# Investigations into Actinomycetes Isolated from Coastal Environments, with a Special Emphasis on the Genus *Micromonospora*

*by*

*Candice Michelle Goodwin*

# INVESTIGATIONS INTO ACTINOMYCETES ISOLATED FROM COASTAL ENVIRONMENTS, WITH A SPECIAL EMPHASIS ON THE GENUS *Micromonospora*

By

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A thesis submitted in partial fulfilment of the requirements for the Master of Science degree in Molecular and Cell Biology in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa.

Cape Town
February 2005

# ACKNOWLEDGEMENTS

I thank my supervisor, Dr Paul Meyers, for offering guidance, and for providing an environment in which I had the freedom to 'drive' my research.

I also thank Professor Jennifer Thomson for her kindness, help, and support. I am also grateful to Marilize le Roes for her patience, her wealth of knowledge and experience, and her friendship and help when I needed it.

I gratefully acknowledge personal financial assistance from the National Research Foundation, and the Department of Molecular and Cell Biology, University of Cape Town.

I thank Juliet and Mark le Roux for the use of their boat to assist with sample collection and for the use of their Hermanus home for processing of all samples. I also thank Jessica Bennett for help with the initial screening experiments.

I thank Donovan S. Porter, Marilize le Roes, and R. Deeya Ballim and Aimeé Whitcroft for passing on the *Micromonospora* strains that they isolated. Furthermore, I thank Colette N. Bezuidenhout, Erna H. Morden, and Marilize le Roes for their assistance.

I also thank the members of the Anaerobe Unit, Department of Molecular and Cell Biology, University of Cape Town for their assistance with the anaerobic chamber, as well as Di James for DNA sequencing and Madhu Chauhan for mol% G+C determination of genomic DNA. Special thanks to Miranda Waldron of the Electron Microscope Unit, University of Cape Town, for Cryo electron microscopy.

I thank my family and my close friends; Meg, Taren, Roger, Jessica, and Carrie, for their endless, unconditional love and support. I also thank Connor for inspiring me.

Finally, I hope that I can pass down my knowledge…

# ABBREVIATIONS

| | |
|---|---|
| AD | Albumin Dextrose |
| BM | Bennett's Medium |
| CHX | Cycloheximide |
| DAP | Diaminopimelic Acid |
| HIV | Human Immunodeficiency Virus |
| *ISP* | International *Streptomyces* Project |
| m172F | Modified 172F Agar |
| MMP | *Micromonospora* Macrolide Production Medium |
| NaCl | Sodium Chloride |
| NAL | Nalidixic Acid |
| OD | Optical Density |
| PKS | Polyketide Synthase |
| SSC | Standard Saline Citrate |
| TB | Tuberculosis |
| TLC | Thin Layer Chromatography |
| w/v | Weight per Volume |
| YEME | Yeast extract-malt extract Agar (*ISP* Medium No. 2) |
| YMEM | *ISP* Medium No. 2 supplemented with 2% NaCl (w/v) |

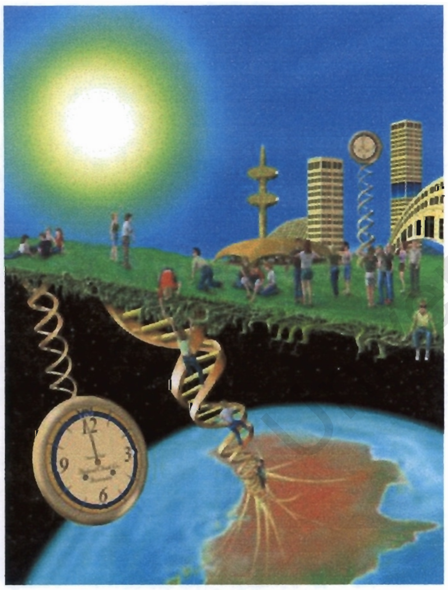
# ABSTRACT

Marine environments were investigated and actinomycetes were isolated on selective media. Thirty-four (34) actinomycete strains were isolated and identified: 21 *Micromonospora* strains, 10 *Streptomyces* strains, and 3 *Pseudonocardia* strains. A polyphasic approach was employed to determine the novelty of the isolates. Potentially, all 21 *Micromonospora* strains are novel, as revealed by an original identification scheme developed to assess quickly and easily the novelty of newly isolated environmental *Micromonospora* strains. Standardized media for testing physiological characters of *Micromonospora* strains were developed, and additional physiological characteristics of 15 of the validly published members of the genus *Micromonospora* are described. Furthermore, 14 of the 15 validly published *Micromonospora* species, and 20 of the 21 environmental *Micromonospora* isolates grew under anaerobic conditions. Eight (8) new *Micromonospora* species are proposed: '*Micromonospora aquatica*' strain MMS5$^{T}$, '*Micromonospora arenincolae*' strain MMS7$^{T}$, '*Micromonospora cinerea*' strain MMS3$^{T}$, '*Micromonospora grateloupicolae*' strain R$_{2}\mu^{T}$, '*Micromonospora hermanusense*' strain MMS6$^{T}$, '*Micromonospora lacunae*' strain MMS4$^{T}$, '*Micromonospora stanfordense*' strain MMS1$^{T}$, and '*Micromonospora terrae*' strain MMS-DA$^{T}$. At least 7 *Streptomyces* strains are novel, on the basis of their morphological and physiological characters. A new *Streptomyces* species is proposed: '*Streptomyces noordhoekensis*' strain BAM4$^{T}$. The antibiotic biosynthetic capability of the strains was assessed by PCR targeted amplification of antibiotic biosynthetic and resistance genes. Five sets of PCR primers (*oxyB*, ARO-PKS, ANSA, *grm*, *fmr*) were used to detect genes involved in the biosynthesis of, or resistance to, various antibiotic types. An aminoglycoside antibiotic susceptibility plate assay and the *grm* and *fmr* PCR primers revealed the presence of aminoglycoside resistance genes in the genome of *Micromonospora* strains and suggested that these strains are capable of synthesizing aminoglycoside-type antibiotics. The antibiotics produced by 5 *Micromonospora* strains were investigated: 2 antibiotic samples were partially purified and their physicochemical properties determined. The antimycobacterial activity of strains MMS2 and MMS7 shows great promise.

# Outline

# Chapter 1

# General Introduction



# Chapter 1

# General Introduction

## 1.1 Background: Actinomycetes, Antibiotics, and Unexplored Niches

Actinomycetes are Gram-positive aerobes that are widely distributed and abundant in soil and are responsible in part for the earthy odour of soil. *Streptomyces* species make up approximately 95% of all actinomycetes found in soil (11). Consequently, they are the easiest actinomycetes to isolate and have thus been extensively studied, resulting in streptomycetes being the best characterised of all actinomycetes (4). Many strains produce one or more antibiotics (14). Colonies are initially relatively smooth surfaced but later develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety (14). A wide variety of pigments are produced that are responsible for the colour of the vegetative and aerial mycelia. On occasion, coloured diffusible pigments are produced.

Streptomycetes do not have specific growth requirements (they are nutritionally versatile) and show tolerance of potentially inhibitory media additives (14). Many competing bacteria and fungi grow or spread more rapidly than actinomycetes on isolation plates. Thus, most selective isolation procedures have been designed as much to inhibit growth of competing microbes as to stimulate development of actinomycetes (14). Whatever the composition of the isolation medium, it is usually necessary to increase its efficiency by adding selective inhibitors.

Morphology has played a major role in distinguishing members of the genus *Streptomyces* from other spore-forming actinomycetes and in their characterization and identification to the species level. The life cycle of a streptomycete offers three features for characterization, namely, substrate mycelium, aerial mycelium bearing chains of arthrospores, and the arthrospores themselves (14). The latter two features provide the most important systematic information.

There are three recognized categories of spore chain morphology (14): *Rectiflexibiles* (straight to flexuous), *Retinaculiaperti* (hooks, loops or spirals with one to two turns), and *Spirales* (spirals possessing many tight turns).

Spore surface ornamentation can be described as smooth, spiny, hairy, warty, or rugose (wrinkled) (14). The ornaments are borne on the spore sheath. Scanning electron microscopy is used to confirm the spore chain morphology (observed by light microscopy) and to determine the spore surface ornamentation.

Streptomycetes are also macroscopically characterized according to the colour of the spore mass, pigmentation of the substrate mycelium, and diffusible pigments. The spore mass can be assigned to one of 7 colour series: blue, grey, green, red, violet, white, or yellow (14). The colour of the substrate mycelium and of the diffusible pigments are allocated to one of 5 categories: yellow-brown (including strains lacking distinctive pigmentation), red-orange, green, blue, or violet (14). The distribution of strains according to these characteristics is unequal and thus striking colours are considered to be of great value as a large proportion of strains are placed in the grey series. Colour determinations are based on standardized media and methodology.

In order to propose that an isolate represents a new species, its morphological and physiological characteristics should not match with the features of any known streptomycete species included in Volume 4 of Bergey's Manual of Systematic Bacteriology (14) and those subsequently described in the literature.

The genus *Micromonospora* has few distinctive morphological characteristics. The mycelium of almost all *Micromonospora* species is coloured orange, turning black upon sporulation. The spore mass becomes mucoid with age. Scanning electron microscopy has revealed that the spores of almost all *Micromonospora* species are blunt spiny surfaced (10) and unlike the genus *Streptomyces*, the spore surface ornamentation is not a useful diagnostic aid for differentiation of *Micromonospora* species.

Actinomycetes produce antibiotics that are antagonistic to other soil microbes (9). This assumes that antibiotics are produced in normal untreated soils in the same way as they are produced in special laboratory media. Competition certainly occurs between organisms in soil, but it is difficult to demonstrate that actinomycetes produce in untreated soils antibiotics at levels high enough to inhibit other soil organisms (9).

It is because of their ability to produce antibiotics and because of their great potential for producing new and useful antibiotics that actinomycetes are perhaps best known. Except for the few antibiotics produced by fungi (most notably, penicillin and cephalosporin) and non-actinomycete bacteria, all other antibiotics that have widespread, useful medical applications are synthesized by actinomycetes (9). By 1970, approximately 85% of all antibiotics discovered in the U.S.A. and Japan were produced by actinomycetes. The most commonly reported new antibiotics were those with antibacterial activity, followed by antifungal agents, antitumour, antiviral, and antiprotozoal antibiotics (9).

The genus *Streptomyces* has been the focus of a long-standing debate with regard to their role in marine environments: it is not clear whether streptomycete isolates are an indigenous component of the marine bacterial community or whether they germinate from spores which washed in from terrestrial habitats (8, 15, 20). The greater abundance of culturable streptomycetes in marine environments located adjacent to terrestrial habitats has been taken as evidence for the germination of spores of terrestrial origin during isolation (8). Moran *et al.* (15) suggested that the higher concentration of culturable streptomycetes in coastal environments than in deepwater marine environments might be explained by the high concentrations of organic detritus, much of which is derived from vascular plants, which characterize many shallow marine environments. Takizawa *et al.* (20) showed that the requirement of seawater for growth of actinoplanete isolates indicates that autochthonous actinomycete populations exist. This is also suggested by the isolation of actinomycetes from deep oceanic sediments distant to land (8).

Moran *et al.* (15) used a 16S rRNA genus-specific probe to determine whether *Streptomyces* populations are an indigenous component of marine sediment bacterial communities. Their method allowed for differentiation between spore- and mycelium-derived DNA. The genus-specific probe indicated that *Streptomyces* spores were not the source of the hybridisation signal, thus indicating that streptomycete isolates found in marine sediment are indigenous to that marine environment. Their studies portrayed a relatively stable bacterial community in which one genus consistently accounts for a predictable fraction of the community rRNA over an entire annual cycle, contrasting with the view of distinct seasonality in species composition. Sediment communities might be expected to be more stable through time because of the constancy of the sediment organic matrix and the regular availability of natural polymers as substrates (15). To be cautious in interpretation of results, the possibility of wash-in from surrounding terrestrial habitats must always be considered.

Rare genera of actinomycetes have gained increasing relevance as potential producers of new antibiotics (12). The development of new methodologies with which to isolate rare actinomycetes is of great importance to extend our understanding of their ecology, taxonomy and bioactivity (17). It has been shown that when selective isolation methods are developed and extensively applied, some genera, such as *Actinomadura*, *Actinoplanes*, *Micromonospora*, and

*Microtetraspora* are not rare at all and can be recovered from many soil samples (8, 12). The current focus is on the isolation of members of such genera, given their potentially promising chemical diversity.

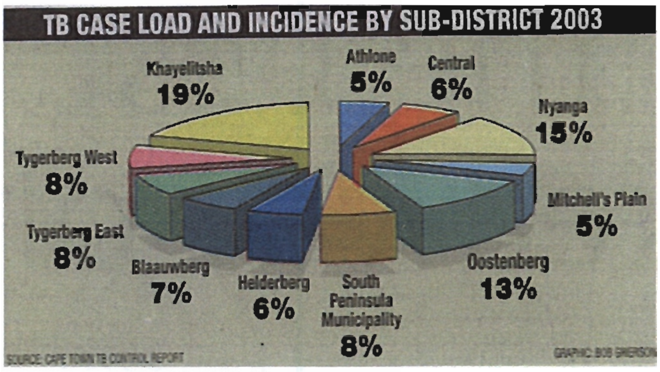
Actinomycetes are routinely screened for new bioactive substances as they have provided many bioactive compounds of great value, including many of medical importance. Presently, there is a severe lack of discovery of novel bioactive compounds, coupled with the growing problem of bacterial resistance to antibiotics used to treat medically important diseases. In a world that expects effective antibiotics available for every disease, the rapid spread of antibiotic resistant bacteria and the threat of bioterrorism are of grave concern (1, 21). Furthermore, inattentiveness to the importance of the need for new antibiotics to treat bacterial infections and the departure of many large pharmaceutical companies from the field of antibiotic development has added to the problem (18, 21). Actinomycetes are abundant in terrestrial soils, a source of the majority of isolates shown to provide bioactive compounds (20). Expanding the source of actinomycetes by carrying out ecological assessments of environments other than terrestrial soils, and investigating the rare, nonstreptomycete genera of actinomycetes as sources of novel compounds, may aid in the discovery of novel bioactive compounds, and help in addressing the problem of bacterial resistance (20). The marine environment is a bountiful resource for the isolation of less exploited microorganisms; marine-derived microorganisms exist in untapped habitats with unique characteristics. The potential contribution of marine sources to the discovery of novel bioactive compounds has been recognized and has prompted the search for new antibiotic-producing microorganisms from marine habitats including marine sediments and organisms (see 8, 15, 19, 20).

The isolation of actinomycin from *Streptomyces antibioticus* in 1940 stimulated extensive screening for bioactive compounds, with proof of novelty of antibiotics frequently resting on the description of the producer as a new species (14, 22). Furthermore, different species and different individuals within a species are distinguished by variations among their organic molecules (3). Hence, screening for the production of new metabolites with new isolates and/or new test methods is the only way to obtain completely new classes of substances (5).

## 1.2 Tuberculosis, a Heavy Burden

The emergence of multidrug-resistant (MDR) bacteria is a phenomenon of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases (6). In the 1980s, pharmaceutical manufacturers believed that infectious diseases were essentially conquered (13). Consequently, the search for additional antibiotics was no longer a priority, and if one drug failed, another in the arsenal would usually work. Now we are facing the threat of increasing bacterial resistance to many antibiotics that once readily cured bacterial diseases. Researchers are searching for novel antibiotics again, however, the few that may reach the market are usually structurally similar to existing antibiotics and could easily encounter bacteria that already have resistance to them (13).

According to a recent report compiled by the World Health Organization (WHO; 24), the total number of new cases of tuberculosis (TB) worldwide in 2002 had risen to approximately 9 million (7). A key driving force of this increase is synergy with the HIV epidemic, and the emergence of strains of *Mycobacterium tuberculosis* (MDR-TB) resistant to all of the first-line drugs in use (7). New drugs are urgently needed with activity against MDR-TB and latent TB, and which would help shorten the duration of treatment from the current 6 to 8 months, to two months or less (7).



SOURCE: CAPE TOWN TB CONTROL REPORT

GRAPHIC: BOB SNERSON

Fig. 1.1. TB infection statistics for the greater Cape Town area, Western Cape, South Africa (2).

Drug resistance poses a serious threat to tuberculosis control programmes in the Western Cape province of South Africa, contributing to the increasing tuberculosis epidemic in the region (23). Tuberculosis (TB) accounts for more than 80% of all communicable diseases notified in South Africa (23). The Western Cape has the ninth highest incidence of TB in the world (2, 24), and in some areas, at least 1 person in every 3 households has had an infectious case of TB in the last 10 years (Fig. 1.1) (2). Cape Town's most recent TB control report indicates that TB is one of the major contributors to the city's burden of disease, with a 66% increase in the number of reported TB cases over the past 6 years (2). An increase in the number of drug-resistant TB cases (especially MDR-TB cases) has been observed both locally and internationally due to ineffective treatment, patient non-compliance, and to the spread of HIV.

The advent of the HIV epidemic in sub-Saharan Africa has seen not only a dramatic increase in the incidence of TB cases in the region, but also a dramatic increase in the number of people dying from what is a curable disease. The HIV epidemic has fuelled the TB epidemic in the region. In the Western Cape, TB is responsible for a third of all deaths in HIV-infected people (2). In the poorer, developing nations, 95% of TB cases occur, 98% of TB deaths occur, and additionally, sub-Saharan Africa is home to over 67% of the HIV-infected people in the world (16). In 1999 it was estimated that 68% of the people worldwide co-infected with HIV and *M. tuberculosis* are living in this region (16). Furthermore, these co-infected people have at least a 30% lifetime risk of developing active TB (16). HIV-compromised immunity increases the susceptibility of individuals to develop TB either from a reactivation of latent infection or a rapid progression of recent infection by the opportunistic *M. tuberculosis* (16). Insidiously, the host's immune response to *M. tuberculosis* enhances HIV replication and might accelerate the natural progression of HIV infection (16). Although education and prevention do help to combat TB, novel antitubercular antibiotics are required to combat the threat posed by MDR *M. tuberculosis* strains.

### 1.3 The Aims of This Study

Selective isolation and enumeration techniques were used to isolate strains of the actinomycete genus *Micromonospora* from unexplored marine habitats, and these strains were characterized. In order to make identification to the species level a quicker process, a simple identification system for *Micromonospora* species needed to be created. The secondary metabolite capabilities of the strains were investigated, not only by investigating the physicochemical properties of the antibiotics produced during fermentation, but also by investigating the biosynthetic capability of the strains at the genetic level.

Importantly, antibiotics from newly discovered strains were tested for antimycobacterial activity, in the search for novel anti-TB drugs.

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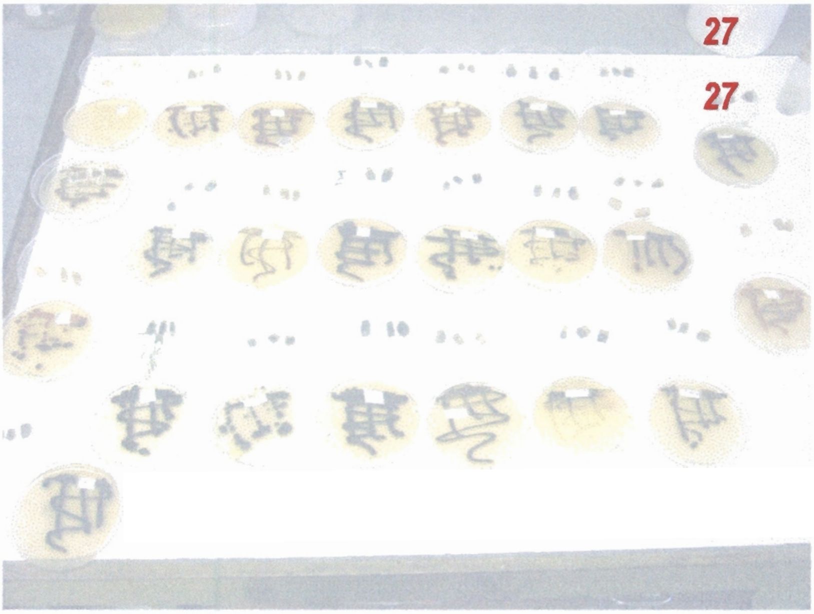
# Chapter 2

# *Micromonospora* Identification and Systematics



# Chapter 2

# *Micromonospora* Identification and Systematics

## 2.1 Introduction: The *Micromonospora* Identification Key

Modern bacterial systematics makes use of a polyphasic approach towards taxonomic determinations. This means that an isolate's physiological characters, further descriptive information (such as chemotaxonomy and morphological features by scanning electron microscopy, in the case of actinomycetes), and 16S rDNA sequence analysis all contribute to determining the novelty of an isolate.

Scanning electron microscopy has revealed that the spores of almost all *Micromonospora* species, including those having been described as having a smooth spore surface, are blunt spiny surfaced (7). It is the size of the blunt spiny projections that shows variation. Unlike the genus *Streptomyces*, the spore surface ornamentation is not a useful diagnostic aid for differentiation of *Micromonospora* species.

Chemotaxonomy, at most, allows one to identify bacteria to the genus level and thus is of little use for differentiating *Micromonospora* species from each other. The cell wall type, whole cell sugar pattern, phospholipid type, fatty acid methyl esters, and the absence of mycolic acids, are similar for all *Micromonospora* species. Perhaps the only useful chemotaxonomic diagnostic aid, are the major menaquinones. Menaquinones with 9, 10, or 12 isoprene units are found in *Micromonospora* strains. The subspecies, *Micromonospora echinospora* subsp. *pallida* is the only *Micromonospora* strain possessing major menaquinones with 12 isoprene units, and thus it was suggested that this subspecies be transferred from *M. echinospora* to new species status (7). However, it was as a result of DNA-DNA hybridisation experiments (6) that *M. echinospora* subsp. *pallida* was elevated to species level, viz. *M. pallida*.

Thus, if one were to disregard scanning electron microscopy and chemotaxonomy in determining the novelty of an isolate, what remains is 16S rDNA sequence analysis. However, if an isolate's 16S rDNA sequence shows greater than 97.5% similarity to another strain, it is recommended that DNA-DNA reassociation (hybridisation) experiments be performed to determine whether the strains belong to the same species (12). Strains that have a DNA relatedness of 70% or more by DNA-DNA hybridisation are considered to belong to the same species (14). Few laboratories are set up for DNA-DNA hybridisation experiments, and having the experiments performed by an outside facility is very costly. Furthermore, when DNA-DNA hybridisation experiments are carried out, two strains sharing 99% 16S rDNA sequence homology often do not show more than 60% DNA similarity, and thus they are not related at the species level (12).

When screening environmental samples for new actinomycete species, the novelty of an isolate can be suggested by the sequencing of the isolate's 16S rDNA (a sequence of more than 1450 nucleotides is sufficient, as it represents more that 90% of the length of 16S rRNA genes). However, even a short, partial 16S rDNA sequence is enough to inform a researcher of the genus to which an isolate belongs. For actinomycete researchers in developing countries, such as South Africa, there are difficulties, such as limited financial and technological resources. Due to the number of potentially novel actinomycete isolates obtained from environmental samples, it would be extremely costly to obtain such sequences for all the isolates. The return on effort could be dismal, if it is found that strains of existing species have been re-isolated.

A less expensive option is to examine an isolate's physiological characters. The genus *Streptomyces* has 139 recommended characters that could be examined (8). The genus *Micromonospora* could also be examined to such an extent. However, it is unnecessary to use so many characters to determine the novelty of environmental *Micromonospora* isolates. A few discerning characters can be used.

Using physiological testing to discern quickly between species may be the answer to the problem. Having a defined set of physiological characters to examine would be inexpensive, easy to perform, and could allow one to differentiate between *Micromonospora* isolates. It would be important that for these physiological characters to be tested, the media used be standardized and that every strain be tested on the specific standardized medium for a specific physiological character. Furthermore, the selected tests should allow one to determine whether a newly isolated strain is likely to be a strain of a known species, or a probable new species.

A dichotomous key for the identification of *Micromonospora* strains was developed to allow one to determine easily and inexpensively the novelty of a *Micromonospora* isolate in a period of 21 days from the time of inoculation of the test media. Due to the lack of attention attributed to the genus *Micromonospora*, standardized media were carefully chosen, or developed, for each physiological test to be performed for the proposed identification key.

## 2.2 Materials and Methods

### 2.2.1 Strains Used in This Study

*Micromonospora aurantiaca* NRRL B-16091$^T$, *Micromonospora carbonacea* subsp. *carbonacea* NRRL 2972$^T$, *Micromonospora chalcea* subsp. *chalcea* NRRL B-2344$^T$, *Micromonospora echinospora* subsp. *echinospora* NRRL 2985$^T$, *Micromonospora halophytica* subsp. *halophytica* NRRL 2998$^T$, *Micromonospora inositola* NRRL B-16095$^T$, *Micromonospora nigra* NRRL 3097$^T$, *Micromonospora olivasterospora* NRRL 8178$^T$, *Micromonospora echinospora* subsp. *pallida* NRRL 2996$^T$, *Micromonospora purpureochromogenes* NRRL B-16094$^T$, and *Micromonospora rosaria* NRRL 3718$^T$ were kindly provided by Dr David P. Labeda, curator of the Actinobacterial Culture Collection at the U.S. Department of Agriculture Agricultural Research Service, Peoria, Illinois, United States of America. *Micromonospora chersina* DSM 44151$^T$, *Micromonospora coerulea* DSM 43143$^T$, and *Micromonospora matsumotoense* DSM 44100$^T$ were purchased from the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Micromonospora endolithica* strain AA-459$^T$ was kindly provided by Prof. emer. Dr. Peter Hirsch, Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität Kiel, Germany. *Micromonospora auratinigra* could not be obtained and thus information from the publication describing this species was used (13).

### 2.2.2 Physiological Testing and Media Used Specifically for the *Micromonospora* Identification Key (Table 2.1)

All cultures were incubated at 28°C – 30°C (unless otherwise stated) for 3 weeks, and were examined at 7, 14, and 21 days. Maximum sodium chloride tolerance (% w/v) was performed as recommended by Locci (8) on Bennett's agar (BM) (1), containing (g/L): glucose (10.0), casitone (Difco; 2.0), yeast extract (1.0), beef extract (1.0), agar (20.0), pH 7.0, and the required amount of sodium chloride. Growth at 37°C was determined on Bennett's agar as recommended by Locci (8). The production of diffusible pigments were tested on *ISP* Medium No. 2 (11), Bennett's-glycerol agar (10 g/L glucose was replaced with 10 g/L glycerol), and on modified 172F agar, containing (g/L): glucose (10.0), yeast extract (5.0), starch (10.0), casitone (Difco; 5.0), $MgSO_4{\cdot}7H_2O$ (2.5), $CaSO_4{\cdot}2H_2O$ (2.0), agar (15.0), pH 7.2. The utilization of compounds as sole carbon sources (1.0% w/v) was performed as recommended by Locci (8) and Shirling and Gottlieb (11). Growth on *ISP* Medium No. 4 was determined according to the methods of Shirling and Gottlieb (11).

Table 2.1. A list of selected physiological tests to be performed and the corresponding standardized media to be used for identification of *Micromonospora* strains.

| Physiological Characters to be Tested: | Standardized Media to be Used (Abbreviations in parentheses): |
|---|---|
| Maximum Sodium Chloride Tolerance (% w/v):<br>1.5%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, and 7.0%.<br>Growth at 37°C. | Bennett's Agar (BM-Glucose) |
| Production of Diffusible Pigments and,<br>Diagnostic Mycelial Pigment Colour. | Bennett's Agar, with glucose replaced by glycerol (BM-Glycerol);<br>*ISP* Medium No. 2 Agar (YEME);<br>Modified 172F Agar (m172F). |
| Growth and Colour of Mycelia. | *ISP* Medium No. 4 Agar (*ISP*4) |
| Utilization of Glycerol (1.0% w/v) and *myo*-Inositol (1.0% w/v) as Sole Carbon Sources [a negative control containing no carbon source, and a positive control, glucose (1.0% w/v) must be included]. | *ISP* Medium No. 9 Agar (*ISP*9) |

### 2.2.3 Other Physiological Tests, Morphological Observations, and Anaerobic Growth Studies

International *Streptomyces* Project (*ISP*) media were prepared according to the methods of Shirling and Gottlieb (11). Controls were used for each test: positive controls, negative controls and/or uninoculated controls. *Micromonospora* morphological characteristics were determined on YEME, Bennett's agar (1), and modified 172F agar. Production of diffusible pigments by *Micromonospora* strains was tested on YEME, Bennett's agar, modified 172F agar, and glycerol-asparagine agar (*ISP* Medium No. 5). The pH sensitivity of both the substrate mycelium and diffusible pigments was assessed by noting any colour changes induced by the addition of 50 mM HCl or 50 mM NaOH.

Physiological characteristics were determined as recommended by Locci (8). For *Micromonospora* strains, growth was determined on inorganic salts-starch agar (*ISP* Medium No. 4), Middlebrook 7H9 agar [(Difco Laboratories, Detroit, Michigan) AD supplement was not added, but glucose was added to a final concentration of 10 mM], Czapek-solution agar (1), and on potato slice and potato slice plus $CaCO_3$. Potatoes were bought from a local supermarket and sliced. Half of the sliced potatoes were autoclaved in a container, and the other half were autoclaved in another container after the addition of $CaCO_3$ to the slices.

Growth at 4°C, 37°C, 45°C, at pH 4.3, and in the presence of 0.1% 2-phenylethanol was determined as recommended by Locci (8) and was tested on Bennett's agar. Degradation of adenine, allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, L-tyrosine, xanthine, and xylan was determined as recommended by Locci (8). Tween 80 degradation was determined according to Sierra (10), and degradation of urea was determined according to Gordon *et al.* (3). Melanin pigment production was determined on peptone-yeast extract-iron agar (*ISP* Medium No. 6) and on tyrosine agar (*ISP* Medium No. 7). Hydrolysis of aesculin, arbutin, hippurate, and pectin, $H_2S$ production, and nitrate reduction were determined as recommended by Locci (8).

Antibiotic resistance was determined by incorporation of the antibiotics into Bennett's agar plates (1). Sole nitrogen sources were tested at a concentration of 0.1% (w/v), as recommended by Locci (8).

All tests were carried out at 28 – 30°C (except for testing growth at 4, 37 and 45°C). *Micromonospora* strains were incubated for 21 days (except for degradation of starch and gelatin, and nitrate reduction, which were incubated for 7 days and 14 days, respectively).

Anaerobic growth of *Micromonospora* strains was tested on the following media: BM-Glycerol agar, modified 172F agar, YEME agar, and *ISP* Medium No. 9 with sucrose (1.0%) as the sole carbon source. A second set of plates of BM-Glycerol agar and *ISP* Medium No. 9 containing sucrose were made with the addition of an indicator, bromocresol purple, as recommended by Gordon *et al.* (3) to detect acid production. This indicator is purple above pH 5.5 and is yellow below pH 5.5 (4). Plates were inoculated in duplicate: one set of plates was incubated under aerobic conditions at 37°C, while the other set was incubated under anaerobic conditions at 37°C in an anaerobic chamber (Forma Scientific, Model 1024) in an atmosphere of 5% $H_2$, 10% $CO_2$, and 85% $N_2$. An incubation temperature of 37°C was employed, as this was the incubator temperature in the anaerobic chamber.

## 2.3 Results and Discussion

### 2.3.1 The Identification Key

The Identification Key is presented in Figure 2.1 and Tables 2.2 – 2.5. The Key is based on the 16 validly published members of the genus *Micromonospora* as at December 20, 2004 (2).

Before using the *Micromonospora* Identification Key, strains must be identified as members of the genus. Environmental isolates were identified as strains of the genus *Micromonospora* on the basis of colony morphology and ARDRA (see 3.2.6).

Maximum sodium chloride tolerance is a very important physiological characteristic of the genus *Micromonospora* as this is the character that most readily differentiates strains into groups. The diagnostic mycelial pigment colour, as well as diffusible pigment colour, are also very significant characters. Since the mycelial pigment colour, prior to sporulation, of the majority of *Micromonospora* strains, is orange, a different mycelial pigment colour is very useful in distinguishing species. The diagnostic mycelial pigment colours of *M. coerulea, M. echinospora, M. olivasterospora*, and *M. pallida*, and the diffusible pigments of *M. aurantiaca*, *M. carbonacea*, *M. chalcea*, *M. halophytica*, *M. olivasterospora*, *M. purpureochromogenes*, and *M. rosaria*, are well-known by taxonomists as easily identifiable characters of these species. To date, *M. inositola* is the only *Micromonospora* species able to utilize inositol as a sole carbon source, and *M. purpureochromogenes* is the only species able to utilize glycerol as a sole carbon source. Thus, it was important that these significant characters be included in the identification key. *M. matsumotoense* is the only species of the 16 members that does not sporulate. This is also a significant determinant and was included in the identification key.

| **1. Maximum Sodium Chloride Tolerance (% w/v) is:** | | | | |
|---|---|---|---|---|
| **1.5%:** | **2.0%:** | **3.0%:** | **4.0%:** | **Less than 1.5% Or greater than 4.0%:** |
| If YES, go to Table 2.2. | If YES, go to Table 2.3. | If YES, go to Table 2.4. | If YES, go to Table 2.5. | If YES: Probable New Species |
| **VPM of the '1.5% Group':**<br>*M. echinospora*<br>*M. purpureochromogenes* | **VPM of the '2.0% Group':**<br>*M. auratinigra*<br>*M. chersina*<br>*M. coerulea*<br>*M. endolithica*<br>*M. inositola*<br>*M. matsumotoense* | **VPM of the '3.0% Group':**<br>*M. aurantiaca*<br>*M. carbonacea*<br>*M. nigra*<br>*M. olivasterospora*<br>*M. pallida*<br>*M. rosaria* | **VPM of the '4.0% Group':**<br>*M. chalcea*<br>*M. halophytica* | |

Fig. 2.1. The crucial first step of the identification key is to determine an isolate's maximum sodium chloride tolerance on BM-Glucose. Illustrated in this scheme is to which 'maximum sodium chloride tolerance group', the validly published members (VPM) of *Micromonospora* belong.

Table 2.2. Dichotomous key for the identification of *Micromonospora* strains of the '1.5% NaCl Group'.

| | |
|---|---|
| 2.1 Is the diagnostic mycelial pigment a **purple** colour on BM-Glucose? | |
| (a) Yes | Probably *M. echinospora*. |
| (b) No | Go to 2.2. |
| 2.2 Produces a ***dark brown*** diffusible pigment on BM-Glucose? | |
| (a) Yes | Go to 2.3. |
| (b) No | Probable New Species. |
| 2.3 Is glycerol (1.0% w/v) utilized as a sole carbon source? | |
| (a) Yes | Probably *M. purpureochromogenes*. |
| (b) No | Probable New Species. |

Table 2.3. Dichotomous key for the identification of *Micromonospora* strains of the '2.0% NaCl Group'.

| | |
|---|---|
| 3.1 Growth on *ISP*4? | |
| (a) Yes | Probably *M. auratinigra.* |
| (b) No | Go to 3.2. |
| 3.2 Is there an absence of sporulation? | |
| (a) Yes | Probably *M. matsumotoense*. |
| (b) No | Go to 3.3. |
| 3.3 Is the diagnostic mycelial pigment a **blue-black** colour? | |
| (a) Yes | Probably *M. coerulea.* |
| (b) No | Go to 3.4. |
| 3.4 Is *myo*-inositol (1.0% w/v) utilized as a sole carbon source? | |
| (a) Yes | Probably *M. inositola.* |
| (b) No | Go to 3.5. |
| 3.5 Growth at 37°C? | |
| (a) Yes | Probably *M. endolithica.* |
| (b) No | Probably *M. chersina.* |

Table 2.4. Dichotomous key for the identification of *Micromonospora* strains of the '3.0% NaCl Group'.

4.1 Produces a bright yellow diffusible pigment on m172F?

| | |
|---|---|
| (a) Yes | Probably *M. aurantiaca*. |
| (b) No | Go to 4.2. |

4.2 Produces a **brown** (but *not* reddish-brown) diffusible pigment on m172F?

| | |
|---|---|
| (a) Yes | Probably *M. carbonacea*. |
| (b) No | Go to 4.3. |

4.3.Produces a **wine-red** diffusible pigment on m172F **and** YEME agars?

| | |
|---|---|
| (a) Yes | Probably *M. rosaria*. |
| (b) No | Go to 4.4. |

4.4 Is the diagnostic mycelial pigment an **olive-green** colour on BM-Glucose?

| | |
|---|---|
| (a) Yes | Go to 4.5. |
| (b) No | Go to 4.6. |

4.5 Produces an **olive-green** diffusible pigment on BM-Glucose **and** YEME agars?

| | |
|---|---|
| (a) Yes | Probably *M. olivasterospora*. |
| (b) No | Probable New Species. |

4.6 Is the diagnostic mycelial pigment a **pale white/ivory** colour on BM-Glucose?

| | |
|---|---|
| (a) Yes | Probably *M. pallida*. |
| (b) No | Go to 4.7. |

4.7 Grows on *ISP*4 producing **orange** mycelia?

| | |
|---|---|
| (a) Yes | Probably *M. nigra*. |
| (b) No | Probable New Species. |

Table 2.5. Dichotomous key for the identification of *Micromonospora* strains of the '4.0% NaCl Group'.

| | |
|---|---|
| 5.1 Produces a yellow diffusible pigment on m172F? | |
| (a) Yes | Probably *M. chalcea*. |
| (b) No | Go to 5.2. |
| 5.2 Produces a **reddish-brown** diffusible pigment on m172F? | |
| (a) Yes | Probably *M. halophytica*. |
| (b) No | Probable New Species. |

### 2.3.2 Additional Characteristics of the Validly Published *Micromonospora* Species

Tables 2.6 and 2.7 show the results of the tests performed on the validly published members of the genus *Micromonospora*. Many of the published descriptions do not fully examine the physiological characteristics of the species and thus many of the characteristics shown and discussed in this section have not been widely used in *Micromonospora* taxonomy. These tests, listed in Table 2.6, will aid in differentiating between species. The results of tests that have been previously used in taxonomy agree with published data. Maximum sodium chloride tolerance tests were performed three times and do not correlate with previously published values for all species as the media used to assess the sodium chloride tolerance varied between publications. In this study, standard media were used to assess the maximum sodium chloride tolerance and the production of diffusible pigments (see Table 2.1).

It must be noted that potatoes are acidic and have a pH between 5.5 and 6.5 depending on the type of potato used (9). Calcium carbonate is added to neutralize the acidity.

*Micromonospora coerulea* is said to have a blue-green diagnostic mycelial pigment, however *M. coerulea* DSM 43143$^T$ appeared blue-black on all media examined.

The red diffusible pigment produced by *M. pallida* mentioned in Bergey's Manual of Systematic Bacteriology Volume 4, was only seen on *ISP* Medium No. 7 (the red pigment is not melanin, which is brown or black in colour).

The brown-red pigment typically seen during tyrosine degradation was produced by *M. aurantiaca*, *M. echinospora*, and *M. pallida*. However, no pigment was produced by *M. nigra*, which strongly degraded tyrosine.

The diffusible pigments and substrate mycelial pigments were tested for sensitivity to acid and base. The diffusible pigment and the substrate mycelium of *M. halophytica* changed colour from reddish-brown to yellow on addition of 50 mM HCl and 50 mM NaOH. The purple coloured substrate mycelium of *M. echinospora* changed to blue upon addition of NaOH and to pink upon addition of HCl. The olive-green substrate of *M. olivasterospora* (grown on Bennett's and modified 172F agars) changed to a light green with NaOH and to blue with HCl. The substrate mycelium of *M. purpureochromogenes* (grown on Bennett's agar) changed from brown to maroon upon addition of NaOH. On YEME agar, the wine-red diffusible pigment characteristic of *M. rosaria* changed colour to yellow upon addition of HCl.

If one examines the published descriptions of the current 16 validly published members of the genus, only *M. rosaria* is said to have tufts or "blooms" of grey aerial mycelia (5). However, many strains were found to produce aerial mycelia, which appeared in very visible tufts close to the periphery of the growing strains. This was more closely examined with a light microscope. The agar medium, modified 172F, appears to be a very good medium for examining the morphological features of the *Micromonospora* species and seemed to promote the appearance of aerial mycelia. Thus on m172F agar, *M. rosaria* produces grey-white aerial mycelia, and *M. nigra* and *M. carbonacea* produce grey aerial mycelia.

Some *Micromonospora* species were tested on other media not listed in the tables. The additional taxonomic features are:

### (i) ***Micromonospora chalcea***

No diffusible pigment is produced on *ISP* Medium No. 5, nor is melanin produced on *ISP* Media No. 6 and No. 7. *M. chalcea* grows well on Middlebrook 7H9 agar and at pH 4.3 but does not grow in the presence of 0.1% 2-phenylethanol. Pectin is weakly hydrolysed and hippurate is not hydrolysed. Gelatin and Tween 80 are degraded, while adenine, allantoin, cellulose, guanine, hypoxanthine, urea, and xanthine are not. Xylan is weakly degraded and casein is very strongly degraded.

### (ii) ***Micromonospora endolithica***

*M. endolithica* was isolated from the Antarctic, thus testing was carried out at 4, 20, 30 and 37°C to find an optimal temperature for growth. Interestingly, the species does not grow on Bennett's agar, modified 172F or YEME agars at 4°C. Moderate growth was observed at 20°C, excellent growth was observed at 30 and 37°C, and no growth was observed at 45°C on BM-Glucose agar. The maximum sodium chloride tolerance is 2.0% (tested at 20 and 30°C). The species does not grow on *ISP* Medium No. 4 and does not produce diffusible pigments on BM-Glucose, YEME or m172F agars.

### (iii) ***Micromonospora inositola***

A diffusible pigment is not produced on *ISP* Medium No. 5, nor is melanin produced on *ISP* Media No. 6 and No. 7. The species grows well on Middlebrook 7H9 agar, but not at pH 4.3 or in the presence of 0.1% 2-phenylethanol. Hippurate and pectin are not hydrolysed. Casein, gelatin, and Tween 80 are degraded, but adenine, allantoin, cellulose, guanine, hypoxanthine, urea, xanthine, and xylan are not.

### (iv) ***Micromonospora nigra***

*M. nigra* does not produce a diffusible pigment on *ISP* Medium No. 5. The species does not grow at pH 4.3, and grows very weakly on Middlebrook 7H9 agar. Melanin is not produced on *ISP* Media No. 6 and No. 7. Starch is hydrolysed, gelatin is degraded but cellulose is not.

### (v) ***Micromonospora pallida***

*M. pallida* does not produce a diffusible pigment on *ISP* Medium No. 5, nor does the species produce melanin on *ISP* Media No. 6 and No. 7. However, a red pigment is produced on *ISP* Medium No. 7. The species does not grow at pH 4.3, and grows very weakly on Middlebrook 7H9 agar. Starch is hydrolysed, gelatin is degraded but cellulose is not.

**(vi) *Micromonospora purpureochromogenes***

No diffusible pigment is produced on *ISP* Medium No. 5, and melanin is not produced on *ISP* Media No. 6 and No. 7. The species grows weakly on Middlebrook 7H9 agar, and does not grow at pH 4.3 or in the presence of 0.1% 2-phenylethanol. Hippurate and pectin are not hydrolysed. Casein, gelatin, and Tween 80 are degraded, but adenine, allantoin, cellulose, guanine, hypoxanthine, urea, xanthine, and xylan are not.

Table 2.6. Physiological characteristics of 14 of the validly published *Micromonospora* species.

| **Characteristics:** | *M. aurantiaca* | *M. carbonacea* | *M. chalcea* | *M. chersina* | *M. coerulea* | *M. echinospora* | *M. halophytica* | *M. inositola* | *M. matsumotoense* | *M. nigra* | *M. olivasterospora* | *M. pallida* | *M. purpureochromogenes* | *M. rosaria* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maximum NaCl Tolerance (%, w/v) | 3.0 | 3.0 | 4.0 | 2.0 | 2.0 | 1.5 | 4.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.0 | 1.5 | 3.0 |
| Nitrate reduction | s+ | – | s+ | – | – | – | s+ | + | + | s+ | s+ | – | s+ | – |
| $H_2S$ production | s+ | s+ | + | – | – | s+ | – | + | + | s+ | + | + | – | s+ |
| L-Tyrosine degradation | s+ | – | + | – | – | s+ | – | + | – | s+ | – | + | – | – |
| Growth at: | | | | | | | | | | | | | | |
| 4°C | + | – | – | – | – | – | – | – | + | – | – | – | – | – |
| 37°C | s+ | s+ | s+ | – | – | s+ | + | + | – | s+ | s+ | s+ | – | s+ |
| 45°C | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Growth on: | | | | | | | | | | | | | | |
| Potato slice | + | – | + | – | – | – | – | + | – | + | – | + | + | – |
| Potato slice plus $CaCO_3$ | + | – | + | – | – | – | – | + | – | + | – | + | + | – |
| Czapek-solution agar | + | – | – | – | – | + | + | – | – | + | + | + | + | + |
| Antibiotic Susceptibility: | | | | | | | | | | | | | | |
| Cephaloridine (100 μg/ml) | – | – | – | – | – | – | + | – | – | – | – | – | – | – |
| Lincomycin (100 μg/ml) | – | – | – | – | – | – | – | – | – | – | – | – | – | + |
| Oleandomycin (100 μg/ml) | + | – | + | – | – | + | + | – | – | – | – | + | – | + |
| Penicillin G (10 I.U.) | – | – | + | – | – | – | + | – | – | + | + | + | – | – |
| Rifampicin (50 μg/ml) | – | – | – | – | – | – | + | – | – | – | – | – | – | – |
| Tobramycin (50 μg/ml) | – | – | – | – | – | – | – | – | – | – | + | – | – | – |
| Vancomycin (50 μg/ml) | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Abbreviations: –, negative result/no growth; +, positive result/growth; s, strongly.

Table 2.7. Sole carbon and nitrogen source utilization of 14 of the validly published *Micromonospora* species.

| **Characteristic:** | *M. aurantiaca* | *M. carbonacea* | *M. chalcea* | *M. chersina* | *M. coerulea* | *M. echinospora* | *M. halophytica* | *M. inositola* | *M. matsumotoense* | *M. nigra* | *M. olivasterospora* | *M. pallida* | *M. purpureochromogenes* | *M. rosaria* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon Sources: | | | | | | | | | | | | | | |
| D(+)-Glucose | + | +++ | +++ | + | + | + | + | + | + | + | +++ | + | + | + |
| Adonitol | + | + | + | + | + | + | – | + | – | – | + | + | – | – |
| L(+)-Arabinose | + | + | + | + | – | + | + | + | + | + | ++ | + | + | + |
| Glycerol | – | – | – | – | – | – | – | – | – | – | – | – | + | – |
| *myo*-Inositol | – | – | – | – | – | – | – | + | – | – | – | – | – | – |
| α-Melibiose | + | +++ | +++ | – | – | – | + | + | + | + | + | + | + | + |
| D(+)-Melezitose | + | – | + | + | – | – | – | – | – | – | – | – | – | – |
| L(+)-Rhamnose | + | – | – | – | – | + | – | – | – | – | – | + | – | + |
| D(–)-Ribose | + | – | – | – | – | – | – | – | + | – | – | + | – | + |
| Sucrose | + | + | + | + | + | + | + | + | + | + | – | + | + | + |
| Nitrogen Sources: | | | | | | | | | | | | | | |
| L-Arginine | + | +++ | +++ | – | + | +++ | – | – | – | + | ++ | – | – | – |
| DL-Ornithine | + | +++ | +++ | + | + | + | + | + | + | +++ | +++ | + | + | + |
| L-Serine | + | ++ | ++ | + | + | ++ | + | ++ | + | ++ | ++ | ++ | ++ | ++ |
| L-Threonine | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

Abbreviations: –, no growth/no utilization; +, weak growth/utilization; ++, moderate growth/utilization; +++, good growth/utilization.

### 2.3.3 Anaerobic Studies on the Validly Published *Micromonospora* Species

The results of the anaerobic studies on the validly published *Micromonospora* species are shown in Table 2.8. *Micromonospora endolithica* was tested on two media (BM-Glycerol and sucrose agars) and did not grow under anaerobic conditions. It could be assumed that *M. endolithica* cannot grow under anaerobic conditions, but the strain should be incubated on other media to reveal if this is truly the case. There was no pattern regarding the changing of the colour of the indicator plates: very few strains produced acid to change the colour of the plates and those same strains did not produce acid under both aerobic and anaerobic conditions. Complex (BM-Glycerol, m172F, and YEME agars) and defined (sucrose agar) media were chosen and in general the degree of growth was the same or poorer under anaerobic conditions as compared to aerobic conditions. Of further interest is that the strains grew well under anaerobic conditions for 14 days, but thereafter growth stopped and the orange mycelial pigments disappeared/faded over the next 7 days. This suggests that the strains may have been using endogenous metabolism for survival due to nutrient depletion after 14 days.

Filamentous actinomycetes are generally regarded and treated as aerobes, and thus these findings are very interesting. This was a preliminary investigation into the anaerobic growth of members of the genus *Micromonospora*, and the results do not allow determination of whether the facultatively anaerobic *Micromonospora* species grew by anaerobic respiration or fermentation.

Table 2.8. Anaerobic and aerobic growth of 15 of the validly published *Micromonospora* species grown on different media, with or without an indicator, at 37°C.

| | BM-Glycerol | | BM-Glycerol with Indicator | | Sucrose as Sole Carbon Source | | Sucrose as Sole Carbon Source with Indicator | | m172F | | YEME | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Micromonospora* species:** | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: |
| *M. aurantiaca* | +++ | + | P | P | ++ | + | P | P | +++ | + | ++ | + |
| *M. carbonacea* | +++ | + | P | Y | +++ | + | Y | P | +++ | + | ++ | + |
| *M. chalcea* | + | + | P | P | +++ | +++ | P | P | +++ | + | ++ | + |
| *M. chersina* | + | – | P | P | – | – | P | P | +++ | + | ++ | – |
| *M. coerulea* | – | – | P | P | – | + | P | P | – | – | ++ | – |
| *M. echinospora* | +++ | + | P | P | +++ | – | P | P | +++ | + | ++ | + |
| *M. endolithica* | +++ | – | P | P | + | – | P | P | N.T. | N.T. | N.T. | N.T. |
| *M. halophytica* | +++ | + | P | P | – | – | P | P | ++ | + | + | – |
| *M. inositola* | +++ | + | P | Y | – | – | P | P | +++ | + | ++ | + |
| *M. matsumotoense* | – | – | P | P | – | + | P | P | – | – | ++ | – |
| *M. nigra* | +++ | + | P | Y | – | + | P | P | +++ | + | ++ | + |
| *M. olivasterospora* | +++ | + | P | P | +++ | + | P | P | +++ | + | ++ | + |
| *M. pallida* | +++ | + | P | P | ++ | + | P | P | +++ | + | ++ | + |
| *M. purpureochromogenes* | +++ | – | Y | P | – | – | P | P | – | + | ++ | – |
| *M. rosaria* | +++ | – | Y | P | ++ | – | P | P | +++ | + | ++ | – |

Abbreviations: –, no growth; +, weak growth; ++, moderate growth; +++, good growth; P, indicator remained unchanged (purple in colour); Y, indicator changed in colour to yellow; N.T., not tested.

## 2.4 Conclusion

This study has shown that a *Micromonospora* Identification Key based on a small number of phenetic characteristics is an easy and inexpensive method to determine the potential novelty of *Micromonospora* isolates obtained from environmental samples. As a result of this study, standardized media have been developed for testing maximum sodium chloride tolerance and for the production of diffusible pigments by *Micromonospora* strains. This Identification Key is proposed to be used to decide whether an isolate is worth investigating further, and is thus not intended as a stand-alone taxonomic tool. It is recommended that when *Micromonospora* strains are isolated, the isolates' sodium chloride tolerance be determined first. If the maximum sodium chloride tolerance is less than 1.5% or greater than 4.0%, the isolate is presumptively identified as a novel species and is worth investigating further. If the maximum sodium chloride tolerance is 1.5%, 2.0%, 3.0%, or 4.0%, the other physiological tests, indicated in Table 2.1, should be carried out.

It must also be emphasized that a strain having a diagnostic mycelial pigment colour other than orange is significant. If a strain is isolated with an olive-green mycelial pigment colour and its maximum sodium chloride tolerance is *not* 3.0%, it cannot be *M. olivasterospora* according to the Identification Key, and thus is a potentially novel species. *M. olivasterospora* is currently the only species having an olive-green mycelial pigment.

The additional physiological tests performed on the validly published *Micromonospora* members provide more characters to be used for species delineation thus allowing a more comprehensive description of new species. Results of growth at 4°C, 45°C, antibiotic susceptibility testing, hydrolysis and degradation testing, growth on Middlebrook 7H9 agar and growth in the presence of 0.1% 2-phenylethanol, are physiological tests not reported for species of *Micromonospora*.

It is of great interest that 14 of the 15 members of the genus *Micromonospora* grew under anaerobic conditions, however it is unknown whether anaerobic respiration or fermentation is used for growth.

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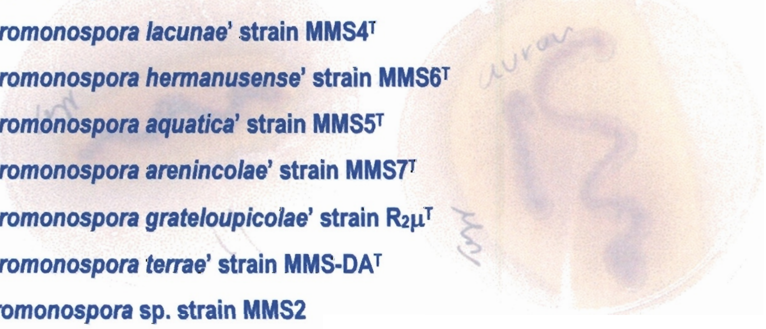
# Chapter 3

# Isolation, Enumeration, and Systematics of Actinomycete Strains



# Chapter 3

## Isolation, Enumeration, and Systematics of Actinomycete Strains

### 3.1 Introduction: This Study

### 3.1.1 The Hermanus Lagoon: A Dynamic Estuarine Environment

The Klein Estuary, better known as the Hermanus lagoon, was investigated as a source of actinomycetes to screen for the production of novel bioactive compounds. The estuary is located on the southwest coast of South Africa and was selected as an ecosystem from which to isolate actinomycetes because it has a wide range of salinities, from freshwater at the head of the estuary (➡) to seawater at the mouth (●) (Fig. 3.1). In addition, a large amount of soil carrying spores of terrestrial actinomycetes enters the Hermanus lagoon (◆), via the Kleinrivier at the head of the estuary and by run-off from surrounding farmland. There is growing concern over the large quantities of fertilizer entering the estuary from the surrounding farmland due to winter rains. This has already caused excessive overgrowth of *Zostera capensis* (seaweed) beds as well as other *Chlorophyta* (green algae) and *Phaeophyta* (brown algae) (6). The result is a shift in the bacterial community to bacteria employing anaerobic respiration (6). Sampling of different niches in an ecosystem will yield the greatest diversity of microbial types, and biological diversity is assumed to imply chemical diversity.

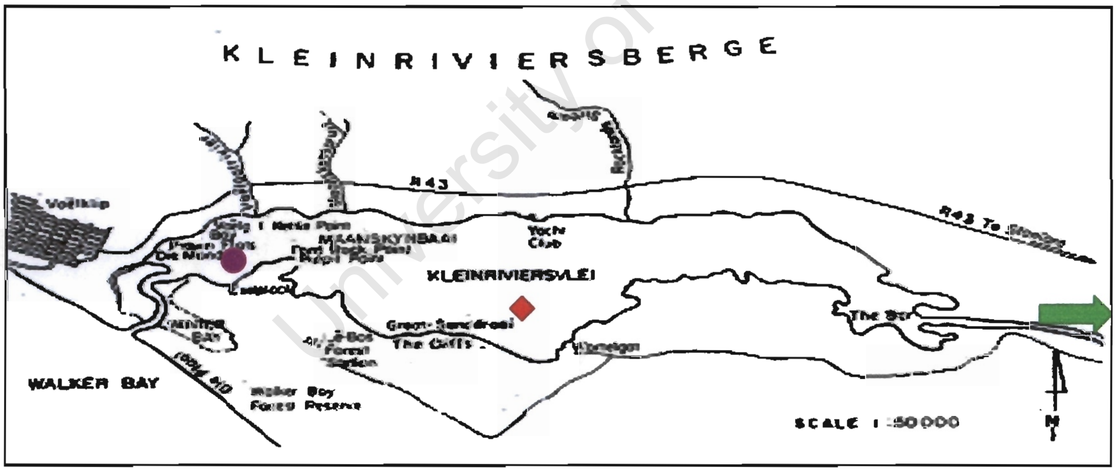


Fig. 3.1. Location of sampling areas in the Kleinriviersvlei (the Hermanus estuary). ●, The mouth of the Klein Estuary (the sea); ◆, the main lagoon channel of the estuary; ➡, the head of the estuary (the Kleinrivier). The estuary lies at 34°24′S, 19°18′E, between the towns of Hermanus and Stanford on the southwest coast of South Africa. The estuary drains into Walker Bay in the Atlantic Ocean. Map taken from reference 6.

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Fig. 3.2. A diagram showing maps of the areas from which samples were taken and the areas to which the 34 actinomycete strains were isolated. World map obtained from http://go.hrw.com/atlas/norm_htm/world.htm; map of South Africa obtained from http://go.hrw.com/atlas/norm_htm/safrica.htm; map of Thailand obtained from http://go.hrw.com/atlas/norm_htm/thailand.htm; map of Bazaruto, Mozambique, obtained from http://www.e-gnu.com/frameset.asp?pagename=maps/maputo_inhaca_map.html.

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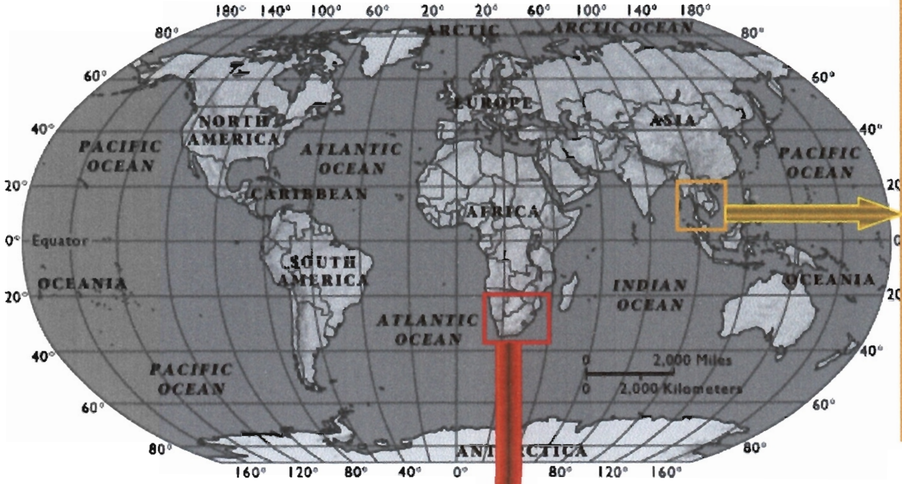
## Strains from Thailand:

**The River Kwai (Kanchanaburi):** *Pseudonocardia* sp. strains RKVB-VW, RKVB-LW, RKVB-3CBM2G.

**Phuket Island:** *Micromonospora* sp. strain MMS19; *Streptomyces* sp. strains LMP-TD, LMP-BRP.



**From the southern most point of the Bazaruto Island:** *Streptomyces* sp. strain PB.

## Strains from South Africa:

**Hermanus:** '*Micromonospora aquatica*', '*Micromonospora arenincolae*', '*Micromonospora cinerea*', '*Micromonospora hermanusense*', '*Micromonospora lacunae*', '*Micromonospora stanfordense*'; *Micromonospora* sp. strains MMS2, MMS8, MMS9, MMS10, MMS11, MMS12, MMS14, MMS16, MMS17; *Streptomyces* sp. strain HMS88.

**Noordhoek Beach:** '*Streptomyces noordhoekensis*'; *Streptomyces* sp. strains BAM1, BAM3, BAM7.

**Plettenberg Bay:** *Micromonospora* sp. strains MMS15, MMS18; *Streptomyces* sp. strain Kit2004.

**Other Locations:** *Streptomyces* sp. strain SVM65; '*Micromonospora grateloupicolae*', '*Micromonospora terrae*'; *Micromonospora* sp. strain MLR.

Other environments, terrestrial and marine, were also investigated in an attempt to isolate novel actinomycete strains (Fig. 3.2). Noordhoek and Plettenberg Bay are coastal marine and estuarine environments, also located along the southwest coast of South Africa. Bazaruto Island is a small island located off the coast of Mozambique, one of South Africa's neighbouring countries.

Samples were also collected in Thailand, South East Asia: from Patong Beach, Phuket Island (now devastated by the tsunami event in December 2004), and from the historical River Kwai, located in Kanchanaburi, inland Thailand.

### 3.1.2 Isolation Media and Selective Inhibitors

The range of media that have been used to isolate actinomycetes is extensive and many were designed primarily to discourage growth of other bacteria, although probably not allowing the growth of all actinomycetes in the sample. Competition from fungi is effectively dealt with by incorporation of antifungal antibiotics in the medium. Actinomycetes are generally insensitive to these compounds at concentrations of 50 – 100 μg/ml (13). Cycloheximide (CHX) has been widely used and has the advantage of being heat stable (13). CHX is an antibiotic isolated from streptomycin-producing strains of *Streptomyces griseus* and inhibits eukaryotic protein synthesis (18). Nalidixic acid (NAL) has been used to reduce contamination of isolation plates by Gram-negative bacteria common to marine and estuarine environments (called swarmer cells) and allow isolation of as broad a range of actinomycetes as possible (8). NAL was added to both terrestrial and marine media to prevent Gram-negative bacteria from interfering with the isolation of actinomycetes as pure cultures. NAL is a quinolone, containing the 4-quinolone ring and is a synthetic antimicrobial agent (18). NAL inhibits bacterial DNA synthesis by inhibiting the bacterial DNA gyrase (18). Although Gram-negative bacteria exist in both the marine and terrestrial environment, Gram-negatives are more prevalent in the marine environment and thus NAL is more useful as a selective inhibitor of swarmer cells from such environments.

### 3.1.3 The Aim of This Study

In view of the potential importance of marine actinomycetes as a source of novel bioactive compounds, methods were used to improve the isolation of actinomycetes from marine and estuarine environments.

Good classification of actinomycetes is important as it provides an essential base for accurate identification to the genus level and ultimately to the species level.

Thirty-four (34) actinomycete strains, mostly from rare actinomycete genera, were isolated from the various environments investigated. To attempt to describe these isolates as new species, a polyphasic approach to bacterial taxonomy was applied.

## 3.2 Materials and Methods

### 3.2.1 Sampling

Terrestrial and marine samples were collected from various environments in South Africa and other countries from May 2002 to July 2004. The South African areas were: The Hermanus Lagoon (HMS); Napier (collected by Marilize le Roes); Noordhoek Beach; Plettenberg Bay (BS), and Silvermine (SVM). Samples from a seaweed, *Grateloupia filicina*, were collected from Kommetjie and St. James, Cape Town, South Africa by Donovan Porter over the period of April 2000 to March 2001. Samples from the southern-most point of Bazaruto Island were collected during January 2003 by Dane Goodwin.

Samples from Thailand: the beaches of Phuket Island (LMP), and the banks of the River Kwai, Kanchanaburi (RKVB), were collected during June 2004. Every sample of soil (including beach sand) was taken at a depth of 0 – 2 cm and transferred to a sterile container. Sediment samples were collected from the Kleinriviersvlei (the Hermanus estuary) on 20 July 2002 by using the boat anchor to pull up sediment from the estuary bottom. A sample (2 g) of sediment was removed from the anchor and suspended in 10 ml of a sterile 2% (w/v) NaCl solution to mimic the ionic strength of the seawater. Samples were processed within 75 min of collection.

### 3.2.2 Sample Treatment, Isolation Media, and Selective Inhibitors

In the case of soil and beach sand samples, 0.25 g of sample was weighed out into a sterile eppendorf, suspended in 1 ml of sterile water and vortexed for 2 min. Soils were also air-dried at 30°C for 2 days and stored in a Petri dish sealed with Parafilm at room temperature.

To isolate terrestrial actinomycetes from soil samples, diluted (10-fold, stepwise to $10^{-5}$) samples were spread on Middlebrook 7H9 agar (containing no AD supplement but glucose was added to a final concentration of 10 mM; Difco Laboratories, Detroit, Michigan), a defined medium. To isolate actinomycetes from aquatic samples (beach sand), diluted (10-fold, stepwise to $10^{-5}$) samples were spread on the following agar media: GM (4); GOT (25); modified 172F agar (see 2.2.2); and yeast extract-malt extract agar (*ISP* Medium No. 2)(21) supplemented with 2% (w/v) NaCl and pH adjusted to 7.3 (YMEM).

All isolation media contained nalidixic acid (NAL) (Sigma) and cycloheximide (CHX) (Sigma) at final concentrations of 10 and 50 μg/ml, respectively.

Plates were incubated at 30°C for 5 to 7 days. Single colonies were subcultured onto fresh plates of the medium on which the colonies were isolated. Colonies were selected on the basis of the presence of aerial mycelium, or a hard, furrowed orange appearance, and allowed to grow for a further week (7 days) at 30°C.

Estuarine sediment samples were vortexed for 2 min. Marine actinomycetes were isolated by spread-plating 10-fold dilutions of sediment samples onto the following agar media: M2 (11); glycerol-asparagine (3); starch-casein (3); and YMEM.

For the Hermanus plates, after 11 days of growth at ambient temperature (approximately 22°C), colonies characteristic of actinomycetes were subcultured onto YMEM agar containing natural extract in place of water. Water from the sampling areas (the natural extract) was removed on 20 July 2002, autoclaved and added to the media. The addition of 2% (w/v) NaCl and natural extract to the media, as well as the incubation temperature, was performed in an effort to mimic the ecophysiological parameters of the Klein Estuary. For all other locations, marine actinomycetes were subcultured onto plates of the medium on which the colonies were isolated. Colonies were selected on the basis of the presence of aerial mycelium, or a hard, furrowed orange appearance, and allowed to grow for a further week (7 days) at 30°C.

### 3.2.3 Morphological Observations, Physiological Tests, Cryo Electron Microscopy, and Anaerobic Growth Studies

International *Streptomyces* Project (*ISP*) media were prepared according to the methods of Shirling and Gottlieb (21). Controls were used for each test: positive controls, negative controls and/or uninoculated controls. *Micromonospora* morphological characteristics were determined on YEME, Bennett's agar (2), and modified 172F agar. Production of diffusible pigments by *Micromonospora* strains was tested on YEME, Bennett's agar, modified 172F agar, and glycerol-asparagine agar (*ISP* Medium No. 5). The pH sensitivity of both the substrate mycelium and diffusible pigments were assessed by noting any colour changes caused by the addition of 50 mM HCl or 50 mM NaOH.

For *Streptomyces* strains, spore chain morphology, spore surface ornamentation, spore mass colour, and colour of the substrate mycelium were determined after 14 days of growth on inorganic salts-starch agar (*ISP* Medium No. 4) (13). Spore mass colour and the colour of the substrate mycelium were determined by visual inspection. Spore chain morphology was determined by light and scanning electron microscopy (SEM). Spore surface ornamentation was determined by SEM of the cultures used for the examination of spore chain morphology. Production of diffusible pigments was detected on glycerol-asparagine agar (*ISP* Medium No. 5). The pH sensitivity of both the substrate mycelium and diffusible pigments was assessed by noting any colour changes caused by the addition of 50 mM HCl or 50 mM NaOH.

Physiological characteristics were determined as recommended by Locci (13). For *Micromonospora* strains, growth was determined on inorganic salts-starch agar (*ISP* Medium No. 4), Middlebrook 7H9 agar (containing no AD supplement but glucose was added to a final concentration of 10 mM), Czapek-solution agar (2), and on potato slice and potato slice plus $CaCO_3$.

Growth at 4°C, 37°C, 45°C, at pH 4.3, in the presence of 0.1% 2-phenylethanol, and in the presence of 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 11.0 % sodium chloride (%, w/v) was determined as recommended by Locci (13) and was tested on Bennett's agar. Degradation of adenine, allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, L-tyrosine, xanthine, and xylan was determined as recommended by Locci (13). Tween 80 degradation was determined according to Sierra (22), and degradation of urea was determined according to Gordon *et al.* (9). Melanin pigment production was determined on peptone-yeast extract-iron agar (*ISP* Medium No. 6) and on tyrosine agar (*ISP* Medium No. 7). Hydrolysis of aesculin, arbutin, hippurate, and pectin, $H_2S$ production, nitrate reduction, and lecithinase, lipolysis, and proteolysis (on egg-yolk medium) were determined as recommended by Locci (13).

Antibiotic resistance was determined by incorporation of the antibiotics into Bennett's agar plates (2). All sole carbon sources for carbon-utilization were tested at a concentration of 1.0% (w/v), and sole nitrogen sources were tested at a concentration of 0.1% (w/v), as recommended by Locci (13). The utilization of organic compounds was tested at a concentration of 0.1% (w/v) as recommended by Gordon *et al.* (9).

All tests were carried out at 28 – 30°C (except for testing growth at 4, 37 and 45°C) and were incubated for the time periods recommended by Locci. *Micromonospora* strains were incubated for 21 days (except for degradation of starch and gelatin, and nitrate reduction, which were incubated for 7 days and 14 days, respectively).

For Cryo scanning electron microscopy, the *Micromonospora* strains were grown on modified 172F agar for 4 weeks at 28°C and the *Streptomyces* strains grown on *ISP* Medium No. 4 for 14 days at 30°C. The procedure was performed as follows: The cold stage and preparation chamber of the scanning electron microscope (SEM) were cooled to –150°C. The plunging chamber was filled with liquid nitrogen and pumped down until the liquid nitrogen turned slushy. A copper stub was screwed onto the transfer rod and the sample (an approximately 1 cm by 1 cm agar plug, with the bacterial strain) was glued on using cryo glue mixed with carbon graphite. The chamber was vented with nitrogen gas and the sample plunged into the slushy nitrogen. The transfer chamber was attached to the side of the SEM and the sample on the copper stub was transferred onto the cold stage via the preparation chamber, using the transfer rod. The samples were viewed uncoated at 5 KV.

Anaerobic testing was performed as described in 2.2.3.

### 3.2.4 Chemotaxonomic Observations

The method of Minnikin *et al.* (16) was used to determine the presence of mycolic acids. The whole cell sugar pattern and cell wall chemotype was determined according to the methods of Hasegawa *et al.* (10), except that dried cells were used instead of colonies from agar plates. The mol% G+C of the genomic DNA was determined in 1.0 × SSC by the method of Mandel and Marmur (15). Genomic DNA was extracted according to the method of Wang *et al.* (26).

### 3.2.5 PCR Amplification and DNA Sequencing

PCR amplification of 16S rDNA was performed as described by Cook and Meyers (5). The 16S rDNA was sequenced using a MegaBACE 500 Automated Capillary DNA Sequencing System (Molecular Dynamics) and a DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE.

### 3.2.6 Rapid Molecular Identification: Amplified Ribosomal DNA Restriction Analysis (ARDRA)

This method was performed according to Cook and Meyers (5). Purified amplicons were digested with enzymes supplied by Roche. DNA fragments were separated on 2% agarose gels (20). The $\lambda$*Pst*DNA ladder (the molecular mass marker) was included. The bands were visualized on a 254 nm transilluminator (UVP©) and photographs taken.

### 3.2.7 Phylogenetic Analysis

An unrooted phylogenetic tree was constructed from 16S rDNA sequences of the 16 validly published species of the genus *Micromonospora* that were on the Approved Lists of Bacterial Names, last updated on 20 December 2004 (7). Eight *Micromonospora* strains described in this study were also included. All sequences were edited to produce the longest sequence region (1311 bp) common to all 24 sequences. Bootstrapping analysis was used to test the significance of the proposed phylogenetic affiliations. The tree was generated by neighbour-joining (19), with pairwise distances calculated by the Kimura two-parameter estimation, and the percentage bootstrap values obtained by using ClustalX (24), Version 1.81 and MEGA2, Version 2.1 (12). The GenBank sequence accession numbers are indicated in brackets after the type strain names.

The 16S rDNA sequences of 9 of the environmental strains can be obtained from GenBank: '*Micromonospora stanfordense*' strain MMS1$^{T}$ (nucleotide accession number: AY723730), '*Micromonospora cinerea*' strain MMS3$^{T}$ (AY723731), '*Micromonospora lacunae*' strain MMS4$^{T}$ (AY765353), '*Micromonospora hermanusense*' strain MMS6$^{T}$ (AY765354), '*Micromonospora aquatica*' strain MMS5$^{T}$ (AY863216), '*Micromonospora arenincolae*' strain MMS7$^{T}$ (AY862317), '*Micromonospora grateloupicolae*' strain R$_{2}\mu^{T}$ (AY862318), '*Micromonospora terrae*' strain MMS-DA$^{T}$ (AY862315), and '*Streptomyces noordhoekensis*' strain BAM4$^{T}$ (AY634625).

**3.2.8 Strains Used in This Study** (See 2.2.1)

## 3.3 Results and Discussion

The results of the isolation and enumeration techniques shall be discussed followed by the application of the Identification Key to environmental *Micromonospora* isolates, and the study on the anaerobic growth of the environmental *Micromonospora* strains. Since the morphological features of *Micromonospora* are not as diverse as those of *Streptomyces*, the physiological characteristics of the genus play an important role in their classification. The strain descriptions of the 34 actinomycete isolates will then be presented. All isolates were Gram positive. The antimicrobial activity of the isolates will be discussed in Chapter 5. The isolates will be listed as follows: from the 'most characterized' strains to the 'least characterized'. If 16S rDNA sequences were not obtained for a strain, ARDRA was performed in order to identify accurately the strain to the genus level. The selection of restriction enzymes used was based on a rapid identification of filamentous actinomycetes scheme proposed by Cook and Meyers (5).

### 3.3.1 Isolation and Enumeration

The addition of the selective inhibitors to the isolation media proved to be extremely useful in limiting the number of Gram negative bacteria and contaminating fungi. The media used in this study were chosen because the growth of actinomycete strains should be favoured. Middlebrook 7H9 and YEME agars favoured the growth of *Streptomyces* strains which were identified on the basis of the presence of aerial mycelia. *Micromonospora* strains were isolated from YMEM and modified 172F agars and were identified on the basis of orange to brown colonies, often including black mucoid spore masses, characteristic of the genus. On all other media, the dominant, if not only, bacteria isolated, belonged to the genus *Gordonia*. These isolates exhibited pastel orange colonies, which changed to shades of a darker orange after exposure to light, a feature characteristic of the genus. As *Gordonia* strains are often human or plant pathogens, they were not further pursued. The bacterial community of the Hermanus estuary has shifted to bacteria employing anaerobic respiration (6). This was evident as the distinctive rotten egg stench of hydrogen sulphide production was overwhelming when sediment samples were removed from the lagoon bottom. It is thus perhaps no surprise that the *Micromonospora* strains isolated from the Hermanus lagoon grew very well under anaerobic conditions.

### 3.3.2 Application: Using the Identification Key

Twenty-one (21) environmental *Micromonospora* strains were isolated by the author. Table 3.1 shows 8 of those strains and their physiological characters pertinent to the Identification Key. As described later in this chapter, 16S rDNA sequences are available for these 8 strains and it is proposed that these strains are novel species.

Table 3.1. Testing the Identification Key with unpublished *Micromonospora* isolates.

| *Micromonospora* strains:<br>Physiological Characteristics: | MMS1 | MMS3 | MMS4 | MMS5 | MMS6 | MMS7 | $R_{2\mu}$ | MMS-DA |
|---|---|---|---|---|---|---|---|---|
| **Maximum Sodium Chloride Tolerance (% w/v):** | 5.0 | 4.0 | 3.0 | 5.0 | 5.0 | 4.0 | 4.0 | 5.0 |
| **Production of Diffusible Pigments On:** | | | | | | | | |
| m172F | – | Red Brown | Reddish-brown | Light Orange | – | – | – | Light Olive-brown |
| BM-Glucose | – | Bright Yellow | – | – | – | Light Brown | – | Light Olive-green |
| YEME | Light Brown | Bright Yellow | Yellow-brown | Light Brown | – | Light Brown | Yellow-brown | Light Orange-brown |
| **Diagnostic Mycelial Pigment Colour** | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange |
| **Growth on *ISP4*** | – | + | – | – | – | – | – | – |
| **Growth at 37°C** | + | + | + | + | + | + | + | + |
| **Carbon Source Utilization (1.0% w/v):** | | | | | | | | |
| Glycerol | ±[a] | – | + | ± | ± | ± | – | ± |
| *myo*-Inositol | ± | – | + | + | + | + | + | + |
| **Probable New Species According to:** | | | | | | | | |
| The Identification Key | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 16S rDNA Sequence Analysis | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

[a] ±, weakly positive utilization.

The Identification Key revealed that *Micromonospora* strain MMS3 could be a strain of *M. halophytica*, while the 16S rDNA sequence analysis suggested that strain MMS3 is probably a new *Micromonospora* species. The physiological characters of strain MMS3 should be compared to those of *M. halophytica* (available in the species description). Strain MMS3 readily produces tufts of grey aerial mycelia on many agar media, while *M. halophytica* does not produce aerial mycelia on any of the media tested. Furthermore, *M. halophytica* does not produce diffusible pigments on BM-Glucose and YEME.

Strains MMS8, MMS9, MMS11, and MMS17 have a maximum sodium chloride tolerance of 5.0%, while strain MMS19 has a maximum sodium chloride tolerance of 6.0%, and strain MMS18 cannot tolerate sodium chloride (i.e. 0.0%), and thus the Identification Key suggests that these strains are probable new species.

Table 3.2 shows the application of the Identification Key to the remaining 7 strains.

Table 3.2. Identification of 7 *Micromonospora* strains by using the Identification Key.

| *Micromonospora* strains: | MMS2 | MLR | MMS10 | MMS12 | MMS14 | MMS15 | MMS16 |
|---|---|---|---|---|---|---|---|
| Physiological Characteristics: | | | | | | | |
| **Maximum Sodium Chloride Tolerance (% w/v):** | 3.0 | 3.0 | 3.0 | 4.0 | 2.0 | 3.0 | 4.0 |
| **Production of Diffusible Pigments On:** | | | | | | | |
| m172F | – | Brown | – | – | – | – | – |
| BM-Glucose | – | Light Yellow | – | – | – | Yellow-brown | – |
| YEME | Light Brown | Yellow | – | – | Orange-brown | Yellow-brown | – |
| **Diagnostic Mycelial Pigment Colour** | Orange | Orange | Orange | Ivory | Orange | Orange | Orange |
| **Growth on *ISP4*** | + | – | + | – | + | + | + |
| **Growth at 37°C** | + | ±[a] | + | + | + | + | + |
| **Carbon Source Utilization (1.0% w/v):** | | | | | | | |
| Glycerol | ± | ± | + | + | + | + | + |
| *myo*-Inositol | + | – | + | + | + | ± | ± |
| **Probable New Species According to:** | | | | | | | |
| The Identification Key | ✓ | × | ✓ | ✓ | × | ✓ | ✓ |

[a]±, weak growth was observed.

The Identification Key indicates that strain MLR could be *M. carbonacea*. However, *M. carbonacea* grows very well on *ISP*4, and very well at 37°C. It can thus be argued that strain MLR is a potentially novel species.

The Identification Key suggests that strain MMS14 could be *M. auratinigra*. *M. auratinigra* is negative for nitrate reduction, grows very well on Czapek-solution agar, and does not utilize L-rhamnose as a sole carbon source (23). Strain MMS14 is positive for nitrate reduction, does not grow on Czapek-solution agar, but does utilize L-rhamnose as a sole carbon source (Tables 3.6 and 3.7).

### 3.3.3 Anaerobic Studies on Environmental *Micromonospora* Strains

The results of the anaerobic studies are shown in Table 3.3. Strain MMS18 did not grow on either of the media chosen, under aerobic or anaerobic conditions. Strain MMS18 utilizes sucrose as a sole carbon source and grows on BM-Glycerol under the standard incubation temperature, 30°C. The strain does not grow at 37°C. To assess if strain MMS18 can grow under anaerobic conditions, the temperature of the anaerobic chamber would have to be kept at 30°C. There is no pattern regarding the changing of the colour of the indicator plates: very few strains produced acid to change the colour of the plates and those same strains did not produce acid under both aerobic and anaerobic conditions. Complex and defined media were chosen and in general the strains showed the same degree of growth or poorer growth under anaerobic conditions as compared to aerobic conditions. The sucrose medium was chosen as most members of the genus *Micromonospora* should strongly utilize sucrose as a sole carbon source. Strains grew well under anaerobic conditions for 14 days, but thereafter growth stopped and the orange mycelial pigments disappeared/faded over the next 7 days. Some strains did grow better under anaerobic conditions, such as strains R2μ, MLR, and MMS17, on the sucrose medium. Since almost all of the author's strains and the validly published species grew under anaerobic conditions, this characteristic can be said to be widely distributed in the genus, not just to strains isolated from oxygen poor environments. It is unknown at this time whether anaerobic respiration or fermentation allows growth under these conditions.

Table 3.3. Anaerobic and aerobic growth of 21 environmental *Micromonospora* strains grown on different media, with or without an indicator, at 37°C.

| | BM-Glycerol | | BM-Glycerol with Indicator | | Sucrose as Sole Carbon Source | | Sucrose as Sole Carbon Source with Indicator | |
|---|---|---|---|---|---|---|---|---|
| Strains: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: | Aerobic: | Anaerobic: |
| **MMS1** | +++ | + | P | P | ++ | + | P | P |
| **MMS2** | +++ | + | P | P | + | – | P | P |
| **MMS3** | +++ | + | P | P | + | + | P | P |
| **MMS4** | + | + | P | P | – | – | P | P |
| **MMS5** | +++ | + | P | Y | + | + | P | P |
| **MMS6** | +++ | + | P | P | + | + | P | P |
| **MMS7** | +++ | + | P | P | + | + | P | P |
| **$R_2\mu$** | +++ | + | P | P | – | + | P | P |
| **MMS-DA** | +++ | + | P | P | + | + | P | P |
| **MLR** | + | + | Y | P | – | + | P | P |
| **MMS8** | +++ | + | P | Y | ++ | + | P | P |
| **MMS9** | +++ | + | P | P | +++ | +++ | Y | P |
| **MMS10** | ++ | + | P | P | +++ | + | Y | P |
| **MMS11** | +++ | + | P | P | ++ | + | Y | P |
| **MMS12** | +++ | + | P | P | +++ | + | P | P |
| **MMS14** | +++ | + | P | P | +++ | +++ | Y | P |
| **MMS15** | + | + | P | P | +++ | +++ | Y | P |
| **MMS16** | ++ | – | P | P | ++ | – | P | P |
| **MMS17** | +++ | + | P | Y | – | + | P | P |
| **MMS18** | – | – | P | P | – | – | P | P |
| **MMS19** | – | – | Y | P | + | + | Y | P |

Abbreviations: –, no growth; +, weak growth; ++, moderate growth; +++, good growth; P, indicator remained unchanged (purple in colour); Y, indicator changed in colour to yellow.

### 3.3.4 The Group A *Micromonospora* Strains: MMS-1 to -7, MMS-DA, MLR, and $R_2\mu$

The physiological characteristics of these 10 strains are shown in Tables 3.4 and 3.5.

It must be noted that electron microscopy is performed primarily to show the single spores characteristic of the genus *Micromonospora*. Spore surface ornamentation could not be seen clearly.

The cell walls of all 10 *Micromonospora* strains contained *meso*-diaminopimelic acid (*meso*-DAP) and glycine. Mycolic acids were absent. The genus *Micromonospora* has 2 sugars in the cell wall that are characteristic of the genus: the pentoses xylose and arabinose.

These 10 strains were placed in the genus *Micromonospora* based on ARDRA and 16S rDNA sequence analysis. The novelty of all 21 of the environmental *Micromonospora* strains investigated in this study is suggested by the application of the Identification Key (3.3.2).

Table 3.4. Physiological characteristics of the Group A *Micromonospora* strains.

| **Characteristics:** | **MMS1** | **MMS2** | **MMS3** | **MMS4** | **MMS5** | **MMS6** | **$R_2\mu$** | **MMS7** | **MLR** | **MMS-DA** |
|---|---|---|---|---|---|---|---|---|---|---|
| Diffusible pigment on *ISP* Medium No. 5 | – | – | – | – | – | – | – | – | – | – |
| Growth on: | | | | | | | | | | |
| Czapek-solution agar | w+ | + | + | w+ | w+ | w+ | w+ | w+ | vs+ | w+ |
| Potato slice | – | – | + | + | – | + | s+ | s+ | s+ | + |
| Potato slice plus $CaCO_3$ | + | + | – | + | + | + | s+ | s+ | s+ | + |
| Aesculin hydrolysis | + | s+ | + | + | vs+ | vs+ | + | + | vs+ | + |
| Arbutin hydrolysis | + | + | + | – | vs+ | vs+ | + | + | + | + |
| Hippurate hydrolysis | – | – | – | – | – | + | – | – | – | – |
| Pectin hydrolysis | + | + | + | – | + | + | + | – | – | – |
| Nitrate reduction | – | – | + | – | – | + | – | – | – | + |
| $H_2S$ production | + | + | w+ | + | + | + | + | + | + | + |
| Maximum NaCl tolerance (%, w/v) | 5.0 | 3.0 | 4.0 | 3.0 | 5.0 | 5.0 | 4.0 | 4.0 | 3.0 | 5.0 |
| Adenine degradation | NG | – | – | – | – | – | – | – | NG | – |
| Allantoin degradation | – | – | – | – | – | w+ | – | – | – | – |
| Casein degradation | + | + | + | + | + | + | + | + | + | + |
| Cellulose degradation | – | – | – | – | – | – | – | – | – | – |
| Gelatin degradation | + | + | + | + | + | + | + | + | + | + |
| Guanine degradation | – | – | – | – | – | + | – | – | – | – |
| Hypoxanthine degradation | – | – | – | – | – | – | – | – | – | – |
| Starch degradation | + | + | + | + | + | + | + | + | + | + |
| Tween 80 degradation | + | + | + | + | + | + | + | + | – | + |
| L-Tyrosine degradation | + | + | – | + | + | + | + | + | – | + |
| Urea degradation | – | – | – | w+ | w+ | s+ | – | – | – | – |
| Xanthine degradation | – | – | – | – | – | – | – | – | – | – |
| Xylan degradation | + | – | + | + | + | + | – | + | – | w+ |
| Melanin on *ISP* Medium No. 6 | – | – | – | – | – | – | – | – | – | – |
| Melanin on *ISP* Medium No. 7 | – | – | – | – | – | – | – | – | – | – |
| Growth at 4°C | – | – | – | – | – | – | + | – | – | – |
| Growth at 37°C | + | + | + | + | + | + | + | + | vw+ | s+ |
| Growth at pH 4.3 | – | – | – | – | – | – | – | + | – | + |
| Growth in the presence of 0.1% 2-Phenylethanol | – | – | – | – | – | – | – | – | – | – |
| Growth on a defined medium: Middlebrook 7H9 Agar | vw+ | + | vw+ | w+ | vw+ | vw+ | vw+ | vw+ | + | – |
| Antibiotic Susceptibility: | | | | | | | | | | |
| Cephaloridine (100 µg/ml) | – | – | – | – | – | – | – | – | – | – |
| Lincomycin (100 µg/ml) | – | + | – | + | – | + | – | – | – | + |
| Oleandomycin (100 µg/ml) | – | + | – | – | – | + | – | + | – | + |
| Penicillin G (10 I.U.) | – | – | – | – | – | – | – | – | – | – |
| Rifampicin (50 µg/ml) | – | – | – | – | – | – | – | – | – | + |
| Tobramycin (50 µg/ml) | – | – | – | – | – | – | – | – | – | – |
| Vancomycin (50 µg/ml) | – | – | – | – | – | – | – | – | – | – |
| Lecithinase activity | – | – | – | – | – | – | – | – | – | – |
| Lipase activity | + | + | + | – | + | – | + | + | – | + |
| Protease activity | + | + | + | – | + | – | + | + | – | + |

Abbreviations: +, positive/growth; –, negative/no growth; w, weak growth; vw, very weak growth; s, strong growth; vs, very strong growth/reaction; NG, no growth.

Table 3.5. Group A *Micromonospora* sole carbon and nitrogen source utilization.

| **Characteristic:** | **MMS1** | **MMS2** | **MMS3** | **MMS4** | **MMS5** | **MMS6** | **R2μ** | **MMS7** | **MLR** | **MMS-DA** |
|---|---|---|---|---|---|---|---|---|---|---|
| Carbon Sources: | | | | | | | | | | |
| Adonitol | – | – | ± | ± | ± | – | – | ± | ± | ± |
| L-Arabinose | ++ | ++ | + | ++ | + | – | – | + | ++ | + |
| Glycerol | ± | ± | – | ++ | ± | ± | – | ± | ± | ± |
| *myo*-Inositol | ± | ++ | – | ++ | + | + | + | + | – | + |
| Inulin | ± | ± | ± | ++ | + | ± | ± | + | ± | ± |
| D-Mannitol | – | + | – | ++ | + | + | + | + | – | ++ |
| D(+)-Melezitose | ± | ± | – | ++ | – | ± | ± | – | ± | ± |
| α-Melibiose | + | + | + | ++ | + | + | + | + | ++ | + |
| Raffinose | + | + | + | ++ | + | + | + | + | ± | + |
| L(+)-Rhamnose | ± | ± | – | ± | – | + | + | ± | – | + |
| D(–)-Ribose | + | – | ± | – | – | – | – | – | ± | – |
| Sucrose | ++ | ++ | ± | ++ | + | + | + | + | ± | + |
| Nitrogen Sources: | | | | | | | | | | |
| L-Arginine | + | + | + | + | + | – | + | + | + | + |
| L-Cysteine | – | ± | – | ± | – | – | – | ± | ± | ± |
| DL-Ornithine | – | – | + | – | – | + | + | – | + | + |
| L-Serine | ++ | + | + | – | + | – | + | + | + | ++ |
| L-Threonine | ++ | + | + | + | + | ± | ± | – | + | + |

Abbreviations: –, no growth/no utilization; ±, weak growth/utilization; +, moderate growth/utilization; ++, good growth/utilization.

### (1) '*Micromonospora stanfordense*' strain MMS1$^T$

*Micromonospora stanfordense* (stan.for.den'se L. fem. adj. *stanfordense*, of Stanford, a town) was isolated from a sediment sample obtained from the Stanford River, which feeds the Klein Estuary. The diagnostic mycelial pigment colour of this strain is orange, turning black and mucoid upon sporulation. Spores are spherical, appear to be smooth, and are borne singly on long or short sporophores [Fig. 3.3 (a)]. Growth does not occur on potato slice unless $CaCO_3$ is added, indicating sensitivity to acidic conditions. Strain MMS1$^T$ does not grow on the medium used for testing adenine degradation. Maximum sodium chloride tolerance for this strain is 5.0% (no growth occurs at 6.0%). The pentoses arabinose and xylose are the only sugar constituents of the cell wall, although there is a greater amount of arabinose present than xylose. The 16S rDNA nucleotide sequence accession number (available from GenBank) is AY723730.

A BLAST search (1) of the GenBank database revealed that *M. stanfordense* strain MMS1$^T$ showed 98% homology to the 16S rDNA sequences of *M. chalcea* DSM 43026$^T$ (sequence accession number: X92594), *M. halophytica* DSM 43171$^T$ (X92601), and *M. purpureochromogenes* DSM 43821$^T$ (X92611) over 1424 bases. However, when the sequences were edited to the same length and pairwise alignments were performed in DNAMAN (Version 4.13, Lynnon Biosoft), the percentage identities dropped to 94.54% (over 1434 nucleotides), 94.53% (over 1434 nucleotides), and 94.32% (over 1423 nucleotides), respectively.

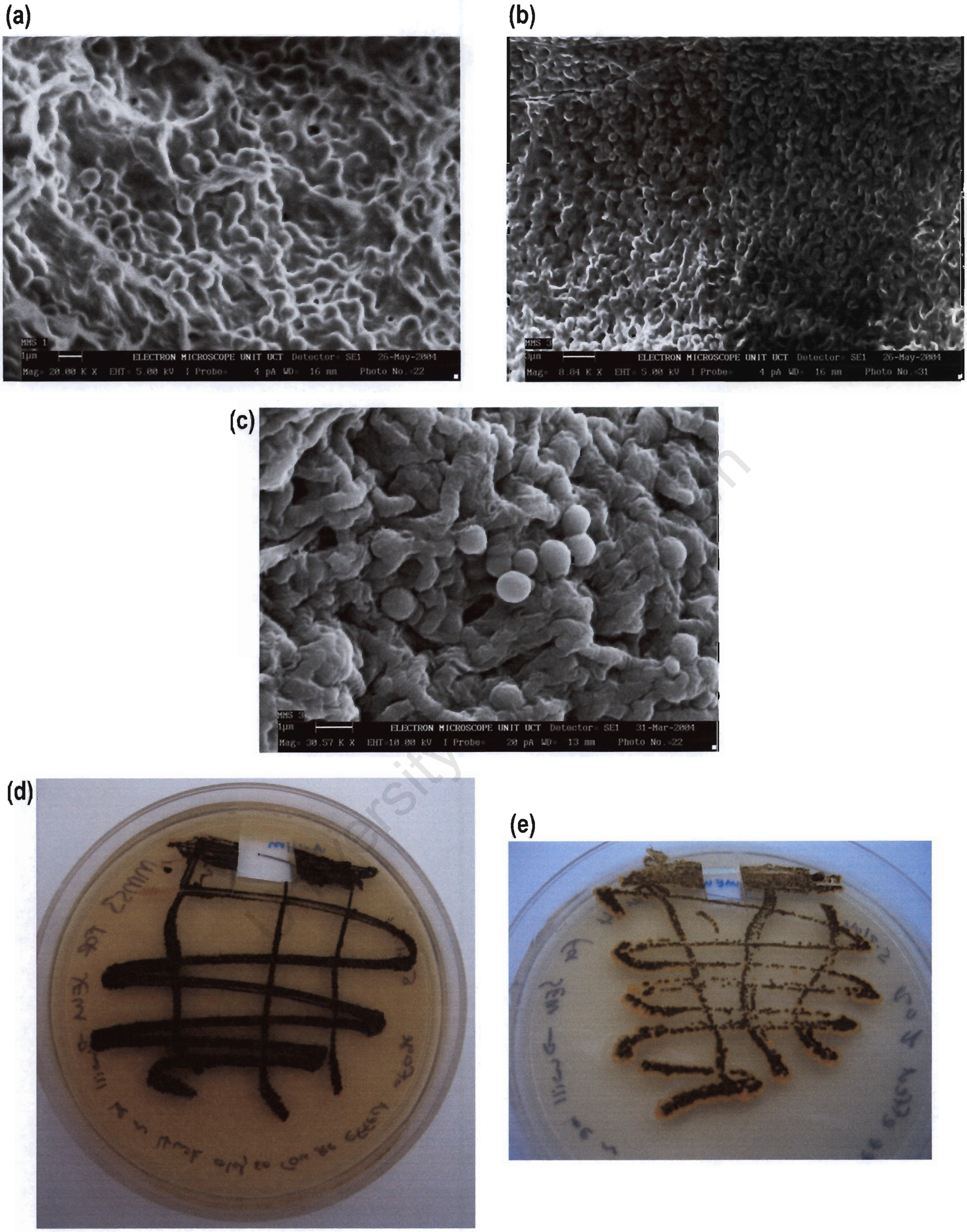


Fig. 3.3. Scanning electron micrographs of (a) '*Micromonospora stanfordense*' strain MMS1$^T$, and (b, c) '*Micromonospora cinerea*' strain MMS3$^T$. Photographs of (d) strain MMS3 and (e) '*Micromonospora lacunae*' strain MMS4$^T$ grown on YMEM agar for 4 weeks at 30°C.

The closest phylogenetic neighbours of strain MMS1$^T$ are *M. carbonacea, M. matsumotoense,* and '*M. cinerea*' strain MMS3$^T$. Strain MMS1$^T$ differs from *M. carbonacea* and '*M. cinerea*' strain MMS3$^T$ with respect to their maximum sodium chloride tolerances, which are 5.0%, 3.0%, and 4.0%, respectively. Strain MMS1$^T$ sporulates whereas *M. matsumotoense* does not.

### (2) '*Micromonospora cinerea*' strain MMS3$^T$

*Micromonospora cinerea* (ci.ne′ re.a. L. fem. adj. *cinerea*, ashy) was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary, and shows an ash-like morphology on some media, including on $\alpha$-melibiose and raffinose agars. Spores are abundant, and are borne singly on short sympodial (having lateral 'branches') sporophores [Fig. 3.3 (b) and (c)]. Spores are spherical and appear smooth. Characteristic gray blooms of short, sterile aerial mycelia are produced on some media. Strain MMS3$^T$ produces a bright yellow, pH sensitive diffusible pigment on Bennett's agar and *ISP2*, which changes colour from bright yellow to red on addition of 50 mM NaOH. A red-brown diffusible pigment is produced on modified 172F agar, which is not pH sensitive. The diagnostic mycelial pigment colour of strain MMS3$^T$ is orange on all media. The strain shows strong growth on autoclaved potato slice but does not grow on potato slice plus $CaCO_3$, indicating an unusual preference for acidic conditions. Most micromonosporas prefer neutral or basic conditions (14). Maximum sodium chloride tolerance is 4.0% for strain MMS3$^T$ (no growth occurs at 5.0%). Strain MMS3$^T$ was susceptible to all antibiotics tested. Fig. 3.3 (d) shows a photograph of strain MMS3$^T$ on YMEM agar medium. The sugar constituents of the cell wall are arabinose, xylose, galactose, and ribose. The mol% G+C content is 72.8%. The 16S rDNA nucleotide sequence accession number is AY723731.

A BLAST search revealed that *M. cinerea* strain MMS3$^T$ showed 98% homology to the 16S rDNA sequences of *M. carbonacea* DSM 43168$^T$ (X92599), *M. chalcea* DSM 43026$^T$ (X92594), and *M. purpureochromogenes* DSM 43821$^T$ (X92611) over 1421 bases. However, when the sequences were edited to the same length and pairwise alignments were performed in DNAMAN, the percentage identities dropped to 93.23% (over 1526 nucleotides), 93.04% (over 1527 nucleotides), and 93.03% (over 1524 nucleotides), respectively.

The closest phylogenetic neighbour is *M. matsumotoense*, and strain MMS3$^T$ can readily be distinguished from *M. matsumotoense* on the basis that strain MMS3$^T$ sporulates.

### (3) '*Micromonospora lacunae*' strain MMS4$^T$

*Micromonospora lacunae* (la.cu′nae L. fem. fig. *lacunae*, lagoon) was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary. Fig. 3.3 (e) shows a photograph of strain MMS4$^T$ on YMEM agar medium. Spores are abundant, spherical, and are borne singly on short sporophores [Fig. 3.4 (a)]. The sugar constituents of the cell wall are arabinose, xylose, and galactose. The mol% G+C content is 74.3%. The 16S rDNA nucleotide sequence accession number is AY765353.

A BLAST search revealed that *M. lacunae* strain MMS4$^T$ showed 98% homology to *M. endolithica* DSM 44398$^T$ (AJ560635), *M. chalcea* DSM 43026$^T$ (X92594), and *M. rosaria* DSM 803$^T$ (X92631) over 1407 bases. However, in DNAMAN, the percentage identities dropped to 94.66% (over 1421 nucleotides), 94.66% (over 1422 nucleotides), and 94.52% (over 1421 nucleotides), respectively.

### (4) '*Micromonospora hermanusense*' strain MMS6$^T$

*Micromonospora hermanusense* (her.ma.nu.sen'se L. fem. adj. *hermanusense*, of Hermanus, a town) was isolated from a sediment sample obtained from the mouth (the sea) of the Klein estuary. Spores are abundant, spherical, and are borne singly on short sporophores [Fig. 3.4 (b)]. The sugar constituents of the cell wall are arabinose, xylose, glucose, and mannose. The mol% G+C content is 73.8%. The 16S rDNA nucleotide sequence accession number is AY765354.

A BLAST search revealed that *M. hermanusense* strain MMS6$^T$ showed 98% homology to *M. endolithica* DSM 44398$^T$ (AJ560635), *M. chalcea* DSM 43026$^T$ (X92594), and *M. rosaria* DSM 803$^T$ (X92631) over 1272 bases. However, in DNAMAN, the percentage identities dropped to 83.56% (over 1400 nucleotides), 83.84% (over 1404 nucleotides), and 83.78% (over 1403 nucleotides), respectively.

### (5) '*Micromonospora aquatica*' strain MMS5$^T$

*Micromonospora aquatica* (a.qua'ti.ca L. fem. adj. *aquatica*, living in water) was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary. Spores are abundant, spherical, and are borne singly on short sporophores [Fig. 3.4 (c)]. The sugar constituents of the cell wall are arabinose, xylose, and galactose. The 16S rDNA nucleotide sequence accession number is AY862316.

A BLAST search revealed that *M. aquatica* strain MMS5$^T$ showed 97% homology to *M. aurantiaca* DSM 43813$^T$ (X92604), *M. chalcea* DSM 43026$^T$ (X92594), *M. halophytica* DSM 43171$^T$ (X92601), and *M. purpureochromogenes* DSM 43821$^T$ (X92611), over 1436 bases.

The closest phylogenetic neighbour of strain MMS5$^T$ is *M. aurantiaca*. MMS5$^T$ has a maximum sodium chloride tolerance of 5.0% while *M. aurantiaca* has a maximum tolerance of 3.0%. Strain MMS5$^T$ (Table 3.4) differs from *M. aurantiaca* (Table 2.6) in that strain MMS5$^T$ cannot grow on potato slice unless calcium carbonate is added, does not reduce nitrate and cannot grow at 4°C. The strains' carbon and nitrogen utilization patterns are also very different (Tables 3.5 and 2.7).

### (6) '*Micromonospora arenincolae*' strain MMS7$^T$

*Micromonospora arenincolae* (a.re.nin'co.lae L. fem. *arenincolae*, inhabits sand) was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary. Spores are abundant, spherical, and are borne singly on short sympodial sporophores [Fig. 3.4 (d)]. The sugar constituents of the cell wall are arabinose, xylose, and galactose. The 16S rDNA nucleotide sequence accession number is AY862317.

A BLAST search revealed that *M. arenincolae* strain MMS7$^T$ showed 97% homology to *M. endolithica* DSM 44398$^T$ (AJ560635), *M. chalcea* DSM 43026$^T$ (X92594), *M. rosaria* DSM 803$^T$ (X92631), and *M. chersina* DSM 44151$^T$ (X92628), over 1364 bases.

### (7) '*Micromonospora grateloupicolae*' strain R$_2\mu^T$

*Micromonospora grateloupicolae* (gra.te.lou.pi'co.lae L. fem. adj. *grateloupicolae*, living on *Grateloupia*, a seaweed) was isolated from the surface of the seaweed *Grateloupia filicina*, found along the coast of Cape Town, by Donovan Porter. Spores are abundant, spherical, and are borne singly on short and long sporophores [Fig. 3.4 (e) courtesy of D. Porter]. The sugar constituents of the cell wall are arabinose, xylose, and galactose. Of note is that strain R$_2\mu^T$ is the only environmental isolate to grow at 4°C. The 16S rDNA nucleotide sequence accession number is AY82318.

A BLAST search revealed that *M. grateloupicolae* strain R$_2\mu^T$ showed 97% homology to *M. endolithica* DSM 44398$^T$ (AJ560635), *M. rosaria* DSM 803$^T$ (X92631), and *M. chalcea* DSM 43026$^T$ (X92594) over 1407 bases.

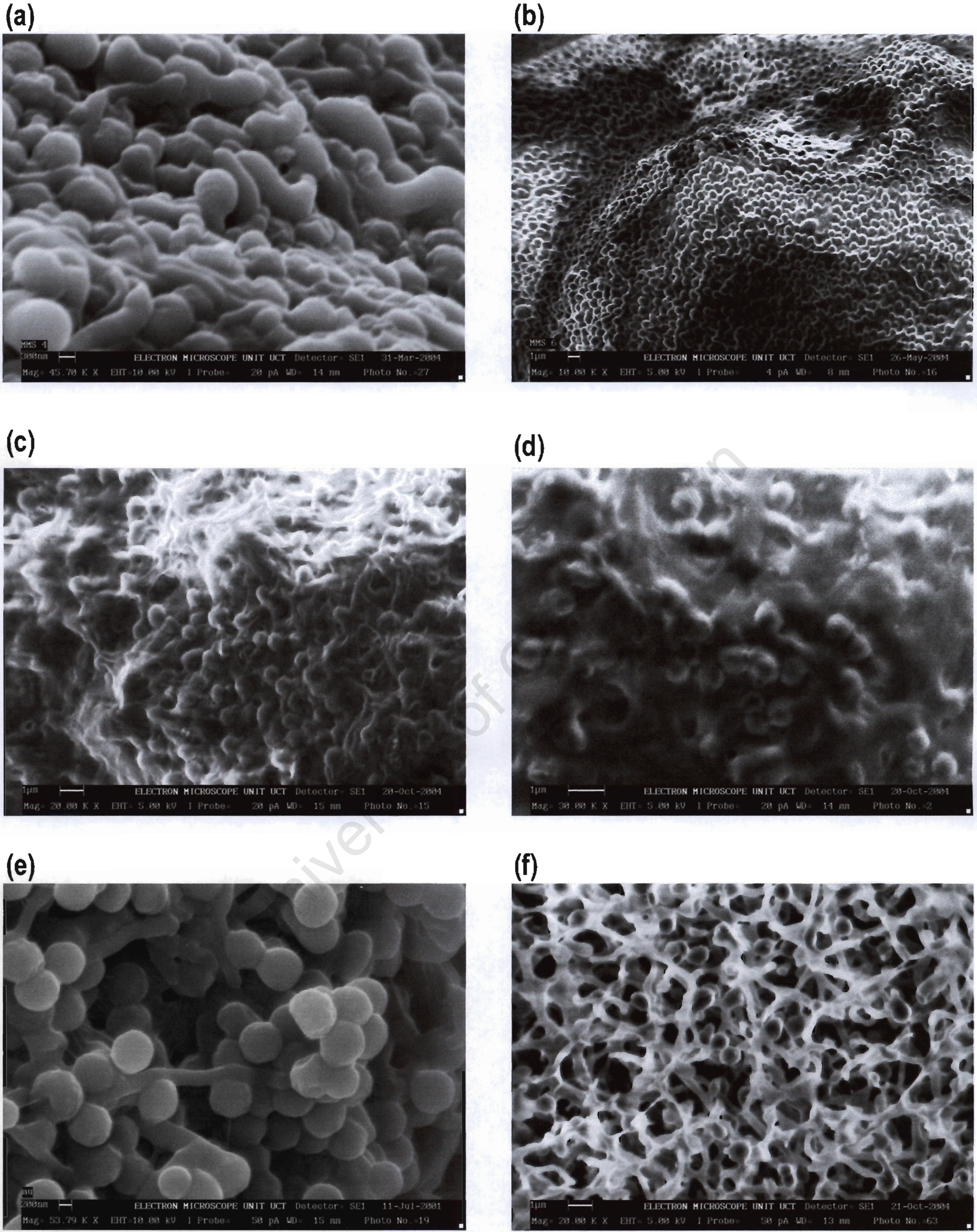


Fig. 3.4. Scanning electron micrographs of (a) '*Micromonospora lacunae*' strain MMS4$^T$, (b) '*Micromonospora hermanusense*' strain MMS6$^T$, (c) '*Micromonospora aquatica*' strain MMS5$^T$, (d) '*Micromonospora arenincolae*' strain MMS7$^T$, (e) '*Micromonospora grateloupicolae*' strain $R_2\mu^T$, and (f) '*Micromonospora terrae*' strain MMS-DA$^T$.

### (8) '*Micromonospora terrae*' strain MMS-DA$^T$

*Micromonospora terrae* (ter'rae L. fem. *terrae*, from soil) was isolated from garden soil in Cape Town by Deeya Ballim and Aimeé Whitcroft in August 2003. Spores are abundant, spherical, and are borne singly on short and long sporophores [Fig. 3.4 (f)]. The sugar constituents of the cell wall are arabinose and xylose, present in equal amounts. The 16S rDNA nucleotide sequence accession number is AY862315.

A BLAST search revealed that *M. terrae* strain MMS-DA$^T$ showed 97% homology to *M. endolithica* DSM 44398$^T$ (AJ560635), *M. chalcea* DSM 43026$^T$ (X92594), and *M. chersina* DSM 44151$^T$ (X92628), over 1331 bases.

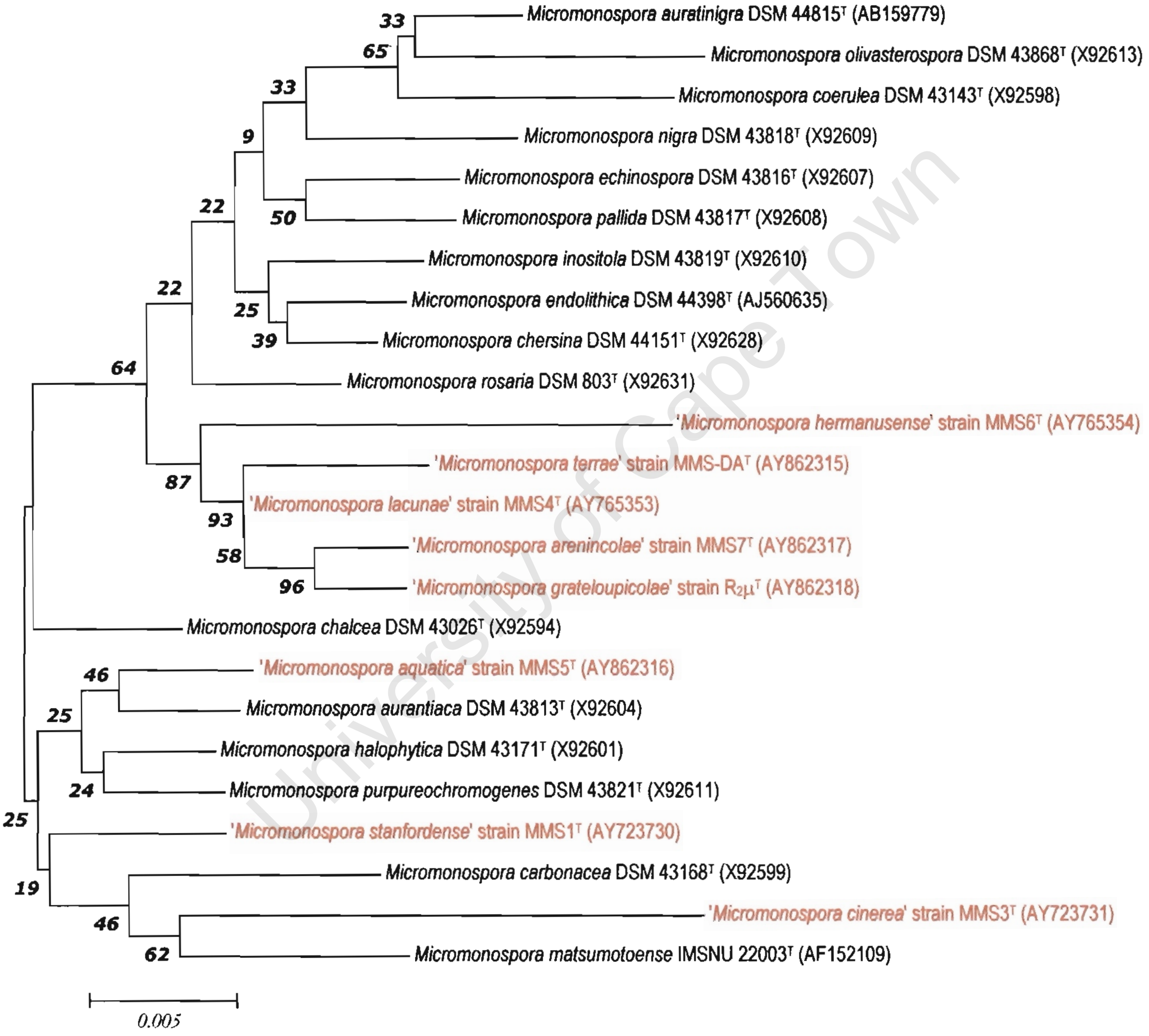


Fig. 3.5. An unrooted neighbour-joining phylogenetic tree constructed from the 16S rDNA sequences of the 16 validly published members of the genus *Micromonospora* and 8 potentially new species (indicated in orange) from this study. The tree was constructed with 1311 bp of alignable sequence. The GenBank sequence accession numbers are indicated in brackets. Nodes are labelled with the percentage bootstrap replications (out of 100) in which the node was found. The scale bar represents an average of 0.005 nucleotide substitutions per site.

The phylogenetic tree (Fig. 3.5) shows that 5 of the South African environmental isolates group together (strains MMS6$^T$, MMS-DA$^T$, MMS4$^T$, MMS7$^T$, and R$_2$μ$^T$).

### (9) *Micromonospora* sp. strain MMS2

Strain MMS2 was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary. Spores are abundant, spherical, and are borne singly on short sporophores [Fig. 3.6 (a)]. The sugar constituents of the cell wall are arabinose and xylose. Two non-overlapping 16S rDNA sequences were obtained.

A BLAST search of the F38-F2 sequence (covering two-thirds of the 5′ end of the 16S rRNA gene) [Fig 3.7 (a)] revealed 98% homology to *M. endolithica* DSM 44398$^{T}$ (AJ560635) and *M. chalcea* DSM 43026$^{T}$ (X92594) over 978 bases, while the BLAST search of the R5 sequence (covering the 3′end of the 16S rRNA gene) [Fig. 3.7 (b)], revealed 96% homology to *M. chersina* DSM 44151$^{T}$ (X92628), *M. auratinigra* DSM 44815$^{T}$ (AB159779), *M. matsumotoense* IMSNU 22003$^{T}$ (AF152109), *M. endolithica* DSM 44398$^{T}$ (AJ560635), *M. rosaria* DSM 803$^{T}$ (X92631), and *M. echinospora* DSM 43816$^{T}$ (X92607).

Sequencing primer F38 binds to position 8 of the *E. coli* 16S rDNA sequence. Primer F2 binds to position 338, and primer R5 binds to position 1492.

(a)

```
SEQ  F38-F2 ED Consensus MMS2: 981 bp;
Composition  213  A; 241  C; 351  G; 176  T; 0 OTHER
Percentage:  22%  A; 25%  C; 36%  G; 18%  T; 0%OTHER

Molecular Weight (kDa): ssDNA: 305.30    dsDNA: 604.9
ORIGIN
1       TAACATGCAG TCGAGCGGAA AGGCCTTCGG GGTTACTCGA GCGGCGAACG GGTGAGTTAA
61      CACGTGAGCA CCTGCCCCAG GCTTTGGGGA TAACCCCGGG AAACCGGGGC TAATACCGAA
121     TATGACCTTG CACCGCATGG TGTTTGGTGG AAAGTTTTTC GGCTTGGGAT GGGCTCGCGG
181     CCTATCAGCT TGTTGGTGGG GTGATGGCCT ACCAAGGCGA CGACGGGTAG CCGGCCTGAG
241     AGGGCGACCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTG
301     GGGAATATTG CACAATGGGC GGAAGCCTGA TGCAGCGACG CCGCGTGAGG GATGACGGCC
361     TTCGGGTTGT AAACCTCTTT CAGCAGGGAC GAAGCGTAAG TGACGGTACC TGCAGAAGAA
421     GCGCCGGCCA ACTACGTGCC AGCAGCCGCG GTAAGACGTA GGGCGCGAGC GTTGTCCGGA
481     TTATTGGGCG TAAGAGCTCG TAGGCGGCTT GTCGCGTCGA CTGTGAAAAC CCGCAGCTCA
541     ACTGCGGGCC TGCAGTCGAT ACGGGCAGGC TAGAGTTCGG TAGGGGAGAC TGGAATTCCT
601     GGTGTAGCGG TGAAATGCGC AGATATCAGG AGGAACACCG GTGGCGAAGG CGGGTCTCTG
661     GGCCGATACT GACGCTGAGG AGCGAAAGCG TGGGGAGCGA ACAGGATTAG ATACCCTGGT
721     AGTCCACGCT GTAAACGTTG GGCGCTAGGT GTGGGGGGCC TCTCCGGTTC CCTGTGCCGC
781     AGCTAACGCA TTAAGCGCCC CGCCTGGGGA GTACGGCCGC AAGGCTAAAA CTCAAAGGAA
841     TTGACGGGGG CCCGCACAAG CGGCGGAGCA TGCGGATTAA TTCGATGCAA CGCGAAGAAC
901     CTTACCTGGG TTTGACATGG CCGCAAAACT GTCAGAGATG GCAGGTCCTT CGGGGGCGGT
961     CACAGGTGGT GCATGGCTGT C
```

(b)

```
SEQ  MMS2 RC-R5 ED: 261 bp;
Composition  63   A; 70   C; 80   G; 48   T; 0 OTHER
Percentage:  24%  A; 27%  C; 31%  G; 18%  T; 0%OTHER

Molecular Weight (kDa): ssDNA: 80.86     dsDNA: 160.9
ORIGIN
1       GGGTCTGATG CTGAATTAAC CACGGGTGAG TGATGGAAGG CCGAAAAATT CCCAAAAAGC
61      CGGATCGTCA GTTCGGATTC GGGGTTCTGC AACTCGACCC CGTGAAGTCG GAGTCGCTAG
121     TAAATCGCAG ATCAGCAACG CTGCGGTGAA TACGTTCCCG GGCCTTGTAC AACACCGCCC
181     GTCACGTCAC GAAAGTCGGC AACACCCGAA GCCGGTGGCC CAACCATTGT GGAGGGAGCC
241     GTCGAATGTG GGCTGCGTGA C
```

Fig. 3.7. Strain MMS2 partial 16S rDNA sequences: (a) sequence obtained from sequencing primers F38 and F2; (b) reverse complementary sequence obtained from sequencing primer R5.

### (10) *Micromonospora* sp. strain MLR

Strain MLR was isolated from a vegetable patch in Napier, South Africa, by Marilize le Roes. Spores are abundant, spherical, and are borne singly on short sporophores [Fig. 3.6 (b)]. The sugar constituents of the cell wall are arabinose and xylose, present in equal amounts.

A BLAST search of the F2 sequence (Fig. 3.8) revealed 98% homology to *M. purpureochromogenes* DSM 43821$^T$ (X92611), *M. chalcea* DSM 43026$^T$ (X92594), and *M. aurantiaca* DSM 43813$^T$ (X92604).

```
SEQ  F2 MLR ED: 803 bp;
Composition  173  A; 197  C; 285  G; 148  T; 0 OTHER
Percentage:  22%  A; 25%  C; 35%  G; 18%  T; 0%OTHER

Molecular Weight (kDa): ssDNA: 249.84    dsDNA: 495.1
ORIGIN
1       TGGGATTTGC CAATGGGCGG AGCCTGATCC AGCGACGCCG CGTGAGGGAT GACGGCCTTC
61      GGGTTGTAAA CCTCTTTCAG CAGGGACGAA GCGAAAGTGA CGGTACCTGC AGAAGAAGCG
121     CCGGCCAACT ACGTGCCAGC AGCCGCGGTA AGACGTAGGG CGCGAGCGTT GTCCGGATTT
181     ATTGGGCGTA AAGAGCTCGT AGGCGGCTTG TCGCGTCGAC CGTGAAAACC TGGGGCTCAA
241     CCCCAGGCCT GCGGTCGATA CGGGCAGGCT AGAGTTCGGT AGGGGAGACT GGAATTCCTG
301     GTGTAGCGGT GAAATGCGCA GATATCAGGA GGAACACCGG TGGCGAAGGC GGGTCTCTGG
361     GCCGATACTG ACGCTGAGGA GCGAAAGCGT GGGGAGCGAA CAGGATTAGA TACCCTGGTA
421     GTCCACGCTG TAAACGTTGG GCGCTAGGTG TGGGGGGCCT CTCCGGTTCC CTGTGCCGCA
481     GCTAACGCAT TAAGCGCCCC GCCTGGGGAG TACGGCCGCA AGGCTAAAAC TCAAAGGAAT
541     TGACGGGGGC CCGCACAAGC GGCGGAGCAT GCGGATTAAT TCGATGCAAC GCGAAGAACC
601     TTACCTGGGT TTGACATGGC CGCAAAACTC GCAGAGATGT GAGGTCCTTC GGGGGCGGTC
661     ACAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGGAGA TGTTGGGTTT AAGTCCCGCA
721     ACGAGCGCAA CCCTCGTTCG ATGTTGCCAG CGCGTTTATG GCGGGGACTC ATCGAAGACT
781     GCCGGGGTCA ACTCGGAAGA AAG
```

Fig. 3.8. Partial 16S rDNA sequence of strain MLR obtained from sequencing primer F2.

### 3.3.5 Group B: *Micromonospora* Strains MMS-8 to -12 and MMS-14 to -19

Strain MMS17 was isolated from a sediment sample obtained from the main lagoon channel of the Klein Estuary. Strains MMS-8 to -12, MMS14, and MMS16 were isolated from a sediment sample obtained from the Stanford River, which feeds the Klein Estuary. Strains MMS15 and MMS18 were isolated from a sediment sample obtained from the Beacon Isle lagoon, Plettenberg Bay, South Africa. Strain MMS19 was isolated from near-shore ocean sediment, Phuket, Thailand and is the only *Micromonospora* strain to grow at 45°C. There is no strain MMS13.

Strain MMS8 produces black-grey aerial mycelia on modified 172F agar, while grey aerial mycelia are produced by MMS14 (on YEME agar) and MMS15 (on BM-Glucose agar). The substrate mycelia and the diffusible pigments of these 11 strains are not pH sensitive. The brown-red pigment typically seen during tyrosine degradation was produced by strains MMS8, MMS9, MMS11, MMS15, and MMS16. These 11 strains were placed in the genus *Micromonospora* on the basis of colony morphology and ARDRA. Physiological characteristics are listed in Tables 3.6 and 3.7. All of the environmental isolates in Group B utilized glycerol (1.0% w/v) as a sole carbon source. This is particularly interesting, as *M. purpureochromogenes* is the only validly published member of the genus to use glycerol as a sole carbon source.

Table 3.6. Physiological characteristics of the Group B *Micromonospora* strains.

| **Characteristics:** | **MMS8** | **MMS9** | **MMS10** | **MMS11** | **MMS12** | **MMS14** | **MMS15** | **MMS16** | **MMS17** | **MMS18** | **MMS19** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Maximum NaCl Tolerance (%, w/v) | 5.0 | 5.0 | 3.0 | 5.0 | 4.0 | 2.0 | 3.0 | 4.0 | 5.0 | 0.0 | 6.0 |
| Nitrate reduction | – | s+ | s+ | + | + | s+ | + | s+ | + | – | – |
| $H_2S$ production | s+ | s+ | + | + | + | + | + | + | + | – | + |
| L-Tyrosine degradation | s+ | + | – | s+ | – | – | + | + | – | – | + |
| Growth at: | | | | | | | | | | | |
| 4°C | – | – | – | – | – | – | – | – | – | – | – |
| 37°C | s+ | s+ | + | s+ | s+ | + | + | s+ | s+ | – | + |
| 45°C | – | – | – | – | – | – | – | – | – | – | + |
| Growth on: | | | | | | | | | | | |
| Potato slice | + | + | + | + | s+ | – | – | – | + | – | + |
| Potato slice plus $CaCO_3$ | + | s+ | + | + | s+ | – | – | – | + | – | + |
| Czapek-solution agar | + | + | s+ | – | w+ | – | + | + | w+ | – | + |
| *ISP* Medium No. 4 | w+ | + | + | + | – | + | + | + | – | – | + |
| Antibiotic Susceptibility: | | | | | | | | | | | |
| Cephaloridine (100 µg/ml) | – | – | – | – | + | – | + | + | – | – | – |
| Lincomycin (100 µg/ml) | – | – | – | – | + | – | + | – | – | – | – |
| Oleandomycin (100 µg/ml) | + | + | – | + | + | + | + | + | + | + | + |
| Penicillin G (10 I.U.) | + | + | – | + | + | + | + | + | + | – | – |
| Rifampicin (50 µg/ml) | – | – | – | – | – | + | – | – | + | – | – |
| Tobramycin (50 µg/ml) | – | – | – | + | + | – | – | – | + | – | – |
| Vancomycin (50 µg/ml) | – | – | – | – | – | – | – | – | – | – | – |
| Organic Compound Utilization: | | | | | | | | | | | |
| *n*-Butanol | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| *meso*-Erythritol | ++ | ++ | + | + | ++ | ++ | ++ | ++ | ++ | ++ | + |
| Ethanol | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| Isoamylalcohol | ++ | ++ | ++ | + | ++ | ++ | + | ++ | ++ | + | + |
| α-Methyl-D-glucoside | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | – | + | ++ |
| Mucic acid | ++ | ++ | ++ | ++ | + | + | – | – | + | – | – |
| Sodium acetate | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |
| Sodium butyrate | ++ | ++ | ++ | + | ++ | ++ | + | + | ++ | ++ | + |
| Sodium citrate | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | + |
| Sodium formate | ++ | ++ | ++ | + | ++ | + | ++ | ++ | – | – | – |
| Sodium lactate | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ |
| Sodium salicylate | + | ++ | + | + | ++ | ++ | + | + | – | – | – |
| Sodium L(+)-tartrate | ++ | ++ | ++ | + | ++ | ++ | ++ | + | ++ | + | + |

Abbreviations: –, no growth/utilization; w+, weakly positive; +, moderate growth/utilization; ++, good growth/utilization; s+, strongly positive.

Table 3.7. Sole carbon and nitrogen source utilization of the Group B *Micromonospora* strains.

| **Characteristic:** | **MMS8** | **MMS9** | **MMS10** | **MMS11** | **MMS12** | **MMS14** | **MMS15** | **MMS16** | **MMS17** | **MMS18** | **MMS19** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon Sources: | | | | | | | | | | | |
| D(+)-Glucose | + | + | + | + | ++ | + | – | + | + | + | + |
| Adonitol | ++ | ++ | + | + | ++ | + | + | + | + | + | + |
| L(+)-Arabinose | + | + | ++ | ++ | ++ | ++ | + | +++ | – | + | + |
| Glycerol | + | + | ++ | + | ++ | ++ | + | + | + | + | + |
| *myo*-Inositol | + | + | ++ | + | – | ++ | ++ | +++ | – | – | + |
| α-Melibiose | + | ++ | +++ | + | ++ | +++ | + | +++ | + | + | + |
| D(+)-Melezitose | ++ | ++ | ++ | ++ | ++ | + | + | + | + | + | + |
| L(+)-Rhamnose | ++ | ++ | ++ | + | + | + | + | + | + | + | + |
| D(–)-Ribose | + | + | + | – | + | + | – | – | – | – | + |
| Sucrose | ++ | +++ | +++ | + | +++ | +++ | ++ | +++ | + | + | + |
| Nitrogen Sources: | | | | | | | | | | | |
| L-Arginine | + | + | + | ++ | – | ++ | – | – | – | – | + |
| DL-Ornithine | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | +++ | + | + |
| L-Serine | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | + | + |
| L-Threonine | ++ | + | +++ | ++ | +++ | +++ | + | + | + | – | – |

Abbreviations: –, no growth/utilization; +, weak growth/utilization; ++, moderate growth/utilization; +++, good growth/utilization.

Some strains were tested on other media not listed in the tables. The results are as follows.

### (i) *Micromonospora* sp. strain MMS10

Strain MMS10 has an orange-coloured mycelium, however, once sporulation occurs, a green layer of spores develops. This can be seen on the medium testing for cellulose degradation, *ISP* Medium No. 4, and on Czapek-solution agar. This has not been reported for any other *Micromonospora* strain and since the morphological features of the members of this genus play an important role in discerning between species, both strains MMS10 and MMS12 might be considered new species. It must be noted that that the mycelial colour of *M. olivasterospora* is olive-green before and after sporulation. As can be seen in Table 3.6 and 3.7, strains MMS10 and MMS12 are different from each other. Strain MMS10 appears black on L-arabinose agar, and does not produce a diffusible pigment on *ISP* Medium No. 5. The strain grows weakly on Middlebrook 7H9 agar and does not grow at pH 4.3. Melanin is not produced on *ISP* Media No. 6 and No. 7. Starch is hydrolysed. Gelatin is degraded but cellulose is not.

### (ii) *Micromonospora* sp. strain MMS12

Strain MMS12 is orange-coloured, turning green upon sporulation on BM-Glycerol agar, erythritol and sodium lactate agars. This strain has a distinctive ocean smell (such as a coastal seaweed smell) on all media. A diffusible pigment is not produced on *ISP* Medium No. 5, nor is melanin produced on *ISP* Media No. 6 and No. 7. Good growth occurs on Middlebrook 7H9 agar but no growth occurs at pH 4.3. Starch is hydrolysed. Gelatin is degraded but cellulose is not.

**(iii) *Micromonospora* sp. strain MMS14**

A diffusible pigment is not produced on *ISP* Medium No. 5, nor is melanin produced on *ISP* Media No. 6 and No. 7. Strain MMS14 grows very weakly on Middlebrook 7H9 agar and does not grow at pH 4.3. Starch is hydrolysed. Gelatin is degraded but cellulose is not.

**(iv) *Micromonospora* sp. strain MMS17**

A diffusible pigment is not produced on *ISP* Medium No. 5, nor is melanin produced on *ISP* Media No. 6 and No. 7. Strain MMS17 grows very weakly on Middlebrook 7H9 agar and does not grow at pH 4.3. Starch is hydrolysed. Gelatin is degraded but cellulose is not.

### 3.3.6 The 7 Streptomycetes

These 7 strains were placed in the genus *Streptomyces* by morphology and ARDRA. Mycolic acids were absent. Table 3.8 shows the important morphological features of these 7 streptomycetes. Tables 3.9 and 3.10 list the physiological characters of the strains. It is interesting to note that members of the genus *Streptomyces* can tolerate much higher sodium chloride concentrations than members of the genus *Micromonospora*. The thin-layer chromatography (TLC) plate of the whole cell sugars of some of the *Streptomyces* strains is shown in Fig. 3.13 (c). The genus *Streptomyces* does not have characteristic sugars useful for descriptive purposes. All 7 strains produced a variety of coloured diffusible pigments on the various agar plates used for testing sole carbon and nitrogen source utilization.

Table 3.8. Morphological characteristics of the 7 *Streptomyces* strains.

| | On *ISP* Medium No. 4: | | By Cryo Electron Microscopy: | | |
|---|---|---|---|---|---|
| *Streptomyces* Strains: | Aerial Mycelium Colour: | Substrate Mycelium Colour: | Spore Chain Morphology: | Spore Surface Ornamentation: | Diffusible Pigment Colour on *ISP* Medium No. 5: |
| BAM1 | Blue | Navy blue | *Spirales* | Spiny | Peach |
| BAM3 | Blue | Blue-green | *Spirales* | Spiny | Green |
| BAM4 | Blue | Red-orange | *Rectiflexibiles* | Smooth | Dark brown to red-orange |
| BAM7 | Blue | Blue-green | *Spirales* | Spiny | Green |
| SVM65 | Blue | Yellow-brown | *Spirales* | Hairy | Yellow |
| PB | Blue | Dark pink | *Spirales* | Spiny | Pink-red |
| HMS88 | Yellow | Yellow-brown | *Rectiflexibiles* | Smooth | Pink |

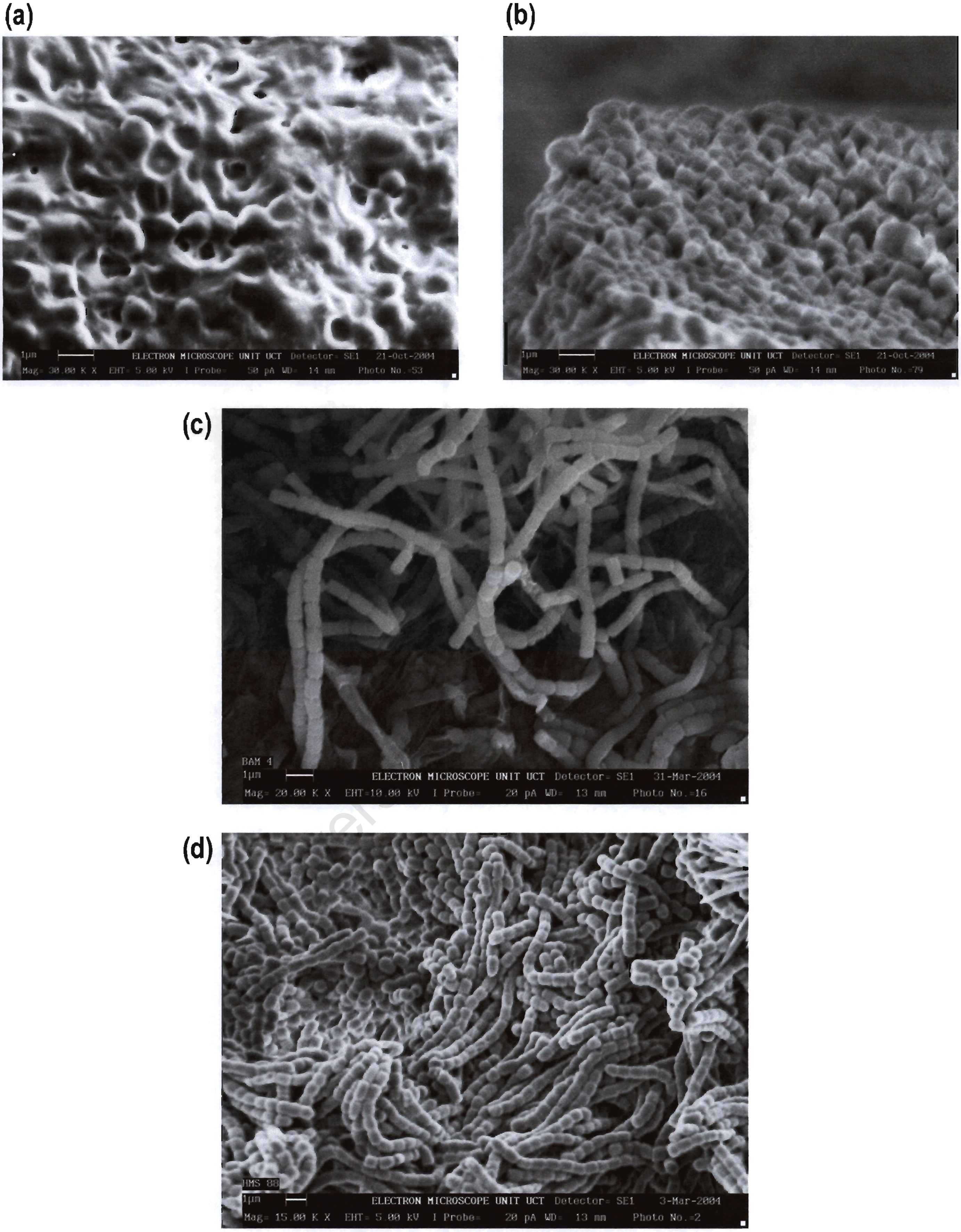


Fig. 3.6. Scanning electron micrographs of *Micromonospora* sp. strains MMS2 (a) and MLR (b), and of '*Streptomyces noordhoekensis*' strain BAM4$^T$ (c) and *Streptomyces* sp. strain HMS88 (d).

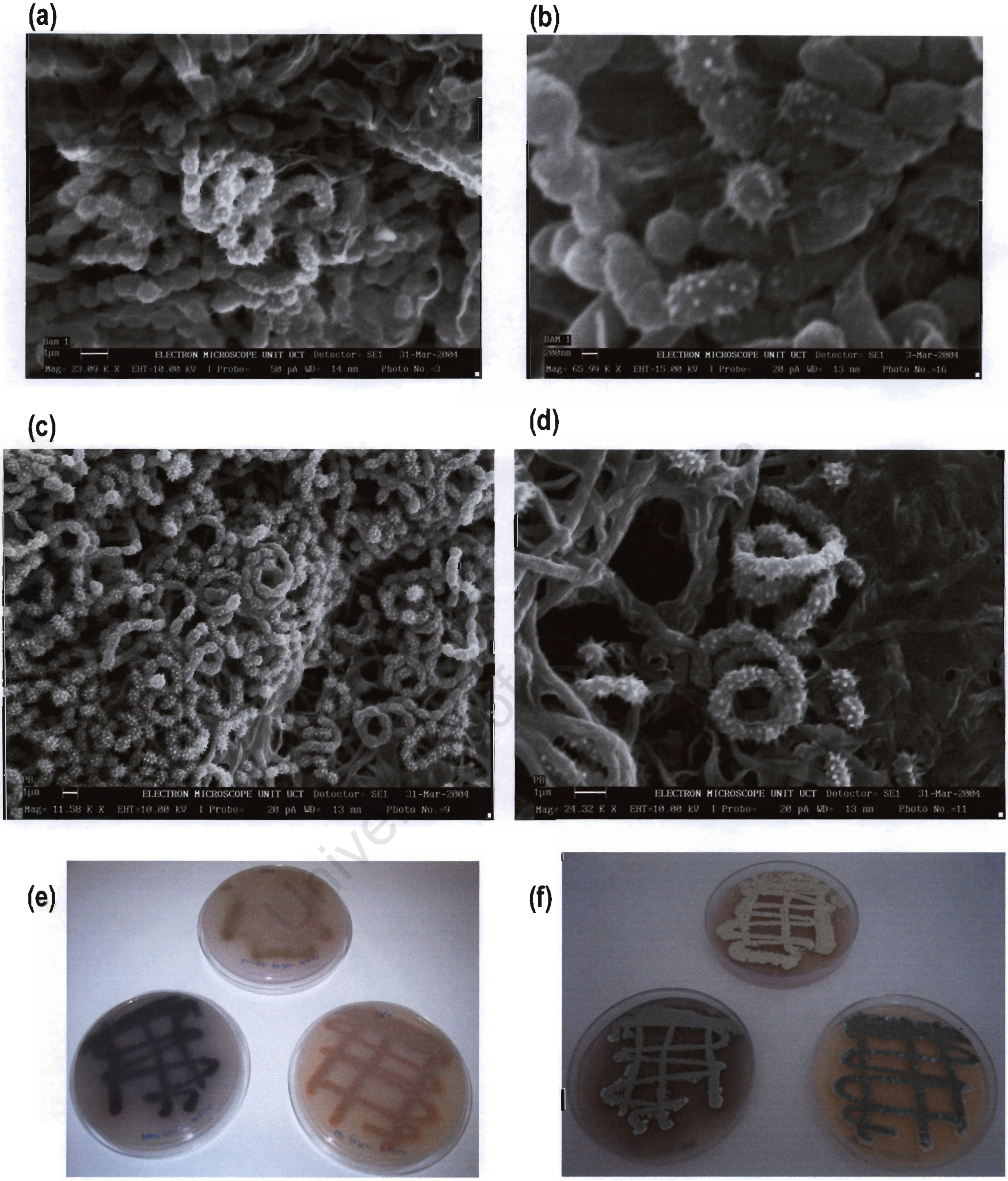


Fig. 3.9.Scanning electron micrographs of *Streptomyces* sp. strains BAM1 (a, b) and PB (c, d) showing on the left, the *Spirales* spore chain morphology, and on the right, a closer view of the spiny spore surface ornamentation. The features of strains HMS88 (top), BAM1 (left), and PB (right) are shown in photographs of the substrate mycelial colour (e) and the aerial mycelia color (f) of the strains grown on *ISP* Medium No. 4 for 2 weeks at 30°C.

### (1) '*Streptomyces noordhoekensis*' strain BAM4$^{T}$

*Streptomyces noordhoekensis* (noord'hoek.ensis L. masc. *noordhoekensis*, of Noordhoek, a coastal suburb in Cape Town) was isolated from a beach sand sample. The features seen by electron microscopy are shown in Fig. 3.6 (c). On *ISP* Medium No. 5, the substrate mycelium of strain BAM4$^{T}$ changes colour from brown to yellow-brown upon addition of 50 mM HCl. This strain produces a dark brown diffusible pigment on Bennett's agar, tyrosine, and egg yolk medium agars, as well as a light brown-orange pigment on *ISP* Medium No. 4.

A BLAST search revealed that the streptomycetes with highest 16S rDNA sequence homology to strain BAM4$^{T}$ are: *S. lateritius* JCM 4389$^{T}$ (AF454764), with 99% homology (1405 over 1408 bases); and *S. venezuelae* JCM 4526$^{T}$ (AB045890), also with 99% homology (1408 over 1412 bases). The percentage pairwise identities in DNAMAN are 98.39% (over 1410 nucleotides) and 94.94% (over 1412 nucleotides), respectively. Because of the high 16S rDNA homology, DNA-DNA reassociation (hybridisation) studies will have to be performed to prove that strain BAM4$^{T}$ is a novel species, despite the many differences in physiological characteristics. BAM4$^{T}$ has blue aerial mycelia, a red-orange substrate mycelium, smooth spores, and a *Rectiflexibiles* spore chain morphology. *S. lateritius* differs from BAM4$^{T}$ in that it has red aerial mycelia and a *Spirales* spore chain morphology, while *S. venezuelae* differs from BAM4$^{T}$ by having yellow aerial mycelia. Strain BAM4$^{T}$ is differentiated from *S. lateritius* by the following physiological tests: strain BAM4$^{T}$ was sensitive to penicillin G (10 I.U.); it utilized L-histidine as a sole nitrogen source but did not utilize L-rhamnose as a sole carbon source. Strain BAM4$^{T}$ is differentiated from *S. venezuelae*, which is a subjective synonym of *S. violaceus*, by the following physiological tests: strain BAM4$^{T}$ did not have lecithinase activity; was resistant to oleandomycin (100 µg/ml), and was sensitive to rifampicin (50 µg/ml) and penicillin G (10 I.U.); it grew in the presence of 7.0% (w/v) NaCl; it utilized L-histidine as a sole nitrogen source but did not utilize L-phenylalanine; it did not utilize raffinose and D-melibiose as sole carbon sources.

### (2) *Streptomyces* sp. strain HMS88

Strain HMS88 was isolated from a sediment sample obtained from the Stanford River, which feeds the Klein Estuary. *Streptomyces* strains isolated from marine environments often have yellow aerial mycelia and a smooth spore surface (17). Fig. 3.6 (d) shows the features seen by electron microscopy. Fig. 3.9 (e) and (f) show photographs of strain HMS88 on *ISP* Medium No. 4. This strain produces a pink pigment on *ISP* Medium No. 4, which diffuses throughout the agar plate.

A BLAST search of the F2 sequence (Fig. 3.10) of strain HMS88 revealed 97% homology to the following *Streptomyces* strains over 860 bases: *S. caviscabies* ATCC 51928$^{T}$ (sequence accession number: AF112160); *S. cyaneus* ISP 5108$^{T}$ (AJ399460); *S. flavogriseus* CBS 101.34$^{T}$ (AJ494864); *S. griseus* (AY207580); and *S. setonii* ATCC 25497$^{T}$ (D63872; a subjective synonym of *S. anulatus*). Strain HMS88 has yellow aerial mycelia, a yellow-brown substrate mycelium, *Rectiflexibiles* spore chain morphology and the spore surface is smooth. *S. griseus* and *S. setonii* share those features but a 97% homology is low enough to suggest that strain HMS88 may be a new species.

```
SEQ  HMS88 F2 ED: 960 bp;
Composition  230  A; 239  C; 310  G; 181  T; 0 OTHER
Percentage:  24%  A; 25%  C; 32%  G; 19%  T; 0%OTHER

Molecular Weight (kDa): ssDNA: 298.07    dsDNA: 591.9
ORIGIN
1       GGAAATTGCC AATGGGCGAA GCCTGATCCA GCGACGCCGC GATGAGGGAT GACGGCCTTC
61      GGGTTGTAAA CCTCTTTCAG CAGGGAAGAA GCGAAAGTGA CGGTACCTGC AGAAGAAGCG
121     CCGGCTAACT ACGTGCCAGC AGCCGCGGTA ATACGTAGGG CGCAAGCGTT GTCCGGAATT
181     ATTGGGCGTA AAGAGCTCGT AGGCGGCTTG TCACGTCGGA TGTGAAAGCC CGGGGCTTAA
241     CCCCGGGTCT GCATTCTATA CGGGCTAGCT AGAGTGTGGT AGGGGAGATC GGAATTCCTG
301     GTGTAGCGGT GAAATGCGCA GATATCAGGA GGAACACCGG TGGCGAAGGC GGATCTCTGG
361     GCCATTACTG ACGCTGAGGA GCGAAAGCGT GGGGAGCGAA CAGGATTAGA TACCCTGGTA
421     GTCCACGCCG TAAACGTTGG GAACTAGGTG TTGGCGACAT TCCACGTCGT CGGTGCCGCA
481     GCTAACGCAT TAAGTTCCCC GCCTGGGGAG TACGGCCGCA AGGCTAAAAC TCAAAGGAAT
541     TGACGGGGGC CCGCACAAGC AGCGGAGCAT GTGGCTTAAT TCGACGCAAC GCGAAAAACC
601     TTACCAAGGC TTGACATATA CCGGAAAGCA TCAGAGATGG TGCCCCCCTT GTGGACGGTA
661     TACAGGTGGT GCATGGCCTG TCCTCAGCTC GTGTCTGGAG ATGTTGGGTT AAGTCCCGCA
721     ACAAGCGCAA CCCTTGCTCT GTGTTGCCAC CATGCCCTTC GGGGTGATGG GGACTCAAGG
781     AGACTGCCGG GGTCAACTCG GAGGAGGTGG GGAAACAACA AGTCCTCATG CCCCTTTGTC
841     TGGGGCCTGA CACGTGCTAC AATGCCGGTA AATGGGTGCG AGCCCGGGGG GAACCAATTC
901     AAAACCGGCC CCCATCCGAT TGGGGTCGCA CTCGCCCCTG AACCGGATTG TAAAAAAAAA
```

Fig. 3.10. Partial 16S rDNA sequence of strain HMS88 obtained from sequencing primer F2.

### (3) *Streptomyces* sp. strain BAM7

Strain BAM7 was isolated from Noordhoek beach. Fig. 3.11 (a) and (b) show the features of this strain revealed by electron microscopy. On *ISP* Medium No. 5, the substrate mycelium of strain BAM7 changes colour from green to purple upon addition of HCl.

A BLAST search of the F2 sequence (Fig. 3.12) revealed that the streptomycetes with highest homology to BAM7 are: *S. olivochromogenes* DSM 40451$^T$ (AY094370) and *S. cyaneus* NRRL B-2296$^T$ (AF346475), both showing 99% homology (542 over 547 bases). Some members of the *S. cyaneus* species group have blue aerial mycelia, spiny spores, and *Spirales* spore chain morphology (13). BAM7 differs from *S. cyaneus* as it has a blue-green substrate mycelium and produces a green diffusible pigment, whereas *S. cyaneus* has neither feature. All strains of *S. cyaneus* produce melanin on *ISP* Medium No. 6 and utilize D-melibiose and raffinose as sole carbon sources (13). BAM7 shares these features except that D-melibiose is weakly utilized. *S. olivochromogenes* is a subjective synonym of *S. diastaticus*, having grey aerial mycelia and does not produce melanin nor has it a green substrate mycelium.

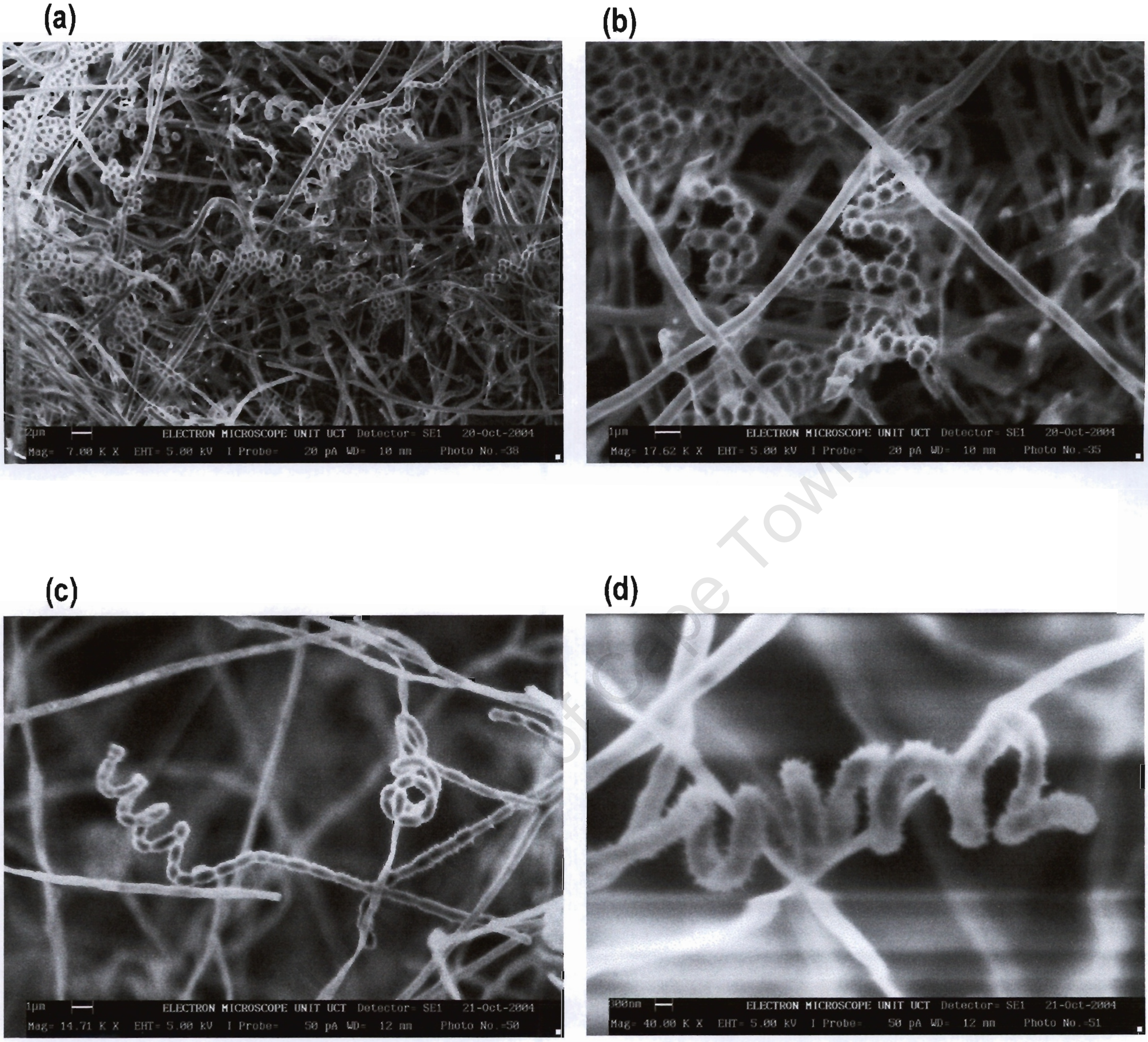


Fig. 3.11. Scanning electron micrographs (a, b) of *Streptomyces* sp. strain BAM7 showing the *Spirales* spore chain morphology and spiny spore surface ornamentation. (c, d) Scanning electron micrographs of *Streptomyces* sp. strain BAM3; this strain is also spiny with a *Spirales* spore chain morphology.

```
SEQ  BAM7 F2 ED: 675 bp;
Composition  171  A; 161  C; 219  G; 124  T; 0 OTHER
Percentage:  25%  A; 24%  C; 32%  G; 18%  T; 0%OTHER

Molecular Weight (kDa): ssDNA: 209.80    dsDNA: 416.2
ORIGIN
1       ATGACATGGG CGAAAGCTGA TGCAGCGACG CCGCGTGAGG GATGACGGCC TTCGGGTTGT
61      AAACCTCTTT CAGCAGGGGA AGAAGCGAAA GTGACGGTAC CTGCAGAAGA AGCGCCGGCT
121     AACTACGTGC CAGCAGCCGC GGTAATACGT AGGGCGCAAG CGTTGTCCGG AATTATTGGG
181     CGTAAAGAGC TCGTAGGCGG CTTGTCACGT CGGGTGTGAA AGCCCGGGGC TTAACCCCGG
241     GTCTGCATTC GATACGGGCT AGCTAGAGTG TGGTAGGGGA GATCGGAATT CCTGGTGTAG
301     CGGTGAAATG CGCAGATATC AGGAGGAACA CCGGTGGCGA AGGCGGATCT CTGGGCCATT
361     ACTGACGCTG AGGAGCGAAA GCGTGGGGAG CGAACAGGAT TAGATACCCT GGTAGTCCAC
421     GCCGTAAACG GTGGGAACTA GCGTGTTGGC GACATTCCAC GTCGTCGGTG CCGCAGTCTA
481     ACGCATTAAG TTCCCCGTCT GGGGAGTACG GCCGCAAGGC TAAAACTCAA AGGAATTGAC
541     GGGGGCCCGC AACAATGCCG ATGGAAGTAC ACTGTGGCTT AATTCGAACG CAAACGACGA
601     AAGAACCTTA CCAACGGGCT TGACACATAC AACCCGCGAA AAGCTCATTC TCAGAATTCG
661     TAGGCCACAC GCTTG
```

Fig. 3.12. Partial 16S rDNA sequence of strain BAM7 obtained from sequencing primer F2.

### (4) ***Streptomyces* sp. strain PB**

Strain PB was isolated from the southern shore of Bazaruto Island, off the coast of Mozambique. The features of strain PB revealed by electron microscopy are shown in Fig. 3.9 (c) and (d). Fig. 3.9 (e) and (f) show photographs of strain PB, or 'Plum Bug', on *ISP* Medium No. 4. On *ISP* Medium No. 5, the substrate mycelium of strain PB is pH sensitive, changing colour from plum to red upon addition of HCl. This strain produces a plum-coloured pigment on *ISP* Medium No. 4, and a dark red diffusible pigment on glucose, galactose, and mannitol agars. Strain PB does not share morphological features in common with other blue streptomycetes, thus this strain is a potentially novel species.

### (5) ***Streptomyces* sp. strain BAM1**

Strain BAM1 was isolated from Noordhoek beach. Fig.3.9 (a) and (b) show the features seen by electron microscopy. Fig. 3.9 (e) and (f) show photographs of strain BAM1 on *ISP* Medium No. 4. Strain BAM1 produces a purple-blue diffusible pigment on Bennett's agar; a dark violet pigment on *ISP* Medium No. 4, casein, xanthine, and tyrosine agars, and in the medium used for the nitrate reduction test. Strain BAM1 is a potentially novel species as there is no known streptomycete having both blue aerial and substrate mycelia.

### (6) ***Streptomyces* sp. strain BAM3**

Strain BAM3 was isolated from Noordhoek beach. Fig. 3.11 (c) and (d) show the features seen by electron microscopy. On *ISP* Medium No. 5, the substrate mycelium of strain BAM3 changes colour from green to purple upon addition of HCl. Strain BAM3 is not a strain of *S. cyaneus*: it has a blue-green substrate mycelium and produces a green diffusible pigment, whereas *S. cyaneus* has neither feature. Furthermore, although strain BAM3 produces melanin on *ISP* Medium No. 6 and utilizes raffinose as a sole carbon source, it differs from the *S. cyaneus* species group in that it does not utilize D-melibiose as a sole carbon source. *S. viridochromogenes* has blue aerial mycelia, spiny spores, and *Spirales* spore chain morphology. However, BAM3 differs from this species as its green diffusible pigment is not pH sensitive, as well as having other differences in physiological characters. *S. viridochromogenes* is resistant to rifampicin (50 μg/ml), sensitive to penicillin G (10 I.U.), utilizes *meso*-inositol, L-rhamnose, and D-melibiose but does not utilize sucrose and L-phenylalanine (13).

### (7) ***Streptomyces* sp. strain SVM65**

Strain SVM65 was isolated from a soil sample obtained from a nature reserve at Silvermine, an area near Noordhoek. Fig. 3.13 (a) and (b) show the features seen by electron microscopy. This strain produces a lime green pigment on *ISP* Medium No. 4, which diffuses throughout the agar plate. Strain SVM65 does not share morphological features in common with other blue streptomycetes, thus this strain is a potentially novel species.

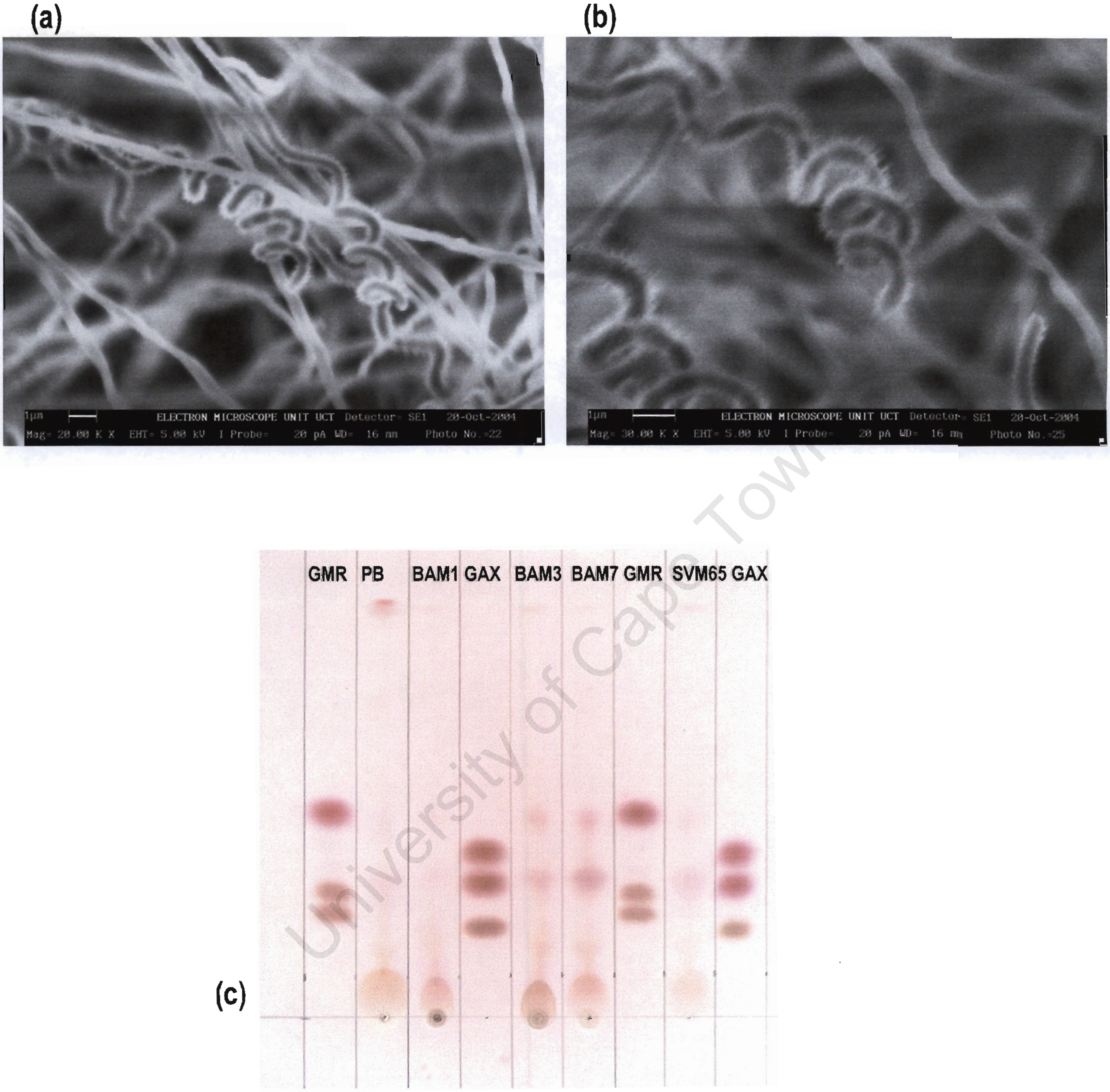


Fig. 3.13. (a, b) Scanning electron micrographs of *Streptomyces* sp. strain SVM65 showing the *Spirales* spore chain morphology and hairy spore surface ornamentation. (c) Determination of the whole cell sugar pattern of *Streptomyces* sp. strains PB, BAM1, BAM3, BAM7, and SVM65. The 1% sugar standards are: GMR, glucose, mannose, and ribose; GAX, galactose, arabinose, and xylose.

Table 3.9. Physiological characteristics of the 7 *Streptomyces* strains.

| **Characteristics:** | **BAM1** | **BAM4** | **BAM3** | **BAM7** | **PB** | **SVM65** | **HMS88** |
|---|---|---|---|---|---|---|---|
| Diffusible pigment on *ISP* Medium No. 5 | + | + | + | + | + | + | + |
| Aesculin hydrolysis | vs+ | vs+ | + | vs+ | vs+ | vs+ | vs+ |
| Arbutin hydrolysis | + | + | + | + | + | + | + |
| Hippurate hydrolysis | – | – | – | – | – | – | – |
| Pectin hydrolysis | + | – | + | + | + | + | – |
| Nitrate reduction | + | + | – | – | + | – | + |
| $H_2S$ production | + | + | + | + | + | + | + |
| Growth in the presence of NaCl (%, w/v): | | | | | | | |
| 7.0 | + | + | + | + | + | + | + |
| 11.0 | – | – | – | – | + | – | + |
| Adenine degradation | + | + | + | + | + | + | + |
| Allantoin degradation | – | – | – | – | + | – | – |
| Casein degradation | + | + | + | + | + | + | + |
| Cellulose degradation | – | – | – | – | – | – | w+ |
| Gelatin degradation | + | + | + | + | + | + | + |
| Guanine degradation | – | + | – | – | – | + | – |
| Hypoxanthine degradation | + | + | – | – | + | + | – |
| Starch degradation | + | + | + | + | – | + | + |
| Tween 80 degradation | + | + | + | + | + | + | + |
| L-Tyrosine degradation | + | + | – | – | + | – | – |
| Urea degradation | w+ | – | – | – | + | – | – |
| Xanthine degradation | – | + | + | – | – | – | – |
| Xylan degradation | – | – | – | – | – | – | – |
| Melanin on *ISP* Medium No. 6 | + | – | + | + | + | – | – |
| Melanin on *ISP* Medium No. 7 | + | – | – | – | – | – | – |
| Growth at 4°C | – | – | – | – | – | – | – |
| Growth at 37°C | + | + | + | + | + | + | + |
| Growth at pH 4.3 | – | – | + | + | + | + | – |
| Growth in the presence of 0.1% 2-Phenylethanol | – | – | – | – | – | – | – |
| Antibiotic Susceptibility: | | | | | | | |
| Cephaloridine (100 μg/ml) | – | – | – | + | – | – | + |
| Gentamicin (100 μg/ml) | – | – | – | – | – | – | – |
| Kanamycin (10 μg/ml) | – | – | – | – | – | – | – |
| Lincomycin (100 μg/ml) | + | – | – | + | + | + | + |
| Neomycin (50 μg/ml) | – | – | – | – | – | – | – |
| Oleandomycin (100 μg/ml) | + | + | + | + | + | + | + |
| Penicillin G (10 I.U.) | + | – | + | + | + | – | + |
| Rifampicin (50 μg/ml) | – | – | – | + | + | + | + |
| Streptomycin (100 μg/ml) | – | – | – | – | – | – | – |
| Tobramycin (50 μg/ml) | – | – | – | – | – | – | + |
| Vancomycin (50 μg/ml) | – | – | – | – | – | – | – |
| Lecithinase activity | + | – | + | + | + | + | – |
| Lipase activity | + | – | – | – | – | – | + |
| Protease activity | + | – | + | + | – | – | + |

Abbreviations: +, positive result/growth; –, negative result/no growth; vs, very strong growth; w, weak growth.

Table 3.10. *Streptomyces* sole carbon and nitrogen source utilization.

| **Characteristic:** | **HMS88** | **SVM65** | **BAM1** | **PB** | **BAM4** | **BAM7** | **BAM3** |
|---|---|---|---|---|---|---|---|
| Carbon Sources: | | | | | | | |
| L(+)-Arabinose | ++ | + | + | ++ | + | + | ++ |
| D(+)-Cellobiose | + | + | + | ++ | ++ | + | ++ |
| D(–)-Fructose | + | ± | + | ++ | ± | + | + |
| D(+)-Galactose | + | + | + | + | + | + | ++ |
| Glycerol | + | + | – | – | ++ | + | + |
| *meso*-Inositol | – | + | + | ++ | ± | ++ | – |
| D-Lactose | ± | ++ | + | ++ | ± | + | ++ |
| Maltose | + | ± | – | – | + | ± | – |
| D-Mannitol | + | + | + | + | – | + | ++ |
| D(+)-Melezitose | – | – | – | ++ | – | ± | – |
| D(+)-Melibiose | – | ± | – | ++ | – | ± | – |
| Raffinose | – | + | + | ++ | – | + | ++ |
| L(+)-Rhamnose | + | ± | – | + | – | + | – |
| Salicin | ± | ± | – | – | ± | + | – |
| Sodium benzoate | – | – | – | – | – | – | – |
| Sodium citrate | ± | ± | – | – | ± | – | – |
| Sodium malonate | – | – | – | – | – | – | – |
| Sodium propionate | ± | ± | – | – | ± | ± | – |
| D-Sorbitol | – | – | – | – | – | – | – |
| L(–)-Sorbose | – | – | – | – | – | – | – |
| Sucrose | – | + | + | + | – | ++ | ++ |
| Trehalose | ++ | + | + | ++ | ++ | ++ | + |
| Xylitol | – | – | – | – | – | – | – |
| D(+)-Xylose | ++ | ± | – | ++ | ± | + | – |
| Nitrogen Sources: | | | | | | | |
| DL-α-amino-n-butyric acid | ± | ± | – | – | + | ± | + |
| 4-amino-n-butyric acid | + | + | ++ | + | ± | + | + |
| L-Arginine | + | ++ | ++ | ++ | + | + | + |
| L-Asparagine | + | + | + | + | + | + | + |
| L-Cysteine | – | – | – | – | – | – | ± |
| L-Histidine | + | ± | ++ | ++ | + | + | + |
| L-Hydroxyproline | – | ± | + | + | + | ± | ++ |
| L-Methionine | – | + | ++ | – | ± | ± | + |
| DL-Ornithine | – | – | ++ | + | – | + | + |
| L-Phenylalanine | + | + | ++ | + | – | ± | + |
| Potassium nitrate | + | + | ++ | + | + | ± | ++ |
| L-Serine | + | + | ++ | ++ | + | + | + |
| L-Threonine | ± | + | ++ | + | + | + | + |
| L-Valine | ± | ++ | + | + | + | + | ++ |

Abbreviations: –, no growth/utilization; ±, weak growth/utilization; +, moderate growth/utilization; ++, good growth/utilization.

### 3.3.7 Six Other Strains of Interest: 3 *Streptomyces* and 3 *Pseudonocardia* Strains

The 3 *Pseudonocardia* strains were isolated from the River Kwai, Thailand, while *Streptomyces* sp. strains LMP-TD and LMP-BRP were isolated from the island of Phuket, Thailand. *Streptomyces* sp. Kit2004 was isolated from the Beacon Isle lagoon, South Africa. As these strains were isolated from previously unexplored environments, they should be further investigated to assess their potential novelty, especially since the streptomycetes show promising antimicrobial activity (see Chapter 5).

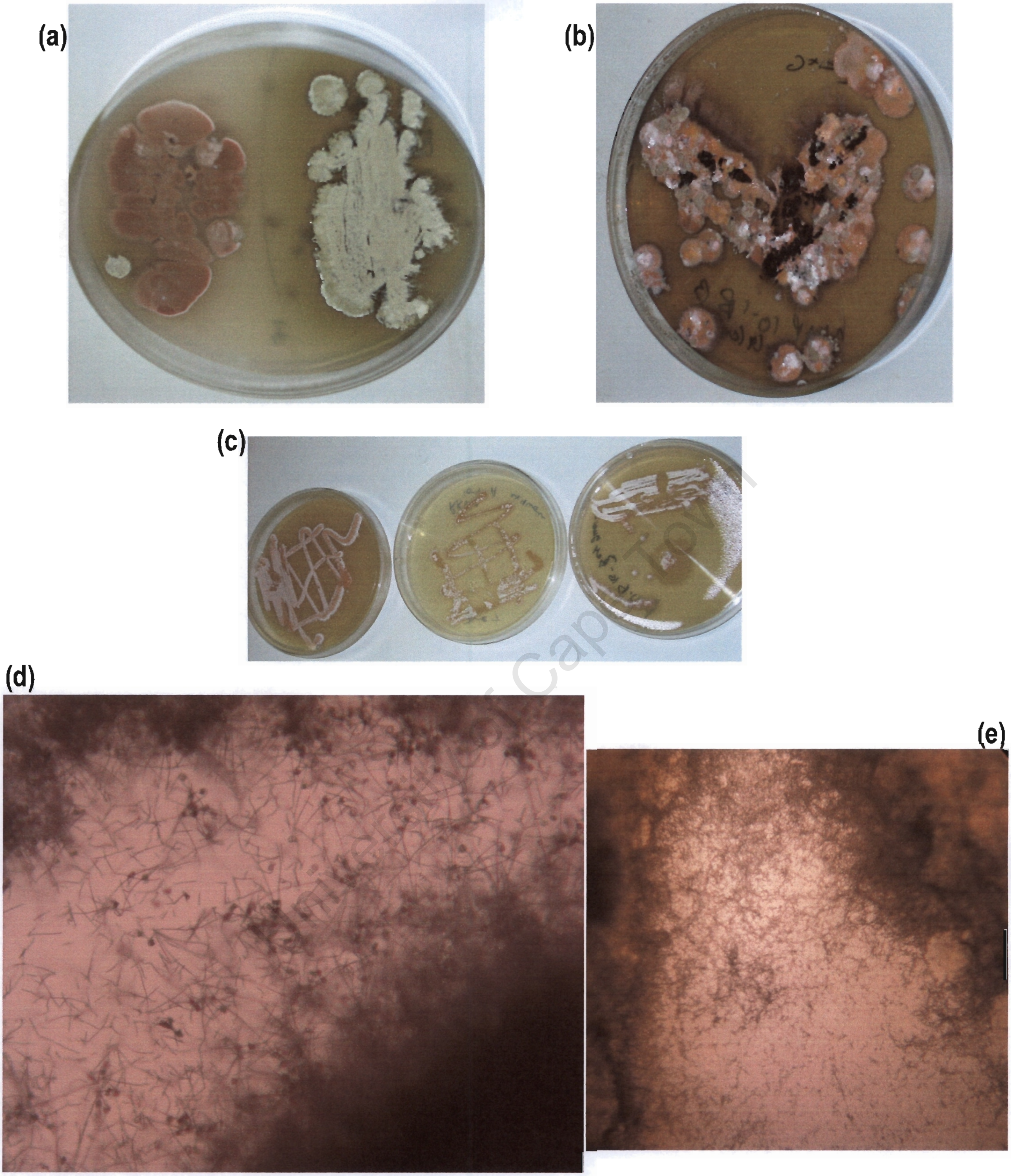


Fig. 3.14. Photograph of the colour of the aerial mycelia of (a) *Streptomyces* sp. strain LMP-TD (left half of the agar plate) and *Streptomyces* sp. strain Kit2004 (right), grown on *ISP* Medium No. 4 for 2 weeks at 28°C. (b) Photograph of the aerial mycelial colour of *Streptomyces* sp. strain LMP-BRP grown on Bennett agar. (c) The 3 *Pseudonocardia* sp. strains grown on YEME agar: strain RKVB-VW (left), strain RKVB-LW (middle), and strain RKVB-3CBM2G (right). Digital photographs taken through the eye lens of a light microscope: (d) The tightly curled spirals at the ends of the long mycelia of strain LMP-TD can be seen; (e) The dense mycelia of strain RKVB-LW. The magnification of the light microscope was 1000×.

The genus level of these 6 strains was determined by ARDRA. Photographs of these 6 strains are shown in Fig. 3.14 (a), (b), and (c). Photographs of the aerial mycelia of strains LMP-TD and RKVB-LW, grown on *ISP*4 and YEME agar plates, respectively, seen through a light microscope, are shown in Fig. 3.14 (d) and (e). *Streptomyces* sp. strain LMP-TD has a brown-red aerial mycelium and brown substrate mycelium on *ISP* Medium No. 4. The spore chain morphology, seen through a light microscope, was *Spirales*. *Streptomyces* sp. strain Kit2004 has a green aerial mycelium and dark brown-green substrate mycelium on *ISP* Medium No. 4. A dark green diffusible pigment is also produced. The spore chain morphology is *Rectiflexibiles*. *Streptomyces* sp. strain LMP-BRP grew poorly on *ISP* Medium No. 4 but grew very well on other media such as Bennett's and modified 172F agars. On *ISP* Medium No. 4 the aerial mycelium is orange and the substrate mycelium is yellow-brown. The spore chain morphology is *Rectiflexibiles*. As can be seen in Fig. 3.14 (b) this strain has a dark red substrate mycelium and multicoloured aerial mycelia on other agar plates, such as YEME, Bennett's agar, and modified 172F agar.

Electron microscopy will need to be performed on the 3 *Pseudonocardia* strains to be able to see details of the mycelium and the spores. All 3 strains have an orange substrate mycelium; the texture of each strain varies, as does the extent of sporulation (surface area covered by the white aerial mycelia).

## 3.4 Conclusion

The Klein Estuary, or Hermanus Lagoon, is concluded to be an eminently suitable ecosystem from which to isolate novel actinomycetes for screening programs. Strains of the rare actinomycete genus *Micromonospora* were easily isolated on YMEM and modified 172F media, supplemented with nalidixic acid and cycloheximide as selective inhibitors. At this time, and as a result of this study, there are 34 actinomycete isolates that warrant further investigation. There are potentially 7 novel *Streptomyces* strains and potentially 21 novel *Micromonospora* strains. Nine (9) new species are proposed: '*Micromonospora aquatica*', '*Micromonospora arenincolae*', '*Micromonospora cinerea*', '*Micromonospora grateloupicolae*', '*Micromonospora hermanusense*', '*Micromonospora lacunae*', '*Micromonospora stanfordense*', '*Micromonospora terrae*', and '*Streptomyces noordhoekensis*'. The application of the Identification Key to environmental *Micromonospora* isolates proved very successful. None of the strains tested grew on vancomycin (50 μg/ml) plates. Hippurate hydrolysis, guanine degradation, growth at 4 and 45°C, growth at pH 4.3, and sensitivity to rifampicin (50 μg/ml) are physiological tests that have been developed for *Micromonospora* species delineation.

## 3.5 References

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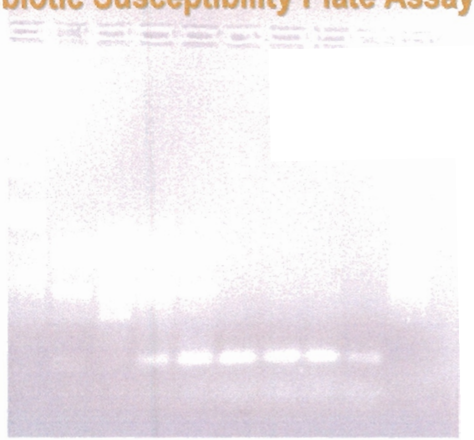
# Chapter 4

# *Micromonospora* Antibiotic Biosynthetic Capability



# Chapter 4

# *Micromonospora* Antibiotic Biosynthetic Capability

## 4.1 Introduction

### 4.1.1 *Micromonospora* Antibiotic Classes in Brief

The genus *Micromonospora* displays diverse secondary metabolite capabilities. Members of *Micromonospora* produce compounds belonging to antibiotic classes such as aminoglycosides, macrolides, ansamysins, and everninomicins (see Table 4.1 and Chapter 5 for more in depth information).

Table 4.1. Validly published *Micromonospora* species, their secondary metabolites, and important information on the strains used in this study.

| **No.:** | **List[a]:** | **Species[b]:** | **Strains Used in This Study And Important Comments[c]:** | **Secondary Metabolite Capabilities Of the Species (Antibiotic Class)[d]:** |
|---|---|---|---|---|
| 1 | AL1980 | *M. aurantiaca* | *M. aurantiaca* NRRL B-16091$^T$<br>➢ Preferred growth medium: m172F Agar. | No antibiotic. |
| 2 | VL2004 | *M. auratinigra* | Information on this species obtained from the publication. | No antibiotic. |
| 3 | AL1980 | *M. carbonacea* | *M. carbonacea* subsp. *carbonacea* NRRL 2972$^T$<br><br>Synonym:<br>*M. carbonacea* subsp. *aurantiaca* NRRL 2997$^T$ | Everninomicin Antibiotic Complex (Everninomicin) |
| 4 | AL1980 | *M. chalcea* (Type Species) | *M. chalcea* subsp. *chalcea* NRRL B-2344$^T$<br>➢ Steroid conversion.<br>➢ '*fmrO*-' and '*fmrT*-type' Negative Control (Nm$^S$).<br>➢ Preferred growth medium: m172F Agar. | Neomycin (Aminoglycoside)<br><br>Tetrocarcin Antitumor and Antimalaria Complex (Tetrocarcin)<br><br>Neorustmicin B (Macrolide)<br><br>Macquarimicin A (Cytotoxic Agent) |
| 5 | VL1992 | *M. chersina* | *M. chersina* DSM 44151$^T$ | Dynemicin[e] (10-membered Enediyne Polyketide) |
| 6 | AL1980 | *M. coerulea* | *M. coerulea* DSM 43143$^T$ | No antibiotic. |
| 7 | AL1980 | *M. echinospora* | *M. echinospora* subsp. *echinospora* NRRL 2985$^T$<br>➢ Gentamicin.<br>➢ '*fmrO*-type' Positive Control.<br>➢ Preferred growth medium: BM Agar. | |

| | | | | |
|---|---|---|---|---|
| | | | *M. echinospora* NRRL 2953$^T$<br>➢ Gentamicins and the restriction endonuclease *MpuI*.<br>➢ '*fmrO*-type' Positive Control.<br>*M. echinospora* subsp. *echinospora* NRRL B-12180<br>➢ No antibiotic product.<br>➢ '*fmrO*-type' Negative Control.<br>Synonym:<br>*M. echinospora* subsp. *ferruginea* NRRL 2995$^T$<br>➢ Gentamicin.<br>Heterotypic Synonyms:<br>*M. purpurea*<br>*M. rhodorangea* | Gentamicin Antibiotic Complex (Aminoglycoside)<br>Liparmacin A and Tiacumicin C (Macrolide)<br>Antibiotic G-418 (Aminoglycoside) |
| 8 | VL2004 | *M. endolithica* | *M. endolithica* strain AA-459$^T$<br>➢ Preferred growth medium: BM Agar.<br>➢ Preferred temperature range for growth: 28°C – 37°C. | No antibiotic. |
| 9 | AL1980 | *M. gallica*<br>(No strain available: Lost) | N/A | N/A |
| 10 | AL1980 | *M. halophytica* | *M. halophytica* subsp. *halophytica* NRRL 2998$^T$<br>➢ Halomicin and vitamin B12.<br>➢ Preferred growth medium: m172F Agar. | Halomicin Antibiotic Complex (Ansamysin)<br>K 13: angiotensin I converting enzyme inhibitor. |
| 11 | AL1980 | *M. inositola* | *M. inositola* NRRL B-16095$^T$<br>➢ XK-41 Complex.<br>➢ Preferred growth medium: BM Agar. | XK-41 Antibiotic Complex (Macrolide) |

| | | | | |
|---|---|---|---|---|
| 12 | VL2000 | *M. matsumotoense* | *M. matsumotoense* DSM 44100$^T$<br>Basonym:<br>*Catellatospora matsumotoense* | No antibiotic. |
| 13 | AL1980 | *M. nigra* | *M. nigra* NRRL 3097$^T$<br>➢ Halomicin and possibly everninomicin.<br>*M. nigra* NRRL B-16696$^T$<br>➢ No antibiotic. | Halomicin Antibiotic Complex<br>(Ansamysin) |
| 14 | 1983 | *M. olivasterospora* | *M. olivasterospora* NRRL 8178$^T$<br>➢ Fortimicins.<br>➢ '*fmrO*-type' Positive Control.<br>➢ Preferred growth medium: BM Agar. | Fortimicin Antibiotic Complex<br>(Aminoglycoside)<br>Fortimicin A ≡ Astromicin. |
| 15 | AL1980 | *M. pallida* | *M. echinospora* subsp. *pallida* NRRL 2996$^T$<br>➢ Gentamicin.<br>➢ Preferred growth medium: BM Agar. | Gentamicin Antibiotic Complex<br>(Aminoglycoside) |
| 16 | AL1980 | *M. purpureochromogenes* | *M. purpureochromogenes* NRRL B-16094$^T$<br>➢ Possible product: Gentamicin.<br>➢ Preferred growth medium: BM Agar.<br>Heterotypic Synonym:<br>*M. brunnea* | No antibiotic. |
| 17 | 1986 | *M. rosaria* | *M. rosaria* NRRL 3718$^T$<br>➢ Rosaramicin<br>➢ Preferred growth media: YEME and m172F agars. | Rosaramicin<br>(Macrolide)<br>Sch23831<br>(Ansamysin) |

Abbreviations: AL, approved list; VL, validation list; BM, Bennett's Agar; N/A, not applicable; $Nm^S$, sensitive to neomycin.

[a] Information obtained from reference 5.

[b] Information obtained from reference 9.

[c] Information obtained during the course of this study, and from the NRRL (14), DSMZ (3), and reference 5.

[d] Information obtained from the NRRL (14), and references 4 and 5.

[e] Information obtained from reference 11.

Of greatest interest in this study are the aminoglycosides. Many *Micromonospora* strains produce aminoglycoside antibiotics and thus if a new *Micromonospora* species is isolated, the strain may produce an aminoglycoside-type antibiotic. Of most significance is that aminoglycoside biosynthesis has been well documented in the literature, which lends a greater understanding to how aminoglycoside antibiotics are produced. In line with the interests of this laboratory, viz. the search for *novel antitubercular antibiotics, it must be noted that two aminoglycosides (kanamycin and streptomycin) are used to treat* tuberculosis. However, *M. tuberculosis* has evolved resistance to these drugs. Thus the search for novel antitubercular drugs *could* begin with searching for novel aminoglycoside antibiotics.

In a 1976 review article (20), Rinehart *et al.* divided aminoglycoside antibiotics into 5 distinct groups. With the discovery of fortimicin in 1977, it was found that this aminoglycoside was a unique member of the antibiotic class. While all other aminoglycosides are pseudotrisaccharides or pseudotetrasaccharides, fortimicin is a unique pseudodisaccharide (8). Aminoglycoside antibiotics can now be divided into 6 groups based on differences in their chemical structures (Table 4.2).

Table 4.2. The 6 groups of the aminoglycoside antibiotic class.

| | Aminoglycoside Group Designated as: | Examples of Antibiotics Belonging to the Group: |
|---|---|---|
| 1 | 4, 5-disubstituted deoxystreptamine | Neomycins |
| 2 | 4, 6-disubstituted deoxystreptamine | Kanamycins<br>Tobramycin<br>Nebramycins<br>Gentamicins<br>G-418<br>Sagamicin<br>G-52 |
| 3 | Monosubstituted deoxystreptamine | Hygromycin B<br>Apramycin |
| 4 | Aminoglycosides derived from Streptidine, Bluensidine, and Actinamine. | Spectinomycin<br>Bluensomycin<br>Streptomycin |
| 5 | Monoaminocyclitol | Hygromycin A |
| 6 | 1, 4-diaminocyclitol | Fortimicin |

### 4.1.2 Aminoglycoside Antibiotic Biosynthesis and Resistance Genes

In order for antibiotic producers to avoid 'suicide', they must have a 'self-defence mechanism' against their own antibiotics. Antibiotic biosynthetic genes are clustered in specific regions in the genome, and the genes conferring resistance to an organism's own antibiotics are located in or near the biosynthetic gene clusters (17).

In a 1993 study by Ohta *et al.* (17), aminoglycoside producing strains were found to possess resistance genes belonging to one of two 'types', based on differences in their aminoglycoside resistance profiles and DNA homologies. The strains used in the study were representatives of the genera *Micromonospora* and *Streptomyces*, and species *Saccharopolyspora hirsuta* and *Dactylosporangium matsuzakiense*. The aminoglycoside producing strains were inoculated onto media containing different concentrations of aminoglycoside antibiotics (fortimicin, gentamicin, neomycin, kanamycin, and streptomycin) in an attempt to assess the sensitivity of aminoglycoside producing strains to various aminoglycosides. As expected, strains were not sensitive to their own products, e.g., the fortimicin producers were resistant to fortimicin in the agar media. Furthermore, it was found that all of the strains tested were highly resistant to kanamycin, but highly sensitive to streptomycin.

They had found a link between the genotype and the phenotype of the strains (Table 4.3). The first resistance type was designated the '*fmrT*-type', showing resistance to *neomycin*, fortimicin and kanamycin, and sensitivity to gentamicin and streptomycin. The second resistance type, designated the '*fmrO*-type' showed resistance to *gentamicin*, fortimicin, and kanamycin, and sensitivity to neomycin and streptomycin. Importantly, they found that the '*fmrO*-type' resistance genes are located adjacent to the biosynthetic gene clusters in the strains examined (17).

Table 4.3. The resistance profiles of the two types of aminoglycoside resistance genes.

| Resistance Genotype: | Resistance Phenotype: | Examples of Strains With This Resistance Type: |
|---|---|---|
| '*fmrO*-type'<br>($Gm^{R}$-$Km^{R}$;<br>$Nm^{S}$-$Sm^{S}$) | Resistant to: Gentamicin, Fortimicin and Kanamycin.<br>Sensitive to: Neomycin and Streptomycin. | *Micromonospora olivasterospora*<br>*Micromonospora echinospora*<br>*Micromonospora pallida* |
| '*fmrT*-type'<br>($Nm^{R}$-$Km^{R}$;<br>$Gm^{S}$-$Sm^{S}$) | Resistant to: Neomycin, Fortimicin and Kanamycin.<br>Sensitive to: Gentamicin and Streptomycin. | *Streptomyces tenebrarius*<br>*Streptomyces tenjimariensis*<br>*Saccharopolyspora hirsuta* |

Abbreviations: $^{R}$, resistant; $^{S}$, sensitive; Gm, Gentamicin; Km, Kanamycin; Nm, Neomycin; Sm, Streptomycin.

One way to predict the antibiotic class before attempting purification is to screen for the presence of specific biosynthetic genes. By using PCR primers, specifically designed to amplify a particular biosynthetic gene, or the resistance genes, belonging to the aminoglycoside biosynthetic cluster, an amplified fragment would indicate the presence of this gene in the bacterium's genome, providing presumptive evidence of the ability to synthesize an aminoglycoside type antibiotic. This strategy acts as a tool to assess rapidly whether or not any strain under investigation has the genes for an aminoglycoside-type antibiotic. This saves on time and effort as knowing the class of antibiotic allows one to consult the literature for a purification procedure that will work for that specific antibiotic type.

It must be noted that targeting resistance genes for PCR amplification presents some limitations: not all resistance genes are located in the biosynthetic cluster, and strains may possess acquired resistance to antibiotics due to gene transfer between strains in their natural environment (i.e. strains may exhibit antibiotic resistance, but lack the genes for antibiotic biosynthesis).

### 4.1.3 This Study

In this study, the aim was to attempt to ascertain what type of antibiotic the *Micromonospora* strains under investigation have the potential to produce in an effort to make the purification step easier. Various genes were analysed and PCR primers, specific for the amplification of ansamysin, glycopeptide, and aromatic antibiotic biosynthetic genes, were used. Primers were designed to amplify the '*fmrO*-' and '*fmrT*-type' aminoglycoside resistance genes, and an antibiotic susceptibility plate assay was used to confirm the PCR results.

## 4.2 Materials and Methods

### 4.2.1 Phylogenetic Analysis

Phylogenetic trees were constructed from 16S rRNA gene sequences, *gyrB* gene sequences, and aminoglycoside resistance gene sequences. Species of the genus *Micromonospora* used were those on the Approved Lists of Bacterial Names prior to May 2004 (5). Multiple sequence alignments were performed using DNAMAN, Version 4.13 (Lynnon BioSoft). All sequences were edited to produce the longest sequence region common to all sequences in each data set. The GenBank sequence accession numbers are indicated in brackets after the type strain names. Bootstrapping analysis was used to test the significance of phylogenetic affiliations. The trees were generated by the neighbour-joining method (21), with pairwise distances calculated by the Kimura two-parameter estimation, and the percentage bootstrap values obtained by using CLUSTAL X, Version 1.81 (24) and MEGA2, Version 2.1 (12).

### 4.2.2 DNA Extraction

DNA was extracted according to the method of Cook and Meyers (2) and by the Freeze-Thaw Method (Goodwin, unpublished). Cultures were grown in *ISP* Medium No. 2 broth (23), Bennett's broth or 172F broth (see 3.2.2). Broth cultures were incubated with shaking for 4 – 6 days at 30°C and 37°C. The Freeze-Thaw Method was performed as follows: 1 ml of broth culture was pipetted into a 1.5 ml microfuge tube, centrifuged for 1 – 2 min, and the supernatant discarded. The cell pellet was washed in 500 µl of 10 mM Tris-HCl–1 mM EDTA (TE) buffer (pH 7.6) and resuspended in 500 µl TE buffer (pH 7.6). The sample was first exposed to –70°C for several hours and then to +70°C for 10 min. This was repeated for three cycles. After the third cycle, the sample was placed in a 100°C water bath for 10 min, allowed to cool and centrifuged for 1 min. Four hundred (400) µl of the supernatant was transferred to a 1.5 ml microfuge tube. The DNA was stored at –20°C.

### 4.2.3 PCR Amplification and Agarose Gel Electrophoresis

The primers used in this study for the amplification of various genes are listed in Table 4.4. All synthetic oligonucleotides were supplied by the Oligonucleotide Synthesising Service of the Molecular and Cell Biology Department, University of Cape Town, Cape Town, South Africa.

Super-Therm thermostable DNA polymerase (*Taq*), 10X Reaction Buffer, and 25 mM $MgCl_2$ were supplied by JMR Holdings, USA. dNTPs were supplied by Roche. Each PCR reaction tube contained the following (giving a total of 50 µl reaction mix): 4 µl $MgCl_2$ (25 mM), 5 µl 10X Reaction Buffer, 2.5 µl of each primer (10 µM each primer), 0.3 µl dNTPs (100 mM), 0.3 µl *Taq* polymerase (5 U/µl), 33.4 µl $H_2O$, and 2 µl DNA. The cycle profile was as follows: initial denaturation (96°C for 2 min); 30 cycles of denaturation (96°C for 45 s), annealing (56°C for 30 s for the 16S rRNA gene and ARO-PKS; 56°C or 54°C for *oxyB*; 50°C or 48°C for *grm*; 60°C or 64°C for *fmr*, 52°C for ANSA), and extension (72°C, for 2 min for the 16S rRNA gene and ANSA; 90 s for ARO-PKS; 75 s or 90 s for *fmr*, 75 s for *oxyB* and *grm*); and a final extension (72°C for 5 min) followed by a cooling step (4°C for 10 min).

PCR products were analysed on agarose gels according to Sambrook *et al.* (22). The bands were visualized on a 254 nm transilluminator (UVP©) and photographs taken. A standard λ*Pst* DNA ladder was used as the marker.

The *oxyB* primers were designed to specifically target the monooxygenase B gene, involved in the biosynthesis of glycopeptide antibiotics (19). The ARO-PKS primers amplify across the junction of the tandem KSα and KSβ genes in the biosynthetic cluster of aromatic polyketide antibiotics specifically using Type II polyketide synthases (PKS) in their biosynthesis. However, these primers may also amplify spore pigment genes as there is an evolutionary link between biosynthetic genes encoding aromatic polyketide antibiotics and those encoding spore pigments (1). The only way to know which has been amplified is to sequence the PCR product. The ANSA primers amplify the gene encoding AHBA synthase, the unique starter molecule that generates the ansamysin antibiotic (6, 13). The *grm* primers amplify '*fmrO*-type' aminoglycoside resistance genes and the *fmr* primers amplify '*fmrT*-type' aminoglycoside resistance genes.

Table 4.4. List of PCR primers used for the amplification of specific genes.

| Primer Sequences:<br>(Listed as Forward and Reverse primers, respectively; I = inosine.) | Expected Fragment Size: | % Agarose Used: |
|---|---|---|
| **16S rRNA gene** (F1 and R5 modified from primers fD1 and rP2, respectively, of Weisburg, *et al.* [25])<br>F1: 5′–AGAGTTTGATCITGGCTCAG–3′<br>R5: 5′–ACGGITACCTTGTTACGACTT–3′ | 1500 bp | 1.2 |
| ***oxyB*** (designed by Dr Paul Meyers)<br>Targets the glycopeptide monooxygenase (*oxyB*) gene.<br>Foxy: 5′–CTGGTCGGCAACCTGATGGAC–3′<br>Roxy: 5′–CAGGTACCGGATCAGCTCGTC–3′ | 591 bp | 1.2 |
| **ARO-PKS** (designed by Dr Paul Meyers)<br>Targets PKS Type II KSI-KSII tandem genes.<br>ARO-PKS-F: 5′–GGCAGCGGITTCGGCGGITTCCAG–3′<br>Binds in the 3′ half of the ketosynthase (KS) gene: KSI/KSα.<br>ARO-PKS-R: 5′–CGITGTTIACIGCGTAGAACCAGGCG–3′<br>Binds in the 5′ half of the chain-length-factor gene: KSII/KSβ. | 492 – 630 bp<br>(But mostly in the region of 500 bp.) | 1.2 |
| ***grm*** (≡*fmrO*) (*grm1* and *grm2*, respectively, of Ohta, *et al.* [15])<br>grm-F: 5′–CAGGCCATCACCAAGAGCCGGCGCTACCAG–3′<br>grm-R: 5′–GTAAATCAGCTCGTTGCCGATCTCCAGTCG–3′ | 734 bp | 2.0 |
| ***fmr*** (≡*fmrT*) (redundant primers, designed by Candice Goodwin and Dr Paul Meyers)<br>fmrT-F: 5′–ATGCC(GC)TGGGG(GC)AGCCTGCTGCGCGG–3′<br>fmrT-R: 5′–G(CA)CG(CG)CGGGTCCA(CG)GAGGT(CG)(GT)CCAG–3′ | 284 bp | 2.0 |
| **ANSA** (redundant primers, designed by Dr Paul Meyers)<br>Targets the ansamysin 3-amino-5-hydroxybenzoic acid (AHBA) synthase gene.<br>ANSA-F: 5′–CC(CG)GC(GC)TTCAC(CG)TTCATCTC–3′<br>ANSA-R: 5′–AI(GC)(CT)GGAICATIGCCATGTAG–3′ | 741 bp | 1.5 |

### 4.2.4 Aminoglycoside Antibiotic Susceptibility Assay on Solid Agar Media

BM-Glucose Agar (see 3.2.2) was used as a basal medium, and the antibiotics were added to the medium after autoclaving. The following antibiotics and concentrations were used: gentamicin (100, 50, 20, 10, and 5 μg/ml) (Sigma), kanamycin (10 and 2 μg/ml) (Sigma), neomycin (20, 10, 7.5, 5, and 4 μg/ml) (Intramed), and streptomycin (100 μg/ml) (Calbiochem). Plates were incubated for 7 days at 28–30°C. Growth at any of these concentrations was taken as resistance to the antibiotic.

### 4.2.5 Strains Used in This Study

DNA was extracted from the following strains for this study: *Micromonospora halophytica* subsp. *halophytica* NRRL 2998$^T$, *Micromonospora nigra* NRRL 3097$^T$, *M. nigra* NRRL B-16696$^T$, *Micromonospora olivasterospora* 8178$^T$, *Micromonospora echinospora* subsp. *pallida* 2996$^T$, '*Streptomyces tenebrarius*' NRRL B-12390$^T$, and *Saccharopolyspora hirsuta* NRRL B-5792$^T$ were kindly provided by Dr David P. Labeda, curator of the Actinobacterial Culture Collection at the U.S. Department of Agriculture Agricultural Research Service, Peoria, Illinois, United States of America. The other strains used in this study are listed in Chapter 2 (2.2.1).

## 4.3 Results and Discussion

### 4.3.1 Phylogenetic Analysis

Phylogenetic relationships deduced from the nucleotide sequences of 16S rRNA genes often do not agree with the DNA reassociation values determined by DNA hybridisation, which is the criterion used to define a species (26). This is because the resolution of 16S rDNA sequence analysis is low for closely related organisms. To resolve the phylogenetic relationships of closely related strains, it is necessary to use other gene sequences that provide a higher resolution than that of the 16S rRNA gene (26).

DNA gyrase (*gyrB* encodes the DNA gyrase B subunit) is the enzyme responsible for introducing negative supercoils into bacterial chromosomal DNA, playing a crucial role in chromosome replication, and thus is essential for a bacterium's survival. Furthermore, protein-encoding genes have been reported to evolve much faster than rRNAs, and horizontal transfer of these genes may be as rare as that of rRNA genes (26). Kasai *et al.* (9) performed a phylogenetic analysis on the genus *Micromonospora* using *gyrB* sequences in order to provide a higher resolution than an analysis using 16S rRNA gene sequences (there is no *gyrB* sequence available for *M. matsumotoense*).

A 16S rDNA phylogenetic tree was constructed and the secondary metabolite capabilities of the *Micromonospora* species were highlighted to determine whether there is a link between taxonomic position and the type of antibiotic produced (Fig. 4.1). It was interesting to note that the macrolide producers formed a clade of their own, however, of more interest were the aminoglycoside producers. Referring to Table 4.2, neomycin, gentamicin, and fortimicin are classified as belonging to three different groups of aminoglycoside antibiotics, viz. 4, 5-disubstituted deoxystreptamine, 4, 6-disubstituted deoxystreptamine, and 1, 4-diaminocyclitol, respectively. It is interesting that both the 16S rRNA and *gyrB* (Fig. 4.2) phylogenetic trees reflect this: *M. echinospora* and *M. pallida* both produce gentamicin and group together (most noticeable in the *gyrB* tree). *M. olivasterospora*, the producer of the unique fortimicin, is isolated in a deep branch in both trees. *M. chalcea*, the neomycin producer, is phylogenetically unrelated. Thus, having their secondary metabolites belonging to three structurally different aminoglycoside groups, could explain why the species' phylogenetic positions are separate, as opposed to a single clade consisting of all the aminoglycoside producers.

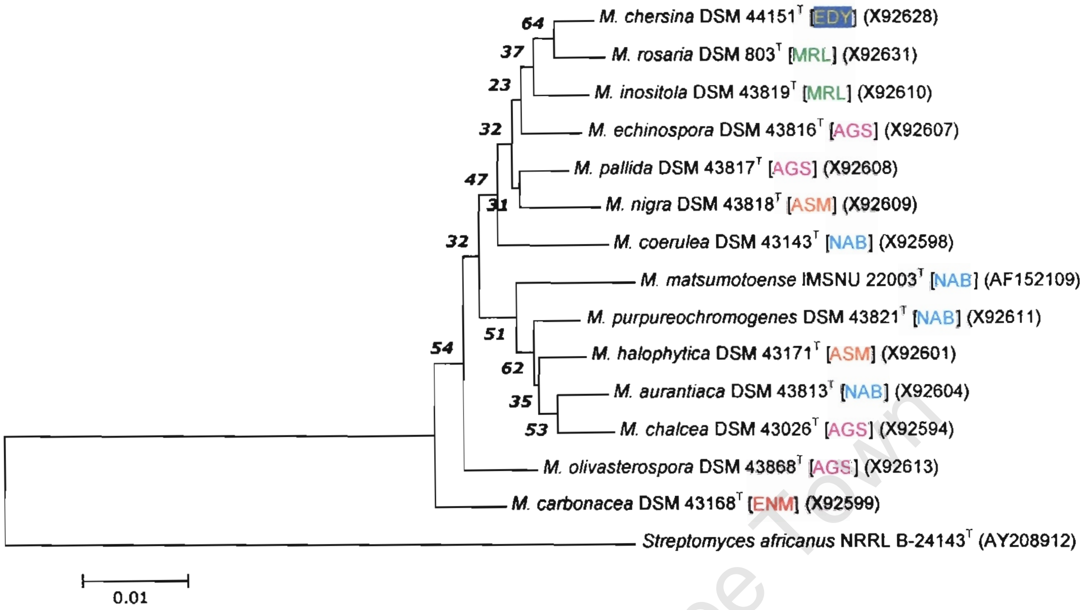


Fig. 4.1. An unrooted phylogenetic tree of 14 of the validly published members of the genus *Micromonospora* based on 16S rRNA gene sequences, and showing the secondary metabolites produced. The tree was constructed with 1412 bp of alignable 16S rRNA gene sequence, with *Streptomyces africanus* NRRL B-24143$^T$ as the outgroup. The GenBank sequence accession numbers are indicated in brackets. The abbreviations of the antibiotic classes to which the secondary metabolites belong are indicated in square brackets: aminoglycoside (AGS), ansamysin (ASM), enediyne polyketide (EDY), everninomicin (ENM), macrolide (MRL), and no antibiotic product (NAB). Nodes are labelled with the percentage bootstrap replications (out of 100) in which the node was found. The scale bar represents an average of 0.01 nucleotide substitutions per site.

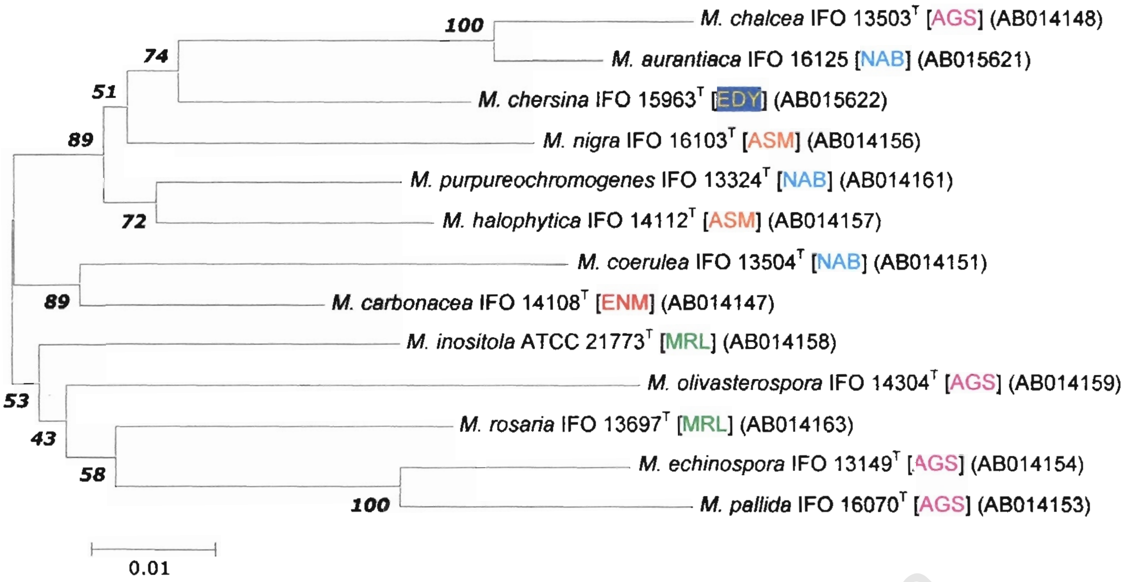


Fig. 4.2. An unrooted phylogenetic tree of 13 of the validly published members of the genus *Micromonospora* based on *gyrB* gene sequences and showing the secondary metabolites produced. The tree was constructed with 1179 bp of alignable *gyrB* gene sequence. The GenBank sequence accession numbers are indicated in brackets. The abbreviations of the antibiotic classes to which the secondary metabolites belong are indicated in square brackets: aminoglycoside (AGS), ansamysin (ASM), enediyne polyketide (EDY), everninomicin (ENM), macrolide (MRL), and no antibiotic product (NAB). Nodes are labelled with the percentage bootstrap replications (out of 100) in which the node was found. The scale bar represents an average of 0.01 nucleotide substitutions per site.

Having examined the phylogenetic trees, it was thought that if a new strain's 16S rDNA sequence was added to the phylogenetic tree, the strain's phylogenetic position may reveal the type of antibiotic the strain may be capable of producing. However, as revealed in Chapter 3 (see Fig. 3.5), it was found that our laboratory's strains formed a clade of their own, and the antibiotic type cannot be predicted. Perhaps the finding may be different if the *gyrB* genes were sequenced for the strains, as well as for *M. matsumotoense*.

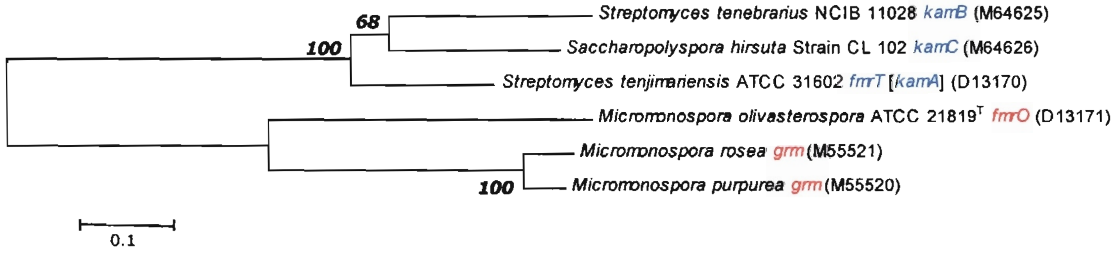


Fig. 4.3. Phylogenetic analysis of the aminoglycoside resistance genes of 6 strains of 3 actinomycete genera. The resistance genes are classified into 2 groups on the basis of DNA homology: '*fmrT*-type' (in blue) and '*fmrO*-type' (in red). The GenBank sequence accession numbers are indicated in brackets. Nodes are labelled with the percentage bootstrap replications (out of 100) in which the node was found. The scale bar represents an average of 0.1 nucleotide substitutions per site.

Fig. 4.3 shows the phylogenetic analysis of the aminoglycoside resistance genes of representatives of the two aminoglycoside resistance types. The tree is neatly divided into two clades, the '*fmrO*-type' clade in red, and the '*fmrT*-type' clade in blue. The gene abbreviations are as follows: *kam*, *k*anamycin-*a*pramycin resistance *m*ethylation; *fmr*, *f*ortimicin-*r*esistance gene; *grm*, *g*entamicin *r*esistance-encoding *m*ethyltransferase (7, 16).

### 4.3.2 Selective Amplification of Antibiotic Biosynthetic and Resistance Genes by PCR

The results of the various PCRs are summarized in Table 4.5, showing the PCR screening of 14 environmental *Micromonospora* isolates. Appropriate control strains were used for the different PCRs. Strains from our laboratory, '*Amycolatopsis umgenii*' strain No. 16 and '*Streptoallomorpha polyantibiotica*' strain SPR were used as the positive controls for the *oxyB* and ARO-PKS PCRs, respectively. *Micromonospora halophytica* and *Micromonospora nigra* NRRL 3097$^T$, the ansamysin producers, were used as positive controls for the ANSA PCR. The non-producing strain, *M. nigra* NRRL B-16696$^T$, was used as the negative control for the ANSA PCR. *Micromonospora olivasterospora* and *Micromonospora pallida* were used as the positive controls for the *grm* PCR, and '*Streptomyces tenebrarius*' and *Saccharopolyspora hirsuta* were the positive controls for the *fmr* PCR.

An amplified PCR product indicates the presence of a specific gene in the bacterium's genome, thus providing presumptive evidence of the ability to synthesise the specific antibiotic type, or to have resistance to aminoglycoside antibiotics (*grm* and *fmr*).

Table 4.5. Table of PCR results for the selective amplification of antibiotic biosynthetic and resistance genes.

| PCR:<br>Strain: | *oxyB* | ARO-PKS | *grm* | *fmr* | ANSA |
|---|---|---|---|---|---|
| MMS1 | – | – | – | – | – |
| MMS2 | – | + | + | + | – |
| MMS3 | – | + | – | – | – |
| MMS4 | – | + | + | + | – |
| MMS5 | – | + | – | – | + |
| MMS6 | – | + | + | – | – |
| MMS7 | – | + | + | – | – |
| $R_2\mu$ | – | + | – | – | – |
| MLR | – | – | – | – | – |
| MMS-DA | – | + | – | – | – |
| MMS10 | – | – | – | – | – |
| MMS12 | – | – | – | – | – |
| MMS14 | – | – | – | + | – |
| MMS17 | – | + | – | – | – |
| *M. nigra* 3097$^T$ | – | + | – | – | + |
| *M. nigra* NRRL B-16696$^T$ | – | – | – | – | – |
| *M. halophytica* | – | N.T. | N.T. | N.T. | + |
| *M. olivasterospora* | N.T. | N.T. | + | – | N.T. |
| *M. pallida* | N.T. | + | + | – | – |
| '*Streptomyces tenebrarius*' | N.T. | N.T. | – | + | N.T. |
| *Saccharopolyspora hirsuta* | N.T. | N.T. | – | + | N.T. |

Abbreviations: –, PCR result is negative; +, PCR result is positive; N.T., not tested.

It is interesting to note that two of the *Micromonospora* control strains were positive for the ARO-PKS PCR. *M. pallida* may thus have the biosynthetic capability to produce an aromatic polyketide antibiotic **and** an aminoglycoside antibiotic. *M. nigra* NRRL 3097$^T$ was positive for the ansamysin and ARO-PKS PCRs, however *M. nigra* NRRL B-16696$^T$, mutated to lose the ability to produce halomicin, an ansamysin, was negative for both the ANSA and ARO-PKS antibiotic type PCRs, suggesting that this strain has also lost the ability to produce the aromatic polyketide (antibiotic or spore pigment).

The ARO-PKS primers do not distinguish between the Type II Polyketide antibiotic subclasses and thus the PCR products should be sequenced to determine to which subclass the aromatic polyketide antibiotic belongs, and to rule out the possibility of the primers having amplified a spore pigment gene.

The *grm* positive controls were positive for the *grm* PCR and negative for the *fmr* PCR, while the *fmr* positive controls were negative for the *grm* PCR, showing that the primers do discern between the two aminoglycoside resistance types.

Interestingly, strains MMS2 and MMS4 were positive for *both* the *grm* and *fmr* PCRs. Since it has not been documented that a strain can possess genes for both resistance types, one of the genes amplified may not be associated with a biosynthetic cluster. Since many workers have found that the genes targeted by the *grm* PCR primers are adjacent to the biosynthetic cluster, the explanation may lie with the specificity of the *fmr* primers. The strains may have acquired the '*fmrT*-type' resistance genes from other bacteria in the Hermanus lagoon via conjugation, or transduction via phages (10).

Fig. 4.4 shows some of the positive results obtained for the *grm* PCR. It would be interesting to sequence the PCR products and expand the phylogenetic tree, shown in Fig. 4.3, to determine if all the *grm* positives form a clade with the existing '*fmrO*-type' resistance genes.

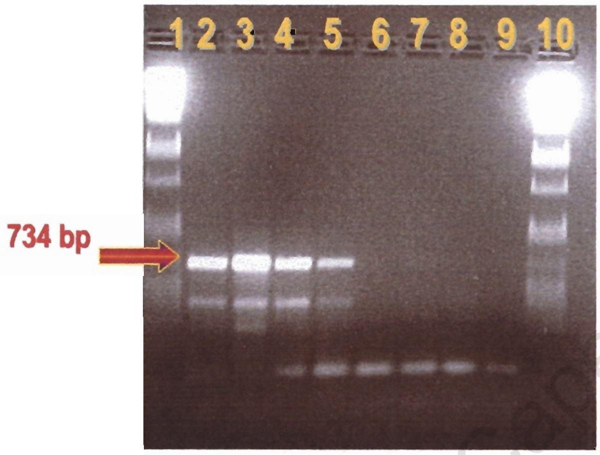


Fig. 4.4. Agarose gel photograph showing the results of the *grm* PCR amplification. The red arrow indicates the position of the expected fragment produced by PCR amplification. Lanes 1 and 10: λ*Pst* Ladder (marker); Lane 2: *Micromonospora* sp. strain MMS2; Lane 3: *Micromonospora* sp. strain MMS4; Lane 4: *Micromonospora* sp. strain MMS6; Lane 5: *Micromonospora* sp. strain MMS7; Lane 6: No DNA control; Lane 7: *Micromonospora* sp. strain MMS1; Lane 8: *Micromonospora* sp. strain MMS5; Lane 9: *Micromonospora* sp. strain MMS-DA.

### 4.3.3 Aminoglycoside Antibiotic Susceptibility Plate Assay

The results of the assay are shown in Tables 4.6 and 4.7.

Table 4.6. Comparison of the aminoglycoside antibiotic susceptibility plate assay results with the *grm* and *fmr* PCR results[a].

| Antibiotic Concentration (µg/ml): | Sm | Km | | Gm | | | | | Nm | | | | | Resistance Type Suggested By The Plate Assay: | PCR Confirms? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain: | 100 | 2 | 10 | 5 | 10 | 20 | 50 | 100 | 4 | 5 | 7.5 | 10 | 20 | | |
| MMS1 | – | + | + | – | – | – | – | – | + | + | + | – | + | *fmrT* | × |
| MMS2 | – | + | + | + | + | – | – | – | – | + | + | – | + | *fmrO* AND *fmrT* | ✓ |
| MMS3 | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. | ✓ |
| MMS4 | – | + | + | ++ | – | – | – | – | – | + | + | + | – | *fmrO* AND *fmrT* | ✓ |
| MMS5 | – | + | + | – | – | – | – | – | – | + | + | + | + | *fmrT* | × |
| MMS6 | – | + | + | ++ | – | – | – | – | – | + | + | + | + | *fmrO* AND *fmrT* | ✓ AND × |
| MMS7 | – | + | + | + | – | – | – | – | – | + | + | – | + | *fmrO* AND *fmrT* | ✓ AND × |
| $R_2\mu$ | – | + | + | – | – | – | – | – | – | – | + | + | + | *fmrT* | × |
| MLR | – | + | – | – | – | – | – | – | – | + | + | + | – | *fmrT* | × |
| MMS-DA | – | + | + | – | – | – | – | – | + | – | + | + | + | *fmrT* | × |
| MMS10 | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. | ✓ |
| MMS12 | – | + | – | – | – | – | – | – | + | + | – | – | + | *fmrT* | × |
| MMS14 | – | – | – | – | – | – | – | – | – | – | + | – | – | *fmrT* | ✓ |
| MMS17 | – | + | + | – | – | – | – | – | + | + | + | – | + | *fmrT* | × |
| *M. nigra* | – | + | + | – | – | – | – | – | + | + | + | – | – | *fmrT* | × |
| *M. olivasterospora* | – | + | + | + | + | + | + | + | – | – | – | – | – | *fmrO* | ✓ |
| *M. pallida* | – | + | – | – | – | – | – | – | + | – | + | – | + | *fmrT* | × |
| *'Streptomyces tenebrarius'* | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* | ✓ |
| *Saccharopolyspora hirsuta* | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* | ✓ |

[a] See Table 4.5.

Abbreviations: Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Nm, neomycin; +, growth; –, no growth.

Table 4.7. Plate assay results for the other MMS strains investigated, and for the other validly published members of the genus *Micromonospora*.

| Antibiotic Concentration (μg/ml): | Sm | Km | | Gm | | | | | Nm | | | | | Resistance Type Suggested by the Plate Assay: |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain: | 100 | 2 | 10 | 5 | 10 | 20 | 50 | 100 | 4 | 5 | 7.5 | 10 | 20 | |
| MMS8 | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* |
| MMS9 | – | + | + | – | – | – | – | – | + | + | – | – | – | *fmrT* |
| MMS11 | – | + | + | – | – | – | – | – | + | + | + | – | – | *fmrT* |
| MMS15 | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* |
| MMS16 | – | + | + | – | – | – | – | – | + | + | – | – | – | *fmrT* |
| MMS18 | – | – | – | – | – | – | – | – | + | – | – | – | – | *fmrT* |
| MMS19 | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. |
| *M. aurantiaca* | – | + | + | – | – | – | – | – | + | – | – | – | – | *fmrT* |
| *M. carbonacea* | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* |
| *M. chalcea* | – | + | + | – | – | – | – | – | + | + | + | + | + | *fmrT* |
| *M. chersina* | – | + | – | – | – | – | – | – | – | – | – | – | – | Neither. |
| *M. coerulea* | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. |
| *M. echinospora* | – | + | + | – | – | – | – | – | – | + | – | – | – | *fmrT* |
| *M. halophytica* | – | + | + | – | – | – | – | – | + | – | – | – | – | *fmrT* |
| *M. inositola* | – | + | + | – | – | – | – | – | – | – | – | – | – | Neither. |
| *M. matsumotoense* | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. |
| *M. purpureochromogenes* | – | – | – | – | – | – | – | – | – | – | – | – | – | Sensitive to ALL Aminoglycoside Antibiotics. |
| *M. rosaria* | – | + | + | – | – | – | – | – | + | + | – | – | – | *fmrT* |

Abbreviations: Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Nm, neomycin.

It can be seen in Table 4.6 that the plate assay revealing a '*fmrO*-type' corresponds exactly with the *grm* PCR results (except for *M. pallida*). Strains MMS3 and MMS10, which were negative for both the *grm* and *fmr* PCRs, were sensitive to all of the antibiotics tested, suggesting that they have no resistance mechanisms against aminoglycoside antibiotics. Generally, kanamycin (2 μg/ml) and streptomycin (100 μg/ml) proved useful as positive and negative control plates, respectively, for this assay (see 4.1.2). As noticed with the PCR experiments, '*fmrT*-type' resistance seems to be widely spread in the plate assay. Researchers have not looked for '*fmrT*-type' resistance genes in species of *Micromonospora*. As can be seen in Fig. 4.3, the '*fmrT*-type' clade is formed from sequences of *Saccharopolyspora* and *Streptomyces* species. Thus, the *fmr* PCR primers were designed using non-*Micromonospora* sequences. If *Micromonospora* strains do have '*fmrT*-type' aminoglycoside resistance genes, then the finding of *Micromonospora* strains having both the *fmrO* and *fmrT* resistance types may not be surprising. The *fmr* PCR products of the *Micromonospora* strains should be sequenced and compared with those sequences used to design the primers, and the phylogenetic tree should be expanded. If the *Micromonospora* sequences are phylogenetically related to the *Saccharopolyspora* and *Streptomyces* sequences, and they all share conserved regions, then the *Micromonospora* strains do possess the '*fmrT*-type' aminoglycoside resistance gene. If not, the strains may possess resistance genes acquired from elsewhere and are not indicative of a resistance gene related to an antibiotic biosynthetic cluster. Although the *fmr* PCR does not seem to be reliable (because the results do not correlate with the plate assay findings), the *grm* PCR presents reasonable results as it verified the plate assay results in all but one case (*M. pallida*). If both the *grm* PCR and the plate assay point to the '*fmrO*-type', then the strains show resistance to their own antibiotic type and thus it is recommended that purification protocols for gentamicin-type aminoglycoside antibiotics be used (e.g. for strains MMS2, MMS4, MMS6, and MMS7).

If one looks at the neomycin results, one can see a strange phenomenon: some strains grow at intermediate concentrations of the antibiotic but not at the highest or lowest concentration. The experiment was performed three times and the same result was observed each time. Piendl *et al.* (18) investigated the involvement of 16S rRNA in aminoglycoside resistance by aminoglycoside producers and stated that "in systems programmed by mRNA, inhibition of protein synthesis observed in the presence of low or high concentrations of aminoglycosides may be obscured at intermediate drug concentrations due to stimulated incorporation of amino acids associated with misreading of mRNA codons."

## 4.4 Conclusion

The *grm* primers and the plate assay seem worthy of being used to predict the ability to synthesize a gentamicin-type aminoglycoside antibiotic. This should be taken into account when deciding on antibiotic purification protocols to be used. Furthermore, the positive *grm* PCR products should be sequenced and the phylogenetic affiliations examined to better understand this type of resistance gene. The problems with the *fmr* PCR and plate assay should be further examined by sequencing the *fmr* PCR products and comparing the sequences to those used to design the primers. Conserved regions between the sequences should be seen, indicating that the '*fmrT*-type' resistance gene has been amplified in *Micromonospora* strains.

Many strains were positive for the ARO-PKS primers and thus the PCR products should be sequenced to determine which subclass of aromatic polyketide antibiotic is produced and purification protocols sought.

Table 4.8 summarizes the PCR screening results, suggesting the type of antibiotic that the strains may produce and the purification procedures to be used. It must be carefully noted that bacteria may have antibiotic biosynthetic genes that are not expressed. This means that a primer may amplify a biosynthetic gene but the gene is not expressed and thus the bacterium will not produce the antibiotic. Relying on PCR results alone may be a gamble but a worthy one as time and effort could be saved on antibiotic purification.

Table 4.8. A summary of strains showing antibiotic biosynthetic capabilities and aminoglycoside resistance genotypes.

| Strains | **MMS5:** | **MMS2 and MMS4:** | **MMS6 and MMS7:** | **MMS14** | **MMS3, MMS-DA, MMS17, $R_2\mu$:** |
|---|---|---|---|---|---|
| Positive for which PCRs? | ANSA and ARO-PKS. | ARO-PKS, *grm*, and *fmr*. | ARO-PKS and *grm*. | *fmr* | ARO-PKS |
| Antibiotic Type Possibly Produced? | Ansamysin and Aromatic antibiotics. | Aromatic, gentamicin-type, and neomycin-type Aminoglycoside antibiotics. | Aromatic and gentamicin-type Aminoglycoside antibiotics. | Neomycin-type Aminoglycoside antibiotic. | Aromatic antibiotics. |

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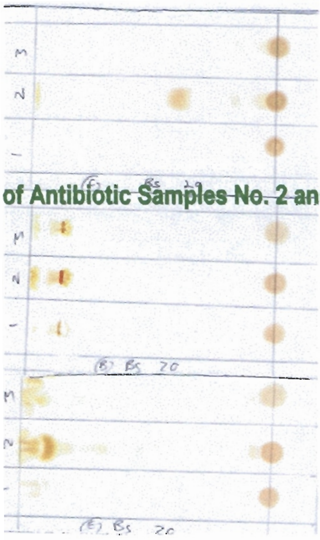
# Chapter 5

# Antibiotic Isolation and Partial Purification



# Chapter 5

# Antibiotic Isolation and Partial Purification

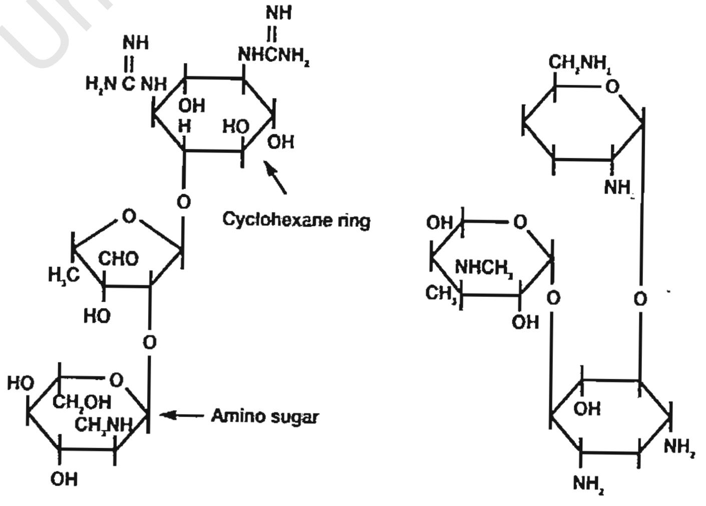
## 5.1 Introduction

### 5.1.1 Antibiotic Classes from *Micromonospora*

#### (i) Aminoglycosides

Several important aminoglycoside antibiotics are produced by *Streptomyces* species and by *Micromonospora* species. For example, streptomycin, kanamycin, neomycin, and tobramycin are produced by *Streptomyces* species, while the gentamicins, fortimicins, sagamicin, and antibiotics G-52 and G-418 are produced by *Micromonospora* species. Neomycin B is produced by both *Streptomyces* and *Micromonospora* (18). Although there is considerable variation in structure among aminoglycosides, the common feature is that they all contain a cyclohexane ring and amino sugars (Fig. 5.1). Their mode of action is the inhibition of protein synthesis in at least two ways: the antibiotic binds to the 30S ribosomal subunit and prevents the elongation of the protein chain, as well as preventing the recognition of the triplet code on mRNA (11). Aminoglycosides are bactericidal and tend to be most active against Gram-negative pathogens, and thus are used to treat infections caused by *Proteus* spp. and *Pseudomonas* spp. (18). Antibiotic G-418 is of particular interest because of its excellent antiparasitic activity against both protozoa and helminths (18).

Aminoglycosides are water-soluble, basic antibiotics, which are purified by means of ion-exchange chromatography. Hydrolytic patterns of the base form of the antibiotics are revealed by acid hydrolysis and the antibiotics give a positive ninhydrin result (17). One of the most common solvent systems used for separation of components by silica TLC is by using the lower (organic) phase of a solvent mixture of chloroform: isopropanol: 17% ammonium hydroxide in a ratio of 2:1:1 (v/v) (10).



Streptomycin

Gentamicin C

Fig. 5.1. Representative aminoglycoside antibiotics (12).

### (ii) Macrolides

Macrolides contain a 12- to 22-carbon macrocyclic lactone ring, linked to a sugar and aminosugar. The sugar residues are important for activity (11). Macrolides are usually bacteriostatic and bind to the 23S rRNA of the 50S ribosomal subunit, inhibiting peptide chain elongation during protein synthesis (12). The macrolides are relatively broad-spectrum antibiotics, effective against Gram-positive and Gram-negative bacteria (12). The most well-known example of a macrolide antibiotic is erythromycin (Fig. 5.2), produced by *Saccharopolyspora erythraea*; it was used against penicillin-resistant staphylococci, and is now the drug of choice against legionnaire's disease (11). Examples of macrolides produced by *Micromonospora* species are the megalomicins, rosaramicin, the M-4365 complex, and erythromycin B (18). An inhibitor of starfish embryonic development, micromonospolide A, is a new macrolide from an uncharacterised *Micromonospora* species (9).

Macrolides are mostly basic, but some are neutral; some are soluble in water, while others are insoluble in water but soluble in ethanol (16). Furthermore, the affinity of binding of macrolides to ribosomes is enhanced by the presence of ethanol (16). Macrolides are commonly purified by organic extraction with chloroform. They give a negative ninhydrin result (13).

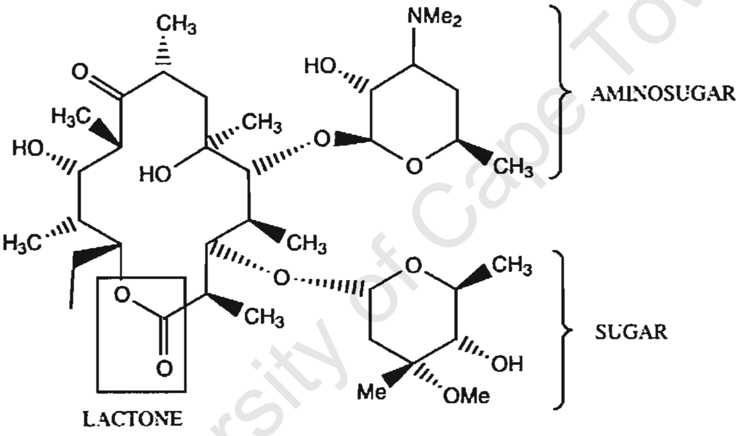


Fig. 5.2. Erythromycin, highlighting the important structural features of macrolide antibiotics (11).

### (iii) Ansamysins

The ansamysins possess an aliphatic chain (the ansa bridge) that is connected to an aromatic nucleus at two, nonadjacent positions (18) (Fig. 5.3). The halomicins and rifamycins, produced by *Micromonospora* species, are members of one of the two ansamysin subgroups in which the ansa bridge is attached to a substituted naphthalene or naphthoquinone moiety (18). The ansamysins are often brightly coloured antibiotics (orange or red in colour, for example) and are solvent extractable (18). The halomicins and rifamycins are active against Gram-positive bacteria and very importantly, *Mycobacterium tuberculosis*, but are not active against Gram-negative bacteria (18). The mode of action is by binding non-covalently to RNA polymerase and inhibiting RNA synthesis, thus impairing protein synthesis (11).

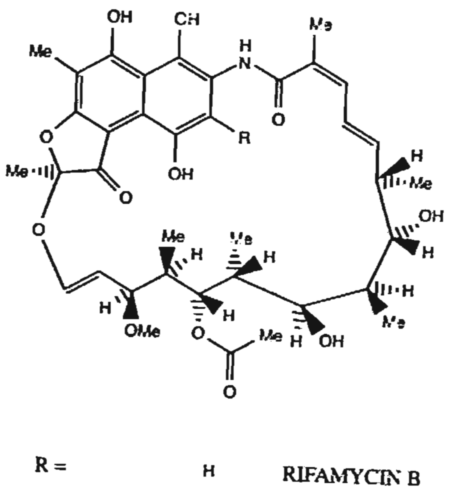


Fig. 5.3. The structure of an ansamysin antibiotic (11).

### (iv) Everninomicins

Everninomicins (Fig. 5.4) are a class of oligosaccharide antibiotics produced by *Micromonospora carbonacea*, which possess highly potent activity against Gram-positive bacteria and are solvent extractable. Most importantly, they demonstrate antibacterial activity against multiply-resistant strains of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (2).

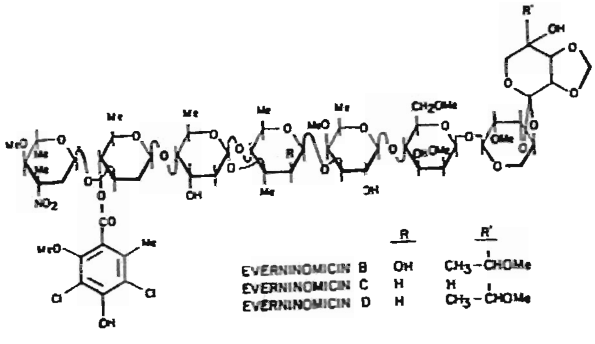
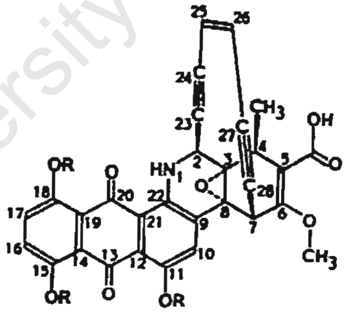


Fig. 5.4. The structures of the everninomicins (18).

### (v) Other *Micromonospora* Antibiotics

The cyclic enediynes, and tetrocarcins, new classes of antitumour antibiotics, are receiving increasing attention because they exhibit remarkable antitumour activity, and in the case of the enediynes, are DNA-cleaving agents (15). These antibiotics show extremely strong inhibition of the growth of Gram-positive bacteria, such as *Bacillus subtilis* (6). Dynemicin A (Fig. 5.5), a novel, violet-coloured antibiotic produced by *Micromonospora chersina*, is unique among the cyclic enediynes as it contains both a cyclic enediyne ring and anthraquinone chromophore (5, 15).



**Dynemicin A** R=H

Fig. 5.5. The structure of dynemicin A (6).

An uncharacterised *Micromonospora* species also produces the galbonolides, antifungal polyketide natural products (14). The orange mycelial pigments of *Micromonospora* strains are extractable in organic solvents and appear to possess antimicrobial activity of their own (7).

### 5.1.2 This Study

The antimicrobial activities of all 34 environmental isolates were investigated by performing sloppy-agar overlays and testing for activity against 5 specifically chosen test bacteria. *Micromonospora* strains showing potential as antibiotic producers were further investigated. The antibiotics were isolated, initial purification attempted, and some physicochemical properties of the most promising antibiotics determined.

## 5.2 Materials and Methods

### 5.2.1 Solid Media Overlays: The Sloppy-agar Method

The antimicrobial activity exhibited by the 34 environmental actinomycete strains was assessed by the use of the sloppy-agar overlay technique. By using sterile toothpicks, the actinomycete strains were stab-inoculated into the centre of agar plates and incubated at 30°C for 5 and 11 days. The antimicrobial activity of the strains was tested against each of five test bacteria: *Escherichia coli* ATCC 25922 (a standard antibiotic-susceptibility testing strain; Gram-negative), *E. coli* CA 84-39 (streptomycin-resistant strain), a vancomycin resistant clinical isolate of *Enterococcus faecium* (Van A; Gram-positive), *Bacillus subtilis* subsp. *subtilis* NCIB 3610 (Gram-positive), and *Mycobacterium aurum* A+ (acid fast). On day 4 and day 10, each test bacterium was inoculated into 10 ml 2YT broth (1), except for *E. coli* CA 84-39, which required 10 ml 2YT broth supplemented with 2 μl of streptomycin (100 μg/ml), and incubated overnight at 37°C with shaking. The test bacteria were Gram stained to verify the purity of the cultures. The optical density at 600 nm ($OD_{600}$) of each culture was measured using a Beckman DU-64 spectrophotometer. Cultures were diluted, before taking the reading, as follows: *E. coli*: diluted 1-in-10 in sterile 2YT broth; *E. faecium, B. subtilis*: diluted 1-in-4 in sterile 2YT broth; and *M. aurum*: diluted 1-in-6 in sterile 2YT broth. Empirically determined calculations were used to ensure that there would be the same concentration of test bacterium cells in the sloppy-agar from one overlay experiment to the next. The number of microlitres of bacterial culture used per sloppy-agar test tube was calculated as follows (solve for $x$): $OD_{600}$ × test bacterium's dilution factor × ($x$ μl) = test bacterium's constant. The values vary for the different test bacteria: *E. coli*: $OD_{600} \cdot 10x = 4$; *E. faecium, B. subtilis*: $OD_{600} \cdot 4x = 160$; *M. aurum*: $OD_{600} \cdot 6x = 160$. The calculated volume (in μl) of test culture was pipetted into test tubes containing 6 ml 2YT sloppy-agar (1), vortexed gently, and poured onto the 5 and 11 day old stab inoculated plates, ensuring that the sloppy-agar was distributed evenly over the entire surface of the plate. These plates were incubated overnight (14 – 18 h) at 37°C for *E. coli, E. faecium*, and *B. subtilis*, and for 40 – 45 h at 37°C for *M. aurum*. The diameter (in mm) of each colony was measured before overlaying. The diameter (in mm) of the zone of growth inhibition of the test bacteria was measured if a strain showed antimicrobial activity. The area of the inhibition zone in $mm^2$ was calculated. The arbitrary assignment of strength of antimicrobial activity was as follows: very weak (<100 $mm^2$); weak (100 – 1000 $mm^2$); moderate (1001 – 2000 $mm^2$); strong (2001 – 3000 $mm^2$); and very strong (>3000 $mm^2$).

The degree of antimicrobial activity against the five test bacteria was compared for 7 different growth media: Middlebrook 7H9 agar (7H9), *ISP* Medium No. 2 [yeast extract-malt extract agar (YEME)], BM-Glucose, BM-Glycerol and modified 172F agars (see 3.2.2); MMP-S and MMP-F agars (see 5.2.4).

### 5.2.2 Solvents Used in This Study

Acetone, benzene, *n*-butanol, chloroform, absolute and technical ethanol, *n*-hexane, methanol and toluene were supplied by Saarchem, while acetic acid was supplied by Merck.

### 5.2.3 Fermentation and Methods For Obtaining Antibiotic Samples Produced by the *Micromonospora* Strains

BM-Glycerol broth was used for initial fermentations. Fermentation was carried out at 30°C with shaking.

A preculture of each strain was grown in a 200 ml Erlenmeyer flask containing 20 ml BM-Glycerol for 5 days. The cultures were then transferred to 500 ml Erlenmeyer flasks containing 50 ml BM-Glycerol and incubated with shaking for 7 days, at which point the liquid medium (the supernatant) was removed and filtered (using coffee filters). Gram stains were performed to verify the purity of the culture.

Cells of each strain were scraped off of the filter paper and left overnight to dry. The following day, 10 ml methanol or chloroform was added to each dried cell sample and stirred overnight by using a magnetic stirrer. The solvent was removed the following day and transferred to a universal/standard container. The solvent was left to evaporate in a fume hood and then redissolved in methanol or chloroform so that samples were concentrated 50 times with respect to the original extract.

Organic extraction of antibiotics from the culture supernatants was performed with chloroform. The chloroform layer was removed, allowed to evaporate and was concentrated 50 times with chloroform. The supernatant (aqueous layer, post extraction with chloroform) was freeze-dried.

All culture filtrates were freeze-dried. The freeze-dried samples were redissolved in sterile water so that the samples had been concentrated 50 times.

### 5.2.4 The *Micromonospora* Macrolide Protocol

The protocol for investigating macrolide antibiotic production by *Micromonospora* was modified from a method of Kawamoto *et al.* (5). The two production media developed were as follows (Goodwin, unpublished). *Micromonospora* Macrolide Production Medium S (MMP-S), the 'seed medium', contained the following (g/L): soluble starch (11), glucose (11), casitone (Difco; 5), yeast extract (6), $CaCO_3$ (1), pH 7.2. For solid media, 15 g/L agar was added. *Micromonospora* Macrolide Production Medium F (MMP-F), the 'fermentation medium', contained the following (g/L): soluble starch (20), casitone (Difco; 6.5), yeast extract (6), $K_2HPO_4$ (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.3), $CaCO_3$ (1), pH 8.0. For solid media, 20 g/L agar was added.

The fermentation protocol was as follows. A heavy inoculum of the strain from an agar plate was inoculated into 3 ml of sterile water, then transferred to 10 ml of MMP-S in a 100 ml Erlenmeyer flask and incubated for 2 days at 30°C with shaking. The culture was then transferred to 30 ml of MMP-S in a 250 ml Erlenmeyer flask and incubated at 30°C for 4 days with shaking. The culture was then transferred to 100 ml of MMP-F in a 1 L Erlenmeyer flask and left shaking at 30°C for 70 hours. At 70 hours, the cell mass was separated from the broth (supernatant) as described in 5.2.3.

Purification of antibiotics from the cell mass was performed as follows: the cell (mycelial) mass was covered with 22 ml of chloroform and left to stir vigorously overnight. The chloroform layer was removed, allowed to evaporate, and concentrated 3 times with chloroform.

Antibiotic purification from the filtrate (supernatant) of the fermentation broth was performed as follows: the pH of the supernatant was adjusted to pH 9.5 with 2 N NaOH. To the supernatant, 65 ml of chloroform was added, and left to stir vigorously overnight. The step was repeated once more. The chloroform layers from each step were pooled, allowed to evaporate, and concentrated 3 times with chloroform. Twenty two (22) ml of distilled water (pH 4.0; adjusted with 2 N HCl) was added to the chloroform concentrate and left to stir vigorously overnight. The water layer was removed and its pH was adjusted to pH 9.5 with 2 N NaOH. Twenty two (22) ml of chloroform was added to the water layer and left to stir vigorously overnight. The chloroform layer was removed and concentrated. At this step, partially purified antibiotic should be obtained from the chloroform layer. Samples were spotted on silica TLC and bioautographed (see 5.2.6).

### 5.2.5 Thin Layer Chromatography

Thin Layer Chromatography (TLC) plates used in this research were TLC aluminium-backed silica gel 60 $F_{254}$ (Merck; Catalogue Number: 1.05554.0001). A 254 nm transilluminator (UVP©) was used to visualize UV absorbing spots, which were marked on the TLC plate with a pencil. Solvents used in TLC solvent systems for the separation of compounds in antibiotic samples are listed in 5.2.2. TLC plates were developed in glass beakers covered with tinfoil.

### 5.2.6 Bioautography for the Visualization of Antibiotically Active Spots

Cultures were grown as described in 5.2.1. The cultures were diluted with sterile 2YT broth to give an $OD_{600}$ of 0.5.

Antibiotic samples were spotted on TLC plates (see 5.2.5) and/or spotted, dried, and developed in a solvent system. Once the solvent had evaporated, the test bacteria were applied to the TLC plate by dabbing with sterile cotton wool until the entire plate was covered with test bacterium. The TLC plates were transferred to a plastic container, the bottom of which was lined with wet paper towel to create a moist environment, and placed face up on the paper towel. The container was sealed and incubated at 37°C for at least 6 hours. TLC plates were removed from the container and sprayed with 0.25% (w/v) MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (SIGMA) dissolved in Phosphate Buffered Saline (pH 7.3). The paper towel was removed and the TLC plates were returned to the container, which was sealed and left at 37°C for at least 45 min, so that a colour change was observed. The activity spots on TLC plates are visualized by using MTT, which acts as an oxidising agent, serving as the final electron acceptor in the bacterial electron transport chain. If MTT is reduced, a colour change from yellow to purple-blue is observed. The presence of the purple-blue colour indicates where the test bacteria are actively respiring, whereas areas lacking the purple-blue colour indicate the existence of an antibiotically active spot that the test bacteria are sensitive to.

The $R_f$ values were calculated as follows: distanced moved by the centre of the solute zone (activity spot , UV spot, or colour component) ÷ the distance moved by the solvent front.

### 5.2.7 Physicochemical Properties and Antibacterial Spectrum

Fehling's reagent (8) was used to test for the presence of reducing sugars. The positive control was 10% glucose (Merck) and the negative control was 10% sucrose (Merck). Exposure to iodine vapours was used for the detection of organic compounds having carbon-carbon double bonds. Cerium (IV) ammonium sulphate (6.3% dissolved in 3% concentrated sulphuric acid; Saarchem) and 50% sulphuric acid (Merck) were also used to detect organic compounds. To detect for the presence of free amino groups in antibiotic samples, 0.1 g ninhydrin (Riedel-de Haën) was dissolved in 100 ml acetone (Saarchem) and used as the visualization reagent. Antibiotic samples were spotted on silica TLC plates, developed with 95% chloroform: 5% technical ethanol (96.4%), and then subjected to testing with the reagents described in this section.

Sugar hydrolysis was performed as follows: 40 µl of antibiotic sample was added to an equal volume of 6M HCl (NT Laboratory Supplies), and heated at 100°C in a water bath for 2 hours. Five (5) µl of each sample (and 1 µl of the 1% sugar standards: glucose, mannose, ribose; and galactose, arabinose, xylose) was spotted on cellulose TLC aluminium-backed sheets (Merck; Catalogue Number: 1.0552.). The solvent system and visualization agent used was as described by Hasegawa *et al.* (4).

All TLC plates were heated in an oven at 130°C for at least 5 minutes to allow the spots to develop.

pH sensitivity was tested as follows: 40 µl of antibiotic sample was added to each of three 1.5 ml microfuge tubes. One tube acted as the control, while 40 µl of 50 mM HCl and 50 mM NaOH were added to the other two tubes and a colour change observed.

Antibiotics used in this study as controls were: oleandomycin (Sigma), gentamicin (Sigma), kanamycin (Sigma), neomycin (Intramed), and streptomycin (Calbiochem).

Test bacteria used for examining the antimicrobial spectrum are the 5 strains listed in 5.2.1, as well as *Acinetobacter calcoaceticus* C91, *Pseudomonas aeruginosa* ATCC 27853 (a standard antibiotic-susceptibility test strain), *Staphylococcus aureus* ATCC 25923 (a standard antibiotic-susceptibility test strain), and *Mycobacterium bovis* BCG (Tokyo). All strains were grown overnight in 10 ml of 2YT broth (1) at 37°C, except for *M. bovis*, which was grown for 2 weeks at 37°C in Middlebrook 7H9 broth supplemented with AD (0.2% glucose and 0.5% BSA) and 0.05% Tween 80 (1).

### 5.2.8 The Modified Eli Lilly Procedure to Test for Antimicrobial Activity

The procedure performed was modified from a method obtained from the Internet: http://www.accessexcellence.org/AE/AEC/AEF?1995?goudie_soil.html.

Eleven-day-old cultures of the strains grown on agar media were used. Small blocks of agar (approximately 1 cm × 1 cm) were cut out with a scalpel and placed on silica TLC plates. A few drops of a solvent were pipetted onto the agar pieces. The TLC plate was left at 4°C for 20 min. The agar blocks were then removed, the TLC plate allowed to dry, and then covered with test bacterium and bioautographed as outlined in 5.2.6.

## 5.3 Results and Discussion

### 5.3.1 Solid Media Overlays

Results of the sloppy-agar overlay technique are shown in Table 5.1. The table shows only the strains that exhibited antibacterial activity. Numerical values indicate the area of the zone of inhibition in $mm^2$. Overlay experiments were performed at 5 days and 11 days; the general trend was for activity to increase over time, viz. the greatest activity was shown at 11 days relative to 5 days. Of the agar media used in this study (see 5.2.1), 7H9 was used as a standard medium for the production of antibiotics by *Streptomyces* species, while YEME, BM-Glucose and BM-Glycerol were used as standard media for the production of antibiotics by *Micromonospora.* Strains of *Micromonospora* showed excellent growth on modified 172F agar and thus this medium was used to test for a correlation between good growth and good antibiotic production. The MMP media were included as they enhance the production of macrolides (5) and thus any antimicrobial activity shown on these media may be the result of a macrolide antibiotic. *B. subtilis* subsp. *subtilis* NCIB 3610 is recommended as the test organism for bioautography showing antimicrobial activity caused by macrolides. *M. aurum* A+ has a similar antibiotic-susceptibility profile to *M. tuberculosis* (3). *M. aurum* also grows faster than *M. tuberculosis* and is non-pathogenic. Activity against *M. aurum* was of most value and the antibiotics of such strains were further pursued as potential antitubercular agents.

Table 5.1. Strains exhibiting antimicrobial activity with an inhibition zone of greater than 1000 mm² in the agar overlays.

| Test Bacterium:<br>Strains: | *Escherichia coli* ATCC 25922 | *E. coli* CA 84-39 | *Bacillus subtilis* subsp. *subtilis* NCIB 3610 | *Mycobacterium aurum* A+ |
|---|---|---|---|---|
| ***Micromonospora* spp.:** | | | | |
| MMS1 | | | 11 days:<br>BM-Glucose: 3798 (+s)<br>YEME: 2749 (+s) | |
| MMS2 | | 11 days:<br>MMP-S: 1442 (–s) | | |
| MMS3 | | | 11 days:<br>YEME: 1477 (+s)<br>BM-Glycerol: 1005 (+s) | 11 days:<br>MMP-S: 1282 (+s) |
| MMS4 | | | | 11 days:<br>m172F: 4033 (+s)<br>MMP-S: 3487 (+s)<br>MMP-F: 2808 (+s)<br>BM-Glucose: 1979 (+s) |
| MMS-DA | | 11 days:<br>BM-Glucose: 2859 (s+) | | 11 days:<br>YEME: 1512 (+s) |
| MMS8 | | | 11 days:<br>YEME: 2695 (+s) | |
| ***Streptomyces* spp.:** | | | | |
| BAM1 | | | 11 days:<br>7H9: 1697 (+s) | |
| BAM3 | | | 11 days:<br>7H9: 1193 (+s) | 11 days:<br>MMP-S: 1225 (+s) |
| BAM7 | | | 11 days:<br>172F: 1178 (–s) | |
| PB | | | 11 days:<br>7H9: 2128 (+s)<br>BM-Glucose: 1512 (+s) | |
| Kit2004 | 11 days:<br>m172F: 1119 (+s) | | 11 days:<br>BM-Glucose: 1298 (+s)<br>m172F: 1030 (+s) | |
| LMP-TD | | | 11 days:<br>YEME: 1159 (+s) | 11 days:<br>MMP-S: 3212 (+s)<br>m172F: 1100 (+s) |
| LMP-BRP | | | 11 days:<br>BM-Glycerol: 2205 (+s)<br>BM-Glucose: 2118 (+s) | |

Abbreviations: (+s), presence of sporulation; (–s), absence of sporulation.

Since the results of activity against *M. aurum* were of most interest and the emphasis of the study lay with the *Micromonospora* strains, strains MMS3, MMS4 and MMS-DA were chosen to investigate further. For strains MMS3 and MMS4, it could be suggested that the antibiotics may be macrolides because the activity was shown on the MMP media. However, one must keep in mind that strain MMS4 was positive for the *grm* primers, indicating the presence of the aminoglycoside resistance gene. Furthermore, we do not have evidence of the presence or absence of macrolide biosynthetic genes in strain MMS4's genome.

### 5.3.2 Initial Fermentation Experiments

Initial fermentation (see 5.2.3) was carried out on only the Group A *Micromonospora* strains (Table 5.2). The concentrated antibiotic samples were spotted in triplicate on TLC plates to test for activity against three test bacteria (TLC is a much more sensitive method than the agar overlays). This fermentation was carried out in one medium only (BM-Glycerol) and was incubated with shaking for a total of 12 days (see 5.2.3).

Table 5.2. Preliminary investigation of the antimicrobial potential of the Group A *Micromonospora* strains.

| Fifty Times Concentrated Antibiotic Sample From: | Activity of *Micromonospora* Strains Against Test Bacteria: MMS1 | MMS2 | MMS3 | MMS4 | MMS5 | MMS6 | MMS7 | $R_2\mu$ | MLR | MMS-DA |
|---|---|---|---|---|---|---|---|---|---|---|
| Bennett's Broth Supernatant: | – | *Ma, Ef, Ec* | *Ec* | *Ec* | *Ef* | – | *Ma, Ef* | – | – | – |
| Bennett's Broth Supernatant Post Organic Extraction (i.e. aqueous layer after extraction with chloroform): | – | *Ma, Ef* | *Ec* | – | – | – | *Ef* | – | – | – |
| Chloroform (organic) Layer: | – | *Ma, Ef* | – | – | – | – | *Ma* | – | – | *Ef* |
| Methanol Organic Extract of the Cell Mass: | – | *Ma, Ef* | – | – | – | – | – | – | – | – |

Abbreviations: *Ec, Escherichia coli* ATCC 25922; *Ef, Enterococcus faecium* (VanA); *Ma, Mycobacterium aurum* A+.

These results show that there may be multiple antibiotics produced by strain MMS2 and MMS7: some antibiotics remained in the aqueous phase while others moved into the organic phase.

Strains MMS2, MMS7 and MMS-DA all showed that antibiotics were extracted into the chloroform layer. Strains MMS2 and MMS7 were also positive for the *grm* primers, however, aminoglycosides are not solvent extractable. On the other hand, macrolide antibiotics readily extract into chloroform. The antibiotics of strains MMS2 and MMS7, which remained in the aqueous phase, could be aminoglycosides.

### 5.3.3 The *Micromonospora* Macrolide Protocol

The macrolide protocol was performed on strains MMS2, MMS7, and MMS-DA as previous results (Table 5.2) showed that antibiotics extracted into the chloroform layer during initial fermentation experiments. The protocol was performed with the media outlined in 5.2.4, but also with BM-Glycerol (the medium used for the initial fermentations). Aqueous and organic phases from each step of the protocol were concentrated, spotted on TLC plates and bioautographed against *M. aurum* (the test bacterium of most interest) and *B. subtilis* (the macrolide-sensitivity test organism). The results are shown in Table 5.3.

Table 5.3. Antimicrobial activity resulting from the macrolide protocol performed on strains MMS2, MMS7 and MMS-DA grown in BM and MMP media.

| Fifty Times Concentrated Antibiotic Sample From: | MMS2 activity against test bacteria in: | | MMS7 activity against test bacteria in: | | MMS-DA activity against test bacteria in: | |
|---|---|---|---|---|---|---|
| | BM: | MMP: | BM: | MMP: | BM: | MMP: |
| Chloroform Organic Extract of the Cell Mass: | *Ma, Bs* | *Ma, Bs* | *Bs* | *Ma, Bs* | *Bs* | *Ma, Bs* |
| Chloroform Layer 1, Post $H_2O$ Extraction (i.e. chloroform layer after water extraction step): | *Bs, Ef* | *Ma* (w), *Bs, Ef* | *Ma* (w), *Bs, Ef* | *Ma, Bs, Ef* | *Bs* | *Ma, Bs* |
| $H_2O$ Layer | | | | *Ma* | | *Ma* |
| Chloroform Layer 2 (i.e. final step of protocol): | *Ef* | *Ef, Bs* | *Ef, Bs* | *Ma* (w), *Bs, Ef* | *Ef, Bs* (w) | *Ma, Ef, Bs* (w) |

Abbreviations: (w), the activity was weak; *Bs*, *Bacillus subtilis* subsp. *subtilis* NCIB 3610; *Ec*, *Escherichia coli* ATCC 25922; *Ef*, *Enterococcus faecium* (VanA); *Ma*, *Mycobacterium aurum* A+; BM, Bennett's broth; MMP, *Micromonospora* Macrolide Production medium.

The samples showing activity against *M. aurum* (not including the water layer samples) were developed in 100% chloroform and bioautographed against *M. aurum* and *B. subtilis* (Table 5.4).

Table 5.4. Results of antibiotic samples from the macrolide protocol developed in 100% chloroform as the solvent system on silica TLC plates.

| | | *Mycobacterium aurum* A+ | | *Bacillus subtilis* subsp. *subtilis* NCIB 3610 | |
|---|---|---|---|---|---|
| MMS Strain: | Antibiotic Sample (50 times concentrated) From: | $R_f$ of Activity Spot: | Remarks: | $R_f$ of Activity Spot: | Remarks: |
| MMS2 | BM Cell Mass Extract (= antibiotic sample No. **1**) | 0.21 | Activity spot and UV spot overlap. | N.D. | Weak activity spot, overlapping with UV spot, on the solvent front. |
| | MMP Cell Mass Extract (= antibiotic sample No. **2**) | 0.23 | Activity spot and UV spot overlap. | N.D. | Weak activity spot, overlapping with UV spot, on the solvent front. |
| | MMP Chloroform Layer 1 | N.A. | | N.D. | 1. Weak activity spot, overlapping with UV spot, on the solvent front.<br>2. Strong activity spot overlaps UV spot on the origin. |
| MMS7 | MMP Cell Mass Extract (= antibiotic sample No. **3**) | 0.24 | Activity spot overlaps with a colour component. | 0.18 | Strong activity spot overlaps with a colour component. |
| | BM Chloroform Layer 1 | N.A. | | 0.15 | Weak activity spot. |
| | MMP Chloroform Layer 1 | N.A. | | 0.15 | Weak activity spot, overlapping with UV spot. |
| | MMP Chloroform Layer 2 | N.A. | | 0.15 | Weak activity spot. |
| MMS-DA | MMP Cell Mass Extract | N.A. | | N.D. | Strong activity spot overlaps UV spot just above the origin. |
| | MMP Chloroform Layer 1 | N.A. | | N.A. | |
| | MMP Chloroform Layer 2 | N.A. | | N.A. | |

Abbreviations: N.A., no activity; N.D., not determined; BM, Bennett's broth; MMP, *Micromonospora* Macrolide Production medium.

Based on the anti-*M. aurum* activity, 3 antibiotic samples were chosen to be investigated further. These are indicated in red: the chloroform cell mass extract of strain MMS2 grown in BM (antibiotic sample No. 1), grown in MMP (antibiotic sample No. 2) and the chloroform cell mass extract of MMS7 grown in MMP (antibiotic sample No. 3).

It is a rule of thumb that an optimal solvent system is one that gives an $R_f$ value of 0.5 for the active spot, indicating that the spot has moved half way up of the TLC plate. This is a good position as many other compounds exist in a partially purified antibiotic sample that are not of interest and often travel to a position near the solvent front or near the origin. Thus, given the data in Table 5.4, a more suitable solvent system needed to be found to increase the $R_f$ value. Many solvent systems were tested (Table 5.5), but the optimal solvent system proved to be 95% chloroform: 5% technical ethanol (96.4%). By the end of this search, the antibiotic samples were all depleted and more sample had to be attained. Unfortunately, after many attempts, it was found that strain MMS2 had lost the activity previously extracted into chloroform from the cell mass grown in BM (antibiotic sample No. 1). Thus, further work continued with antibiotic samples No. 2 and No. 3 only. Their respective $R_f$ values in the optimal solvent system were 0.50 and 0.53, and further indicated that the two samples potentially contained different antibiotic compounds.

Table 5.5. List of solvent systems used for purification of antibiotics from *Micromonospora* strains.

| Solvent Systems Used for Antibiotic Samples From Strains MMS2 and MMS7: | Solvent Systems Used for Antibiotic Samples From Strains MMS3, MMS4, and MMS-DA: |
|---|---|
| ▪ 100% chloroform<br>▪ 90% chloroform: 10% *n*-butanol<br>▪ 90% chloroform: 10% absolute ethanol<br>▪ *n*-Butanol: acetic acid: water (3:1:1)<br>▪ Water-saturated butanol<br><br>*Optimal* Solvent System:<br>95% chloroform: 5% technical ethanol (96.4%) | Solvent Systems which showed *slight* movement of the activity spots from the origin (low $R_f$ values):<br>• *n*-Butanol: acetic acid: water (3:1:1)<br>• 100% acetone<br>• 100% *n*-butanol<br>• 100% ethanol<br>• 100% methanol<br><br>Solvent Systems which show *good* movement of the activity spots from the origin (higher $R_f$ values):<br>• 80% methanol: 20% *n*-butanol<br>• 50% methanol: 50% chloroform<br>• 50% methanol: 50% water<br>• 80% *n*-butanol: 20% water<br>• 50% *n*-butanol: 50% water |

**5.3.4 Physicochemical Properties and Antibacterial Spectra of Antibiotic Samples No. 2 and No. 3**

Antibiotic samples No. 2 and No. 3 were developed in two different solvent systems (*n*-butanol: acetic acid: water [3:1:1], and water-saturated butanol) along with a known macrolide antibiotic (oleandomycin) and the aminoglycoside antibiotics, kanamycin, neomycin, gentamicin, and streptomycin. When bioautographed against *M. aurum*, the $R_f$ values of samples No. 2 and No. 3 were distinct from the $R_f$ values of the control aminoglycosides and macrolide antibiotic used.

Acid hydrolysis revealed that the aminoglycoside controls and samples No. 2 and No. 3 all shared a common hydrolysate spot on the TLC plate. No spot was observed for oleandomycin. Furthermore, the ninhydrin result was positive for all samples except oleandomycin, which was as expected since macrolides do give a negative result. Thus, these two experiments indicate that samples No. 2 and No. 3 may be aminoglycoside-type antibiotics, distinct from gentamicin, kanamycin, neomycin, and streptomycin (based on the different $R_f$ values).

The pattern of spots produced by cerium (IV) ammonium sulphate and by the use of 50% sulphuric acid was similar. This suggests that there were many organic compounds in the samples and further purification attempts are most certainly required. Iodine vapours revealed many spots positive for compounds having C=C bonds. Antibiotic samples No. 2 and No. 3 were positive for the reduction of Fehling's reagent, however, since the samples were not pure, it could not be determined which component was responsible for the reaction, as many contaminating compounds were in the samples.

Figures 5.6 and 5.7 show the features of the anti-*M. aurum* active spot of interest in samples No. 2 and No. 3.

Sample No. 2 showed pH sensitivity when treated with HCl and NaOH: the colour of the sample changed from a dark orange to light yellow colour in each case. Sample No. 3 changed colour from dark red-orange to light orange when treated with HCl and NaOH.

The samples were spotted on silica TLC and developed in the optimal solvent system [95% chloroform: 5% technical ethanol (96.4%)], and bioautographed against the 9 test bacteria (see 5.2.7). The TLC plate showing activity against *M. aurum* A+ is shown in Fig. 5.6 and the plates showing activity against *B. subtilis* subsp. *subtilis* NCIB 3610 and *P. aeruginosa* ATCC 27853 are shown in Fig. 5.8. *E. coli* ATCC 25922, *E. coli* CA 84-39, and *E. faecium* (VanA) were extremely sensitive to the antibiotic samples (not shown). Multiple activity spots along the length of the TLC plate were seen for *S. aureus* and for *A. calcoaceticus* C91 (not shown). Only sample No. 2 showed activity against *Mycobacterium bovis* BCG (Tokyo): a single activity spot with an $R_f$ value of 0.51 (not shown). The $R_f$ value of the single activity spot of sample No. 2 against *M. aurum* was 0.50. This antimycobacterial activity is promising and should be tested against *Mycobacterium tuberculosis*.

There are many opportunistic pathogens involved in outbreaks of hospital infections, which are becoming totally resistant to antibiotics such as aminoglycosides and β-lactams (19, 20). These include vancomycin-resistant *Enterococcus faecium* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter* and *Pseudomonas aeruginosa* (19). Thus there is a need for novel antibiotics to treat such infections. Samples No. 2 and No. 3 could be potentially useful to treat infections caused by *Enterococcus*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas* spp.

Samples No. 2 and No. 3 may be considered broad-spectrum antibiotics, such as macrolide antibiotics.

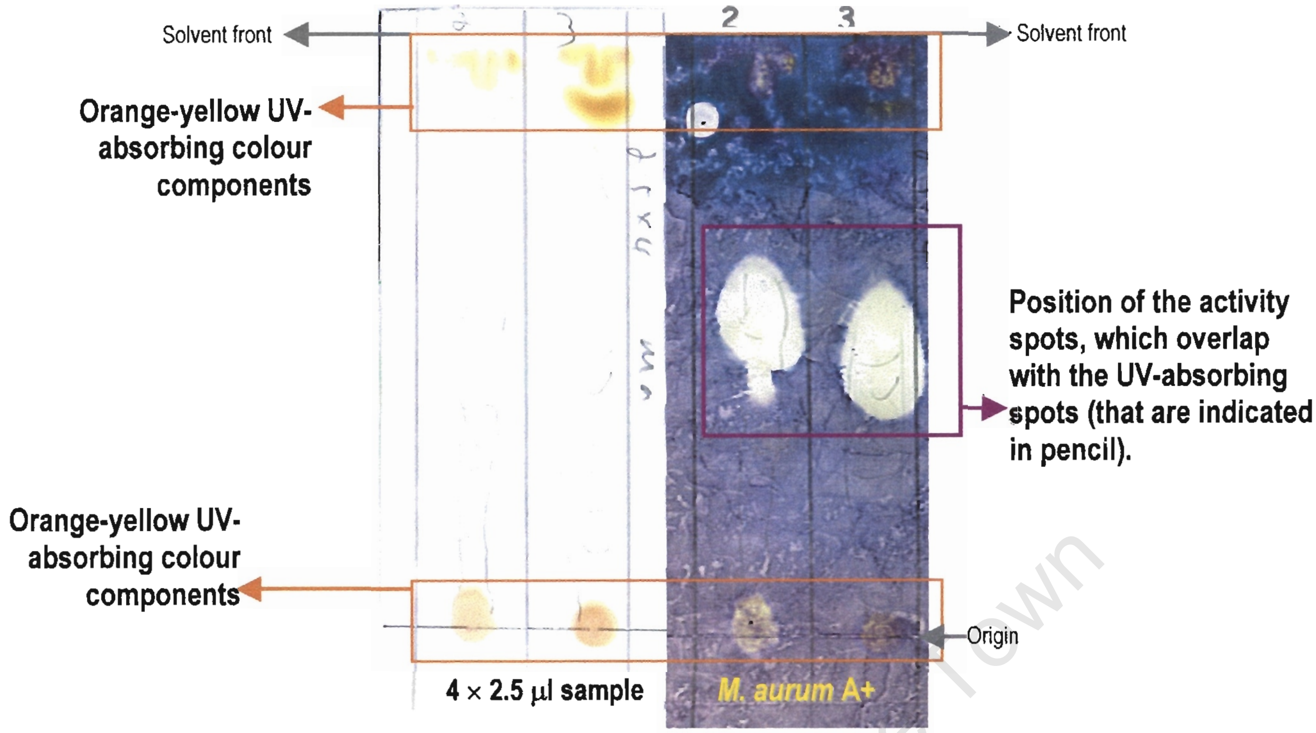


Fig. 5.6. Silica TLC plates: Left, the colour components of antibiotic samples No. 2 and No. 3; Right, bioautography with *M. aurum*, showing the antimicrobial activity of the antibiotic samples, and the position of the UV-absorbing spots. Solvent system: 95% chloroform: 5% technical ethanol (96.4%).

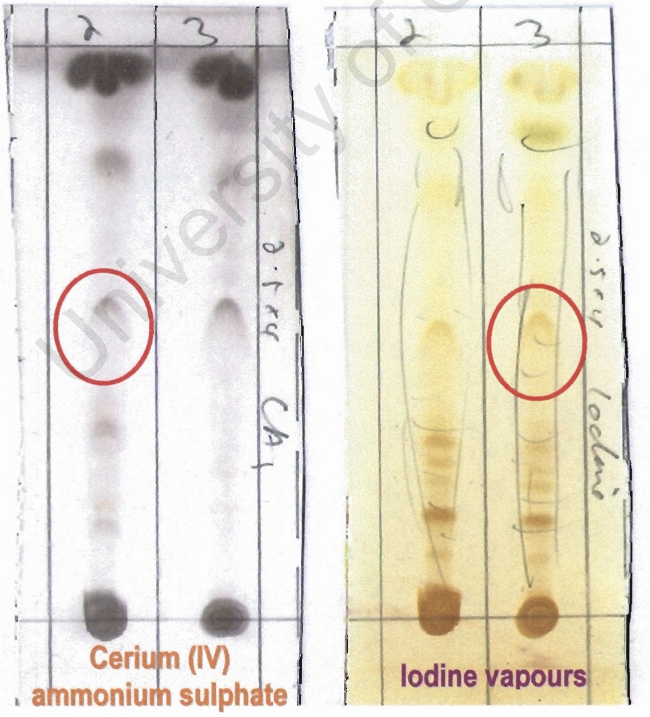


Fig. 5.7. TLC plates showing some physicochemical properties of antibiotic samples No. 2 and No. 3: cerium (IV) ammonium sulphate (left) detects organic compounds in the sample (black-grey spots); iodine vapours (right) detect organic compounds having carbon-carbon double bonds (shades of brown). The red circles indicate the approximate position of the activity spot of interest against *M. aurum*.

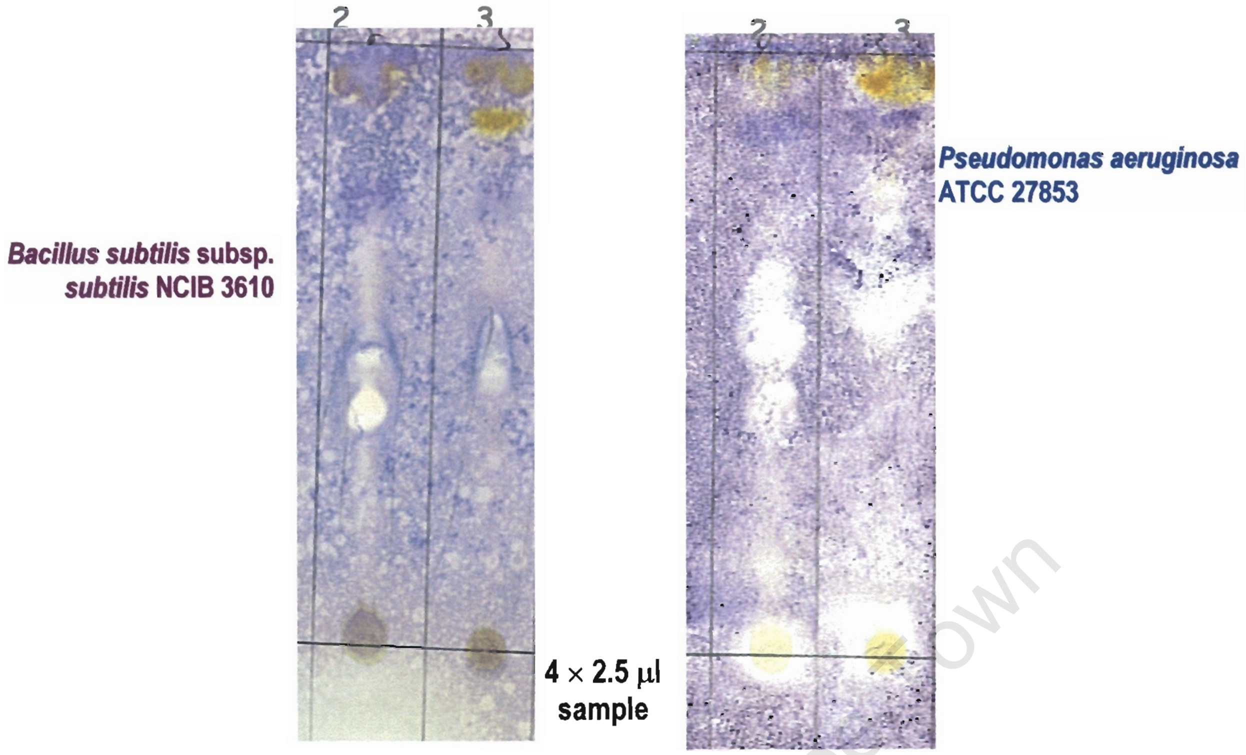


Fig. 5.8. Silica TLC plates showing the antimicrobial activity (zones of clearing) of antibiotic samples No. 2 and No. 3 against *B. subtilis* (left) and *P. aeruginosa* (right). Solvent system: 95% chloroform: 5% technical ethanol (96.4%).

### 5.3.5 Antibiotics from Strains MMS3, MMS4, and MMS-DA

The results of the overlay experiments suggested that strains MMS3, MMS4, and MMS-DA be further pursued (see 5.3.1). The strains were grown in the optimal media indicated by the solid agar overlay results. Broth culture conditions were the same as for the agar overlays: the strains were grown at 30°C for 11 days. Bioautography was performed with *M. aurum*, the test bacterium of interest. The results are shown in Table 5.6.

Table 5.6. Antibiotic activity of strains, grown in the optimal medium suggested by the overlay experiments, at 30°C for 11 days and bioautographed against *Mycobacterium aurum* A+.

| Fifty Times Concentrated Antibiotic Sample From: | MMS4 activity in: | | | | MMS3 activity in MMP-S: | MMS-DA activity in YEME: |
|---|---|---|---|---|---|---|
| | BM-Glucose: | m172F: | MMP-S: | MMP-F: | | |
| Methanol Organic Extract of the Cell Mass: | ++ | ++ | ++ | – | – | +++ |
| Chloroform Organic Extract of the Cell Mass: | – | – | – | – | – | +++ |
| Specific Medium's Supernatant: | + | ++ | +++ | +++ | – | – |

Abbreviations: –, no activity; +, weak activity; ++, moderate activity; +++, strong activity.

It is noticeable that strain MMS3 did not show any activity after being grown in liquid medium under the same conditions the agar overlay experiment was performed. Thus, a modified Eli Lilly procedure (5.2.8) was performed using 11 solvents of differing polarity, in an effort to detect the antibacterial activity observed in the agar overlays (Table 5.7).

Table 5.7. Table of Eli Lilly procedure results, bioautographed against *Mycobacterium aurum* A+.

| | MMS3 on MMP-S at: | | MMS-DA on YEME at: | | MMS4 on BM-Glucose at: | | MMS4 on m172F at: | | MMS4 on MMP-S at: | | MMS4 on MMP-F at: | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Solvents: | 30°C | 37°C | 30°C | 37°C | 30°C | 37°C | 30°C | 37°C | 30°C | 37°C | 30°C | 37°C |
| Water | + | – | – | – | – | – | – | – | – | – | – | – |
| Technical Ethanol (96.4%) | – | – | – | – | – | – | – | – | – | – | – | – |
| Absolute Ethanol (100%) | + | + | – | – | – | – | + | + | – | + | – | – |
| Acetic Acid | – | – | – | – | – | – | – | – | – | – | – | – |
| Acetone | – | – | – | – | – | – | – | – | – | – | – | – |
| Methanol | – | – | – | – | – | – | – | – | – | – | – | – |
| *n*-Butanol | – | – | + | + | – | – | – | – | – | – | – | – |
| Chloroform | – | – | – | – | – | – | – | – | – | – | – | – |
| Benzene | – | – | + | – | – | – | + | + | + | + | + | + |
| *n*-Hexane | – | – | – | – | – | – | + | + | + | + | + | + |
| Toluene | – | – | – | – | – | – | – | + | – | – | – | – |

Abbreviations: –, no activity; +, activity.

Strain MMS3 exhibited antibacterial activity when water and absolute ethanol (a polar solvent) were used.

The fermentation of the strains in their optimal media was repeated and the supernatant tested for activity against *M. aurum* after development in various solvent systems (Table 5.5). Each of the 6 supernatant samples (MMS3 grown in MMP-S broth; MMS-DA grown in YEME broth; MMS4 grown in BM-Glucose, modified 172F, MMP-S, and MMP-F broths) contained only one active compound, as TLC revealed the presence of one active spot when experimenting with different solvent systems. An optimal solvent system for each of the 6 samples is still to be determined.

### 5.4 Conclusion

Anti-*M. aurum* antibiotics were shown to be produced by *Micromonospora* strains MMS2, MMS3, MMS4, MMS7, and MMS-DA. Thus, 5 out of the 10 Group A strains investigated have antibiotics that should be pursued, as they may have good antitubercular activity. The strains that were not investigated further than the overlay experiments (*Micromonospora* strain MMS8 and 7 *Streptomyces* strains; Table 5.1) should be investigated by performing initial fermentations in the optimal medium suggested by the overlays, and an attempt made to isolate the antibiotics. Since the *Streptomyces* strains were not the focus of this study, but do have antimicrobial activity, it would be worth screening them with the battery of PCR primers discussed in Chapter 4, to attempt to determine the type of antibiotic they may produce.

*Micromonospora* strains MMS2 and MMS7 produce antibiotic samples No. 2 and No. 3, which were the focus of this chapter. By examining the physicochemical properties of the partially purified antibiotic samples, it is clear that many contaminating compounds are present in the samples and that further purification must be carried out to isolate the antibiotic of interest. These samples are of particular interest because of their broad spectrum activity against many pathogenic bacteria.

Since the optimal solvent system was found for samples No. 2 and No. 3, silica column chromatography should be performed using 95% chloroform: 5% technical ethanol (96.4%) as the mobile phase. This will allow one to further purify the antibiotic compounds. Bioautography will confirm the presence of the antibiotics and repeating the physicochemical tests [such as cerium (IV) ammonium sulphate] will give an indication of how many impurities have been removed.

The genome screening in Chapter 4 showed that both strains MMS2 and MMS7 may have the biosynthetic capability to produce gentamicin-type aminoglycoside antibiotics. The results obtained by working with the antibiotic samples did not identify the antibiotic class: the compounds in the samples could be macrolides, aminoglycosides, or novel antibiotic compounds. There is evidence for all three suggestions. Nuclear magnetic resonance (NMR) studies will determine the structure of the compounds once they have been purified.

The antibiotics produced by strains MMS3, MMS4, and MMS-DA require much more work, starting with finding an optimal solvent system and $R_f$ values for the compounds in the six samples obtained from these strains. Strain MMS4 was positive for the *grm* PCR, indicating the potential ability to produce a gentamicin-type antibiotic. However, the overlay experiments showed that great activity was produced on the macrolide production media.

## 5.5 References

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# Chapter 6
# Conclusion and Future Work

The Klein Estuary (Hermanus Lagoon) is a suitable environment for isolating novel *Micromonospora* strains. The other marine environments investigated in this study (Noordhoek Beach; Phuket Island; Plettenberg Bay) yielded additional *Micromonospora* strains, members of the rare actinomycete genus *Pseudonocardia*, and interesting *Streptomyces* strains. Of the 34 actinomycete strains isolated, 28 are potentially novel species. Nine (9) new species are proposed: '*Micromonospora aquatica*', '*Micromonospora arenincolae*', '*Micromonospora cinerea*', '*Micromonospora grateloupicolae*', '*Micromonospora hermanusense*', '*Micromonospora lacunae*', '*Micromonospora stanfordense*', '*Micromonospora terrae*', and '*Streptomyces noordhoekensis*'. Several of the potentially novel strains showed promising antimicrobial activities against pathogenic bacteria. Unexplored marine environments should be further investigated for strains having promising antimicrobial activities. The genus *Micromonospora* was easily isolated on YEME and modified 172F agars supplemented with selective inhibitors to reduce the numbers of contaminating Gram-negative bacteria and fungi, and are thus not so rare. Strains that have not been fully characterised should have their descriptions completed. The menaquinone profile of the *Micromonospora* strains should be investigated, as this is perhaps the only worthwhile chemotaxonomic feature of the genus that could be used to discern between *Micromonospora* species. Complete 16S rRNA gene sequencing should be undertaken on those strains that do not already have complete sequences. Physiological testing on the last 6 strains discussed in Chapter 3 should be completed, especially the *Pseudonocardia* strains, so that their novelty can be assessed.

The genus *Micromonospora* has few distinctive morphological features and species delineation is difficult. Thus an identification key was created to easily determine whether a strain should be pursued by asking if it shares the features of a validly published *Micromonospora* species. The identification key makes use of a few carefully selected physiological tests. The key should be used to assess the novelty of every *Micromonospora* strain isolated, and should be updated and modified when new species are described in the literature. A further benefit of the identification key is that standardized media were developed for the testing of important physiological characters of the genus *Micromonospora*, such as maximum sodium chloride tolerance and the production of diffusible pigments. Additional physiological characters of 15 of the validly published *Micromonospora* species are described, in an attempt to assist with species delineation. It is also of interest that many *Micromonospora* strains do produce aerial mycelia and that almost all of the *Micromonospora* strains investigated can grow under anaerobic conditions. Many of the environmental *Micromonospora* isolates are capable of using glycerol (1.0% w/v) and inositol (1.0% w/v) as sole carbon sources, whereas previously, only *Micromonospora purpureochromogenes* utilized glycerol as a sole carbon source and *Micromonospora inositola* utilized inositol as a sole carbon source.

Many strains showed encouraging antimicrobial activity with the overlay experiments. Of the 10 *Micromonospora* strains investigated beyond overlay experiments, 5 produce antibiotics worth pursuing further. The antimycobacterial activity appears promising and testing against *M. tuberculosis* should be carried out. However, there is no definitive idea at this time as to the identity of the antibiotics in the samples. A macrolide purification protocol was performed on strains MMS2, MMS7, and MMS-DA but did not yield antibiotics with strong anti-*M. aurum* activity. Aminoglycoside purification procedures should be performed, and because these antibiotics can be purified by means of ion-exchange chromatography, one could quickly confirm if charged antibiotic molecules are present in the samples. However, structure determination by NMR will ultimately reveal the class of antibiotic the molecules belong to.

Aminoglycosides are of special interest because the resistance genes to these antibiotics are part of the biosynthetic cluster that synthesises these same antibiotics. The presence of the resistance genes indicates which antibiotic type a strain is capable of producing, provided the biosynthetic cluster is actively transcribed. The resistance gene indicating the biosynthesis of gentamicin-type aminoglycoside antibiotics (*grm*) can be detected with PCR primers and a plate assay, which complement each other, and are reliable. The neomycin-type detection strategy (*fmrT*) needs further investigation: Is the resistance gene specific to the biosynthetic cluster or can the resistance gene be acquired by many strains? The primers for the *fmrT* PCR are degenerate and would need to be improved, however, there are few gene sequences available from which to design primers. The concern when targeting antibiotic resistance genes is that they may not be part of a biosynthetic cluster and thus are not indicative of the ability to produce the specific antibiotic. Genes encoding specific molecules essential for the synthesis of aminoglycosides could be considered as targets for primer design. However, as mentioned in Chapter 4, there are 6 different groups of aminoglycoside antibiotics and it is difficult to target a gene common in all the biosynthetic pathways. A pair of primers would have to be designed for each of the different aminoglycoside sub-groups.