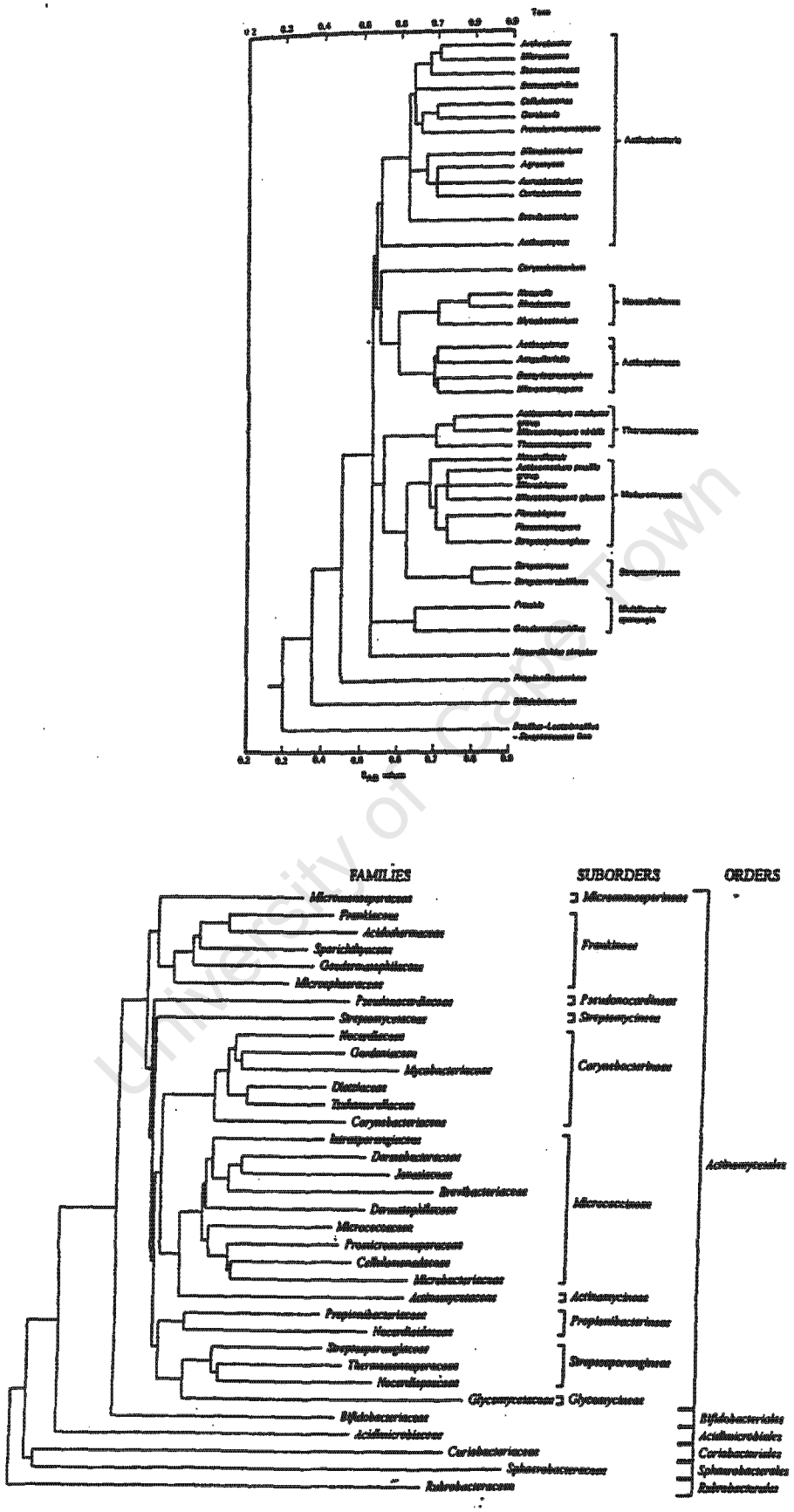


Fig 2. Comparison of two molecular phylogenies of the actinomycetes. See text.

Two 16S rDNA phylogenies are shown in Figure 2. The first is from *Bergey's Manual of Systematic Bacteriology* (1989). The tree is based on partial sequencing of 16S rRNA genes and analysis is by association coefficients. Suprageneric groups are indicated. However, the non-molecular classification system used to delineate these groups led to some genera being placed in a group inconsistent with their placement on the phylogenetic tree. For instance, *Oerskovia* and *Promicromonospora* were grouped with the nocardioforms, even though they were placed among the actinobacteria on the phylogenetic tree (Goodfellow, 1989a).

A new phylogeny of the actinomycetes was published in 1997 (Stackebrandt *et al.*, 1997). This greatly clarified the placement of genera such as *Promicromonospora*. Also, *Sporichthya* and *Intrasporangium*, grouped with *Streptomyces* on chemical evidence, were moved to other suborders, away from *Streptomyces*. The next edition of *Bergey's Manual of Systematic Bacteriology* will classify organisms according to phylogenetic principles, reducing taxonomic confusion (Prescott *et al.*, 2002).

3.1.5 Polyphasic Taxonomy

Polyphasic taxonomy is the use of all available phenotypic and genotypic data to construct a consensus classification (Vandamme *et al.*, 1996). This is a pragmatic approach that enables data to be treated independently of competing hypotheses. A major advantage is the increased stability of the classification as more data is integrated. This is ideal for the study of actinomycetes, where analysis of different characters can give rise to very different classifications (Anderson and Wellington, 2001; Embley and Stackebrandt, 1994).

3.2 Materials and Methods

3.2.1 Physiological Characterisation

Antibiotic resistance was determined by incorporation of the antibiotics into Bennett's Medium agar plates at the concentrations recommended and not by using antibiotic-impregnated filter discs. Degradation of urea and hydrolysis of esculin and arbutin were carried out according to Kutzner (1992). All other physiological tests were carried out according to Locci (1989). All tests (for *Streptomyces* spp.) were carried out at 28°C (unless otherwise indicated) and were read after the recommended incubation periods.

3.2.2 Chemical Taxonomy

Chemical taxonomy was carried out as described by Hasegawa *et al.* (1983) with the exception that dried cells were used instead of colonies from agar plates. Dried cells were prepared from filtered and washed ISP#1 cultures.

For determination of the whole cell sugar pattern (WCSP), 150mg of dried cells were used and 10mg were used for the determination of the DAP isomer. The samples were allowed to stand overnight to rehydrate and were then autoclaved (15 min at 15 psi). The hydrolysed samples were loaded directly onto Polygram® CEL 400 UV_{254} microcrystalline cellulose TLC plates (Macherey-Nagel). The chromatography was carried out using the solvent systems described by Hasegawa *et al.* (1983): for DAP isomer determination, methanol-dH_2O-6N HCL-pyridine (80:26:4:10, v/v) and for WCSP determination, *n*-butanol-dH_2O-pyridine-toluene (10:6:6:1, v/v).

3.2.3 Genomic DNA Extraction

Genomic DNA for use as a template in the polymerase chain reaction (PCR) was extracted as described in Appendix B.

3.2.4 Polymerase Chain Reaction

The 16S rRNA gene of strains P_1m, O_1o, $R_2\mu$ and SS12 was amplified using the universal bacterial 16S rDNA primers (forward primer adapted from primer fD1 of Weisburg *et al.*, 1991; reverse primer adapted from primer rP2 of Weisburg *et al.*, 1991). Primer sequences, reaction set-up and PCR program are given in Appendix C. PCR products were visualised on 0.7% agarose gels and purified using the High Pure™ PCR Product Purification Kit (Roche).

3.2.5 DNA Sequencing

The 16S rDNA was sequenced using the MegaBACE 500 Automated Capillary DNA Sequencing System (Molecular Dynamics) and a DYEnamic Dye Terminator Cycle Sequencing Kit for MegaBACE. The nucleotide sequences were determined by the fluorescent dideoxy chain-termination protocol of Sanger *et al.* (1977), using the universal bacterial 16S-rDNA primers, as per the manufacturer's instructions.

Sequence results were analysed using the DNAman sequence analysis software package (Lynnon BioSoft v. 4.13). Homology searches were carried out using the BLASTn algorithm (Altschul *et al.*, 1990) provided by the Internet service of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/).

3.2.6 Electron Microscopy

Specimens were prepared for scanning electron microscopy as described in Appendix D.

3.3 Results and Discussion

3.3.1 Physiological Characteristics of Antibiotic-Producing Strains

The physiological characteristics of the antibiotic-producing strains isolated are listed in Tables 8-15. In addition, strain HCBα produced a red-orange diffusible pigment that turned blue when covered with 0.05N NaOH. A red pigment produced by E_1e on Bennett's medium containing gelatin was not pH sensitive.

Only 3 strains were found to produce melanin. Also, P_1m did not degrade starch and E_1e did not show proteolytic activity on Egg Yolk medium. C_1l was the only strain found to degrade guanine.

Resistance to Penicillin G (10 I.U. /ml) was common, with only $N_3\tau$ and SS1 being sensitive. Large numbers of strains were found to grow in the presence of 10 and 13 % sodium chloride. Although the salinity of seawater is only 3%, this may indicate a degree of marine adaptation. Strains of many *Streptomyces* sp. are able to grow at 7% NaCl (Locci, 1989). In this study, 7% NaCl formed a "baseline", with only one strain tested (SS13) not being able to grow at that salinity. All strains could grow in the presence of 0.1% 2-phenylethanol and all but 2 strains ($O_1\iota$ and $B_1\xi$) grew at pH 4.3. C_1l, $N_3\tau$ and $I_2\upsilon$ were the only strains that could not grow in the presence of 0.001% potassium tellurite.

L(+)-Arabinose, D(-)-ribose and sodium D-lactate could be utilised as sole carbon sources by all strains tested and only SS12 could not utilise maltose. Eight carbon sources (*meso*-erythritol, D(+)-melezitose, α-methyl-D-glucoside, sodium benzoate, sodium formate, sodium oxalate, sodium salicylate and L(-)-sorbose) could not be utilised by any of the strains tested. Sorbitol could only be used by HCBα and P_1m. N_1q was the only strain to use inulin and tartrate. DL-α-amino-n-butyric acid and L-hydroxyproline could not be utilised as sole nitrogen sources by any of the strains tested. L-cysteine could only be used by $B_1\xi$ and O_1o.

Tentative species assignments on the basis of physiological and morphological characteristics are possible for some strains.

On the basis of non-production of hydrogen sulphide, reduction of nitrate and colour of aerial and substrate mycelia, P_1m may belong to *S. rimosus*, although the antibiotic profile of P_1m suggests otherwise. Another possibility is *S. lydicus*, although, unlike P_1m, this species does not reduce nitrate.

C_1l, $N_3\tau$ and $I_2\upsilon$ display a green aerial mycelium and are sensitive to 0.001% potassium tellurite. This may indicate a relationship to *S. flaveolus*.

Table 8. Melanin production in antibiotic-producing actinomycete strains

Medium	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$I_2\upsilon$	$I_2\varphi$	SS1	SS12	SS13
ISP#6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
ISP#7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-

Table 9. Enzyme activity present in antibiotic-producing actinomycete strains

Enzyme Activity	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$L_2\upsilon$	$L_2\varphi$	SS1	SS12	SS13
Lecithinase	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-
Lipolysis	-	+	+	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+
Proteolysis	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitinolysis	+	-	-	-	+	+	-	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-	+	-	+	-
Pectin hydrolysis	-	-	-	+	-	-	-	+	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	+
Nitrate reduction	+	-	+	-	-	+	-	+	-	-	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-
H_2S production	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+

Table 10. Degradation activity present in antibiotic-producing actinomycete strains

Degradation Activity	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$I_2\upsilon$	$I_2\varphi$	SS1	SS12	SS13
Adenine	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-
Arbutin	+	+	+	-	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-
Casein	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
DNA	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+
Esculin	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+
Gelatin	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Guanine	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypoxanthine	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
L-tyrosine	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	+	-
Urea	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	+	-	-	-
Xylan	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

Table 11. Antibiotic resistance in antibiotic-producing actinomycete strains

Antibiotic	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$I_2\upsilon$	$I_2\varphi$	SS1	SS12	SS13
Neomycin	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-
Oleandomycin	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Penicillin G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Rifampicin	-	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-	+	-	-

Table 12. Resistance of antibiotic-producing actinomycete strains to inhibitory compounds or conditions

Inhibitory Condition/ Compound (%w/v)	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$I_2\upsilon$	$I_2\varphi$	SS1	SS12	SS13
Phenol (0.1)	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
2-Phenylethanol (0.1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(0.3)	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-
Potassium tellurite (0.001)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
(0.01)	-	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	-
Sodium azide (0.01)	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
(0.02)	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+
Sodium chloride (4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(7)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
(10)	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-
(13)	-	+	-	-	+	-	+	+	+	+	-	+	+	+	+	-	+	-	-	+	+	-	-	+	-	-
Growth at pH 4.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+

Table 13. Ability of antibiotic-producing actinomycete strains to utilise specific compounds as sole carbon sources

Carbon Source	HCBα	$E_{2}a$	$G_{3}d$	$E_{1}e$	$P_{2}f$	$P_{1}g$	$G_{3}i$	$E_{4}j$	$P_{2}k$	$C_{1}l$	$P_{1}m$	$P_{3}p$	$N_{1}q$	$U_{2}t$	$E_{3}u$	$U_{2}z$	$A_{1}\delta$	$O_{1}\iota$	$B_{1}\xi$	$O_{1}o$	$N_{3}\tau$	$I_{2}\upsilon$	$I_{2}\varphi$	SS1	SS12	SS13
L(+)-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)-Cellobiose	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	-	+	-	+	-
meso-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D(-)-Fructose	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)-Galactose	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-	+	-
Glycerol	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+
meso-Inositol	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lactose	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	+	+	-	+	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-Mannitol	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
D(+)-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-
D(+)-Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 13. (cont.)

Carbon Source	HCBα	E_2a	G_3d	E_1e	P_2f	P_1g	G_3i	E_4j	P_2k	C_1l	P_1m	P_3p	N_1q	U_2t	E_3u	U_2z	$A_1\delta$	$O_1\iota$	$B_1\xi$	O_1o	$N_3\tau$	$I_2\upsilon$	$I_2\varphi$	SS1	SS12	SS13
Raffinose	+	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+
L(+)-Rhamnose	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
D(+)-Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+
D-Sorbitol	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L(-)-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	+	-	+	+	-	-	-	+
Trehalose	+	-	-	+	-	+	-	-	-	-	+	+	-	+	+	+	-	+	-	+	-	+	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
D(+)-Xylose	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	+		+	+	-	-	-	+	-	-	-
α-Methyl-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 14. Ability of antibiotic-producing actinomycete strains to utilise sodium salts of organic acids as sole carbon sources

Carbon Source	HCBα	E_{2a}	G_{3d}	E_{1e}	P_{2f}	P_{1g}	G_{3i}	E_{4j}	P_{2k}	C_{1l}	P_{1m}	P_{3p}	N_{1q}	U_{2t}	E_{3u}	U_{2z}	$A_{1\delta}$	$O_{1\iota}$	$B_{1\xi}$	O_{1o}	$N_{3\tau}$	$I_{2\upsilon}$	$I_{2\varphi}$	SS1	SS12	SS13
Sodium acetate	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	+	+	-
Sodium benzoate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium butyrate	-	-	-	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	+	-	-	+
Sodium citrate	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
Sodium formate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium DL-malate	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-
Sodium maleate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+
Sodium malonate	+	+	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+
Sodium oxalate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium propionate	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Sodium salicylate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium succinate	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+
Sodium L(+)-tartrate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 15. Ability of antibiotic-producing actinomycete strains to utilise specific compounds as sole nitrogen sources

Nitrogen Source	HCBα	$E_{2}a$	$G_{3}d$	$E_{1}e$	$P_{2}f$	$P_{1}g$	$G_{3}i$	$E_{4}j$	$P_{2}k$	$C_{1}l$	$P_{1}m$	$P_{3}p$	$N_{1}q$	$U_{2}t$	$E_{3}u$	$U_{2}z$	$A_{1}\delta$	$O_{1}\iota$	$B_{1}\xi$	$O_{1}o$	$N_{3}\tau$	$I_{2}\upsilon$	$I_{2}\varphi$	SS1	SS12	SS13
DL-α-Amino-n-butyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Cysteine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
L-Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Hydroxyproline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Methionine	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	+	-	+	-	-	+	+	-	-	-
Potassium nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
L-Phenylalanine	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+
L-Serine	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+	+	-	-
L-Threonine	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
L-Valine	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+

3.3.2 Physiological Characteristics of Strain $R_2\mu$

Despite not exhibiting antibiotic activity, $R_2\mu$ was characterised, as it was thought to belong to the genus *Micromonospora*. Some of these results are compared in Table 16 to the characteristics of species of the genus *Micromonospora* described in Vobis (1989). Table 16 shows comparative data only.

No diffusible pigments were produced on any medium and sporulation appeared in the form of black mucoid specks on the surface of the colonies.

$R_2\mu$ displayed resistance to 0.1% phenylethanol, but not to 0.3%. The strain was resistant to 0.1% phenol, but sensitive to all concentrations tested of sodium azide and potassium tellurite.

$R_2\mu$ did not reduce nitrate or produce hydrogen sulphide. Esculin and arbutin were not degraded and urease activity was not present. Starch, casein and xylan were degraded, but not xanthine, adenine or guanine. Gelatin was not liquefied.

Arabinose, fructose, galactose, glycerol, mannitol, mannose and sucrose could be utilised as sole carbon sources, but not acetate, cellobiose, inositol, maleate, maltose, melibiose, propionate, rhamnose, ribose, salicin, succinate or xylose.

It is not possible using the present data to identify $R_2\mu$ to species level. No diagnostic mycelial pigments or diffusible pigments were produced and no consistent similarities between $R_2\mu$ and species of *Micromonospora* were apparent.

Table 16. Comparison of R2μ to species of *Micromonospora* listed in Vobis (1989)[1]

Characteristic	$R_2\mu$	Species[2]											
		a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**
Growth on Czapek-Solution Agar	+	-	-	+	+	-	-	-	v	+	+	+	+
Growth on Potato Slice	-	+	+	-	-	+	+	+	v	-	-	-	-
α-Melibiose	-	+	+	+	+	+	+	+	+	-	-	-	-
D-Mannitol	+	-	-	-	-	-	+	+	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	+	+	+
Glycerol	+	-	-	-	-	-	-	-	+	-	-	-	-
Inositol	-	-	-	-	-	-	v	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-	-	+	-	+	d
Gelatin	-	+	+	+	+	+	+	-	ND	ND	+	+	+
L-Arabinose	+	+	+	+	+	+	ND	-	-	-	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	ND
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	-	-	ND	ND	-	-	ND	ND	-	-	-	-
Nitrate reduction	-	+	-	+	+	v	-	-	-	-	v	-	+
NaCl tolerance (%)	3	3	3	4	4	5	1.5	1.5	1.5	3	3	3	3

1. Symbols: ND, not determined; d, 11-89% of strains positive; v, strain instability

2. Species: **a**. *M. carbonacea* subsp. *carbonacaea;* **b**. *M. carbonacaea* subsp. *aurantiaca;* **c**. *M. halophytica* subsp. *halophytica;* **d**. *M. halophytica* subsp. *nigra;* **e**. *M. chalcea;* **f**. *M. inositola;* **g**. *M. coerulea;* **h**. *M. purpureochromogenes;* **i**. *M. olivasterospora;* **j**. *M. echinospora* subsp. *echinospora;* **k**. *M. echinospora* subsp. *ferruginea;* **l**. *M. echinospora* subsp. *pallida*

3.3.3 Chemical Taxonomy

The result of the DAP isomer determination is shown in Figure 3. P_1m, O_1o and SS12 were shown to contain LL-DAP in their cell wall, while the peptidoglycan of $R_2\mu$ contained meso-DAP. All four strains appeared to contain glycine in their cell walls.

Glycine and LL-DAP are the major constituents of a type I cell wall, typical of streptomycetes, but also a characteristic of the genus *Nocardioides*. A type II cell wall, containing glycine and meso-DAP is exhibited by $R_2\mu$. This is a characteristic of the actinoplanetes, a group that includes the genus *Micromonospora*.

The result of the whole cell sugar pattern (WCSP) determination is shown in Figure 4. The only sugar distinctly visible in the P_1m, O_1o and SS12 lanes is ribose, a non-diagnostic sugar. This is consistent with these strains being streptomycetes, which do not exhibit a diagnostic WCSP.

$R_2\mu$ exhibits a sugar with a high R_f value that cannot be identified. A second sugar is evident between the positions of arabinose and xylose, the diagnostic sugars of WCSP D.

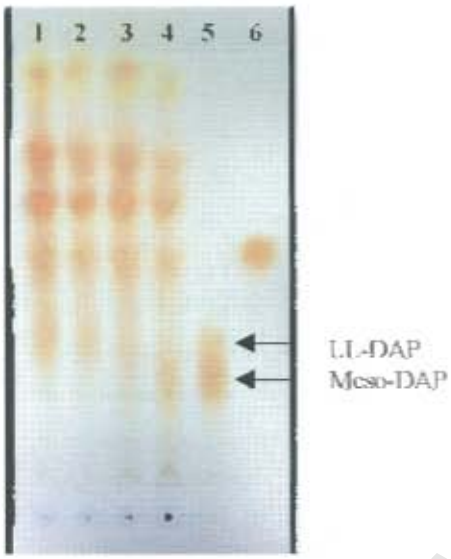


Fig 3. Separation of DAP isomers from actinomycete hydrolysates by thin-layer chromatography **1.** P_1m **2.** O_1o **3.** SS12 **4.** $R_2\mu$ **5.** LL/meso-DAP standard **6.** Glycine standard.

Fig. 4. Separation of whole-cell sugars from actinomycete hydrolysates by thin-layer chromatography. **1.** P_1m **2.** O_1o **3.** SS12 **4.** $R_2\mu$ **5.** Ribose-Mannose-Glucose standard **6.** Xylose-Arabinose-Galactose standard. Order of sugars in standards as seen from top to bottom.

3.3.4 Electron Microscopy

Electron micrographs of P_1m, O_1o, SS12 and $R_2\mu$ are shown in Figures 5-8.

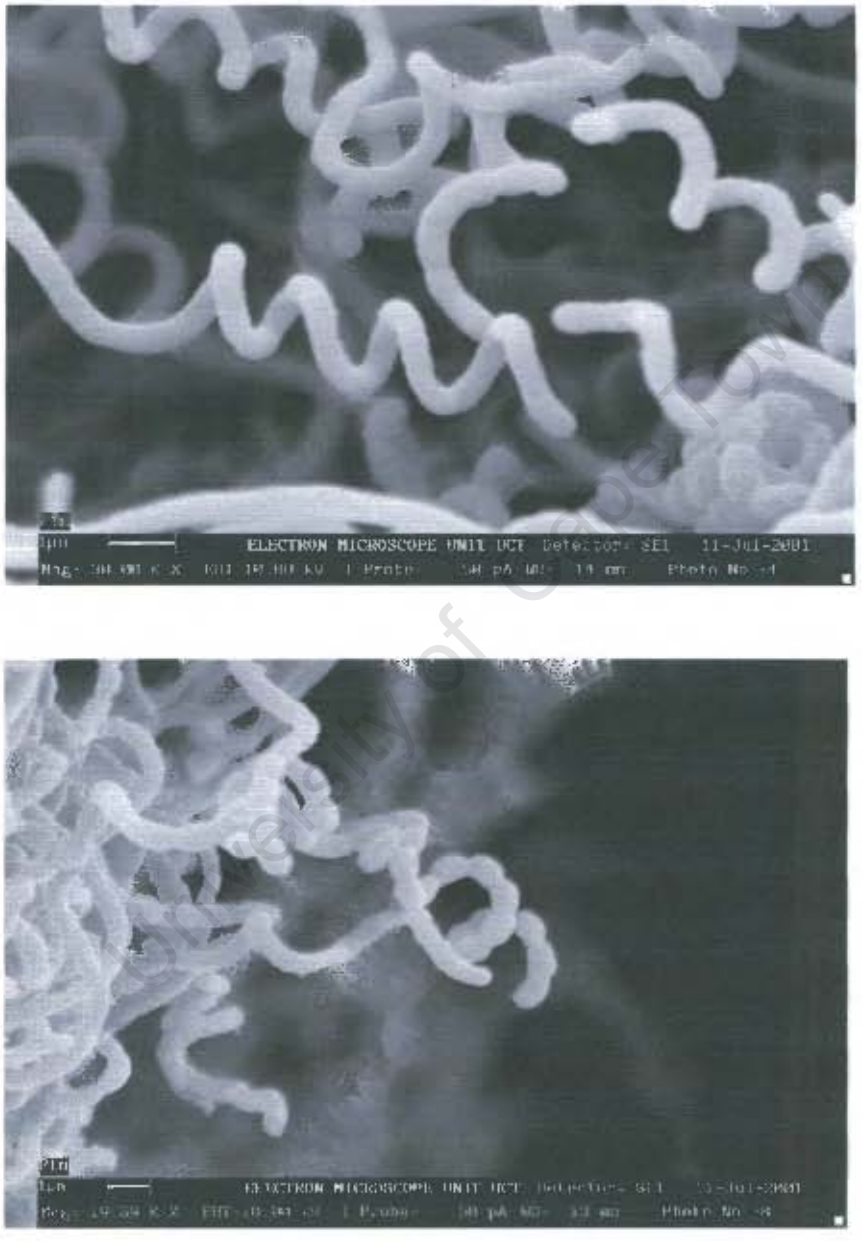


Fig. 5. Scanning electron micrographs of P_1m, showing spore chain morphology (*Spirales*) and spore surface ornamentation (smooth). Scale shown.

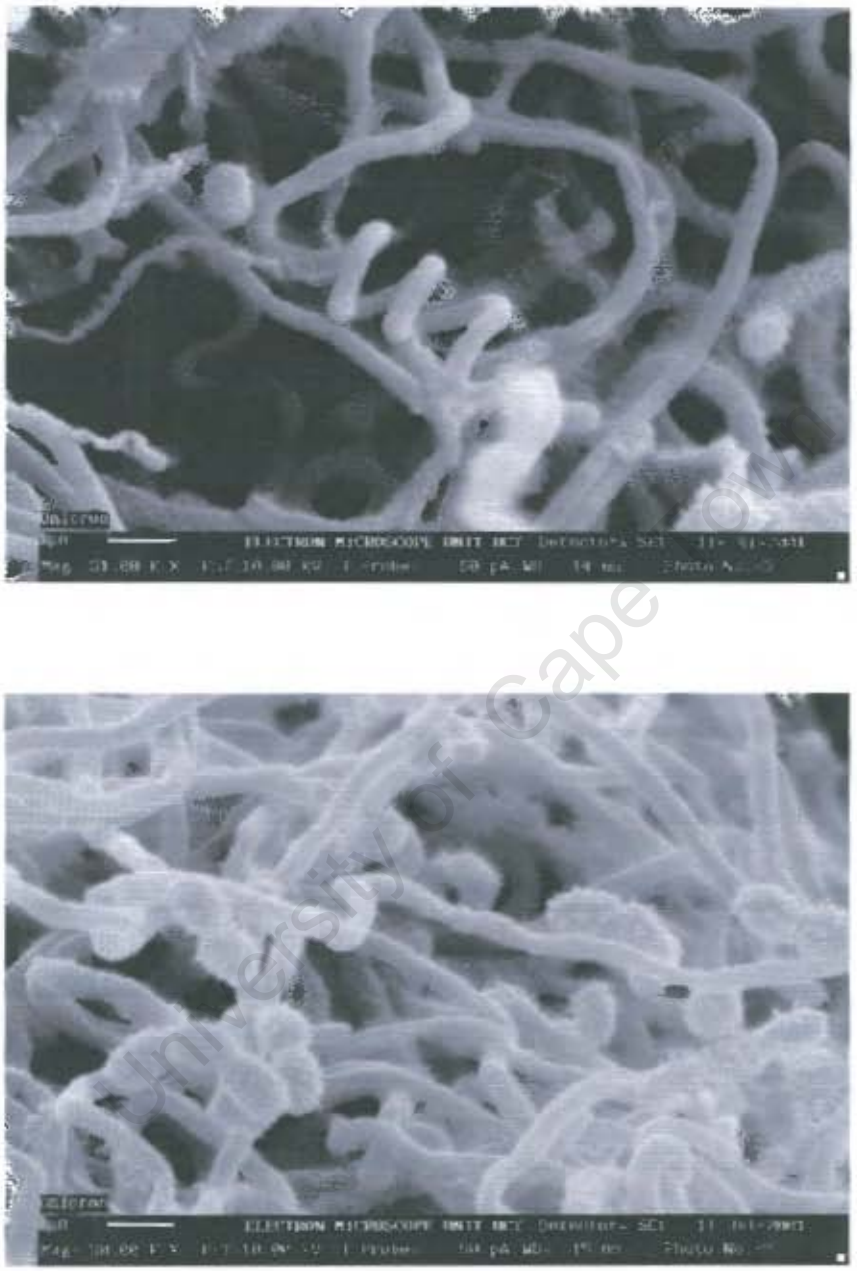


Fig. 6. Scanning electron micrographs of O_1o, showing spore chain morphology (*Spirales*) and spore surface ornamentation (spiny). Scale shown.

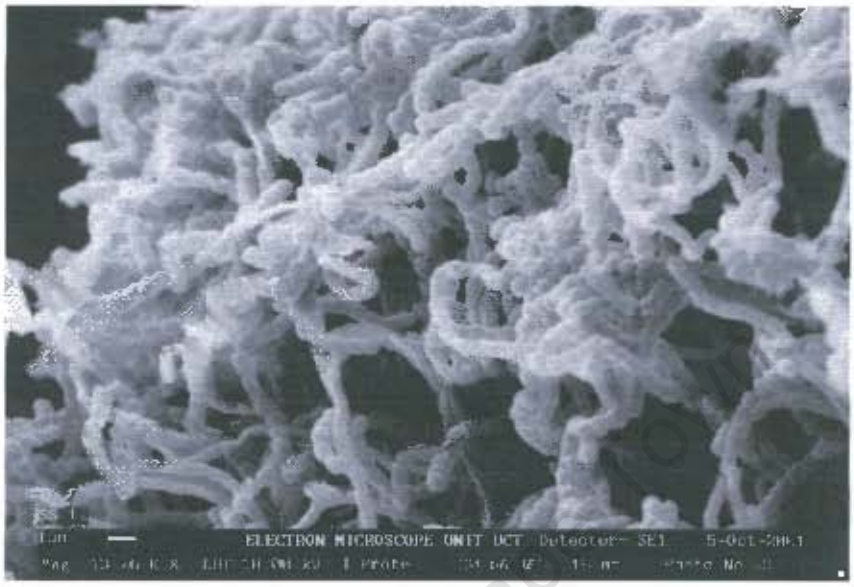


Fig. 7. Scanning electron micrograph of SS12, showing spore chain morphology (*Rectiflexibiles*) and spore surface ornamentation (smooth). Scale shown.

The scanning electron micrographs of $R_2\mu$ (Figure 8) show that it exhibited the morphology typical of the genus *Micromonospora*. Spores were borne singly on the substrate mycelium. Spores were seen as sessile and on short and long sporophores.

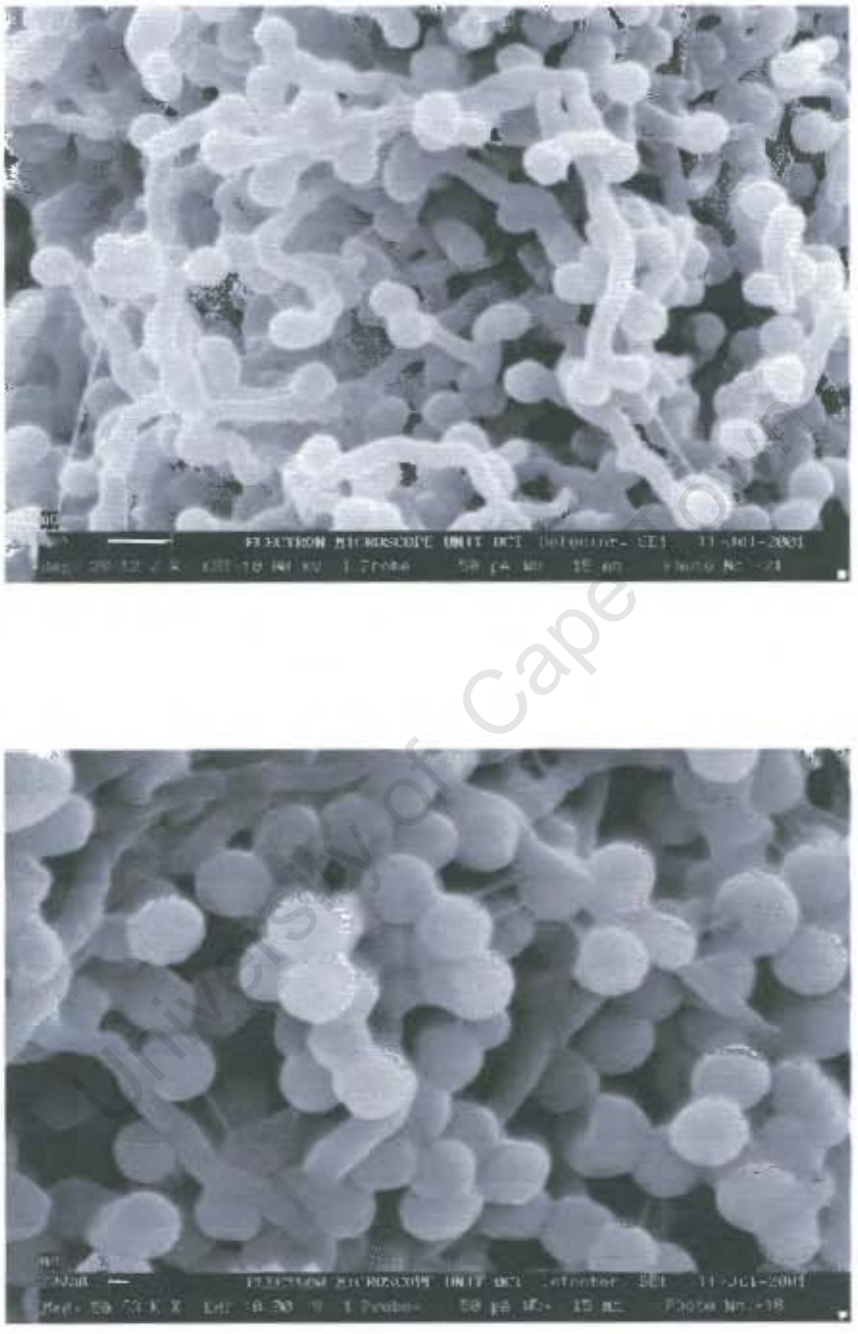


Fig. 8. Electron micrographs of R3μ, showing spore chain morphology and spore surface ornamentation. See text. Scale shown.

3.3.5 DNA Sequencing

Partial DNA sequences of the 16S rRNA genes of P_1m, O_1o, SS12 and $R_2\mu$ are shown in Figures 9-12 respectively. The universal bacterial 16S rDNA-primers were employed for obtaining partial sequences of the PCR products. There is a gap between the 3' and 5' regions of the sequences of P_1m and $R_2\mu$. For P_1m, the first fragment was obtained using the F1 and F2 primers and the second using R4 and R5. For $R_2\mu$, the first fragment was obtained using the F1 and F2 primers and the second using the R3 primer. Sequences in which gaps are present were BLASTed as single sequences. For each strain, the closest BLAST search matches in which database sequences have been ascribed to a species are given in Tables 17-20.

CGGGGTGCTT AACCTGCAAT TTCGAACGAT GAACCGCTTT CGGGCGGGGG ATTAGTGGCG AACGGGTGAG TAACACGTGG
GCAATCTGCC CTGCACTCTG GGACAAGCCC TGGAAACGGG GTCTAATACC TGGATATGAC CGTCTGCCGC ATGGTGGATG
GTGTAAAGCT CCGGCGGTGC AGTGATGAGC CCGCGGCCTA TCAGCTTGTT GGTGAGGTAG TGGCTCACCA AGGCGACGAC
GGGTAGCCGG CCTGAGAGGG CGACCGGCCA CACTGGGACT GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
ATATTGCACA ATGGGCGAAA GCCTGATGCA GGGACGCCGC GTGAGGGATG ACGGCCTTTC GGGTGTAACC TCTTTCAGCA
GGGAAGAAGC GAAAGTGACG GTACCTGCAG AAGAAGCGCC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT ACGTAGGGCG
CAAGCGTTGT CCGGAATTAT TGGGCGTAAA GAGCTCGTAG GCGGCTTGTC ACGTCGGTTG TGAAAGCCCG GGGCTTAACC
CCGGGTCTGC AGTCGATACG GGCAGGCTAG AGTTCGGTAG GGGAGATCGG AATTCCTGGT GTAGCGGTGA AATGCGCAGA
TATCAGGAGG AACACCGGTG GCGAAGGCGG ATCTCTGGGC CGATTACTGA CGCTGAGGAG CGAAAAGGCC GTGGGGGAGG
CGAAACAGGG ATTTAAGAAT A

CTGGTAGAGT GCCCATCAGC CCGAAGGGCA TGCTGGCAAC ACAGGACAAG GGTTGCGCTC GTTGCGGGAC TTAACCCCAA
CATCTCACGA CACGAGCTGA CGACAGCCAT GCACCACCTG TATACCGACC ACAAGGGGGG CACCATCTCT GATGCTTTCC
GGTATATGTC AAGCCTTGGT AAGGTTCTTC GCGTTGCGTC GAATTAAGCC ACATGCTCCG CTGCTTGTGC GGGCCCCCGT
CAATTCCTTT GAGTTTTAGC CTTGCGGCCG TACTCCCCAG GCGGGGAACT TAATGCGTTA GCTGCGGCAC CGACGACGTG
GAATGTCGCC AACACCTAGT TCCCAACGTT TACGGCGTGG ACTACCAGGG TATCTAATCC TGTTCGCTCC CCACGCTTTC
GCTCCTCAGC GTCAGTAATG GCCCAGAGAT CCGCCTTCGC CACCGGTGTT CCTCCTGATA TCTGCGCATT TCACCGCTAC
ACCAGGAATT CCGATCTCCC CTACCACACT CTAGCTAGCC CGTATCGAAT GCAGACCCGG GGTTAAGCCC CGGGCTTTCA
CATCCGACGT GACAAGCCGC CTACGAGCTC TTTACGCCCA ATAATTCCGG ACAACGCTTG CGCCCTACGT ATTACCGCGG
CTGCTGGC

Fig. 9. Partial DNA sequence of the 16S rRNA gene of P_1m (1389 bp).

Table 17. Database matches for the partial DNA sequence of the 16S rRNA gene of P_1m.

Database Match/Accession Number	% Identity
Streptomyces griseus subsp. *griseus* AB045866	97
Streptomyces caviscabies (SAFR-024) AY167847	97
Streptomyces cyaneus (ISP 5108) AJ399460	97
Streptomyces griseus Y15502	97
Streptomyces griseus (ATCC 10137) Y15501	97

Of the five closest matches to the partial DNA sequence of the 16S rRNA gene of P_1m (Table 17), three are to *Streptomyces griseus*, which is now a subjective synonym of *S. anulatus* (Locci, 1989). These results are inconsistent with those obtained by analysis of physiological characteristics, which suggested a species assignment to *S. lydicus* or *S. rimosus*. The former three species typically produce hydrogen sulphide (unlike P_1m) and spore chains are only occasionally *Spirales*. The nucleotide identity is however low, and the possibility that P_1m should be assigned to a new species must not be discounted. Additional data may help to resolve the taxonomic position of P_1m.

TCGGGCGGGG ATAGTGGCGA ACGGGTGAGT AACACGTGGG CAATCTGCCC TGCACTCTGG GACAAGCCCT GGAAACGGGG
TCTAATACCG GATATGACTG TCCATCGCAT GGTGGATGGT GTAAAGCTCC GGCGGTGCAG GATGAGCCCG CGGCCTATCA
GCTTGTTGGT GAGGTAGTGG CTCACCAAGG CGACGACGGG TAGCCGGCCT GAGAGGGCGA CCGGCCACAC TGGGACTGAG
ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCACAATG GGCGAAAGCC TGATGCAGCG ACGCCGCGTG
AGGGATGACG GCCTTCGGGT TGTAAACCTC TTTCAGCAGG GAAAGAAAGC GAAAGTGACG GTACCTGCGA GGGCGAAAGC
TGATGCAGCG ACGCCGCGTG AGGGATGACG GGCCTTTCGG GTTGTAAACC TCTTTCAGCA GGGAAGAAGC GAAAGTGACG
GTACCTGCAG AAGAAGCGCC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT ACGTAGGGCG CAAGCGTTGT CCGGAATTAT
TGGGCGTAAA GAGCTCGTAG GCGGCTTGTC ACGTCGGTTG TGAAAGCCCG GGGCTTAACC CCGGGTCTGC AGTCGATACG
GGCAGGCTAG AGTTCGGTAG GGGAGATCGG AATTCCTGGT GTAGCGGTGA AATGCGCAGA TATCAGGAGG AACACCGGTG
GCGAAGGCGG ATCTCTGGGC CGATACTGAC GCTGAGGAGC GAACAGCGTG GGGAGCGAAC AGGATTAGAT ACCCTGGTAG
TCCACGCCGT AAAACGGTGG GCACTAGGTT TGGGCAACAT TCCACGTTGT CCGTGCCGCA GCTAACGCAT TAAGTGCCCC
GCACTGGGGA GTACGGCCGC AAGGCTAAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGCGGAGCA TGTGGCTTAA
TTCGACGCAA CGCGAAGAAC ACTTACCAAG GCTTGACATA CACCGGAAAC GTCTGGAGAC AGGCGCCCAC CTTGTGGTCA
GGTGTACAGG TGGTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG CAACCCTTGT
CCCGTGTTGC CAGCAGGCAC CATTGTGGTG CTGGGGACTC ACGGGAGACC GCCACAGGGG TCAACTCGGA GGAAGGTGGG
AAAACTCGGT CATCACCCCC GTGG

Fig. 10. Partial DNA sequence of the 16S rRNA gene of O_1o (1224 bp).

Table 18. Database matches for the partial DNA sequence of the 16S rRNA gene of O_1o

Database Match/Accession Number	% Identity
Streptomyces somaliensis (DSM 40267) AJ007400	97
Streptomyces albidoflavus (DSM 46452) Z76685	97
Streptomyces canescens (DSM 40001T) Z76684	97
Streptomyces albidoflavus (DSM 40880) Z76683	97
Streptomyces odorifer (DSM 40347T) Z76682	97

The distinctive morphology of O_1o as determined by electron microscopy (spiny spores on spiral spore chains) greatly reduces the number of the species to which it might belong. Taking physiological characteristics into account, the closest match appears to be *Streptomyces filipinensis*. The only 16S-rDNA sequence available for this species (D44086) is only 120bp long, which makes sequence comparison difficult. Of the nucleotide identity matches, *S. canescens* and *S. odorifer* are subjective synonyms of *S. albidoflavus*. However, the spore chains of this species are *Rectiflexibiles* or *Retinaculiaperti* and the spore surface is smooth. Also, *S. somaliensis* is an unusual actinomycete that causes human actinomycetoma (Locci, 1989). It is thus unlikely that O_1o belongs to this species.

ACGGGCGTGG GATTTATGGC GACGGGTGAG TAACACGTCG GGCAATACTG CCCTGCACTC TGGGACAAGC CCTGGAAACG
GGGTCTAATA CCTGGATATG ACTGTCCATC GCATGGTGGA TGGTGTAAAG CTCCGGCGGT GCACGGATGA GCACCGCGTG
CCTATCAGCT TGTTCGTGTC GAGGGTATGT GGCTCACCAA GGCGACGACG CGAGATAGCC GGCCTGAGGA GGGCGACCGG
CCACCACTGG GACTGAAACA CGGCCCAGAA CTCCTACGGC GTGGAATTGA CAATGGGCGA AAGCTGATGC AGCGACGCCG
CGTGAGGGAT GACGGCTTCG GGTTGTAAAC CTCTTTCAGC AGGGAAGAGC GAAAGTGACG GTACCTGCAG AAGAAGCGCC
GGCTAACTAC GTGCCAGCAG CCGCGGTAAT ACGTAGGGCG CAAGCGTTGT CCGGAATTAT TGGGCGTAAA GAGCTCGTAG
GCGGCTTGTC ACGTCGGTTG TGAAAGCCCG GGGCTTAACC CCGGGTCTGC AGTCGATACG GGCAGGCTAG AGTTCGGTAG
GGGAGATCGG AATTCCTGGT GTAGCGGTGA AATGCGCAGA TATCAGGAGG AACACCGGTG GCGAAGGCGG ATCTCTGGGC
CGATACTCGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC CTGGGTAGTC CACGCCGTAA ACGGTGGGCA
CTAGGTGTGG GCAACATTCC ACCGTTGTCC GTGGCCGCAG CTAACGCATT AAGTGACCCG GCTGGGGATA CGCCGCACAG
GCTAAAAAAC CTTCCACACC CGCCTGGTGG AGTACGGCCG GCAAGGCTAA AACTCAAAGG AATTGACGGG GCGCCCTGCA
CAATGCGGCC GGAGCATGTG GCTTAATTCG ACGCAACGCT GAATGAACCT TACCAAAGGC TTGACATACA CCTGGAAACA
GTCTGGAGAC AGTGCGCCCC CTTGTGGTCG GTGTACAGGT GTGTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG
GTTAAAGTCC CCGCAAACGA GCGCAACCCT TGTCCCGTGT TGCCAGCAGG CCCTTGTGGT GCTGGGGACT CACGGGAGAC
CCCGGGGTCA ACTCGGAGGA AGGTGGGGAC GACGTCAAGT CATCATGCCC CTTATGTCTT GGGCTGCACA CGTGCTACAA
TGGCCGGTAC AATGAGCTGC GATACCGCGA GGTGGAGCGA ATCTCAAAAA GCCGGTCTCA GTTCGGATTG GGGTCTGCAA
CTCGACCCCA TGAAGTCGGA GTCGCTAGTA ATCGCAGATC AGCACTGCTG CGGTGAATAC GTTCCCGGGC CGTTGTACAC
ACCGCCCGTC ACGTCACGAA AGTCG

Fig. 11. Partial DNA sequence of the 16S rRNA gene of SS12 (1385bp).

Table 19. Database matches for the partial DNA sequence of the 16S rRNA gene of SS12

Database Match/Accession Number	% Identity
Streptomyces lividans Strain TK2 AY039029	95
Streptomyces coelicolor A3(2) AL939119	95
Streptomyces coelicolor A3(2) AL939124	95
Streptomyces coelicolor A3(2) 939116	95
Streptomyces coelicolor A3(2) AL939114	95

S. violaceoruber (of which *S. coelicolor* A3(2) is a subjective synonym) has spiral spore chains and spores with smooth surfaces, although spore chains of the *Rectiflexibles* type have also been reported for this species. *S. violaceoruber* however does not produce melanin and displays a blue to violet substrate mycelium (Locci,1989). The data suggests that SS12 may represent a new species of *Streptomyces*.

CGCGAACGGG TGAGTAACAC GTGAGCAACC TGACCCAGGC TTTGGGATAA CCCCGGGAAA CCGGGGCTAA TACCGAATAT
GACCTTGCAT CGCATGGTGT TTGGTGGAAA GTTTTTCGGC TTGGGATGGG CTCGCGTGCC TATCACGCTC TGTTAGGTGG
GGTGATGGCC TACCAATTGC GACGACGGGT AGCCGGCCTG AGAGTGGCGA ACGGCCACAC TGGGACTGAG ACACGGCCAG
ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGCACAATGG GCGACGCGCG TGAGGGATCA CGGCTTTAGG TTGTAAACCT
CTTTCAGCAG TGACGAAGCG TAAGTGACTG GTACCTGCAA AGAAGCGCCG GCCAACTACG TGCCAGCAGC CGCGTGTAAT
GACGTAGGGC GCGATGCTGT TGTCCTGGAT TTATTGGCGC TGTAACATGA TGCTCGTAGA GCTGGCATTG TCAGCCGTCT
GACATGTGAA AACCCGCAGC TCAACTGCGA GGCCTGCATG TCGATACGGG CAGGCTACAG TTCGGTAGGG GA

CACCGGTGGT CGAAGGCGGG TCTCTTGGGC CGATACTGAC GCTGAGGAGC GAAAGCGTGG GGAGCGAACT AGGATTAGAT
ACCCTGGTAG TCCACGCTTG TAAACGGTTG GGGCTAGGTG TGGGGGGCCT CTCCGGTTCC CTGTGCCGCA AGCTAACGCA
TTAAGCGCCC CGCCTGGGGA GTACGGCCGC AAGGCTAAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGCCGGAGCA
TGCGGATTTA GTTCGATGCA ACGCGAA

Fig. 12. Partial DNA sequence of the 16S rRNA gene of $R_2\mu$ (799 bp).

Table 20. Database matches for the partial DNA sequence of the 16S rRNA gene of $R_2\mu$.

Database Match/Accession Number	% Identity
Micromonospora melanospora X92596	95
Micromonospora chalcea U58531	95
Micromonospora chalcea X92594	95
Micromonospora purpurea X92595	95
Micromonospora chersinia X92628	95

$R_2\mu$ is not closely related to any species in particular, as is evidenced by the nucleotide identity. Taken together, the physiological and sequence data strongly suggest that $R_2\mu$ is a new species of *Micromonospora*.

Sequence identities below 99% cannot be utilised for species assignment, even given the incomplete nature of the above sequences. For that reason, physiological and morphological characterisation has been indispensable in the identification of the above strains.

CHAPTER 4:

PARTIAL PURIFICATION OF ACTIVE COMPOUNDS

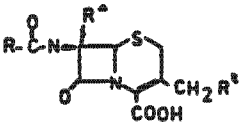
4.1 Introduction: Antibiotics Produced by Actinomycetes

4.1.1 β-Lactam Antibiotics

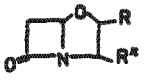
The β-lactam antibiotics (Fig. 17) produced by the actinomycetes belong mainly to the 'nonclassic' structural types i.e. they are not penicillins or cephalosporins (Crueger and Crueger, 1989). *Streptomyces clavuligerus* strains produce several β-lactam antibiotics, such as cephamycins (7-methoxy-cephalosporins) and clavulanic acid, a β-lactamase inhibitor. Carbapenems are produced by several species of the genus *Streptomyces*. *Nocardia uniformis* subsp. *tsuyamenensis* produces monolactam antibiotics called nocardins (Lancini *et al.*, 1995). The β-lactam antibiotics inhibit cell wall synthesis by binding to enzymes involved in peptidoglycan production.

Penam

Ceph-3-em

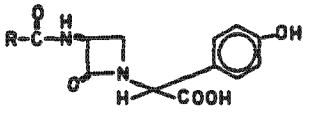


Clavam



Carbapenem

Monolactam (Nocardins)



Monolactam (Monobactams)

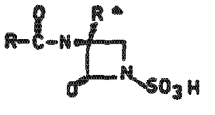


Fig. 17 β-lactam structural types (based on Crueger and Crueger, 1989).

4.1.2 Tetracyclines

Tetracyclines are broad-spectrum bacteriostatic drugs first isolated from *Streptomyces aureofaciens*, but now also produced semisynthetically (Crandall and Hamill, 1986). Protein synthesis is inhibited by the binding of the drug to the small subunit of the ribosome.

4.1.3 Aminoglycosides

Aminoglycosides are mainly used for treatment of severe infections caused by Gram-negative pathogens, but also for treatment of tuberculosis (streptomycin) (Crueger and Crueger, 1989). They are produced by strains of the genera *Streptomyces* (streptomycin, kanamycin) and *Micromonospora* (gentamicin). Like tetracyclines, aminoglycosides bind to the small subunit of the ribosome, inhibiting protein synthesis and causing errors in translation (Lancini *et al.*, 1985). Due to their high toxicity, patients treated with aminoglycosides must be closely monitored.

4.1.4 Macrolides

Macrolides are bacteriostatic antibiotics used to treat infections caused by Gram-positive bacteria and are produced mainly by members of the *Streptomyces* genus (Crueger and Crueger, 1989), but also by strains of *Micromonospora* (Crandall and Hamill, 1986). Inhibition of protein synthesis is by binding of the drug to the large subunit of the ribosome, thereby preventing amino acid linkage (Lancini *et al.*, 1995). Erythromycin, the most widely used macrolide, is a broad-spectrum drug often used to treat patients allergic to penicillins.

4.1.5 Glycopeptides

Vancomycin, well known as the antibiotic of last resort is a glycopeptide produced by *Amycolatopsis orientalis* subsp. *orientalis* (Lancini *et al.*, 1995). Use of vancomycin has increased due to the rise in resistance to drugs such as methicillin (a β-lactam). Glycopeptides inhibit the transpeptidation of the peptidoglycan cell wall, causing it be weak, leading to plasmolysis (Prescott *et al.*, 2002).

4.1.6 Amphenicols

Although now produced synthetically, chloramphenicol was originally isolated from *Streptomyces venezuelae* (Prescott *et al.*, 2002). The mechanism of action is by inhibition of peptidyl transferase through binding of the drug on the large ribosomal subunit. Chloramphenicol is a broad-spectrum drug with high toxicity to humans.

4.1.7 Ansamycins

The most important group of ansamycins are the rifamycins, first isolated from *Streptomyces mediterranei* (now *Amycolatopsis mediterranei*) (Lancini *et al.*, 1995). Rifampicin, an important anti-TB drug, is a semi-synthetic compound derived from these naturally occuring rifamycins. Rifapentine was approved by the Food and Drug Administration for use against tuberculosis in 1999 (Bishai and Chaisson, 2000). Both drugs are inhibitors of bacterial DNA-dependent RNA polymerase (Crueger and Crueger, 1989).

4.2 Materials and Methods

4.2.1 Testing of Culture Supernatant Concentrates

Isolates P_1m, O_1o and SS12 were grown for 10 days at 30°C in Difco 7H9 (1% glucose), International *Streptomyces* Project Medium #1 (ISP#1), Yeast Extract-Malt Extract (YEME) and Mueller-Hinton (MH) broths. Culture supernatants were freeze-dried and redissolved in Millipore milli-RO H_2O to produce a 50-fold concentrate.

Volumes of 20µl of the concentrates were spotted onto Luria agar plates onto which 100µl aliquots of test cultures (*E. faecium* VanA or *M. aurum* A+) had been spread-plated and allowed to dry. Test organisms were grown in 2YT broth overnight with agitation at 37°C. Cultures were diluted as described in section 2.2.2.

After concentrate aliquots had dried, plates were incubated at 37°C for 14-18h (*E. faecium*) or 40-45h (*M. aurum*). Thereafter, plates were examined for zones of inhibition.

4.2.2 Testing of Solvent Extracts

Isolates P_1m, O_1o and SS12 were grown for 10 days at 30°C in Difco 7H9 medium (1% glucose) and filtered through Whatman No.1 filter paper.

Culture supernatants were extracted with 0.1 volume hexane or chloroform or 0.5 volume ethylacetate overnight on a wrist-action shaker at room temperature. The phases were separated with a separating funnel. The organic phase was allowed to dry in a laminar flow hood, with the residue taken up in milli-RO H_2O to produce a 50-fold concentrated extract. The aqueous phase was freeze-dried and reconstituted in milli-RO H_2O to produce a 50-fold concentrated extract. In addition, extractions from supernatants acidified to pH 2.0 with HCl were carried out with ethylacetate. After phase separation,

back-extraction from the ethylacetate was carried out with half a volume of 10mM Tris-HCl at pH 7.0.

Mycelial masses were subjected to solvent extraction overnight with 1 volume methanol or acetone (without shaking). After centrifugation, the organic phase was allowed to dry, with the residue taken up in milli-RO H_2O to produce a 50-fold concentrated extract. In addition, extractions were carried out with acetone acidified to pH 2.0 with HCl. Before drying, the acetone was neutralised with 1M NaOH.

All extracts were stored at -20°C and their antibacterial activity against the test cultures determined as described in section 4.2.1.

4.2.3 Thin Layer Chromatography and Bioautography

Thin layer chromatography (TLC) of solvent extracts was carried out on silica gel 60 F_{254} plates (Merck), using solvent systems consisting of methanol and ethylacetate in various proportions.

M. aurum and *E. faecium* were used as test organisms for bioautography. Cultures were incubated overnight at 37°C and diluted to an OD_{600} of 0.5 using Luria broth. After being dried of all solvent, TLC plates were sprayed with one of the diluted cultures and incubated overnight at 37°C in a sealed container lined with moistened paper towel. After incubation, plates were sprayed with 0.5% (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT) (Sigma) and incubated at 37°C for 1 hour. Plates were then examined for zones of inhibition.

After testing antibacterial activity, TLC plates were viewed under UV light (254nm) with a Cole-Parmer 9815-series 4-watt lamp.

4.3 Results and Discussion

4.3.1 Testing of Growth Media for Antibacterial Activity

P_1m, O_1o and SS12 were grown in 7H9, ISP#1, YEME and MH broths and their concentrated culture supernatants tested for antibacterial activity against *M. aurum and E. faecium*. The results are shown in Table 21. The actinomycete strains grown in Difco 7H9 showed antibacterial activity in all tests with the exception of the O_1o concentrated culture supernatant, which did not exhibit activity against *M. aurum*. No antibacterial activity was detected in any of the extracts derived from MH broth and ISP#1. The only YEME–derived concentrate showing activity was SS12 acting against *E. faecium*. Due to its superiority to the other media in promoting antibacterial activity, 7H9 was chosen as the culture medium for the solvent extraction tests.

Table 21. Antibacterial activity of actinomycete strains in various culture media[1]

	Actinomycete Strain					
Growth Medium	**P_1m**		**O_1o**		**SS12**	
	M. aurum	*E. faecium*	*M. aurum*	*E. faecium*	*M. aurum*	*E. faecium*
7H9	+	+	-	+	+	+
ISP#1	-	-	-	-	-	-
YEME	-	-	-	-	-	+
MH	-	-	-	-	-	-

1. + = Activity; - = No activity

4.3.2 Solvent Extraction of Antibacterial Compounds

The results of the solvent extraction tests are shown in Table 22. The data obtained allows broad speculation on the chemical nature of the compounds extracted. Hexane, the most non-polar solvent used, did not extract antibacterial activity from any of the supernatants. Hence there is no evidence that any of the actinomycete strains are producing a highly non-polar antibacterial compound. Also, the extractions from acidified supernatant or mycelium were successful in extracting antibacterial activity. It thus appears likely that all three actinomycete strains produce compounds that become negatively charged at neutral pH and are reprotonated and non-ionic at pH 2.

The ethylacetate organic phase extract of O_1o was active against *M. aurum* but not *E. faecium*. The chloroform organic phase extract was active against both. This may suggest that different molecules are being extracted in each case, or chloroform may be extracting a greater quantity of the same compound. The possibility of a concentration effect is supported by the ethylacetate data: the extract derived from acidified supernatant was active against both *M. aurum* and *E. faecium*, whereas the organic phase extract (of neutral supernatant) was only active against *M. aurum*.

The antibacterial compound(s) produced by P_1m is extracted only by methanol and acidified acetone. This suggests that the compound(s) is moderately polar. The lack of activity in either the organic or aqueous phase-derived extracts of neutral supernatant may be due to partial extraction. With the active compound(s) being partitioned between the two phases, the activity present in each may not be strong enough to be detectable by the method used. The compound(s) produced by SS12 may be even more polar, as it is not extracted from neutral supernatant by any of the solvents used, but is extracted at low pH.

As acidified acetone extracts of the mycelium of P_1m, O_1o and SS12 were found to show antibacterial activity against *M. aurum* and *E. faecium*, these extracts were used for TLC analysis and bioautography.

Table 22. Solvent extraction of antbacterial compounds[1]

Solvent	Phase Tested	Actinomycete Strain					
		P_1m		O_1o		SS12	
		M. aurum	*E. faecium*	*M. aurum*	*E. faecium*	*M. aurum*	*E. faecium*
Chloroform	Organic[2]	-	-	+	+	-	-
	Aqueous[3]	-	-	-	-	+	+
Ethylacetate	Organic[2]	-	-	+	-	-	-
	Aqueous[3]	-	-	-	-	+	+
	pH 2.0[4]	-	-	+	+	+	+
Hexane	Organic[2]	-	-	-	-	-	-
	Aqueous[3]	-	-	-	-	+	-
Methanol	Mycelium	-	+	-	+	-	-
Acetone	Mycelium	-	-	-	-	-	-
	Mycelium	+	+	+	+	+	+
	pH 2.0[5]						

1. + = Activity; - = No activity

2. The extract derived from the organic phase of the solvent extraction of neutral culture supernatant

3.The extract derived from the aqueous phase of the solvent extraction (post solvent extraction).

4.Antibacterial activity was extracted from acidified supernatant into ethylacetate before being back-extracted into 10mM Tris-HCl (pH 7)

5.The mycelium-acetone slurry was adjusted to pH 2 prior to extraction and neutralised before drying.

4.3.3 Thin Layer Chromatography and Bioautography

TLC of the above extracts was carried out in ethylacetate:methanol solvent systems in ratios of 1:2, 1:1, 2:1, 3:1 and 4:1, whereafter bioautography with *M. aurum* and *E. faecium* was carried out.

Chromatography using ethylacetate:methanol solvent systems in ratios of 1:2, 1:1, 2:1 and 3:1 did not separate compounds in the extract or move them away from the origin. This was determined after bioautography was carried out, where the zones depicting lack of bacterial growth were present only at the origin (data not shown).

The bioautographs of the 4:1 (ethylacetate:methanol) TLC are shown in Figure 18. The plates on which bioautography was carried out are shown exposed to UV light in Figure 19. While most of the antibacterial activity is still located at the origin, slight inhibition is visible between the origin and the mid-point of the bioautograph. As the antibacterial compound(s) for each of the actinomycete strains was shown to be highly polar and most of the activity is still located at the origin, the present solvent system is favouring the migration of less polar molecules. Time constraints prevented further investigation, but it is anticipated that a more polar solvent system (with a higher proportion of methanol) would prove more successful in resolving compounds present with antibacterial activity.

The zone of antibacterial activity shown by O_1o was smaller than those shown by P_1m and SS12. This may be a concentration effect, although given the size of the absorbance spot when viewed under UV light at 254nm, this would indicate that the compound produced by O_1o has a high extinction coefficient than that produced by P_1m. The UV absorbance spots shown by the products of P_1m and O_1o were similar in size, while that of SS12 was visibly smaller, indicating a compound(s) with a lower extinction coefficient.

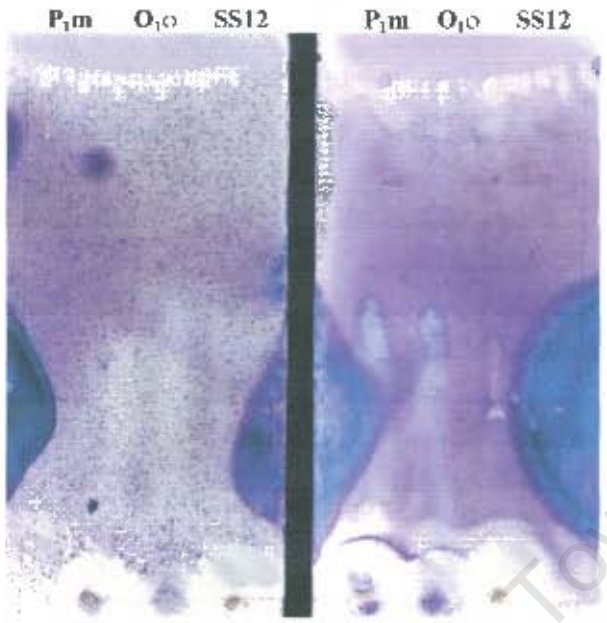


Fig 14. Bioautographic detection of antibacterial compounds produced by actinomycete strains. Zones of activity are at the origin (bottom) in each case. **A**. *M. aurum* **B**. *E. faecium*.

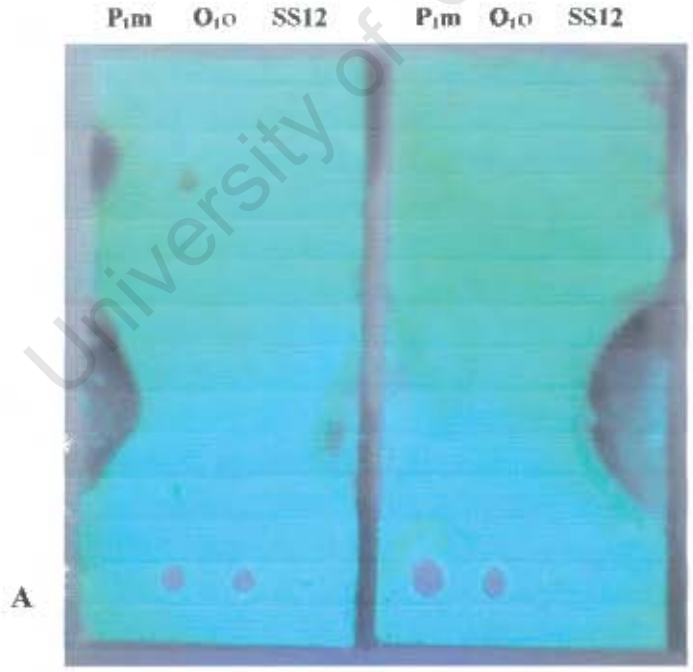


Fig 15. Bioautographs viewed under UV light (254nm). Dark spots at the origin indicate UV-absorbing compounds. **A.** *M. aurum* **B.** *E. faecium*

In the bioautograph, O_1o exhibited greater activity against *E. faecium* than against *M. aurum*. Table 22 showed that O_1o had activity against both *E. faecium* and *M. aurum*. This may be a concentration effect, as described in section 4.3.2.

In summary, P_1m, O_1o and SS12 all produce an antibiotic (or antibiotics) active against *M. aurum* and *E. faecium*. These molecules are at least moderately polar. Since the molecules were not separated by TLC, it is not certain whether UV absorption at 254nm is due to the antibacterial molecules or to other molecules present in the extracts.

CHAPTER 5:
CONCLUSIONS AND FUTURE WORK

In the past, marine natural products have been attributed to the plants (seaweeds) and animals (marine invertebrates) from which they were isolated (Jensen and Fenical, 1994). However, the possibility that at least some of these molecules are of bacterial origin is now a serious subject of study. Most marine actinomycetes are derived from sediment (Takizawa *et al.*, 1993), but it was thought worthwhile to screen the surfaces of 12 seaweed species for the presence of antibiotic-producing actinomycetes. Although the variety of isolates was superficially small, of the 67 strains isolated, 26 exhibited antibacterial activity against *M. aurum* and/or *E. faecium*.

As actinomycete research is dominated by the *Streptomyces* genus, there is an increasing drive to study non-streptomycete actinomycetes in more detail (Anderson and Wellington, 2001; Lazzarini *et al.*, 2000). These genera have been less heavily exploited than the streptomycetes and thus offer a promising avenue of antibiotic discovery. Applications of selective screening protocols have been highly successful in the isolation of 'rare' genera of actinomycetes and will prove useful in future studies. Cook and Meyers (in press) have developed a method to rapidly distinguish between isolates of different actinomycete groups, allowing promising isolates to be identified quickly.

$R_2\mu$ was the single isolate that could be easily identified as a non-streptomycete, in this case as a member of the genus *Micromonospora*. Although it was not found to exhibit antibacterial activity against the test bacteria used in this study, further work on this isolate will be worthwhile to determine whether it exhibits antibacterial activity on other growth media or against other test bacteria. This is especially true in the light of other putative *Micromonospora* isolates having been discovered in this laboratory (Goodwin and Meyers, personal communication).

Three strains (P_1m, O_1o and SS12) were selected for further study due to their very strong antibacterial activity against *M. aurum* in particular. Chemical taxonomy, DNA sequencing and scanning electron microscopy showed them to belong to the *Streptomyces* genus. For each strain, the highest nucleotide identity match was fairly low. Thus the discrepancy between the molecular and physiological data may not be as serious as it may appear. The fact that the DNA sequences were largely fragmentary must also be taken into account. Re-sequencing of the PCR products with multiple coverage of the gene will be useful. These data allow a tentative suggestion of new species to be made.

Analysis of the antibacterial activity exhibited by solvent extracts showed that P_1m, O_1o and SS12 all produce moderately to strongly polar compounds. Resolution of these compounds by TLC was not achieved due to a suitable solvent system not being determined. A larger proportion of methanol in the solvent system than has been used in this study may be able to resolve the compounds present. Antibacterial activity was found to be extracted when the pH was lowered, suggesting the presence of negatively charged groups at neutral pH. Anion-exchange chromatography would thus constitute a useful purification step.

APPENDIX A:
MEDIA AND SOLUTIONS

A.1 Solid Media

A.1.1 Luria Agar

Tryptone	10g
Yeast Extract	5g
NaCl	5g
dH_2O	1000ml
Agar	15g

A.1.2 Starch Casein Agar

Starch	10g
Casein	1g
K_2HPO_4	0.5g
NaCl	20g
dH_2O	1000ml
pH to	7.5
Agar	20g

A.1.3 Yeast Extract-Malt Extract Agar (ISP Medium 2)

Malt Extract	10g
Yeast Extract	4g
Glucose	4g
NaCl	20g
dH_2O	1000ml
pH to	7.2
Agar	20g

A.1.4 Seawater Starch Agar

Starch	10g
Yeast Extract	4g
Peptone	2g
KBr	0.16g
Tris-HCl buffer (1M, pH 8.0)	10ml
NaCl	20g
dH_2O	1000ml
pH to	7.8
Agar	20g

A.1.5 Inorganic Salts-Starch Agar (ISP Medium 4)

Starch	10g
K_2HPO_4	1g
$MgSO_4.7H_2O$	1g
$(NH_4)_2SO_4$	2g
$CaCO_3$	2g
Trace Salts Solution	1ml
NaCl	20g
dH_2O	1000ml
pH to	7.0-7.4
Agar	20g

A.1.6 Glycerol-Asparagine Agar (ISP Medium 5)

L-asparagine	1g
Glycerol	10g
K_2HPO_4	1g
Trace Salts Solution	1ml
NaCl	20g
dH_2O	1000ml
pH to	7.0-7.4
Agar	20g

A.1.7 Peptone-Yeast Extract-Iron Agar (ISP Medium 6)

Peptone	15g
Proteose Peptone	5g
Ferric Ammonium Citrate	0.5g
K_2HPO_4	1g
$Na_2S_2O_3$	0.08g
Yeast Extract	1g
NaCl	20g
dH_2O	1000ml
pH to	7.0-7.2
Agar	15g

A.1.8 Tyrosine Agar (ISP Medium 7)

Glycerol	15g
L-tyrosine	0.5g
L-asparagine	1g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
NaCl	20g
dH_2O	1000ml
pH to	7.2-7.4
Agar	20g

A.1.9 Egg Yolk Agar

Peptone	10g
Glucose	1g
Yeast Extract	5g
NaCl	20g
dH_2O	1000ml
pH to	7.2-7.4
Agar	12g

Egg Yolk Emulsion (Biolab) 50ml added aseptically after autoclaving

A.1.10 Pectin Hydrolysis Agar

Part1: KH_2PO_4	4g
Na_2HPO_4	6g
dH_2O	100ml
Part2: Pectin (from citrus fruit rind)	5g
dH_2O	100ml
Part 3: $(NH_4)_2SO_4$	2g
Yeast Extract	1g
$MgSO_4.7H_2O$	2g
$FeSO_4.7H_2O$	0.0001g
$CaCl_2$	0.0001g
NaCl	20g
dH_2O	800ml
pH to	7.0
Agar	10g

Parts 1,2 and 3 autoclaved separately and mixed

A.1.11 Colloidal Chitin Agar

Chitin	2g
KH_2PO_4	0.3g
K_2HPO_4	0.7g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4$	0.001g
$MnCl_2$	0.001g
NaCl	20g
dH_2O	1000ml
pH to	7.0
Agar	20g

A.1.12 Nitrate Reduction Medium

Nutrient Broth (Oxoid, CM1)	1.3g
KNO_3	0.2g
Agar (Oxoid #1)	0.6g
dH_2O	100ml

A.1.13 Bennett Agar (modified)

Glycerol	10g
Casitone	2g
Yeast Extract	1g
Lab-Lemco Powder (Oxoid)	1g
NaCl	20g
dH_2O	1000ml
pH to	7.2-7.4
Agar	20g

A.1.14 Sierra's Medium

Peptone	10g
$CaCl_2.2H_2O$	0.114g
NaCl	20g
dH_2O	900ml
pH to	7.2-7.4
Agar	15g

A.1.15 Esculin/Arbutin Hydrolysis Basal Medium

Yeast Extract	3g
Ferric Ammonium Citrate	0.5g
NaCl	20g
dH_2O	1000ml
pH to	7.2
Agar	3g

A.1.16 Urea Hydrolysis Basal Medium

Glucose	1g
Casitone	1g
$Na_2HPO_4.2H_2O$	2g
KH_2PO_4	1.5g
$MgSO_4.7H_2O$	0.5g
NaCl	20g
Phenol Red	0.012g
dH_2O	900ml
pH to	6.8
Agar	12g

A.1.17 Carbon Utilisation Medium (ISP Medium 9)

$(NH_4)_2SO_4$	2.64g
KH_2PO_4	2.38g
$K_2HPO_4.3H_2O$	5.65g
$MgSO_4.7H_2O$	1g
NaCl	20g
Pridham & Gottlieb	
Trace Salts Solution	1ml
dH_2O	1000ml
pH to	6.8-7.0
Agar (Oxoid #1)	15g

A.1.18 Nitrogen Utilisation Basal Medium

Glucose	10g
$MgSO_4.7H_2O$	0.5g
NaCl	20g
$FeSO_4.7H_2O$	0.01g
K_2HPO_4	1g
dH_2O	1000ml
pH to	6.8-7.0
Agar (Oxoid #1)	15g

A.1.19 Luria Sloppy Agar

Tryptone	10g
Yeast Extract	5g
NaCl	5g
Agar	7g

A.2 Trace Salts Solutions

A.2.1 Pridham & Gottlieb Trace Salts Solution

$CuSO_4.5H_2O$	0.64g
$FeSO_4.7H_2O$	0.11g
$MnCl_2.4H_2O$	0.79g
$ZnSO_4.7H_2O$	0.15g
dH_2O	100ml

A.2.2 ISP Trace Salts Solution

$FeSO_4.7H_2O$	0.1g
$MnCl_2.4H_2O$	0.1g
$ZnSO_4.7H_2O$	0.1g
dH_2O	100ml

A.3 Liquid Media

A.3.1 Tryptone-Yeast Extract Broth

Tryptone	5g
Yeast Extract	3g
NaCl	20g
dH_2O	1000ml

A.3.2 2YT Broth

Tryptone	16g
Yeast Extract	10g
NaCl	5g
dH_2O	1000ml

A.4 Solutions

A.4.1 1M Tris buffer

Tris base	12.1g
dH_2O	80ml
Adjust to required pH with HCl	
dH_2O to	100ml

A.4.2 5M Acetic Acid

Glacial Acetic Acid	14.3ml
dH_2O	35.7ml

A.4.3 Nitrate Reduction Test Reagent Solution A

Sulphanilic Acid	0.04g
5M Acetic Acid	5ml

A.4.4 Nitrate Reduction Test Reagent Solution B

α-Naphthylamine	0.025g
5M Acetic Acid	5ml

A.4.5 Gel Tracking Dye

Bromophenol Blue	0.25g
Sucrose	40g
EDTA (0.5M)	12.0ml
dH_2O to	100ml

A.4.6 50x TAE (Tris-acetate buffer)

Tris	242g
Glacial Acetic Acid	57.1ml
EDTA (0.5M)	100ml
dH_2O to	1000ml

APPENDIX B:
TOTAL GENOMIC DNA EXTRACTION

Cells from a 600ml 3-4 day culture were harvested by centriguation at 7500 rpm for 20 min and resuspended in 20ml suspension buffer (Appendix A). The cells were then transferred to a 150ml flask whereupon 4mg/ml dry lysozyme (Boehringer-Mannheim) was added to the cell suspension and incubated at 37°C for 1h.

To determine whether lysis was complete, an equal volume of 2x lysing buffer (Appendix A) and 0.25 volumes of 5M NaClO4 (SIGMA) were added. Heating at 50°C resulted in the turbid solution becoming translucent and viscous. The lysate was incubated at 50°C overnight to degrade cellular proteins. Phenol-chloroform solution (15ml) was added to the lysate and shaken on a wrist-action shaker for 20 min. The lysate was centrifuged at 12000 rpm for 10 min at room temperature and the aqueous phase collected. The extraction was repeated twice.

The aqueous phase from the final phenol-chloroform extraction was transferred to a 150ml flask and 0.6 volumes of isopropanol were added. The precipitate was held back with a glass rod and the lysate-isopropanol solution was poured off. Twenty-five millilitres of 76% ethanol was added and allowed to stand for 10 min. The ethanol was poured off and the wash repeated. After the ethanol was removed the precipitate was dried at 37°C for 15 min.

The nucleic acid precipitate was re-dissolved in 20ml TE buffer (pH 7.6, Appendix A) and 250µl of 1mg/ml RNase (SIGMA) was added and the solution incubated at 37°C for 1h. The solution was extracted with 5ml of a chloroform:isoamyl solution, centrifuged at 12000 rpm for 10 min and the aqueous layer saved. To this aqueous solution, 0.1 volumes of 3M sodium acetate (Saarchem) was added and mixed, followed by the addition of 2 volumes of 95% ethanol. The DNA was collected using a glass rod and washed as before

with 76% ethanol and dried at 37°C for 15 min. The precipitate was re-dissolved in 5ml TE buffer (pH 7.6, Appendix A) and left at 4°C overnight. The DNA concentration was determined spectrophotometrically with a 310-220 nm scan.

APPENDIX C

PCR AMPLIFICATION OF ACTINOMYCETE 16S rDNA GENES

C.1 Universal Primers

F1 (Forward Primer; 20-mer): 5' AGAGTTTGATCITGGCTCAG 3'

Binds to positions 8-27 of the 16S rDNA gene (numbering based on E.coli 16S rDNA gene sequence)
PCR Annealing Temperature: 56°C

R5 (Reverse Primer; 21-mer): 5' ACGGITACCTTGTTACGACTT 3'

Binds to positions 1512-1492 of the 16S rDNA gene (numbering based on E.coli 16S rDNA gene sequence)
PCR Annealing Temperature: 56°C

C.2 Reaction Set-up

Template DNA	1µl
MgCl2 (25mM)	2µl
10x buffer	5µl
Taq polymerase (5U/µl)	0.3µl
F1 primer (10µM)	2.5µl
R5 primer (10µM)	2.5µl
dNTPs (25 mM)	0.3µl
H_2O	35µl
	50µl

C.3 PCR Program

1	96°C	2 min
2	96°C	45s
3	56°C	30s
4	72°C	2 min
5	72°C	5 min
6	4°C	10 min

Steps 2-4 were carried out 30 times

APPENDIX D

PREPARATION OF ACTINOMYCETE SPECIMENS FOR SCANNING ELECTRON MICROSCOPY

A 1 cm^2 plug containing the substrate and aerial mycelium was cut out of the agar plate. This plug was fixed in 100mM sodium cacodylate buffer containing 2% glutaraldehyde (pH 7.5 –8.0) overnight at room temperature. After the buffer was discarded, the specimen was washed twice (15-30 min per wash) with 100mM sodium cacodylate buffer (pH 7.5 –8.0) without glutaraldehyde at room temperature. The specimen was post-fixed in 100mM sodium cacodylate buffer containing 1.0% osmium tetroxide (pH 7.5 –8.0) for 1-2 h at room temperature, after which the specimen was washed (10 min per wash) once with 100mM cacodylate (pH 7.5 –8.0) and twice with dH2O at room temperature. The fixed specimen was then dehydrated in a series of increasing ethanol concentrations: 30, 50, 70, 85 and 95% (ethanol diluted in dH2O). The specimen was left in each solution for 10 min before being transferred to the next-most concentrated solution. Thereafter, the specimen was dehydrated further in four changes of 100% ethanol (10 min each). The sample was stored in 100% ethanol and transported to the Electron Microscope Unit (University of Cape Town) for critical-point drying, coating and examination by scanning electron microscopy.

APPENDIX E:
STANDARD EXPERIMENTAL PROCEDURES

E.1 Spectrophotometric Quantitation of DNA

DNA concentrations were determined spectrophotometrically using a Beckman DU-40 spectrophotometer. Suitable dilutions of DNA in Tris-EDTA (TE) buffer were scanned and the UV light absorbance of the samples between 220nm and 310nm were plotted. The absorbance reading at 260nm allowed the concentration of each sample to be determined using the fact that A_{260} =1 for 50 μg/ml dsDNA.

E.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed using a horizontal submerged gel system as described by Sambrook *et al.* (1989). Tris-acetate EDTA (TAE) buffers were used routinely. Sigma Type II agarose was used at 0.8% and gels were run at 60V.

All gels contained 5μl ethidium bromide (10mg/ml) in 100ml of agarose (0.5 μg/ml), so that the DNA could be visualised using a 254nm UVP© transilluminator.

Photographs of the gels were taken using the UVP IMAGESTORE 500 Version 5 (1993). DNA fragment sizes were calculated by extrapolation from the standard curves of the molbility of lambda (λ) DNA fragments electrophoresed on the same gels as DNA samples. Standard λ fragments were obtained by digesting λ DNA with the restriction endonuclease *PstI*.

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