SELECTIVE ISOLATION AND TAXONOMIC ANALYSIS OF THE GENUS *MICROMONOSPORA*

"A thesis submitted to the University of Kent for the degree of Doctor of Philosophy in the Faculty of Science, Technology and Medical Studies"

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September 2000

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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Date: 24/9/00

Carpe diem

Abstract

Micromonosporae were isolated from a diverse range of environmental habitats and geographic locations. The dispersion and differential centrifugation technique, coupled with a phenol pre-treatment and the use of colloidal chitin and M3 agars as selective media proved to be highly efficient for the recovery of micromonosporae from environmental samples. The dispersion and centrifugation technique yielded recoveries of up to 10,000 fold more micromonosporae than the traditional vortex technique. Counts varied between 0 and $9 * 10^4$ colony forming units/g for the terrestrial samples and from 6 to $5 * 10^2$ colony forming units/g for the marine samples. The highest counts of micromonosporae ($9 * 10^4$ colony forming units/g) were recovered from a Namibian sample. A number of alkalitolerant micromonosporae were isolated from the Indonesian samples using the same procedure but with the medium adjusted to pH 10. Counts of alkalitolerant micromonosporae ranged from 0 to $5 * 10^3$ colony forming units/g of sample, with the highest counts being recovered from a rice paddy field. Representative alkalitolerant micromonosporae grew well on media at pH 7 to 8.

Pyrolysis mass spectrometry (PyMS) was used to rapidly screen representatives of *Micromonospora* isolates from all environmental habitats and geographical locations sampled. PyMS revealed a number of pyro-groups containing isolates distinct from the Type strains suggesting that these isolates may represent novel taxa. There was some evidence of isolates forming pyro-groups based on their sample location.

One hundred and eighty representatives of the genus *Micromonospora* and 15 duplicated strains were examined for 134 unit characters and the resultant data analysed using the Jaccard (S_J) and simple matching S_{SM} coefficients. Clustering was achieved using the unweighted pair group method with arithmetic averages algorithm (UPGMA). Good congruence was found between the classifications based on the S_J and S_{SM}, UPGMA analyses.

The numerical phenetic data were difficult to interpret but a measure of confidence can be placed in the numerical classification based on the S_J, UPGMA analysis given the acceptable cophenetic correlation and test error values and the congruence observed with the results from the S_{SM}, UPGMA analysis. The test strains were assigned to major (4 or more strains), minor (2-3 strains) and single membered clusters defined at the 69% similarity level in the S_J, UPGMA classification. There was good agreement between the pyrogroups formed in the PyMS analyses and the clusters generated from the numerical taxonomy analysis.

The PyMS and numerical taxonomic analyses revealed that there is some evidence for *Micromonospora* isolates existing as geovars as both techniques revealed some evidence for isolates grouping according to their sample location.

Attempts to sequence the 16S gene of *Micromonospora* isolates were unsuccessful due to difficulties in obtaining high quantities of pure DNA and because questions have been raised over whether this genus contains more than one rRNA operon. The single strand conformation polymorphism (SSCP) technique demonstrated that differences in 16S rDNA sequences between isolates could be detected and individual profiles for the isolates could be generated. The SSCP technique also highlighted the discrepancies that may exist between supposedly identical type strains in culture collections.

Acknowledgements

I thank my supervisor Alan Bull for his enthusiastic support of my PhD, for giving me a global perspective of this subject and for his fervent encouragement of my overseas projects. I hope one day, to have as many travel stories to tell.

I would like to extend a warm thanks to the many people who collected soil samples for me, or have provided me with samples from their own collections, this project would not have been so interesting and diverse had it not been for the variety of locations and habitats I was able to analyse. So, thanks go to: Dr. Gary Robinson (UKC) for the North American samples, Dr. Steve Heald and company (UKC) for the Dartmoor samples, Professor Ian Swingland (UKC) for the New Zealand and Bangladesh samples, Professor Peter Jeffries (UKC) for the sample from the Czech Republic, Dr. Yadi Setiadi (Institut Pertanian, Bogor) for the samples from Irian Jaya, Sulawesi and Sumatra, Dr. Castro (catholic University, Porto) for the Portuguese samples, Professor Alan Bull for the Australian samples, Dr. Phoebe Barnard and the Ministry of Agriculture, Namibia for the samples from Namibia, Dr. Joy Colquhoun for the Japanese hot spring samples, Dr. Peter Wellsbury (University of Bristol) for the Blake Ridge core sediments. Special thanks go to Dr. David Wynn-Williams (British Antarctic Survey) who kindly gave me some very valuable samples from the Antarctic; it was highly disappointing not to isolate more actinomycetes from them. I am extremely grateful towards Professor Koki Horikoshi and JAMSTEC for the precious marine samples; a study just on marine micromonosporae would have been interesting.

I would like to thank the British Council and the International Institute for Biotechnology for facilitating the collaboration established during this project. It gives me great pleasure to thank Dr. Joedoro Soedarsono and his staff IUC Biotechnology at Universitas Gadjah Mada, not only for his assistance with collecting the Indonesian samples, but also for the invitation to work in his labs. My time in Indonesia was truly rich and rewarding and made especially memorable by the warm hospitality I received. It's a beautiful country and I dearly hope to return soon.

My gratitude and respect goes out to Professor Mike Goodfellow and Dr. Alan Ward at Newcastle University. I thank them for the warm welcome to their labs and for their guidance on the mysteries of numerical taxonomy. I would also like to thank Dr. John Perry at the Freeman Hospital (Newcastle) for his kind invitation to carry out the rapid enzyme profiling in his labs and for his enthusiastic support. Thanks also to Kay, one of his PhD students who spent several afternoons helping me pipette my organisms and substrates into micro-titre plates.

On a note closer to home, my thanks go to Dr. Joy Colquhoun who helped introduce me to the world of actinomycetes, to Dr. Steve Heald for his never ending supply of knowledge regarding processes, techniques and chemicals and for the introduction to gin and tonic! Two other people in the lab have been invaluable along the way; Pedro Brandao for always being organised and Helia Radianingtyas for those endless discussions of our 16S and SSCP work in the tearoom. I thank Dr. Justin Clapp for introducing me to SSCP and being on hand for advice with molecular work. My appreciation goes to Sheila Rearden at Wye College for her enthusiastic help with the SEM; it gave a fascinating insight into my micromonosporae and to Dr. Phillip Murphy with his assistance in trying to sequence the 16S genes for some of my isolates

Finally but in no means least, I thank my family. Thank you, for always being there, for your patience, your encouragement and your belief in me. Mum and Kerry: it was always good to know you were only a phone call away. A special thank you goes to Dad, for his endless help and advice with computers and presentation and enthusiasm for the final big print. And my dear Simon, without whose help I would never have finished this project.

Publications

Original publications

1. **Colquhoun, J. A., J. Mexson, M. Goodfellow, A. C. Ward, K. Horikoshi and A. T. Bull.** (1998) Novel rhodococci and other mycolate actinomycetes from the deep sea. Antonie van Leeuwenhoek **74**: 27-40

2. **Mexson, J. and A. T. Bull.** (2000) Preliminary studies of the diversity of micromonosporae in Indonesian soils and lake sediments. Indonesian Journal of Biotechnology **Special Issue**: 384-390

Oral Presentations

1. **Mexson, J. and A. T. Bull.** (1999) Actinomycetes in deep-sea sediments, p306-312. *In* H-S Xu and R. R. Colwell (ed.), Progress and prospect of marine biotechnology. China Ocean Press, Beijing.

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Abbreviations

µM	Micromole (10^{-6} M)
µm	Micrometre (10^{-6} metre)
µg	Microgram (10^{-6} g)
µl	Microlitre (10^{-6} l)
#	Number
%	Percent
°C	Degrees Celsius
APS	Ammonium persulphate
CFU	Colony forming unit
cm	Centimetre (10^{-2} m)
CVA	Canonical variates analysis
DDC	Dispersion and Differential Centrifugation
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganism und Zellkulturen
16S DNA	16S unit of deoxyribonucleic acid
16S rRNA	16S unit of ribosomal ribonucleic acid
16S rDNA	Deoxyribonucleic acid coding for 16S rRNA
dsDNA	Double stranded deoxynucleic acid
dNTPs	Deoxynucleotide triphosphate
dH_20	Distilled water
EDTA	Ethylene-diamine-tetra acetic acid
eV	Electron volts
g	Gramme
GES	Guanidine thiocyanate, EDTA and Sarkosyl reagent
GYEA	Glucose yeast extract agar
L	Litre
m	Metre
M	Mole
mg	Milligramme (10^{-3} g)
ml	Millilitre (10^{-3} l)
mm	Millimetre (10^{-3} m)

mM	Millimole (10^{-3} M)
NCIMB	National Collection for Industrial and Marine Bacteria
nM	Nanomole (10^{-9} M)
PC	Personal computer
PBS	Phosphate buffered saline
PCA	Principal coordinates analysis
PCR	Polymerase chain reaction
pH	Negative $\log_{10}$ of the hydrogen ion concentration
pM	Picomole (10^{-12} M)
PyMS	Pyrolysis mass spectroscopy
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
ssDNA	Single stranded DNA
SSCP	Single strand conformation polymorphism
TAE	Tris acetate-EDTA buffer
TBE	Tris borate-EDTA buffer
UV	Ultraviolet
v/v	Volume for volume
Vh	Volt hours
w/v	Weight for volume
w/w	Weight for weight

Chapter One

Introduction

1.1. Aims of the study

The isolation and screening of bacteria from diverse environments has led to the discovery of many novel and commercially important exploitable properties (Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992, 2000). However, such screening programmes are often limited to the examination of aerobic, heterotrophic bacteria that grow at ambient temperature and neutral pH, even though these organisms probably represent only a small fraction of the microbial population (Bull *et al.*, 1992). Despite this limited approach, these screening programmes yielded a steady supply of isolates having novel properties and producing novel metabolites. One of the problems with this type of screening is the rediscovery of known compounds, hence it is important to examine unusual environments and habitats (Takizawa *et al.*, 1993; Jensen and Fenical, 1994) and to develop methods for isolating and detecting novel organisms in more prosaic habitats (Bull *et al.*, 2000). Discoveries of important, novel bioactive compounds have been shown increasingly to be dependent on the development of objective strategies for the isolation and characterisation rare and novel organisms for use in new and existing pharmacological screens (Nolan and Cross, 1988; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992, 2000).

Actinomycetes are a unique source of commercially important bioactive compounds and biocatalysts (Goodfellow *et al.*, 1988; Bull *et al.*, 1992). Initially, these organisms were best known for their capacity to synthesize antibiotics (Okami and Hotta, 1988) but more recently have been shown to produce commercially significant enzymes, enzyme inhibitors and immunomodifiers (Peczynska-Czoch and Mordarski, 1988; Umezawa, 1988).

Initial work was focused largely on the genus *Streptomyces* following the discovery of streptomycin from *Streptomyces griseus* (Schatz *et al.*, 1944) but it has become increasingly apparent that actinomycetes other than streptomycetes are important sources of bioactive compounds and biocatalysts (Goodfellow and O'Donnell, 1989). Consequently, the development of new isolation procedures is desirable for ensuring the supply of novel and rare actinomycetes for screening

programmes. New developments in selective isolation procedures and the rapid detection of novel strains have enabled this challenge to be met (Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992, 2000).

Members of the actinomycete genus *Micromonospora* have proved to be an important source of antibiotics (Wagman and Weinstein, 1980; Berdy, 1984a,b). Intensive screening of micromonosporae began in 1963 after the discovery of gentamicin from strains of *Micromonospora purpurea* and *Micromonospora echinospora* (Weinstein *et al.*, 1964). Since then, over 400 antibiotics from micromonosporae have been described (Goodfellow and O'Donnell, 1989). The search for new antibiotics is held back by the limits of selective isolation procedures in recovery of specific parts of the microbial community and the inadequate taxonomy of this genus (Vobis, 1991).

Given this background to the genus *Micromonospora* the detailed aims of this project were defined as follows:

- Selective isolation of *Micromonospora* from a wide range of environmental and geographically derived samples

- Evaluation of different extraction techniques, sample pre-treatments and selective media for the isolation of micromonosporae

- Evaluation of pyrolysis mass spectrometry (PyMS) for the preliminary discrimination of large collections of isolated *Micromonospora* strains

- Comprehensive numerical taxonomic analysis of representatives of pyro-groups defined by the PyMS analysis

- Assessment of rapid enzyme profiling for the classification of micromonosporae

- Genotypic analysis of selected micromonosporae using 16S rRNA gene sequencing and single strand conformation polymorphism (SSCP)

- Assessment of the biogeographic distribution of the genus *Micromonospora*

1.2. Bacterial Systematics

1.2.1. Introduction

Bacterial classification and identification are data dependent and hence are in a progressive state of development as they are influenced by the introduction and application of new taxonomic concepts and methods. Nomenclature, by ensuring that the current internationally recognised scientific names are given to taxa (classification) and unknown strains (identification), covers both disciplines and, as with classification and identification, is being constantly adjusted and refined. Sound classification is a pre-requisite for both stable nomenclature and reliable identification. Classification and identification are core disciplines as they are relevant to both basic and applied research. Nomenclature is central to all aspects of science, as scientists need to know what organisms they are working with before they can pass on information about them. In addition, bacterial taxonomic and metabolic diversity are fundamentally interrelated and therefore important to natural product discovery (Jensen and Fenical, 1994).

Edward Wilson (1985) stated:
"As the study of biological diversity, systematics is sometimes portrayed as the mere classification of organisms, but in fact its range and challenge are amongst the greatest in biology. Systematics remains a fountainhead of discovery and new ideas in biology due to the largely unknown nature of diversity."

Classification and identification of actinomycetes is essentially a two-stage process (Goodfellow and O'Donnell, 1989). Reliable taxonomic criteria are needed to assign organisms to genera prior to the selection of diagnostic tests for identification to constituent species. Identification to the genus level and above can usually be achieved either by using a combination of chemical and

morphological markers (Lechevalier and Lechevalier, 1971; Goodfellow and Cross, 1984; Williams *et al.*, 1989) or by using 16S rDNA/rRNA sequence data (Embley *et al.*, 1988; Chun *et al.*, 1995; Zhang *et al.*, 1998). In contrast, few reliable and well-tested schemes are available for the differentiation of the species, the basic taxonomic unit in bacterial systematics. The nature of the species concept remains a source of argument amongst bacteriologists, sparking frequent debate (Cowan, 1962; Staley and Krieg, 1984; Goodfellow *et al.*, 1997a; Ward, 1998). The traditional view is that correlated phenotypic characters can distinguish prokaryotic species, therefore, members of a given species have a combination of characters specific to that species (Goodfellow and O'Donnell, 1993). In practice, the number of species in a genus is often influenced by the aims of the taxonomist, the extent to which the genus has been studied, and the criteria adopted to define the species (Williams *et al.*, 1984). The subjective nature of the traditional bacterial species concept was summed up by Cowan in 1962, who considered a species to be "a group of organisms defined more or less subjectively by the criteria chosen by the taxonomist to show to best advantage and as far as possible put into practice his individual concept of what a species is".

1.2.2. Classification systems

The most desirable classification system, called a natural classification, arranges organisms into groups whose members share many characteristics and reflects, as much as possible the biological nature of organisms (Staley and Krieg, 1984). Linneaus developed the first natural classification, based largely on anatomical characteristics, in the middle of the eighteenth century. He named thousands of plants and animals and classified them in the systems Plantae and Animalia and published them in several editions of *Systema Naturae* between 1735 and 1759, it was a great improvement over previously employed artificial systems because knowledge of an organism's position in the scheme provided information about many of its properties.

Following the publication in 1859 of Darwin's *On the Origin of Species,* biologists began trying to develop phylogenetic or phyletic classification

systems; systems based on evolutionary relationships rather than general resemblance (the term phylogeny refers to the evolutionary development of a species). Current taxonomic work throughout biology and particularly in microbiology focuses on a comparison of genetic material and gene products such as RNA and proteins.

1.2.3. Phenetic taxonomy

Many taxonomists have maintained that the most natural classification is the one with the greatest information content or predictive value. A good classification should bring order to biological diversity. When viewed in this way, the best natural classification system may be a phenetic system, one that groups organisms together on the basis of their phenetic characteristics: it does not necessarily reflect phyletic relationships. This approach encompasses all measurable features of the organism (e.g. biochemical, chemical, morphological and physiological properties, and percentage base composition of DNA) so that the resultant classification has a high information content. Since phenetic classification is constructed on the basis of correlated characters, it is by definition highly predictive if the group to which an organism belongs is known (Goodfellow and O'Donnell, 1993). Although phenetic studies can reveal possible evolutionary relationships, they are not dependent on phylogenetic analysis. They compare many traits without assuming that any features are more important than others; that is, unweighted traits are employed in estimating general similarity. Obviously, the best phenetic classification is one constructed by comparing as many attributes as possible.

1.2.4. Numerical taxonomy

Early classifications of actinomycetes were generally based on single, or a series of single, characters and were known as monothetic classifications (Sneath, 1962). These artificial classifications were unreliable and resulted in the circumscription of some heterogeneous taxa (Goodfellow and Cross, 1984; Kroppenstadt *et al.*, 1990) as they have low information contents and cannot readily accommodate strain variation or test error (Goodfellow and O'Donnell,

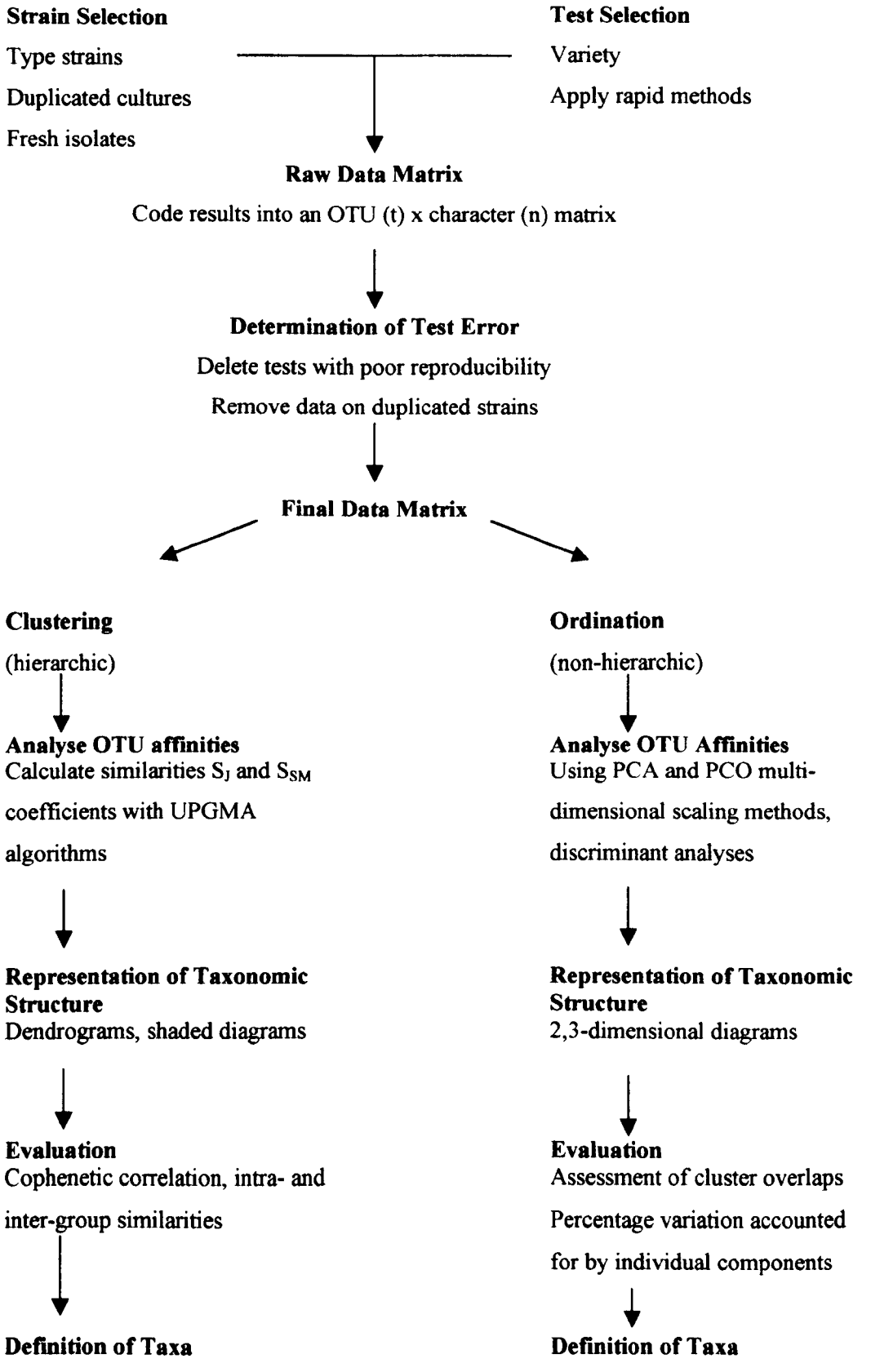
1993). Monothetic classifications also tend to lack uniformity as different taxonomic criteria are used to delineate species belonging to different genera.

There is still no widely accepted definition of the term species in bacteriology. However, it is often useful to distinguish a taxospecies, a group of strains which share a high proportion of phenotypic properties (Sneath, 1989) from a genospecies, a group of organisms capable of genetic exchange; from a genomic species, a group of organism which have a high degree of DNA:DNA relatedness (Murray *et al.*, 1990). Nucleic acid sequence data can be used to generate a hierarchical branching pattern of relationships but the definition of taxonomic boundaries still relies on the discontinuous distribution of phenotypic characters (Murray *et al.*, 1990).

The weakness inherent in these monothetic classifications led some bacterial systematists to believe that stable taxonomies could only be achieved when many bacteria are examined for a large balanced set of properties, for example biochemical, physiological, morphological and nutritional properties. The advent of computer technology allowed large number of strains and tests to be analysed. These studies were introduced as numerical taxonomies by Sneath (1957a) and have a high information content and are often described as general purpose taxonomies as they can be of use to a range of scientists (Goodfellow and O'Donnell, 1993).

Numerical taxonomy is the classification of organisms into structured groups based on their nutritional and physiological requirements, morphological characteristics, and tolerance to chemical inhibitors and resistance to antibiotics. Numerical taxonomy has been proven to be a definitive and effective method for classifying groups of bacteria (Sneath, 1957a, 1957b; Sneath and Sokal, 1962; MacDonnell and Colwell, 1985; Sackin and Jones, 1993). The theoretical basis of numerical taxonomy has been well documented (Sneath, 1971, 1972; Sneath and Sokal, 1973; Goodfellow, 1977; Sneath 1978a, b; Jones and Sackin, 1980; Sackin and Jones, 1993). An outline of the numerical taxonomic procedure is given in Figure 1.1.

Figure 1.1. Stages in the numerical taxonomy procedure*



* Modified from Ferguson 1997.

Essentially numerical classifications are formed when a large number of strains are examined for many characters then classified on the basis of their overall similarities. Such classifications are polythetic as they have high information contents and are based on a complete set of recorded characters and not on the presence or absence of single subjectively weighted properties. They can generally accommodate strain variation and are objective in the sense that they are not overtly sensitive to the addition of more strains or test characters.

The objects to be classified, such as strains, species or genera, are referred to as operational taxonomic units (OTU's). The latter should include several type strains, well-studied reference strains and duplicated cultures to provide a check on test error. It has been proposed that numerical taxonomic studies should include at least 60 but preferably more strains (Sneath and Sokal, 1973; Sackin and Jones, 1993). A balanced battery of tests should be selected that are generally stable and not sensitive to observational uncertainties (O'Brien and Colwell, 1987). The usual practice is to select a set of biochemical, cultural, morphological and physiological characters (Sneath, 1978a). It is recommended that between 100 and 200 characters, equally shared between the tests are studied (Sackin and Jones, 1993).

Numerical taxonomies are only as good as the data upon which they are based. Therefore, it is usual to determine test error by examining duplicated strains under code. The average probability of test error can then be estimated from an analysis of test variance. Individual test variance based on pairs of replicated strains for a test i (Si^2) may be calculated from the equation:

$$Si^2 = \frac{n}{2t}$$ (Equation No. 15; Sneath and Johnson, 1972)

Where, n corresponds to the number of OTU's with discrepancies in the test and t is the total number of strains. The individual test variances may be averaged to give a general estimate of test error. The probability of an erroneous result for an individual test (P_i) is:

$$P_i = \tfrac{1}{2} [1-\sqrt{1-4S_i^2}]$$ (Equation No. 4; Sneath and Johnson, 1972)

Sneath and Johnson (1972) found that when Pi > 0.1 there was a rapid erosion of taxonomic structure. Highly irreproducible tests can be removed from raw data matrices but care needs to be taken to ensure that as a result of test deletion excessive information is not lost. Tests that are all positive or all negative are also removed from the data matrix as they have no group discriminating value but may be useful for identification purposes.

Similarity values, which are determined for each pair of test strains using a resemblance coefficient, are arranged into an unsorted resemblance matrix. The two most commonly used resemblance coefficients are the simple matching (S_{SM}; Sokal and Michener, 1958) and the Jaccard (S_J; Jaccard, 1908) coefficients that measure similarity based on binary data. The S_{SM} coefficient is used to calculate similarity based on both positive and negative matches whereas the S_J coefficient ignores negative matches. The equations for the these two coefficients are:

$$\text{Simple matching } (S_{SM}) = \frac{a+d}{a+b+c+d} \qquad \text{(Sokal and Michener, 1958)}$$

$$\text{Jaccard } (S_J) = \frac{a}{a+b+c} \qquad \text{(Sneath, 1957a)}$$

where a (+ +) and d (- -) are the number of shared positive and negative matches and b (+ -) and c (- +) the number of differences between pairs of OTU's.

Operational taxonomic units are assigned to groups on the basis of shared similarities using clustering algorithms. Hierarchical non-overlapping clustering methods operate on OTU resemblance matrices by treating each OTU as a separate single membered cluster. The two OTU's with the highest overall similarity are grouped together and proximities between each of the remaining OTU's and the initial two membered cluster calculated according to the clustering algorithm used. This process is repeated, with, at each stage, the number of single membered clusters being reduced by one, until a point is reached when all of the OTU's form a single cluster. The various clustering

techniques only differ in the way the proximity between a single OTU and a group containing several OTU's is calculated.

The most commonly used algorithm is the average linkage method, the most popular variant of which is the unweighted pair group method with arithmetic averages linkage (UPGMA; Sokal and Michener, 1958). This algorithm gives equal weight to all of the clusters, regardless of the number of OTU's they contain.

Hierarchical clustering techniques impose a structure upon data that may or may not be a true representation of the original relationships between OTU's as implied by their similarity values. The suitability of data for hierarchical clustering can be assessed by determining the cophenetic correlation coefficient (Sokal and Rohlf, 1962; Sneath 1978a; Sackin and Jones, 1993). In practice complete agreement between dendrograms (a tree-like diagram resulting from cluster analyses, which express relationships between OTU's.) and resemblance matrices cannot be achieved given the taxonomic distortion introduced when representing multidimensional data in a two dimensional form. Typical cophenetic correlation values range from 0.6 to 0.95 (Sackin and Jones, 1993). Values below about 0.7 imply that only limited confidence can be given to relationships depicted in dendrograms.

Potentially useful taxonomic information can be determined from several calculations once OTU's have been assigned to clusters. The compactness of clusters and the degree of separation between them can be determined from intra- and inter- cluster similarities, respectively. The taxon radius is a measure of the volume occupied by a cluster in phenetic hyperspace. A high intra-cluster similarity and a low 95% taxonomic radius indicate a tight homogenous cluster and high inter-cluster similarity denotes poor separation of clusters.

Numerical classifications need to be interpreted with care as similarity values between strains can be distorted by factors such as test and sampling error and the statistics used (Sneath and Johnson, 1972; Goodfellow *et al.*, 1979, 1990a). Most confidence can usually be placed in the major centres of variation defined

in numerical analyses; it is the relationships of strains lying towards the periphery of clusters that are not always clear (Goodfellow and Cross, 1984). It is, therefore, important to evaluate numerical taxonomies in the context of other taxonomic methods such as chemotaxonomy and molecular systematics. It can also be important to identify OTU's that are most typical of each cluster and therefore suitable for representing phena in additional studies. The OTU that is most typical of a phenon and lies closest to the centroid of the cluster is the centrotype; this organism shows the highest average similarity of all the OTU's in the cluster. Centrotype, type strains and additional representative strains should be included where appropriate in analyses designed to evaluate numerical taxonomies.

In most numerical taxonomic surveys, the majority of test strains have been assigned to a small number of major clusters that are often equated with taxospecies (Goodfellow and Dickinson, 1985; Goodfellow *et al.*, 1990a). Single member clusters or phena that include only a few strains tend to be overlooked. However, these minor or single member clusters may represent nuclei of novel groups, genetically unstable strains or organisms of established taxa lacking plasmids (Goodfellow *et al*, 1987a) and need to be given consideration when interpreting numerical taxonomies.

There are two common approaches to evaluating numerical taxonomic studies namely, comparing numerical classifications obtained using different statistics for consistency and evaluating them with those derived from the application of other independent taxonomic criteria, notably chemical and molecular data. Priest and Barbour (1985) demonstrated this approach in their work on *Lactobacillus.* They examined 146 lactobacilli isolated from whisky distilleries and 32 marker strains of *Lactobacillus* for 107 unit characters and analysed their data using the simple matching (S_{SM}) coefficient and the unweighted pair group with arithmetic averages clustering algorithm (UPGMA). The resultant dendrogram showed few distinct clusters. Clusters that are more distinct were recovered when the data were re-examined using the S_{SM} and the complete linkage algorithm. Nineteen of the 27 clusters defined at the 82% similarity level contained distillery isolates, the remainder consisted of the reference strains.

With the exception of the clusters encompassing *Lactobacillus casei* var. *rhamnosus* and *Lactobacillus fermentum* strains, the phena containing the remaining marker strains included representatives of more than one species. Priest and Barbour (1985) concluded that the distillery isolates probably represented undescribed taxa and were not variants of established *Lactobacillus* species. They also found that their classification of the marker strains did not correspond with the established division of *Lactobacillus* into subgenera.

It is well known that hierarchic clustering algorithms give poor representation of relationships between more distantly related clusters but reproduce distances between those with many properties in common quite dependably (Alderson, 1985). Sneath and Sokal (1973) recommended the use of both hierarchical and ordination techniques when generating numerical taxonomies, the latter providing an understanding of the taxonomic structure in detail.

The lack of congruence between the results of the different analyses on lactobacilli suggested that the assignment of the marker strains to clusters was largely random thereby giving confidence to the results of the original S_{SM}, UPGMA analysis where few distinct clusters were obtained.

Priest and Barbour (1985) concluded that their *Lactobacillus* isolates were phenetically homogeneous and that the numerical taxonomic procedures did not yield useful or accurate classifications. In addition, they found that their numerical taxonomic database included few characters that could be weighted for the identification of clusters generated in the S_{SM} UPGMA analysis.

The numerical taxonomic data were also compared with results derived from DNA base composition and DNA relatedness studies (Priest and Barbour, 1985). The evaluation of numerical phenetic classifications using DNA relatedness data assumes that microorganisms belonging to different taxa express a constant proportion of the genome, and that phenetic diversity will be an expression of genetic diversity. Priest and Barbour (1985) concluded from their work on *Lactobacillus* that this assumption was not supported in this instance given the apparent phenetic homogeneity and genetic heterogeneity. The authors

speculated that the apparently anomalous results might reflect a very limited expression of the genome due to adaptation of the strains to a limited and specialised habitat or to convergent evolution resulting in similar apparent phenotypes despite diverse genotypes.

Numerical taxonomic procedures have been used to reclassify several actinomycete taxa notably the genera *Actinomadura* (Athalye *et al.*, 1981), *Actinomyces* (Schofield and Schal, 1981), *Actinoplanes* (Goodfellow *et al.*, 1990a), *Corynebacterium* (Jones, 1975), *Gordona* (Goodfellow *et al.*, 1991), *Mycobacterium* (Goodfellow and Wayne, 1982), *Nocardia* (Goodfellow, 1971; Orchard and Goodfellow, 1980), *Rhodococcus*, (Goodfellow *et al.*, 1990b), *Streptomyces* (Williams *et al.*, 1983; Kampfer *et al.*, 1991), *Thermomonospora* (McCarthy and Cross, 1984) and *Tsukamurella* (Goodfellow *et al.*, 1991).

Relatively few numerical taxonomic studies have included, let alone focused on, *Micromonospora* strains. Johnston (1972) examined 6 marker strains and 300 *Micromonospora* isolates obtained from aquatic and terrestrial habitats for 107 unit characters including biochemical, morphological, nutritional and physiological properties. Most of the taxonomic tests were performed only once and no attempt was made to estimate test error. The representatives of the named *Micromonospora* species were tested twice with variable results. *Micromonospora brunnea* and *Micromonospora chalcea* showed 100% agreement between duplicated strains for all tests. "*Micromonospora rubra*" failed to grow on the majority of the media used and the results obtained were not considered typical of the species.

Bibikova *et al.* (1989) carried out a numerical analysis on 32 *Micromonospora* strains belonging to 9 different groups based on cultural-morphological properties, the ability to produce antibiotics of several chemical classes and sensitivity to different antibiotics. The strains were examined for 102 unit characters. Most of the strains in a given group were recovered in distinct clusters, although two strains were recovered as single membered clusters. Two distinct groups were obtained at a similarity level of 61%. One encompassed strains that were rarely isolated from soil substrates, were rarely sensitive to

antibiotics and exhibited virtually no antibiotic properties. Conversely, the second group of strains were widespread in soil, were resistant to antibiotics and were active antibiotic producers.

Goodfellow and co-workers (1990a) included marker strains of the genus *Micromonospora* in a numerical taxonomic survey of the genus *Actinoplanes* and related taxa. The test strains were assigned to seven major, three minor and twenty-eight single membered clusters defined at the 83% similarity level using the S_{SM} similarity coefficient and the unweighted pair group method with arithmetic averages clustering algorithm. These taxa, in turn, were assigned to two aggregate groups circumscribed at the 68% similarity level.

The first aggregate group encompassed organisms belonging to the genera *Actinoplanes* (including *Ampullariella*), *Dactylosporangium* and *Micromonospora*; the *Micromonospora* strains formed a distinct taxon at the 75% similarity level. This taxon consisted of two clusters, each of which contained 6 unnamed *Micromonospora* isolates, and three single membered clusters, namely *Micromonospora carbonacea* subsp. *aurantiaca* KCC A-0127, *Micromonospora coerula* DSM 43143 and "*Micromonospora parva*" KCC A-0168. The second aggregate group included representatives of the genera *Micromonospora*, *Planobispora*, *Planomonospora*, *Spirillospora* and *Streptosporangium*. The *Micromonospora* strains, namely *Micromonospora halophytica* DSM 43171, "*Micromonospora megalomicea* subsp. *nigra*" KCC A-0107 and *Micromonospora rosaria* KCC A-0159 formed discrete single membered clusters.

High quality test characters in numbers sufficient to discriminate between taxa are necessary pre-requisites for any taxonomic study. The usual practice is to select biochemical, cultural, morphological, nutritional and physiological tests that should be carried out under rigorous, standardised conditions so that a representative sample of the entire phenome is characterised (O'Brien and Colwell, 1987). Where possible, tests should correlate with phenetic characteristics that are genetically stable and relatively insensitive to experimental procedures. This would favour the likelihood that tests yield

consistent results. The presence or absence of a unit character is generally recorded using a 2-state format where '+' or '1' is positive and '-' or '0' is negative. This method of coding has the disadvantage that equal weight is given to each complex character state (Sneath and Sokal, 1973). Therefore, multi-state characters are usually kept to a minimum or avoided (Goodfellow *et al.*, 1990a).

Having compiled a data matrix comprising the test responses for each OTU, measures of resemblance are calculated to give some taxonomic structure to the information.

1.2.5. Antimicrobial profiling

The ability of an organism to inhibit growth of another organism is a characteristic that can provide information on the diversity of a group of isolates (Takizawa *et al.*, 1993). Antimicrobial profiling (characterising the pattern of resistance or sensitivity of test organisms against a given isolate) involves seeding an agar plate with a test organism and observing if its growth is inhibited by the isolate (a plug of agar containing a mature colony of the isolate). The test organisms should include Gram-positive and Gram-negative bacteria and yeasts to give an accurate profile of the organisms the isolate is able to inhibit. Higher numbers of test organisms are preferable for a comprehensive reflection of diversity as the number of antimicrobial profiles increases with the number of test organisms used. The antimicrobial profiles can be used to compute a diversity index:

$$\text{Diversity index} = \frac{\text{number of antimicrobial profiles}}{\text{number of isolates tested}}$$

This index represents the likelihood of a particular colony representing a novel isolate in a particular sample or environment. This method allows the rapid assessment of the diversity in a sample. It is a particularly useful technique for the detection of diversity amongst strains with the potential for the production of bioactive agents as these are usually strain-specific and not species-specific (Takizawa *et al.*, 1993).

1.2.6. Enzyme profiling

The discontinuous distribution of enzymes amongst microbial species can provide information of value for classification and identification. Most early biochemical tests used for bacterial identification were applied empirically, their underlying biochemical basis becoming apparent only later. Early biochemical tests included examination for enzymes of the hydrolase, lyase and oxidoreductase groups, as well as for end products of metabolic pathways.

Tests based on enzymes give an advantage over most other taxonomic criteria due to their ease of performance, flexibility in a variety of situations, e.g. in agar, liquid media and microtitre plates, ability to test diverse organisms in the same study, e.g. fast and slow growing organisms, and the capacity to acquire data quickly. In addition, tests designed to detect individual enzymes may be rapidly performed and are simple in operation.

The specificity of enzymes combined with their ability to catalyse reactions at extremely low concentrations can be exploited as a good source of high quality data for generating taxonomic databases.

Enzymes can be identified according to the reactions they catalyse. A systematic nomenclature recommended by the Enzyme Commission of the International Union of Biochemistry has been modified several times (International Union of Biochemistry, 1965, 1972). This system involves the subdivision of enzymes into 6 major groups according to the type of reaction they catalyse.

Hydrolases: catalyse the hydrolysis of substrates, e.g., esterases:

Carboxylic esterase

$$RCOOR' + H_2O \longrightarrow RCOOH + R'OH$$

Isomerases: catalyse intramolecular rearrangements, e.g., racemases:

Amino acid racemase

$$\text{L-Amino acid} \rightleftharpoons \text{D-Amino acid}$$

Ligases: catalyse the formation of C-C, C-N, C-O or C-S bonds with the splitting of nucleoside triphosphate, e.g. amino acid activating enzymes:

Amino acyl tRNA ligase

Amino acid + ATP + tRNA $\longrightarrow$ Amino acyl tRNA +PPi + AMP

Lyases: catalyse the splitting of C-C, C-N, C-O or C-S bonds other than by hydrolysis e.g., decarboxylases

Decarboxylase

RCOOH $\longrightarrow$ RH + CO_2

Oxidoreductases: catalyse the transfer of H atoms, O atoms or electrons from one substrate to another e.g. alcohol dehydrogenase

Alcohol dehydrogenase

$RCH_2OH + NAD^+ \longrightarrow RCHO + NADH + H^+$

Transferases: catalyse the transfer of a specific group from one substrate to another e.g., hexokinase

Hexokinase

Glucose + ATP $\longrightarrow$ Glucose-6-phosphate + ADP

In order to monitor the progress of an enzyme reaction it is necessary to measure the change in concentration of one of the reactants or products. This may be accomplished by observing a physicochemical property of the substance of interest or indirectly by using a coupled detection system. Methods that are commonly used fall into a number of categories, namely absorptiometric, electrochemical, fluorescence, luminescence, manometric and radiochemical methods.

Enzymes are not distributed uniformly amongst prokaryotes hence their distribution can be weighted for classification and identification. Among the oxidoreductases catalase, cytochrome oxidase and nitrate reductase have been found useful for classification and identification of microorganisms (Kovacs, 1956; Blazevic and Ederer, 1975; Hanker and Rabin, 1975; McFaddin, 1980).

One of the first tests applied in bacterial classification was designed for the detection of β-glucosidase. The naturally occurring compound esculin was used as a substrate (Meyer and Schönfield, 1926). β-glucosidase hydrolyses esculin releasing 6,7-dihydroxycoumarin (esculetin) that forms a dark brown chelate in the presence of ferric ions, which can be incorporated into the medium.

In the search for new diagnostic tests chromogenic hydrolase substrates developed for biochemical applications were adapted for bacterial identification. Phenolphthalein diphosphate has been used to detect alkaline phosphatase activity (Lewis, 1961), and Lowe (1962) applied O-nitrophenyl-β-D-galactoside in a rapid test for lactose fermenting bacteria. Muftic (1967) demonstrated the utility of aminopeptidases in the classification of mycobacteria. Substrates were formed from naphthylamine and free naphthylamine released in the reaction was visualised as the diazonium salt.

Fluorescence techniques have also been employed to detect enzyme activities. Maddocks and Greenan (1975) introduced a simple test procedure that involved the use of 4-methyl-umbelliferyl-glycosides to differentiate between *Escherichia coli* and *Pseudomonas aeruginosa*. This procedure was later applied to mycobacteria (Grange and Clark, 1977). Fluorogenic substrates have been used in the classification of several bacterial groups including the Enterobacteriaceae (Godsey, *et al.* 1981, *Rhodococcus*, (Goodfellow *et al.*, 1988), *Streptomyces* (Goodfellow *et al.*, 1987b) and mycolic acid containing actinomycetes (Goodfellow *et al.*, 1990b, 1991).

There are many types of biochemical test. Originally, biochemical tests were carried out using bottles or tubes containing broth test media. In an effort to increase the efficiency of these tests, the biomedical companies began to produce

diagnostic test kits. Colorimetric liquid media test kits have a series of microcupules containing dehydrated test media into which a suspension of the test organism is inoculated, the kit is incubated, then a reagent is added after which the colour formation is estimated visually or using a colorimeter. Many biochemical tests can be carried out using agar plates. Multipoint inoculated agar plates where an indicator incorporated in the media or reagent added after incubation produces a coloured ring around the positive test colonies allows up to 40 different microbial strains to be examined on a single plate. Fluorometric testing has become increasingly popular as it is a much more sensitive method of detecting enzyme activity than colorimetric based procedures. Colorimetric tests can be adapted for fluorometry by adding a fluorophore with a spectrum that is quenched by a product of the enzyme reaction. Several kits have been developed Microscan (Microscan, California, USA) and Sensititre (Sensititre Ltd., West Sussex, UK) containing fluorogenic enzyme tests. Alternatively, substrates can be dispensed into microtitre trays and the organisms inoculated into the wells and following incubation quantitative results of the fluorescent intensity can be measured using a computer controlled fluorometric plate reader.

Little attempt has been made to determine the enzyme profiles of *Micromonospora* strains. Kawamoto and colleagues (1983b) made the most comprehensive study, and found that activity was usually detected in *Micromonospora* with β-N-acetylglucos-aminidine, β-galactoside, α-glucoside, β-glucoside and *p*-nitrophenyl-glycoside, whereas there was little activity of α-fucosidase, β-fucosidase, β-glucuronidase or α-xylosidase activity. However, the *Micromonospora* strains were found to have different patterns of α-galactosidase, α-mannosidase and β-xylosidase activity indicating the potential value of enzymic tests for the classification and identification of *Micromonospora* species.

More recently, Rowlands (1993) examined ninety six *Micromonospora* strains for their ability to enzymatically degrade thirty four 4-methylumbelliferone and fifty seven 7-amino-4-methylcoumarin substrates. Table 1.1 shows which enzymes the *Micromonospora* strains were positive and negative for.

Table 1.1. Enzymes for which *Micromonospora* isolates are positive or negative

Positive	Negative
4MU-2-Acetamido-4,6-O-benzylidene-2-β-D-glucopyranoside	4MU-N-Acetyl-β-D-glucosamine
4MU-β-D-Cellobiose	4MU-α-L-Arabinofuranoside
4MU-D-N,N'-Diacetylchitobioside	4MU-α-L-Arabinopyranoside
4MU-β-D-Fucopyranoside	4MU-α-D-Galactopyranoside
4MU-β-L-Fucopyranoside	4MU-β-D-Galactopyranoside
4MU-α-D-Mannopyrannoside	4MU-β-D-Glucopyranoside
4MU-6-Sulpho-2-acetamido-2-deoxy-β-D-glucopyranoside	4MU-β-D-Glucoronide
4MU-Elaidate,	4MU-β-D-Lactoside
L-Citrulline-7AMC	4MU-(6-Acetamido-hexanoate)
L-Glutamine-7AMC	4MU-Palmitate
L-Methionine-7AMC	4MU-Pyrophosphate
L-Ornithine-7AMC	β-Asparagine-7AMC
L-Proline-7AMC	α-Glutamate-7AMC
L-Valine-7AMC	L-Lysine-7AMC
BOC-L-Valine-L-leucine-L-lysine-7AMC	L-Phenylalanine-7AMC
Glutaryl-glycine-L-phenylalanine-7AMC	L-Threonine-7AMC
Z-L-Arginine-arginine-7AMC	Acetyl-L-alanine-L-tyrosine-7AMC
Z-Glycine-glycine-L-arginine-7AMC	Glycine-L-arginine-7AMC
Haloxon, 7-methoxy-4-methylcoumarin	Glycine-L-proline-7AMC
7-Methoxycoumarin-4-acetic acid	L-Leucine-L-valine-L-tyrosine-7AMC
7-Nitro-3,4-benzocoumarin	L-Lysine(Z)-7AMC
4MU-Myrsitate	Meosuccinyl-L-alanine-L-phenylalanine-L-lysine-7-AMC
	Proline-L-phenylalanine-L-arginine-7AMC
	Z-L-Arginine-7AMC
	7-(Carboxymethyl)-4-methylcoumarin

1.2.7. Pyrolysis mass spectrometry

Pyrolysis is a process that involves the thermal breakdown of complex organic material, such as whole organisms or cell fractions, in an inert atmosphere or vacuum to produce a series of volatile, lower molecular weight molecules 'the pyrolysate' that consists of chemical compounds which differ in structure, polarity and molecular mass. The breakdown of test material is reproducible under controlled conditions and the resultant fragments are characteristic of the original material. The volatile fragments are ionised and separated by mass spectrometry on the basis of their mass-to-charge ratio (m/z) to give a pyrolysis mass spectrum that can be taken as a 'chemical fingerprint' of the original material. The resultant data are complex and need to be analysed using suitable statistical routines (Gutteridge *et al.*, 1985; Magee, 1993; Goodfellow *et al.*, 1997).

Pyrolysis was first applied to mass spectrometry by Zemany (1952) who discovered that under standardised conditions, complex biological materials were degraded in a reproducible manner. However, this technique proved expensive and was not applied for over twenty years as interest focused on an alternative technique, pyrolysis gas liquid chromatography (Py-GC). This technique involved the separation of the products of thermal degradation on the basis of relative polarity, that is, components of the pyrolysate were separated by differences in retention time in chromatographic columns.

This Py-GC technique enjoyed restricted use but became unpopular since it yielded irreproducible data due to factors such as (a) the finite lifetimes of chromatographic columns and hence inconsistent performances between the original and subsequent columns, (b) inadequate data handling procedures and (c) slow sampling such that the volume throughput of samples was low (Gutteridge *et al.*, 1985; Magee, 1993).

Interest in pyrolysis mass spectrometry was renewed with the introduction of the first dedicated PyMS system developed and built by Meuzelaar and Kistemaker (1973) at the FOM Institute in Amsterdam. Further technical developments led

to the construction of the first fully automated instrument, the Autopyms, which allowed the use of high speed counting and computerised data processing (Meuzelaar *et al.*, 1976). This system served as a model for the production of two commercial instruments, the Extranuclear 5000 (Extranuclear Laboratories, Pittsburgh, USA) and the Pyromass 8-80 (VG Gas Analysis Ltd., Middlewich, Cheshire, UK). The Pyromass 8-80 machine has been described in detail (Gutteridge *et al.*, 1985; Shute *et al.*, 1984a,b) but like the Extranuclear 5000 had restricted use because of problems such as prolonged processing time, which required intensive labour for manual loading thereby resulting in low daily throughput (Gutteridge, 1987). In addition, these instruments were expensive, in excess of £100,000 and therefore not widely available. Limited, early PyMS applications involved classifying and identifying several groups of clinically significant bacteria for example the mycobacteria (Meuzelaar *et al.*, 1976). Unfortunately, the limitations of the PyMS machines were further compounded since new PyMS data could not be compared with existing data in libraries owing to poor long-term reproducibility of the chemical fingerprints. This was a consequence of machine instability termed 'mass spectrometer drift' (Meuzelaar *et al.*, 1982).

It was not until this expensive hardware gave way to cheaper PyMS machines, such as the Horizon 200X and 400X (Horizon Instruments Ltd., Heathfield, UK (see Figure 1.2), coupled with improved software development, that the value of PyMS in microbiology was reappraised (Magee, 1993, 1994; Goodfellow *et al.*, 1994). This current system is based on Curie-point pyrolysis, where the pyrolyser consists of a high-power radiofrequency oscillator driving an induction coil, and the carrier is a foil made from ferromagnetic alloy, in this case a nickel-iron alloy. The foil becomes paramagnetic at a sharply defined temperature (530°C), the Curie point, which is characteristic of the alloy (Magee, 1993). The superior performance of these new PyMS machines, owes much to an improved electron multiplier system resulting in fast analysis times (~2 minutes per sample) as well as improved reproducibility. The merits of these new machines also relates to two factors that have considerable bearing upon the design of a good pyrolysis

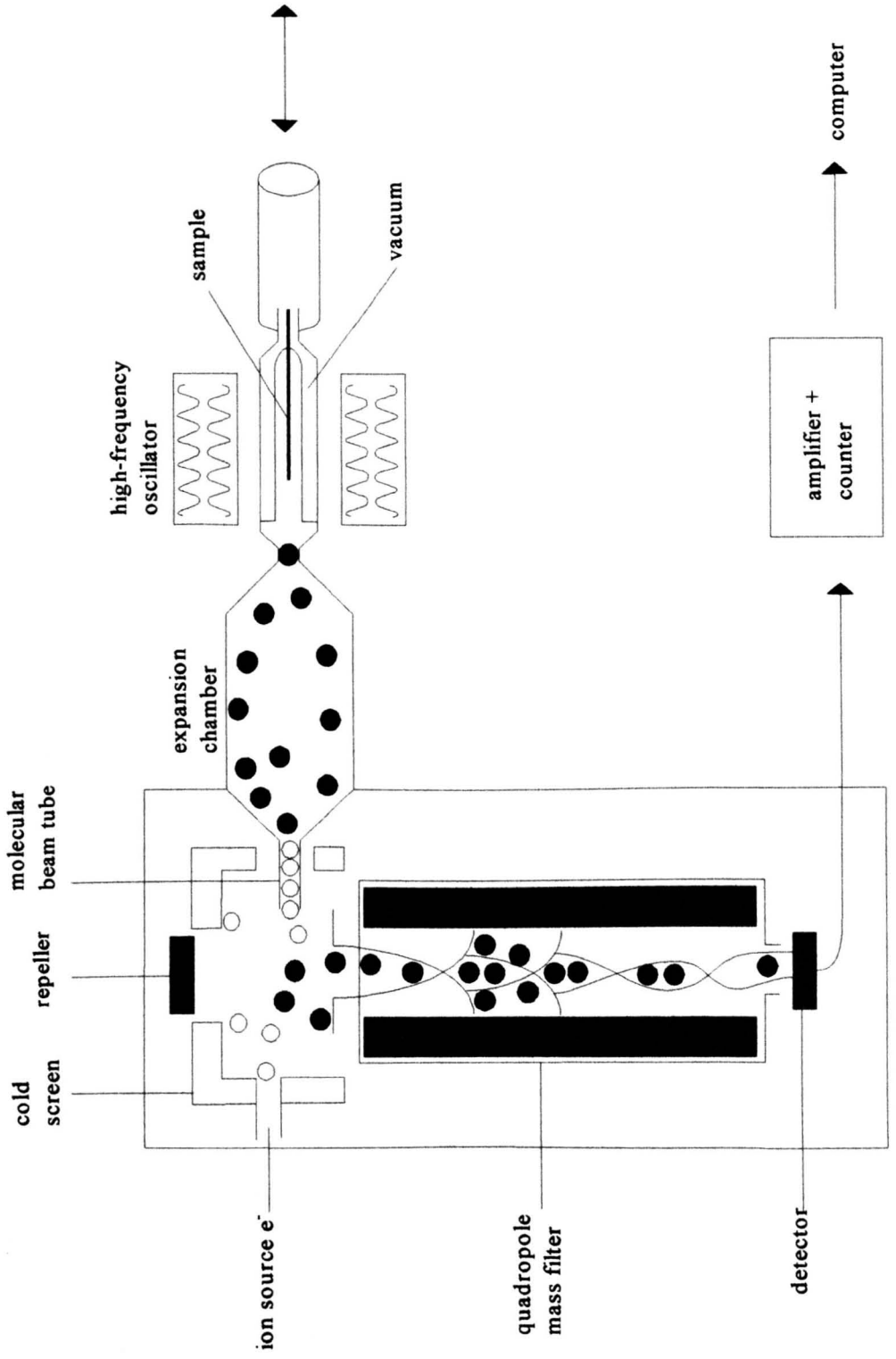


Figure 1.2 Schematic diagram of the Horizon 400X pyrolysis mass spectrometer (Modified from Magee, 1993)

system. They include (a) the time and temperature at which primary decomposition occurs and (b) the fate of primary pyrolysis products.

Sample preparation for PyMS is minimal and relatively inexpensive and with the rapid pyrolytic analysis times up to 300 samples can be analysed per day. An additional advantage with the new machines is that the analytical system for pyrolysis and data acquisition is automated therefore enabling the technique to be applied to a large number of samples with relative ease (Shute *et al.*, 1984a; Magee, 1993).

One of the major advantages of pyrolysis mass spectrometry (PyMS) over comparable taxonomic methods, such as conventional chemotaxonomic procedures (Suzuki *et al.*, 1993; Embley and Wait, 1994; Pot *et al.*, 1994) and nucleic acid probing (Schleifer *et al.*, 1993; Amman *et al.*, 1995) is that it is rapid with respect to single and multiple samples. Pyrolysis techniques, notably Curie-point pyrolysis mass spectrometry, are currently being introduced in diagnostic and industrial screening laboratories (Sanglier *et al.*, 1992; Goodfellow *et al.*, 1997b; Goodacre *et al.*, 1998). This technique has also been used to investigate the relationships between microorganisms isolated from natural habitats (Donisson *et al.*, 1986; Manfio *et al.*, 1995).

To date, the most important application of Curie-point pyrolysis mass spectrometry has been in microbial epidemiology (Goodfellow *et al.*, 1997). Pyrolysis mass spectrometry is not a typing method *per se* as a permanent type designation is not assigned to test strains but it has proved to be a quick and effective method for inter-strain comparisons of bacteria that commonly cause outbreaks of disease. This conclusion is based on studies of clinically significant bacteria; recent examples include *Acinetobacter calcoaceticus* (Freeman *et al.*, 1997), *Bacteroides* spp. (Sultana *et al.*, 1995), *Campylobacter jejuni* (Orr *et al.*, 1995), *Clostridium difficile* (Al-Saif *et al.*, 1998), *Legionella pneumoniae* (Sisson *et al.*, 1991), *Listeria monocyogenes* (Low *et al.*, 1992), *Streptococcus pneumoniae* (Freeman *et al.*, 1991), *Staphylococcus aureus* (Gould *et al.*, 1991), and *Xanthomonas maltophila* (Orr *et al.*, 1991).

It is evident that PyMS can be used to discriminate between strains as accurately as routine typing systems (Goodfellow, 1995). Indeed, in some cases it has been used to separate isolates beyond the resolution of such systems (Freeman *et al.*, 1991; Gould *et al.*, 1991). The results of PyMS analyses have also been shown to correspond to those from molecular based techniques, including DNA:DNA relatedness and 16S rDNA sequencing (Manchester *et al.*, 1995), random amplification of polymorphic DNA analyses (Kay *et al.*, 1994; Trujillo and Goodfellow, 1997), restriction fragment length polymorphism (Low *et al.*, 1992) and ribotyping (Al-Saif *et al.*, 1998).

Pyrolysis mass spectrometry has also been used to classify and identify industrially significant actinomycetes (Saddler *et al.*, 1988; Sanglier *et al.*, 1992). In this latter study, members of representative actinomycetes genera were pyrolysed in order to determine the effects of medium design, incubation time and sample preparation on experimental data; it was concluded that reproducible results could be obtained given rigorous standardisation of growth and pyrolysis conditions. Sanglier and his colleagues (1992) also showed that PyMS data could be used to objectively select strains for pharmacological screens, as unknown or putatively novel actinomycetes appeared as outliers on ordination diagrams. They were also able to distinguish between actinomycetes at and below the species level. In particular, representatives of three closely related *Streptomyces* species strains, namely *Streptomyces albidoflavus* (sub-cluster 1A; Williams *et al*, 1983), *Streptomyces anulatus* (sub-cluster 1B; Williams *et al.*, 1983), and *Streptomyces halstedii* (sub-cluster 1C; Williams *et al.*, 1983) were distinguished. These workers also used the procedure to compare *Streptomyces hygroscopicus* isolates and *bona fide* representatives of *Streptomyces violaceusniger* (cluster 32; Williams *et al.*, 1983). The separation of these numerically circumscribed streptomycete species indicated that PyMS can provide a rapid way of establishing the taxonomic integrity of established or putatively novel actinomycetes species.

A more recent development in analysing PyMS data involves the application of Artificial Neural networks (ANN), a form of pattern recognition analysis, to provide a fast and accurate identification of bacteria. In brief, ANNs are used to

detect complex non-linear relationships in multivariate data (Goodacre, *et al.*, 1994) as exemplified by pyrolysis mass spectra. An ANN can be trained, either in the context of supervised or unsupervised learning (Goodacre and Kell, 1996b) to distinguish between and within species in a very short period of time (Freeman *et al.*, 1994) using commercially available software. PyMS data collected from representative strains of taxonomically defined groups are taken and used as inputs for known identities in the training of an ANN. Thus, trained ANNs can distinguish target or known members of a taxon from unwanted strains. Chun *et al.* (1993a,b) demonstrated the value of ANNs in the analysis of pyrolysis data for the identification of streptomycetes. Pyrolysis profiles derived from representatives of several actinomycete taxa, including the genera *Actinomadura*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora*, *Streptosporangium* and *Streptomyces* were used to train an ANN to recognise the characteristic profiles of representatives of *Streptomyces* groups A, B, C. Successful identification of members of the three *Streptomyces* species-groups was also achieved when data from several different PyMS runs were carried out over a twenty-month period (Chun *et al*, 1997). These findings demonstrate the potential of ANN analyses in the circumscription of *Streptomyces* species.

1.2.8. Phylogenetic taxonomy

Phylogeny is the study of the evolutionary history of organisms. Cladistic relationships (Cain and Harrison, 1960) describe affinities between microorganisms by pathways of ancestry and can imply the pathway by which phenotypes arose, but are not derived from the observable phenotype. Phylogenetic relationships are represented by cladograms, a type of dendrogram, and are inferred from various types of phenetic relationships using assumptions as to how evolution occurs. They express the evolutionary relationships between organisms and thereby reflect the extent of change over time. In the absence of actual time units relationships may be expressed in topological units (numbers of nodes or internodes) or numbers of inferred evolutionary changes (Sneath, 1989). Phenetic classifications will only be congruent with phylogenetic taxonomies given the absence of parallel or convergent evolution. The two approaches will differ if convergent evolution or recent gene transfer gives rise to organisms that

are phenetically similar but have different ancestry (Goodfellow and O'Donnell, 1993).

1.2.9. Molecular systematics

1.2.9.1. Suprageneric classification

The most accurate and informative methods for classifying microorganisms at and above the genus level are based on the determination of precise nucleotide sequences of specific regions of the chromosome. Nucleotide sequencing methods have developed rapidly since their inception, so that comparative sequencing of homologous genes is now a standard procedure in molecular systematics. Conserved genes have been sequenced to establish the position of organisms in the universal tree of life (Woese, 1987) and less conserved genes are used to distinguish between closely related organisms.

Ribosomal RNA genes, which are essential for the survival of all organisms, are highly conserved in eukaryotes and prokaryotes and are widely used to establish suprageneric relationships. Two rationales underline this approach, namely, that lateral gene transfer is a rare event between 16S rDNA genes and that the degree of dissimilarity of 16S rRNA sequences between a given pair of organisms is a measure of the variation shown by the corresponding whole genomes. The congruence found between phylogenies based on 16S rRNA and those derived from analyses of other conserved molecules, such as elongation factors, 23S rRNA and RNA polymerases supports this latter point (Ludwig and Schleifer, 1994; Olsen and Woese, 1993). It also seems likely that lateral gene transfer between 16S rDNA genes will be rare as this gene is responsible for maintaining functional and tertiary structural consistency (Woese, 1987).

Ribosomal RNA operons are transcribed into single pre-rRNA transcripts which contain the following components in the order (5′ to 3′); 16S rRNA, spacer region, tRNA, spacer region, 23S rRNA, spacer region and 5S rRNA (Figure 1.3) (Gürtler and Stanisich, 1996). The 16S rRNA genes are similar in length (about 1.5kb) for members of the bacterial domain and contain both highly conserved

Figure 1.3. Schematic diagram showing the positions of conserved regions within the rRNA operon

The boxed areas represent the various genes of the bacterial rRNA operon. Transcription of the operon is from left to right (5′ to 3′). Some bacteria have two rRNA genes (as shown above); others have one (either $tRNA^{ile}$ or $tRNA^{ala}$) or none. The dark lines represent the spacer regions that separate the various rRNA genes and the dark jagged lines represent breaks in the 16S and 23S genes and have not been shown in full. The 5S rRNA gene that occurs downstream from the 23S rRNA gene is not shown. The thin numbered line shows the nucleotide numbering of the 16S and 23S rRNA genes of *Escherichia coli* with breaks shown as dashed lines.

and variable regions. The locations of changes in the variable regions seem to be either group or species specific (Stackebrandt and Woese, 1981; Woese, 1987).

There are some examples in the literature where more than one rRNA operon has been sequenced for a given organism (Ji *et al.*, 1994; Cillia *et al.*, 1996). The nucleotide sequences of different operons in the same strain tend to be either identical or to show a low level of heterogeneity, about 0.1% difference in nucleotide positions, (Ji *et al.*, 1994). However, there are some cases where higher levels of heterogeneity have been found between different rRNA operons in the same strain (Ninet *et al.*, 1996). *Mycobacterium terra* has two copies of 16S rRNA operons that differ by 18 nucleotide substitutions or 1.2% nucleotide sequence dissimilarity (Ninet *et al.*, 1996). Such degrees of dissimilarity exceed the difference found between the 16S rDNA sequences of some well-established bacterial species (Portaels *et al.*, 1996). It is evident from these results that nucleic acid sequencing methods that do not include a cloning step may be flawed in cases where rRNA operons are heterogeneous. However, the identification of most taxa should not be affected, as multiple copies of the 16S rRNA genes usually show a high level of homogeneity.

The characterisation of the 16S RNA gene is now well established as a standard method in bacterial classification (Woese, 1987; Amann *et al.*, 1995) as representatives of nearly ten thousand different bacterial species have been sequenced (Maidak *et al.*, 1999). The corresponding number of available sequences for the 23S rRNA gene is much lower at 334 (De Rijk *et al.*, 1996). Initially, the 5S rRNA gene was widely studied (Stackebrandt and Liesack, 1993), but it is now rarely sequenced due to the ease of sequencing the more information rich 16S rRNA gene.

Evolutionary relationships between bacteria need to be interpreted with care as phylogenetic inferences are based on relatively simple assumptions when viewed against the complexities of evolutionary processes (Goodfellow *et al.*, 1997). Additional problems, which may affect the interpretation of nucleotide sequence data, include alignment artefacts, non-independence of sites, inequalities in base substitution frequencies between sequences, and lineage-dependent inequalities in rates of change (O'Donnell *et al.*, 1993).

1.2.9.2. Analysis of 16S rRNA gene sequence data

Sequence information for 5S, 16S and 23S rRNA molecules is held in several specialised databases. These include: Genbank (Benson *et al.*, 1997), the European Molecular Biology Laboratories database (EMBL; Stoesser *et al.*, 1999) and the Ribosomal Database Project (RDP; Olsen *et al.*, 1992; Maidak *et al.*, 1999). The sequence entries are usually indexed according to a taxonomic classification based on the family. Access to sequence data is provided in most databases by means of user-friendly interfaces, such as email servers and the World Wide Web (Maidak *et al.*, 1999). These software tools can be used to search and retrieve the required sequences.

1.2.9.3. Alignment of 16S rRNA gene sequence data

Analyses of nucleotide sequence data are based on different conceptual assumptions with phylogenies being strongly influenced by the method used to derive them. However, the concept of homology underpins all phylogenetic

analyses, namely, that ancestry can only be traced by estimating changes in nucleotide sequences of homologous genes from test organisms (Sneath, 1989).

The first stage in phylogenetic reconstruction from nucleotide sequence data is sequence alignment. This involves finding homologous sites, that is, positions derived from the same ancestral organism, in the molecules under study. A set of sequences can be aligned against one another by introducing 'alignment gaps'. The general rule underpinning multiple sequence alignment is that the increase in sequence similarity due to the introduction of alignment gaps must be greater than that which would be expected due to random alignment (Olsen, 1988). Some workers have developed multiple sequence alignment procedures (Feng and Doolittle, 1987). The best alignment between two sequences is the one in which the number of mismatches and gaps is kept to a minimum (Austin and Priest, 1986). These alignment methods are purely computational hence, it is necessary to check alignments manually and if possible, in light of biological function in order to clarify ambiguous regions (Viale *et al.*, 1994).

Computerised algorithms for sequence alignment, such as pairwise and multiple alignments (Clustal) are based on minimising mismatches between sequences by introducing gaps or shifting bases to overcome mismatches. However, such optimal alignment may not be historically correct since base changes in regions of 16S rRNA associated with secondary structural features of the molecule, such as hairpins and pairing between distant regions, cannot be assumed to have the same effect or importance as changes in loops and hypervariable regions that are not associated with the preservation of secondary structure. Manual alignment of sequences by an experienced molecular scientist takes into account secondary structural features namely, base signatures, pairing between distant regions, conserved mismatches, hairpins, gaps and loops and thereby provides more accurate data than those generated by automated computerised algorithms. During manual alignment, some idea about the identity of the sequence can be gained by considering the main line of descent to which it best aligns. Sequence alignments should always be considered as working hypotheses due to the degree of uncertainty about the historical correctness of data. Unalignable regions should ultimately be omitted from phylogenetic analyses.

Once nucleotide sequences have been aligned, similarity matrices can be constructed. In most cases, the main phylogenetic group to which an unidentified sequence shows its highest similarity is determined. The nucleotide sequence can then be compared to those available for members of that group. This comparison can lead to the placement of the sequence at one of various taxonomic levels from family down to species. It is at this stage that a detailed taxonomic knowledge of the group into which the sequence falls is necessary since failure to include nucleotide sequences of all representatives can lead to the erroneous assumption that the sequence represents a member of an unknown or unsequenced taxon.

Most representatives of validly described species can be classified into suprageneric taxa. The discovery of new lines of descent within the main bacterial phyletic lines must be viewed with caution, as sequences are not available for all genera (Stackebrandt and Liesack, 1993).

The restriction of sequence analysis to selected portions of 16S rDNA can cause significant deviation in the branching points of almost all species. The variation of tree topologies is due to significant differences in the similarity values determined for certain regions of the sequence from the same set of organisms. It has already been pointed out that the position of hypervariable regions within the 16S rDNA primary structure differ from taxon to taxon and need to be determined individually for their use in the determination of relatedness between species (Stackebrandt *et al.*, 1993).

It is clear that partial nucleotide sequences should not be used to unravel intergeneric phylogeny and that the phylogenetic positions of bacterial taxa should be based on analysis of complete sequences. However, the 3′ terminal 900 nucleotides, or the first 450 (5′ to 3′) nucleotides can be used for rapid allocation of isolates and clone sequences to higher taxa (Farelly *et al.*, 1995).

There are several pitfalls that can distort phylogenies based on 16S rRNA sequence data. Examples of these include the assignment of taxa to erroneous

taxonomic ranks due to the comparison of 16S rRNA sequences with inadequate reference strains (Stackebrandt and Ludwig, 1994). Classifications biased by the use of nucleotide sequences with higher numbers of ambiguities from reverse transcriptase sequencing, PCR generation of chimeric amplification products (Liesack *et al.*, 1991), omission of data from critical differentiating regions (Ruimy *et al.*, 1994), and oversimplified interpretation of phylogenetic analyses when proposing major taxonomic changes (Embley and Stackebrandt, 1994; Rainey *et al.*, 1995b). It is clear from this that 16S rRNA sequence data must be carefully interpreted.

1.2.9.4. Single strand conformational polymorphism

Single strand conformational polymorphism (SSCP) is a method widely used to detect mutations, especially in human genes (Hayashi, 1991). More recently, it has been adapted to study microbial communities (Schweiger and Tebbe, 1998). Under nondenaturing conditions, single stranded DNA molecules will fold into secondary structures, or conformations, according to their nucleotide sequence and their physicochemical environment (e.g. temperature and ion strength). Different conformations of DNA will have different electrophoretic mobilities and these can be separated on a polyacrylamide gel (Yap *et al.*, 1994). Hence, DNA fragments of identical lengths but different sequences can be separated into different bands in polyacrylamide gel electrophoresis as differences in the sequences will result in the DNA strands folding in different conformations (Hayashi, 1991). Typically, three bands are detectable; two single DNA strands and one double stranded molecule (Schweiger and Tebbe, 1998).

SSCP is a highly sensitive technique, it can be used to detect minor changes, down to one base substitution, in DNA sequences of PCR amplified DNA (Sheffield *et al.*, 1993). SSCP lends itself to the analysis of microbial communities due to its sensitivity, capacity for analysing large numbers of sequences and the speed at which the analysis can be performed. SSCP does have some limitations; there is often a high rate of reannealing of DNA strands after the initial denaturation during electrophoresis, multiple bands can be observed for one sequence where several conformations of one product coexist in

a gel, and the formation of heteroduplex DNA from PCR products with similar sequences occurs frequently (Schwieger and Tebbe, 1998).

Lee *et al.* (1996) used SSCP to study the genetic profiles of bacterial communities in natural ecosystems. They demonstrated that each bacterial strain generated a characteristic band pattern, which could be observed on an electrophoretic polyacrylamide gel. They also demonstrated the sensitivity of SSCP, as they were able to detect a bacterial population that comprised less than 1.5% of the bacterial community.

In this present study, it is proposed to develop this method further by treating 16S rDNA sequences of micromonosporae with restriction enzymes to obtain 4 or 5 DNA fragments prior to the SSCP analysis. The additional restriction yields a number of fragments for each 16S rDNA sequence, thereby allowing each micromonosporae strain to be identified according to its individual banding pattern.

1.2.9.5. Delineation of species

The driving force in bacterial systematics owes much to developments in molecular biology, notably, nucleic acid sequencing. However, several other molecular systematic methods provide valuable data for the classification of bacteria at the species level. These methods include the estimation of the mean overall base composition of DNA and indirect comparisons of nucleotide sequences by DNA:DNA hybridisation. DNA base composition studies can be used to detect heterogeneous species whereas DNA:DNA pairing is the only method currently available for giving a quantitative definition for species (Grimont and Grimont, 1991).

1.2.9.6. DNA:DNA hybridisation

A unique property of DNA and RNA is their ability for reassociation or hybridisation. The complementary strands of DNA once denatured, can, under appropriate experimental conditions, reassociate to reform native duplex structures. The specific pairings are between the base pairs, adenine with

thymine and cytosine with guanine, and the overall pairing of the nucleic acid fragments is dependent on similar linear arrangements of these bases along the DNA. When comparing nucleic acids from different organisms, the amount of molecular hybrid formed and its thermal stability provide an average measurement of nucleotide sequence similarity.

DNA:DNA relatedness data are now extensively used to delineate bacterial species (Stackebrandt and Goebel, 1994). A formal molecular definition of a bacterial species has been proposed, namely, that a species should generally include strains with approximately 70% or more DNA:DNA relatedness (Wayne *et al.*, 1987). Hybridisation values from 30% to 70% reflect a moderate degree of relationship between strains, but values become increasingly unreliable once they fall below the 30% level as they can be attributed to experimental error. These guidelines have been used to circumscribe species in diverse bacterial genera (Stackebrandt and Goebel, 1994).

Experimental procedures for estimating DNA relatedness are based on two key properties of DNA molecules, namely, specificity of base pairing and denaturation-renaturation kinetics at specific temperatures (Johnson, 1985). Double stranded DNA (dsDNA) dissociates into single stranded DNA (ssDNA) either at its melting temperature (Tm) or under highly alkaline conditions and reassociates at temperatures 15 to 30°C below the Tm value and at neutral pH. Single-stranded DNA from one organism will hybridise with ssDNA from another organism under appropriate experimental conditions to form heterologous molecules or duplexes. The extent of hybridisation can be directly quantified by monitoring the rate of the reassociation kinetics or by using labelled reference DNA. The amount of mismatch in heterologous duplexes can also be determined by comparing Tm values with those of corresponding homologous duplexes, the smaller the difference between the thermal stabilities, the fewer mismatches in the hybrid. The stringency of hybridisation reactions can be varied by altering experimental parameters, notably, temperature, salt concentration and added reaction components (Johnson, 1991).

The rationale for using DNA reassociation as the gold standard for species delineation originates from the results of numerous studies where a high degree of correlation was found between DNA similarity and chemotaxonomic, genomic, serological, and numerical phenetic data (Stackebrandt and Goebel, 1994). These studies were based on the original finding that single stranded DNA from two different strains will reassociate to a measurable extent and form a DNA hybrid if the strands contain less than 15% base mispairing.

The *Ad Hoc* Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987) recommended that a difference in thermal stability of 5°C or less was necessary to indicate relationships at the species level. This estimate was made by using experimentally introduced mispairings in short DNA fragments (Stackebrandt and Goebel, 1994). In general, organisms that have 70% or greater DNA similarity will also have at least 96% DNA sequence identity (Stackebrandt and Goebel, 1994).

1.2.9.7. Comparison of 16S rRNA sequences and DNA:DNA relatedness data

Fox and workers (1992) were the first to realise that 16S rRNA molecules of closely related species might not always show molecular clock behaviour since they are so conserved. This important observation meant that strains of related species with almost identical 16S rRNA sequences may belong to different genomic species.

The relationship between 16S rRNA sequence and DNA relatedness data is not a linear one. However, 16S rRNA sequence similarity values below 97% always correspond to DNA relatedness values below 60% though the reverse is not true. A similar relationship between 16S rRNA sequence and DNA relatedness data was presented by Stackebrandt and Goebel (1994) who argued that organisms assigned to a genomic species could be expected to show a 97% similarity value in light of corresponding 16S rRNA sequence data.

DNA sequencing studies are more cost-effective and less laborious than DNA:DNA hybridisation studies due to developments in molecular biology, notably, the use of PCR and the introduction of automatic DNA sequencers (Stackebrandt and Goebel, 1994). However, the two methods should be seen as complementary for the circumscription of bacterial species. 16S rDNA sequencing studies are useful for detecting taxonomic relationships at species or higher taxonomic ranks whereas DNA relatedness studies are only valid at the species or subspecific level (Goodfellow and O'Donnell, 1993).

The best strategy is to use both molecular methods to delineate closely related species. Initial 16S rRNA sequencing studies can be undertaken to show the broad relationships between closely related organisms with DNA:DNA pairing studies used to detect the finer taxonomic structure. The sequential use of the 16S rRNA sequencing and DNA:DNA pairing methods should prove useful in unravelling the taxonomic structure of groups such as the micromonosporae.

1.2.10. Polyphasic taxonomy

The term polyphasic taxonomy was first coined by Colwell in 1970, and was introduced to signify successive or simultaneous taxonomic studies of a group of organisms using an array of techniques designed to yield both molecular and phenotypic data. The polyphasic approach to the circumscription of bacterial taxa only became possible due to the availability of rapid data acquisition systems and improved data handling procedures (Vandamme *et al.*, 1996). It is encouraging that most descriptions of new cultivable bacteria in recent issues of the International Journal of Systematic and Evolutionary Microbiology are based on a selection of genotypic and phenotypic data. For example, several new actinomycete taxa have been proposed based on a polyphasic approach. These taxa include the genus *Verrucosispora* (Rheims *et al.*, 1998) and the species *Corynebacterium confuscum* (Funke *et al.*, 1998) and *Mycobacterium bohemicum* (Reischl, *et al.*, 1998).

Polyphasic taxonomy is now widely practised but little attempt has been made to define which methods should be used to generate phenotypic and genotypic

information. Table 1.2 shows some sources of taxonomic information and the taxonomic rank they define. At present, polyphasic taxonomic studies tend to reflect the interests of individual research groups and the equipment and procedures that they have at their disposal. It is difficult to say exactly which methods should be applied in polyphasic taxonomic studies as those employed depend, to some extent, on the rank of the taxa under investigation.

The polyphasic taxonomic approach to circumscribing bacterial species can be expected to meet most of the primary challenges facing bacterial systematists, namely, the need to generate well defined species, a stable nomenclature and improved identification systems. In the present study a selection of micromonosporae representative of the environmental samples investigated were the subject of comprehensive polyphasic taxonomic studies designed to establish the biogeographic distribution of the genus *Micromonospora* and to identify putative 'hot spots' for novel species.

Table 1.2. Sources of taxonomic information*

Cell component	Analysis	Taxonomic rank		
		Genus or above	Species	Subspecies or below
Chromosomal DNA	Base composition (mol% G+C)	√	√	
	DNA:DNA hybridisation		√	√
	Restriction patterns (RFLP, ribotyping)		√	√
DNA segments	DNA probes	√	√	√
	DNA sequencing	√	√	√
	PCR based DNA fingerprinting (PCR-RFLP, RAPD, SSCP)		√	√
Ribosomal RNA	DNA:rRNA hybridisation	√	√	
	Nucleotide sequences	√	√	
Proteins	Amino acid sequences	√	√	
	Electrophoretic patterns		√	√
	Multiloccus enzyme electrophoresis			√
	Serological comparisons	√	√	√
Chemical markers	Peptidoglycans	√		
	Fatty acids	√	√	
	Isoprenoid quinones	√	√	
	Mycolic acids	√	√	
	Polar lipids	√	√	
	Polyamines	√		
	Polysaccharides	√	√	
	Teichoic acids	√	√	
Whole-organisms	Pyrolysis mass spectrometry		√	√
	Rapid enzyme tests		√	√
Expressed features	Morphology	√	√	
	Physiology	√	√	

* Modified from Bull *et al.* (2000)

Abbreviations: RFLP, restriction fragment length polymorphism; RAPD, randomly amplified pleomorphic DNA fingerprints; PCR, polymerase chain reaction; SSCP, single stranded conformation polymorphism.

1.3. Taxonomic history of the genus *Micromonospora*

The family *Actinoplanaceae* (Couch, 1955) was proposed for actinomycetes that formed spores within sporangia or spore vesicles. Subsequently, sporangiate actinomycetes were found to form a heterogeneous group on the basis of their lipid composition (Kroppenstadt, 1985), cell wall chemotypes (Szaniszlo and Gooder, 1967) and their nucleic acid similarity and sequencing data (Farina and Bradley, 1970; Stackebrandt *et al.*, 1981, 1983). In 1984 Goodfellow and Cross assigned sporangiate actinomycetes to two aggregate groups, the actinoplanetes and maduromycetes.

The actinoplanetes are currently classified in the genera *Actinoplanes*, *Ampuriella*, *Dactylosporangium* and *Pilimelia* (Vobis, 1989). Organisms classified in these taxa are commonly known as cell wall type II organisms (Lechevalier and Lechevalier, 1970) and have *meso* and hydroxy-diaminopimelic acid and glycine in the peptidoglycan, N-glycolated muramic acid (Uchida and Aida, 1977), an AL_{γ} peptidoglycan (Schleifer and Kandler, 1972; Schleifer and Seidl, 1985), whole organism hydrolysates rich in arabinose and xylose (Goodfellow *et al.*, 1988), and a polar lipid pattern that includes phosphatidylethanolamine (phospholipid pattern II; Lechevalier *et al.*, 1977). An identical peptidoglycan structure, cell wall sugar composition and polar lipid composition is typical of members of the genus *Micromonospora* (Lechevalier *et al.*, 1977; Kawamoto *et al.*, 1981). Molecular systematic data support the inclusion of micromonosporae within the actinoplanetes (Stackebrandt and Woese, 1981; Stackebrandt *et al.*, 1981). *Catellospora* (Asano and Kawamoto, 1986) and *Glycomyces* (Labeda *et al.*, 1985), which form non-motile spores, are also classified in the actinoplanete group primarily on the basis of the chemical markers (Vobis, 1989).

Table 1.3. Chemical profiles of actinomycete genera assigned to suprageneric groups (Goodfellow, 1989)

Family/genus [a]	Wall chemotype [b]	Whole-organism sugar pattern [c]	Peptido-glycan type [d]	Fatty acid pattern [e]	Major menaquinone (MK) [f]	Phospho-lipid type [g]	Mol % (G+C) of DNA
ACTINOMYCETACEAE							
* *Actinomyces*	V, VI	-	A4α, A4β	1A, 1C	-10 (H_2, H_4)	II	57-69
Arcanobacterium	VI	-	A5α	1A	-9 (H_4)	ND	48-52
BREVIBACTERIACEAE							
Brevibacterium	III	C	A1γ	2C	-8 (H_2)	I	60-67
CELLULOMONADACEAE							
* *Cellulomonas*	VIII	-	A4β	2C	-9(H_4)	V	71-76
Jonesia	VI	-	A4β	2C	-9(H_4)	ND	56-58
Oerskovia	VI	-	A4β	2C	-9(H_4)	V	70-75
Promicromonospora	VI	-	A4β	ND	-9(H_4)	ND	70-75
CORYNEBACTERIACEAE							
* *Corynebacterium*	IV	A	A1γ	1A	-8 (H_2), -9(H_2)	I	51-63
DERMATOPHILACEAE							
* *Dermatophilus*	III	B	A1γ	1A	-8 (H_4)	I	57-69
FRANKIACEAE							
* *Frankia*	III	B, C, E	ND	1	ND	I	66-71
Geodermatophilus	III	C	A1γ	2B	-9(H_4)	II	73-76
MICROBACTERIACEAE							
* *Microbacterium*	VI	-	$B1_{\alpha}$, $B1_{\beta}$	2C	-11, -12	I	69-75
Agromyces	VII	-	$B2_{\gamma}$	2C	-11, -12	I	71-76
Aureobacterium	VIII	-	$B2_{\gamma}$	2C	-11, -12	I	67-70
Clavibacter	VII	-	$B2_{\gamma}$	2C	-9, -10	I	68-75
Curtobacterium	VIII	-	$B2_{\gamma}$	2C	-9	I	68-75
MICROCOCCACEAE							
* *Micrococcus*	VI	-	$A3_{\alpha}$	2C	-7(H_2), -8(H_2), -9(H_2)	I	65-75
Arthrobacter	VI	-	A3α	2C	-9(H_2)	I	59-66
Renibacterium	VI	-	A3α	2C	-9(H_2)	I	53-54

Table 1.3. Continued.

Family/genus [a]	Wall chemotype [b]	Whole-organism sugar pattern [c]	Peptido-glycan type [d]	Fatty acid pattern [e]	Major menaquinone (MK) [f]	Phospho-lipid type [g]	Mol % (G+C) of DNA
Rothia	VI	-	A3α	2C	-7	I	54-57
Stomatococcus	VI	-	A3α	2C	-7	I	56-60
MICROMONOSPORACEAE							
* *Micromonospora*	II	-	A1γ	3B	-9(H_4), -10(H_4)	II	71-73
Actinoplanes	II	-	A1γ	2D	-9(H_4), -10(H_4)	II	71-73
Ampullariella	II	-	A1γ	2D	-9(H_4), -10(H_4)	II	71-73
Catellospora	II	D	A1γ	ND	-9(H_4), -10(H_4)	II	71-73
Dactylosporangium	II	D	A1γ	3B	-9(H_2, H_6, H_8)	II	71-73
Glycomyces	II	D	ND	2C	-9(H_4), -10(H_4)	I	71-73
Pilimelia	II	-	$A1_\gamma$	2D	-9(H_2,H_4)	II	ND
MYCOBACTERIACEAE							
* *Mycobacterium*	IV	A	$A1_\gamma$	1B	-9(H_2)	II	62-69
NOCARDIACEAE							
* *Nocardia*	IV	A	$A1_\gamma$	1B	-8(H_2), -9(H_2)	II	64-72
Gordona	IV	A	$A1_\gamma$	1B	-8(H_2), -9(H_2)	II	63-69
Rhodococcus	IV	A	$A1_\gamma$	1B	-8(H_2), -9(H_2)	II	63-72
Tsukamurella	IV	A	$A1_\gamma$	1B	-9	II	67-68
NOCARDIOIDACEAE							
Nocardioides	I	ND	$A3_\gamma$	3A	-8(H_4)	I	ND
Pimelobacter	I	ND	$A3_\gamma$	3A	-8(H_4)	I	69-74
Terrabacter	I	ND	$A3_\gamma$	3A	-8(II,III-H_4)	I	ND
PSEUDONOCARDIACEAE							
* *Pseudonocardia*	IV	A	$A1_\gamma$	2B	-8(H_4)	III	79
Actinobispora	IV	A	$A1_\gamma$	ND	-7(H_2), -9(H_2)	IV	71
Actinokineospora	IV	A	ND	ND	-10	II	72
Actinopolyspora	IV	A	$A1_\gamma$	2C	-9(H_4,H_6)	III	64
Amycolata	IV	A	$A1_\gamma$	3E	-8(H_4)	III	68-72
Amycolatopsis	IV	A	$A1_\gamma$	3F	-9(H_2,H_4)	II	66-69
Kibdelosporangium	IV	A	$A1_\gamma$	3C	ND	II	66
Pseudomycolata	IV	A	$A1_\gamma$	ND	-8(H_4)	II	ND
Saccharomonospora	IV	A	$A1_\gamma$	2A	-9(H_4)	II	69-74
Saccharopolyspora	IV	A	$A1_\gamma$	2C	-9(H_4)	III	77

Table 1.3. Continued.

Family/genus [a]	Wall chemotype [b]	Whole-organism sugar pattern [c]	Peptido-glycan type [d]	Fatty acid pattern [e]	Major menaquinone (MK) [f]	Phospho-lipid type [g]	Mol % (G+C) of DNA
STREPTOMYCETACEAE							
* *Streptomyces*	I	-	$A3_\gamma$	2C	-9(H_6,H_8)	II	69-78
Intrasporangium	I	-	$A3_\gamma$	1A	-8	I	ND
Kineosporia	I	-	$A3_\gamma$	ND	-9(H_4)	III	ND
Sporichyta	I	-	$A3_\gamma$	3A	-9(H_6,H_8)	ND	ND
STREPTOSPORANGIACEAE							
* *Streptosporangium*	III	B	$A1_\gamma$	3C	-9(H_2,H_4)	IV	69-71
Microbispora	III	BC	$A1_\gamma$	3C	-9(H_2,H_4,H_6)	IV	67-74
Microtetraspora	III	BC	$A1_\gamma$	3C	-9(H_1,H_2,H_4)	IV	66
Planobispora	III	B	$A1_\gamma$	3C	-9(H_2,H_4)	IV	70-71
Planomonospora	III	B	$A1_\gamma$	3C	-9(H_2)	IV	72
Spirillospora	III	B	$A1_\gamma$	3A	-9(H_4,H_6)	III	69-71
THERMOMONOSPORACEAE							
* *Thermomonospora*	III	C	ND	3E	-10(H_4,H_6)	II	ND
Actinomadura	III	B	$A1_\gamma$	3A	-9(H_6)	I	66-69
Actinosynnema	III	C	ND	3F	-9(H_4), -10(H_4)	II	71-73
Nocardiopsis	III	C	ND	3D	-10(H_2,H_4,H_6)	III	64-69
Saccharothrix	III	C	ND	3F	-9(H_4), -10(H_4)	II	70-76
Streptoalloteichus	III	C	ND	ND	-9(H_6, -10(H_6)	ND	ND

*, Type genus

a; Data from Lechevalier and Lechevalier (1980); Kroppenstadt (1985), Labeda *et al.* (1985); Poschner *et al.* (1985); Asano and Kawamoto (1986); Goodfellow and Williams (1986); Lechevalier *et al.* (1986); Shearer *et al.* (1986a,b); Rocourt *et al.* (1987); Collins *et al.* (1988); Hasegawa (1988); Stackebrandt *et al.* (1988); Akimov *et al.* (1989); Demharter *et al.* (1989); Suzuki and Komagata (1989); Kroppenstadt *et al.* (1990); Jiang *et al.* (1991).

b; Major constituents in wall chemotypes; I, LL-diaminopimelic acid (LL-DAP); II, *meso*-diaminopimelic acid (*meso*-DAP), glycine; III, *meso*-DAP; IV, *meso*-DAP, arabinose and galactose; V, lysine and ornithine with variable presence of aspartic acid and galactose; VII, diaminobutyric acid, glycine, lysine is variable; VII, ornithine; All wall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid (Lechevalier and Lechevalier, 1970, 1980).

c; Whole-organism sugar patterns of actinomycetes containing *meso*-DAP: A, arabinose and galactose; B, madurose (3-0-methyl-D-galactose); C, no diagnostic sugars; D, arabinose and xylose (Lechevalier *et al.*, 1971).

d; The letters A and B refer to the position of the cross-linkage, numbers refer to the type of cross-linkage and greek letters mark the diversity of amino acids in position 3 of the peptide subunit (Schleifer and Kandler, 1972; Schleifer and Stackebrandt, 1983; Schleifer and Seidl, 1985).

e; Fatty acid patterns after Kroppenstadt (1985).

f; Abbreviations exemplified by MK-8(H2), menaquinone having two of the isoprene units hydrogenated.
g; Characteristic phospholipids PI, phosphatidylglycerol (PG) variable; PII, only phosphatidylethanolamine (PE); PIII, phosphatidylcholine (PC with PE, phosphatidylmethylethanolamine (PME) and PG variable, no phospholipids containing glucosamine); PIV, phospholipids containing glucosamine and PG. All preparations contain phosphatidylinositol (Lechevalier *et al.*, 1981).

Goodfellow *et al.* (1990a) proposed that the genera *Actinoplanes*, *Ampuriella*, *Dactylosporangium*, *Micromonospora* and *Pilimelia* be assigned to the family *Micromonosporaceae* (Krassilnikov, 1938) given the genotypic and phenotypic similarities between members of these taxa. The emended family *Micromonosporaceae* can be distinguished from all other actinomycetes families using a combination of morphological and chemical markers (see Table1.3).

The genus *Micromonospora* currently contains Gram-positive, non-acid fast actinomycetes that form well developed, branched, septate mycelia averaging 0.5μm in diameter. Non-motile spores are borne singly on substrate hyphae and are sessile or on long or short sporophores which can often occur in branched clusters. Sporophore development is usually monopodial but can be sympodial. Aerial hyphae are usually absent but in some cases appear irregularly as a restricted white or greyish bloom. Micromonosporae usually grow between 20 and 40°C but not above 50°C. They are sensitive to pH below 6 but will tolerate up to and above pH 10.

Members of the genus *Micromonospora* have a cell wall type II and a whole-organism sugar pattern D (Lechevalier and Lechevalier, 1971). Cell walls contain meso- and/or 3-hydroxydiaminopimelic acid (A_2pm) and glycine, arabinose and xylose are present in whole-organism hydrolysates. Characteristic phospholipids are phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type PII; Lechevalier *et al.*, 1977) and the major isoprenoid quinones are tetra- or hexa-hydrogenated menaquinones with 9, 10 or 12 isoprene units, namely [MK-9(H_4)], [MK-10(H_4)], [MK-10(H_6)] or [MK-12(H_6)]. The mol% guanine (G) plus cytosine (C) content of the DNA is 71-73% though a broader range of 67-76% G+C has been

reported (Kothe, 1987). The type species is *Micromonospora chalcea* (Foulerton, 1905; Ørskov, 1923).

According to the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) twelve species and seven sub-species are listed in the genus *Micromonospora* (see Table 1.4). Kawamoto (1989) reported that eight species and seven sub-species in this genus can be recognised as individual species or sub-species from their phenotypic and genotypic traits. Kawamoto (1989) raised questions concerning the validity of the five species, namely *M. purpurea, M. rhodorangea, M. brunnea, M. aurantiaca* and *M. gallica.* Kasai *et al.* (2000) reclassified the genus *Micromonospora,* using gyrβ sequences, into the following 14 species: *M. aurantiaca, M. carbonacea, M. chalcea, M. chersina, M. coerula, M. echinospora, M. gallica, M. halophytica, M. inositola, M. nigra, M. olivaterospora, M. pallida, M. purpureochromogenes* and *M. rosaria.*

In addition to these taxa that contain aerobic strains, a number of anaerobic isolates have been assigned to the genus. Hungate (1946) described *M. propionici* to accommodate anaerobic cellulolytic bacteria isolated from the gut of a termite. More recently, Pasti and Belli (1985) have isolated *Micromonospora* spp. and other mesophilic actinomycetes taxa with vigorous cellulolytic activity from the gut of termites of the subfamilies *Macrotermitinae* and *Termitinae* using anaerobic media supplemented with cellulose. Micromonosporae have also been isolated from the gut, parent soil and mound materials of the termites *Cubitermes severus* and *Procubitermes aburiensis* (Bignell *et al.*, 1991). Many of the more recent isolates are obligately anaerobic and can be separated from other members of the genus by their ability to utilise cellulose and by the nature of their fermentation products. Three anaerobic species have been described, *M. acetoformici* which is unable to utilise cellulose but can ferment glucose and starch, *M. propionici* (Hungate, 1946), which ferments cellulose and glucose to form acetic and propionic acids and *M. ruminatum* (Maluszyńka and Janota-Bassalik, 1974), a cellulose fermentor.

The genus *Micromonospora* was proposed by Ørskov (1923) for an organism isolated from air by Foulerton (1905) and named *Streptothrix chalcea.* Foulerton

(1905) described his culture using what were then considered to be the essential bacteriological approaches for determining macroscopic colony morphology and physiological reactions whereas Ørskov used what was thought to be a mycological technique, namely following the morphological development of the spore-bearing structures by microscopy.

In 1932, Jensen isolated sixty-seven *Micromonospora* isolates from Australian soils and classified sixty of them as *M. chalcea*. He also proposed the name *M. chalcea* (Foulerton) but it was accepted that Ørskov (1923) made his intention sufficiently clear when proposing the genus *Micromonospora* based upon his observations of *Streptothrix chalcea* to credit him with the new combination. Jensen assigned the seven remaining isolates to three new species, namely *M. coerula*, "*M. fusca*" and "*M. parva*", mainly on morphological criteria. *M. fusca* is now considered to be a subjective synonym of *M. purpureochromogenes* (Luedemann, 1971) while "*M. parva*", which is characterised by scant growth and sporulation, is not considered to be a validly described species (Kawamoto, 1989).

Erikson (1935) noted that an organism isolated from a blood culture of a patient with Banti's disease was morphologically similar to *M. chalcea* but did not classify it in the genus *Micromonospora* given limited segmentation of the substrate mycelium and the presence of a few aerial hyphae. She named the organism *Actinomyces gallicus* but subsequently (Erikson, 1941) regarded it as a *Micromonospora* strain that grew poorly and which resembled "*M. parva*". This organism was classified as *Streptomyces gallicus* in the 6th Edition of Bergey's Manual of Determinative Bacteriology (Waksman and Henrici, 1948) and is currently described as a species *incertae sedis* (Kawamoto, 1989) as the type strain is no longer extant.

Luedemann and Brodsky (1964) described two strains of *Micromonospora* that produced antibiotics assigned to the gentamicin complex (Weinstein *et al.*, 1964). *M. echinospora* was characterised by the presence of spores with blunt spines; two subspecies were recognised by colour and physiological criteria, namely *M. echinospora* subsp. *ferruginea* and *M. echinospora* subsp. *pallida*. A

second gentamicin producing strain was described as *M. purpurea*. This strain differed from *M. echinospora* given the presence of aberrant spore forms and a failure to grow on L-rhamnose as a carbohydrate source. *M. purpurea* is currently considered as a species *incertae sedis* (Kawamoto, 1989) given a lack of descriptive information on single spore formation. If a sporulating variant of this culture were found and the spore and sporophore appeared similar to those of *M. echinospora* then this species would merit reduction to a subspecies of the latter (Luedemann and Brodsky, 1964).

Luedemann and Brodsky (1965) described *M. carbonacea* subsp. *carbonacea* and *M. carbonacea* subsp. *aurantiaca* for organisms that produce the antibiotic everninomicin (Weinstein *et al.*, 1965). *M. carbonacea* was characterised by the production of spores with a charcoal-like colour. *M. carbonacea* subsp. *aurantiaca* is differentiated from *M. carbonacea* subsp. *carbonacea* by its sparse spore production, inability to reduce nitrate to nitrite or to produce a pale yellow pigment on mannose and xylose agars.

M. halophytica subsp. *halophytica* and *M. halophytica* subsp. *nigra* were described by Weinstein *et al.* (1968) for organisms that produced antibiotics belonging to the halomicin complex; the organisms were isolated from mud obtained from the bottom of a salt pool. *M. halophytica* subsp. *halophytica* was distinguished from *M. halophytica* subsp. *nigra* by its capacity to produce a reddish-brown diffusible pigment on certain media.

Sveshnikova *et al.* (1969) assigned isolates from muddy or silty soils collected in Moscow, Odessa and Poland to five new *Micromonospora* species, namely *M. aurantiaca*, *M. brunnea*, "*M. fulvopurpurea*", "*M. lilacina*" and "*M. rubra*". They also emended the descriptions of several existing *Micromonospora* species and proposed that *M. purpurea* (Luedemann and Brodsky, 1964) be considered a subjective synonym of *M. echinospora* (Luedemann and Brodsky, 1964).

Sveshnikova and colleagues (1969) also assigned *Micromonospora* species to several aggregate groups based on their cultural characters on various media and on their ability to produce melanoid pigments, degrade cellulose, utilise sole

carbon compounds for energy and growth, reduce nitrate, and invert sucrose. The "brunnea" group encompassed *M. brunnea*; the "coerula" group, "*M. bicolour*" and *M. coerula*; the "fusca-chalcea" group, *M. aurantiaca*, *M. chalcea*, "*M. fusca*", "*M. globosa*", *M. halophytica* and "*M. parva*"; and the "purpurea" group, "*M. fulvopurpurea*", "*M. lilicana*", *M. purpurea* and "*M. rubra*". "*M. megalomicea*" was found to produce a new macrolide antibiotic assigned to the megalomiceum complex (Weinstein *et al.*, 1969). Two natural colour variants of the species "*M. megalomicea* subsp. *megalomicea*" and "*M. megalomicea* subsp. *nigra*" were distinguished by the degree to which sporulation occurred. Another antibiotic producing strain, "*M. inyoensis*" was isolated from soil collected from the Inyo National Forest in California. This organism produced a new aminoglycoside antibiotic with a spectrum of activity similar to that of gentamicin (Weinstein *et al.*, 1970).

Table 1.4. Validly described species and subspecies of the genus *Micromonospora*

Species	Reference	Strain No.
Micromonospora aurantiaca	Sveshnikova *et al.* (1969)	ATCC 27029
Micromonospora brunnea	Sveshnikova *et al.* (1969)	ATCC 27334
Micromonospora carbonacea	Luedemann and Brodsky (1965)	
subsp. *aurantiaca*		ATCC 27115
subsp. *carbonacea*		ATCC 27114
Micromonospora chalcea	Foulerton (1905) Ørskov (1923)	ATCC 12452
Micromonospora chersina	Tomita *et al.* (1992)	ATCC 53710
Micromonospora coerula	Jensen (1932)	ATCC 27008
Micromonospora echinospora	Luedemann and Brodsky (1965)	
subsp. *echinospora*		ATCC 15837
subsp. *ferruginea*		ATCC 15836
subsp. *pallida*		ATCC 15838
Micromonospora gallica	Erikson (1935) Waksman (1961)	NCTC 4582
Micromonospora halophytica	Weinstein *et al.* (1968)	
subsp. *halophytica*		ATCC 27596
subsp. *nigra*		ATCC 33088
Micromonospora inositola	Kawamoto *et al.* (1974)	ATCC 21773
Micromonospora olivaterospora	Kawamoto *et al.* (1983a)	ATCC 21819
Micromonospora purpurea	Luedemann and Brodsky (1964)	ATCC 15836
Micromonospora purpureochromogenes	Waksman and Curtis (1916) Luedemann (1971b)	ATCC 27007
Micromonospora Rhodorangea	Wagman *et al.* (1974)	ATCC 27932
Micromonospora rosaria	Horan and Brodsky (1986)	ATCC 29337

ATCC, American Type Culture Collection, USA; NCTC, National Collection of Type Cultures, UK.

Luedemann (1971b) examined a number of strains, which were morphologically and physiologically similar to "*M. fusca*" (Jensen, 1932) when searching for a new neotype strain for this organism. These organisms included the type strain of *Streptomyces purpureochromogenes* (Waksman and Curtis, 1916). Luedemann proposed that this latter strain be transferred to the genus *Micromonospora* (Ørskov, 1923) as *Micromonospora purpureochromogenes*

(Waksman and Curtis, 1916) comb. nov. and that *M. fusca* be regarded as a subjective synonym of this taxon. *Micromonospora inositola*, which produces antibiotics active against Gram-positive bacteria, was proposed by Kawamoto *et al.* (1974). The type strain was compared with representative strains of *Micromonospora* species with regard to mycelial and sporulation pattern, spore surface ornamentation, mycelial pigments, diffusible pigments, utilisation of carbohydrates and other physiological properties. *Micromonospora inositola* was considered to belong to the "fusca-chalcea" group defined by Sveshnikova *et al.* (1969).

Wagman *et al.* (1972) described *Micromonospora rosaria* for an organism that produced a new macrolide antibiotic, rosaramicin. A full description of the strain was not given hence the species was not included on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). Following the initial publication *Micromonospora rosaria* NRRL 3718 was used as a reference strain in numerous studies describing new species of antibiotic producing micromonosporae (Hatano *et al.*, 1976; Lee *et al.*, 1976; Furumai *et al.*, 1977; Satol *et al.*, 1980; Wagman and Weinstein, 1980). In an attempt to provide continuity in the literature, the name was revived by Horan and Brodsky (1986) when they presented a full description of the species and designated strain NRRL 3718 as the type strain.

In 1974, Wagman *et al.* proposed *Micromonospora rhodorangea* for an organism that produced a broad-spectrum antibacterial antibiotic that was active against amoebae, protozoa, tapeworm and pinworm infections in mice. The strain was classified as a new species of *Micromonospora* on the basis of its growth properties on a number of standard agar media and its ability to utilise carbohydrates. *Micromonospora rhodorangea* is described as a species *incertae sedis* in Bergey's Manual of Systematic Bacteriology (Kawamoto, 1989).

Micromonospora olivaterospora was described by Kawamoto *et al.* (1983a) on the basis of chemotaxonomic and morphological features for an organism that produced antibiotics belonging to the fortimicin complex. The organism is characterised by an olive-black spore layer, spores with blunt spiny surfaces, an olive-green soluble pigment and its carbon utilisation pattern.

Micromonospora chersina (Tomita *et al.*, 1992) was described for an organism found to produce dynemicin, a novel antitumour antibiotic. The strain was distinguished on the basis of quantitative analysis of menaquinones and fatty acids, cell wall diamino acid composition and physiological characteristics.

It is evident from what has been said that *Micromonospora* species have been described at best by using a few chemical and morphological properties with little attempt to undertake comparative taxonomic studies. One of the more comprehensive studies was performed by T'ao (1958) who found micromonosporae difficult to classify given their delicate structure, the slight morphological differences between strains and the variation observed in physiological reactions. The diagnostic carbohydrate utilisation used was based on an inorganic nitrogen source, which in some instances resulted in a poor dextrose positive control and hence gave difficulty in evaluating comparative results.

Subsequently T'ao and Potter (1960) considered that the morphological properties should be weighted as primary criteria for the classification of *Micromonospora* species. Important physiological characteristics included cellulose degradation, inversion of sucrose and nitrate reduction. In contrast, the colour of the growth and the form of the colony were not considered good taxonomic characters. Reproducibility of colour for a given organism could not be repeated on the same medium.

Sveshnikova *et al.* (1969) concluded that the orange colour of the mycelium of *Micromonospora* strains was not a useful diagnostic character since it was common to most micromonosporae. Nevertheless, they concluded that the capacity of micromonosporae to produce blue, brown, and red pigments was useful for classification and that sporulation type served as a valuable characteristic for differentiating *Micromonospora* species. They acknowledged, however, that it was necessary to acquire more data before the value of physiological reactions in *Micromonospora* systematics could be assessed. Their original aim had been to go beyond the process of cataloguing *Micromonospora* strains given the lack of stable taxonomic criteria.

Luedemann and Brodsky (1964) compared representatives of the genus *Micromonospora* namely, *Micromonospora chalcea* ATCC 12452, *Micromonospora echinospora* NRRL 2985, *M. echinospora* subsp. *ferruginea* NRRL 2995, *Micromonospora echinospora* subsp. *pallida* NRRL 2996, "*Micromonospora fusca*" NRRL B-943, *Micromonospora purpurea* NRRL 2953, and *Micromonospora* sp. ATCC 10026 for their ability to use 24 carbohydrates as sole sources of carbon and energy for growth. Apart from the *Micromonospora echinospora* subsp. *ferruginea*, *Micromonospora echinospora* subsp. *pallida* and *Micromonospora purpurea* strains all of the organisms grew well on α-melibiose. They concluded that the newly described, gentamicin producing *Micromonospora echinospora* and *Micromonospora purpurea* species could be distinguished from other gentamicin-producing *Micromonospora* species on the basis of their carbohydrate utilisation patterns.

In 1970, Luedemann reviewed the taxonomic and ecological literature on the genus *Micromonospora*. At that time, morphology was considered the primary characteristic for the classification of *Micromonospora* species; biochemical, colony and physiological properties were seen as secondary features. He concluded that the relationships between *Micromonospora* species were difficult to ascertain given a dearth of suitable differential properties.

The most current taxonomic assessment of this genus has been carried out by Kasai *et al.* (2000). This study looked at the intrageneric relationships among 15 *Micromonospora* species and 4 sub-species using *gyrB* sequences and DNA:DNA hybridisation techniques. It was found that all but one of the *Micromonospora* strains formed a tight cluster, as had previously been observed using 16S rDNA-based phylogenetic analysis (Koch *et al.*, 1996b). However, the intrageneric relationships revealed by the *gyr*B-based phylogeny were different to those based on their 16S rDNA sequences. DNA:DNA hybridisation studies revealed that the *gyr*B-based phylogeny gave a classification relevant for the genus *Micromonospora*. Kasai et al. (2000) have suggested that *gyr*B-based phylogenies are useful for analysing the phylogenetic relationships of high G+C Gram-positive bacteria at the level of the genomic species.

In general, the taxonomic problems outlined above stem from a lack of widely accepted, reliable characters for classification together with a tendency for *a priori* weighting of characters examined under non-defined conditions. *Micromonospora* have generally been classified using a few subjectively chosen morphological, pigmentation and physiological properties. In contrast, actinomycetes taxa defined in numerical taxonomic surveys are polythetic, that is no single character is either indispensable or sufficient to entitle an organism to group membership.

1.4. Ecology of micromonosporae

1.4.1. Introduction

Actinomycetes are a highly successful group of bacteria that live in a variety of natural environments. Most are strict saprophytes but some form parasitic or mutualistic associations with animals and plants (Williams *et al.*, 1984; Schaal and Lee 1992). The primary natural reservoir of actinomycetes is soil, where strains are believed to play a role in the recycling of nutrients (McCarthy and Williams, 1992). Soil particles carrying spores are widely dispersed and consequently actinomycetes can be isolated from most natural habitats. Soil is continually being washed into streams and deposited in lake sediments where actinomycetes spores represent a high proportion of the 'wash-in' microbiota. It has been suggested that certain actinomycete genera and species have been selected by their ability to grow in aquatic environments so that they form part of a truly aquatic microbial flora (Johnston, 1972), however this is contested by others who consider that actinomycetes found in aquatic habitats are essentially 'wash-in' components (Goodfellow and Haynes, 1984).

1.4.2. Ecology of micromonosporae in terrestrial habitats

Jensen (1932) first reported the occurrence of micromonosporae in soil for Australian soils then in 1939 Kriss found them in Russian soils. Lechevalier (1964) found 2.5% of 5000 isolates from soil samples to be micromonosporae. Ishizawa and workers (1969) found the genus to comprise 14% of the total

actinomycetes in Japanese paddy soils. Shearer (1987) predicted that most soil samples would contain 2 or 3 strains of micromonosporae.

1.4.3. Ecology of micromonosporae in freshwater habitats

The single spores produced by micromonosporae are hydrophilic and easily detached from soil particles by water (Ruddick and Williams, 1978). Cross (1981) considered that *Micromonospora* spores were washed from soils into streams, rivers and lakes where they could survive as dormant propagules for many years.

Erikson (1941) studied the physiology of 10 representatives of *Micromonospora* isolated from two lakes in Wisconsin and found that they actively decomposed cellulose, chitin and lignin. She suggested that micromonosporae might play an important role in lacustrine ecology, as organic substances tend to accumulate in lake muds.

Umbreit and McCoy (1941) studied the actinomycete populations of 15 Wisconsin lakes and found that 10-20% of the total microbial community belonged to the genus *Micromonospora*, though the total microbial population in the water was low (250-3300 organisms per ml). In some cases, the numbers of *Micromonospora* strains formed 40 to 50% of the isolates. They concluded that members of the genus *Micromonospora* were indigenous inhabitants of the waters and muds of fresh water lakes, but they only played a minor role in the decomposition of organic matter, especially in the deeper muds. Other workers subsequently confirmed the occurrence of *Micromonospora* strains in lake systems (Potter and Baker, 1956).

Cross and Collins (1966) described the occurrence and distribution of *Micromonospora* strains in the waters of Blenham Tarn and in the English Lake District. Micromonosporae showed a pronounced distribution as the lake became stratified during the summer, high numbers accumulating beneath the thermocline. Samples taken shortly before overturn gave counts of 100 to 250 *Micromonospora* per ml beneath the thermocline thereby forming approximately

60% of the bacterial population, but only 0 to 10 per ml, or 0.5% of the bacterial population, above the thermocline. After overturn, higher numbers of micromonosporae were isolated from all depths sampled (100 to 1000 per ml); these numbers accounted for approximately 10% of the aerobic bacterial count. The authors suggested that the distribution pattern was indicative of an organism that originated from the lake mud and became suspended in the water by local currents and water movements. Studies on the properties of isolated strains suggested that they might have an important role in lacustrine ecology given their ability to degrade organic substrates such as cellulose and chitin.

Micromonosporae have also been isolated from streams and rivers. Rowbotham and Cross (1977) recorded the numbers of micromonosporae, rhodococci and streptomycetes in streams in Yorkshire and found that micromonosporae represented between 14 and 72% of the population isolated. The combined numbers of micromonosporae, rhodococci and streptomycetes varied from 120 to 1500 cfu/ml. The ability of *Micromonospora* strains to tolerate reduced oxygen tensions (Watson and Williams, 1974) is in line with the view that spores washed into aquatic habitats can survive in littoral and marine sediments for long periods of time (Goodfellow and Williams, 1983).

1.4.4. Ecology of micromonosporae in marine habitats

Micromonosporae have been recovered from many marine habitats, ranging from coastal regions to deep-sea sediments (Weyland, 1969; Takizawa *et al*, 1993; Colquhoun 1999). Hunter and workers (1981) found micromonosporae to be abundant in salt marsh ecosystems in New Jersey (USA). Watson and Williams (1974) examined the actinomycete flora of a coastal sand belt near Formby, Lancashire (UK) and found that *Micromonospora* strains predominated in beach sand and seawater samples forming up to 94 and 71% of the actinomycete populations, respectively.

Weyland (1969, 1981) found that micromonosporae formed the predominant fraction of the actinomycete community in deep-sea sediments. This work was subsequently extended by Goodfellow and Haynes (1984) who found that

actinomycetes formed a small fraction of the bacterial population in marine sediments, with the highest actinomycete counts in sediments collected near the shore with numbers decreasing with increasing distance from the land. All of the actinomycetes isolated by Goodfellow and Haynes (1984) from the marine habitats were presumptively identified as *Micromonospora*, *Rhodococcus* or *Streptomyces* strains on the basis of colony morphology on isolation media. Numbers of micromonosporae, rhodococci and streptomycetes were found to be roughly equal in the littoral and near shore sediments but micromonosporae predominated in the samples collected further from land, constituting between 78 and 100% of the total actinomycete populations at distances between 5 and 45 miles from land.

1.5. Selective isolation of micromonosporae

1.5.1. Introduction

The selective isolation of specific organisms from environmental samples is a procedure of paramount importance in microbiology (Bull *et al.*, 1992, 2000). Selective isolation procedures are influenced by several factors, notably the number and nature of the target organisms and the habitat under study. Many kinds of microorganisms coexist in natural habitats and each has its own function and ecological niche. Figure 1.4 shows the geographic locations for the samples used during this study.

Microorganisms differ in their nutritional requirements hence no culture medium will allow the growth of all kinds of bacteria from a given habitat. Organisms present in large numbers in environmental samples can usually be isolated simply by plating the appropriate serial dilutions of the sample onto suitable non-selective media and incubating at the required temperature (Williams *et al.*,

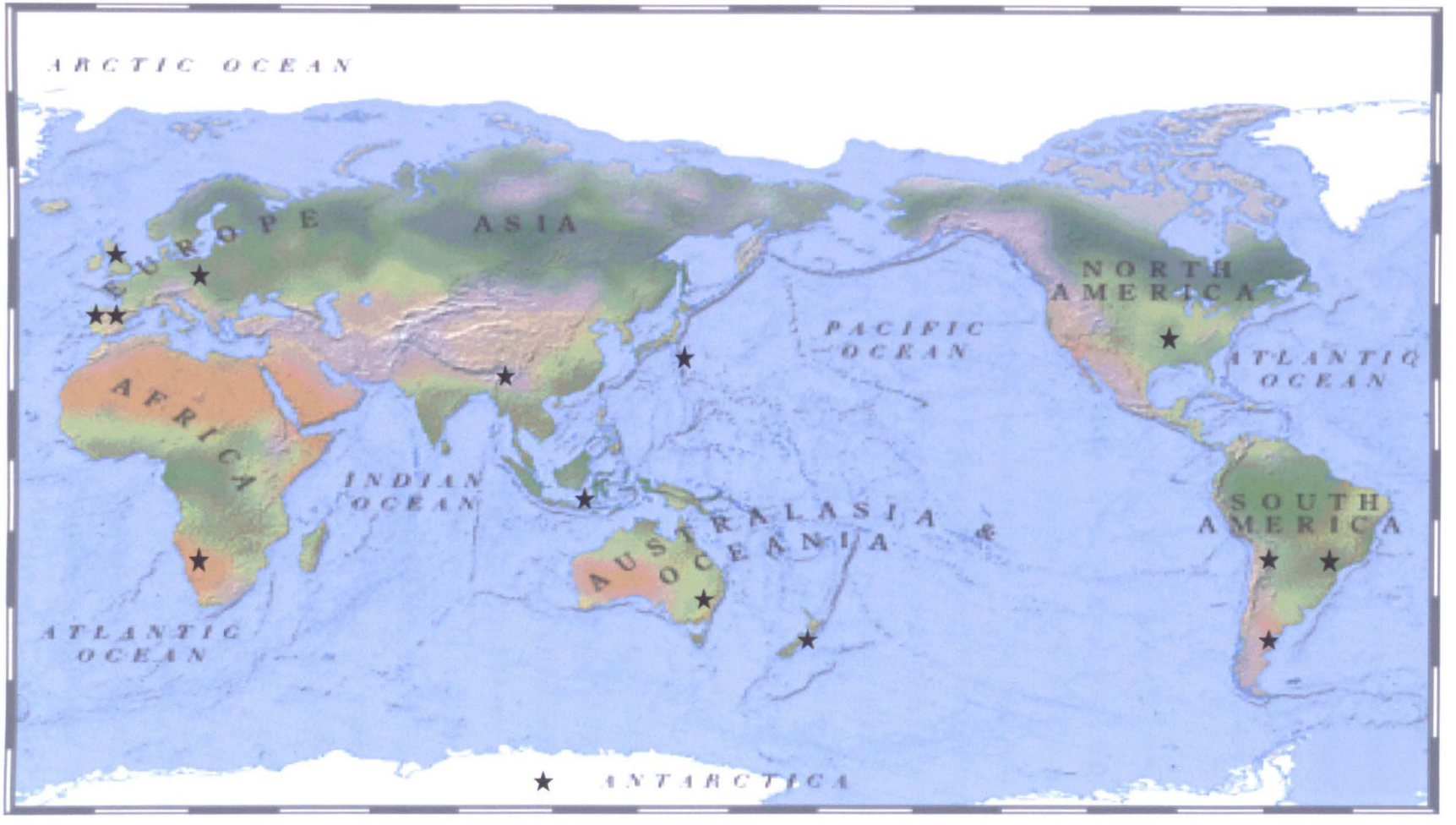


Figure 1.4. Geographic locations of sample sites for micromonosporae (Modified from Dorling Kindersley)

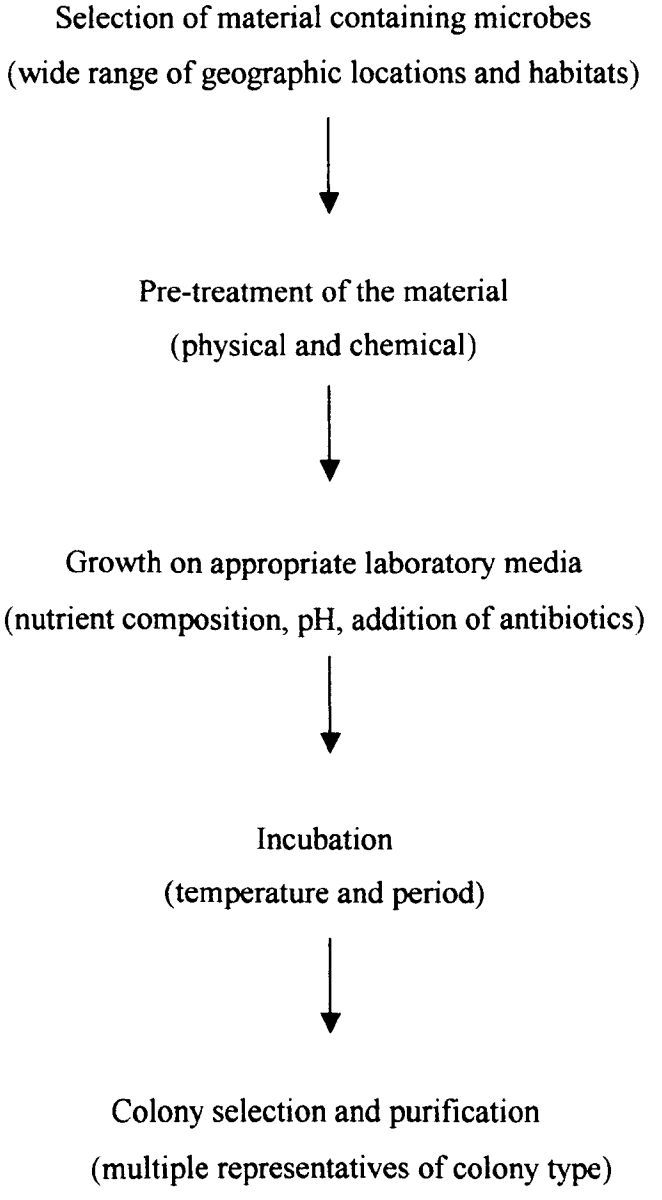
1984). So-called "non-selective media", such as peptone yeast extract and soil extract agars are known to be selective for fast growing bacteria and are unsuitable for the isolation of actinomycetes which are relatively slow growing and tend to be found in low numbers in some natural habitats. The protocols usually adopted in selective isolation are shown in Figure 1.5.

Selective media favour the growth of target microorganisms but not that of unwanted organisms. A number of approaches based on some aspect of the biology of the individuals or groups of organisms can be used to selectively isolate actinomycetes from environmental samples. The organisms may be selected by plating serial dilutions of environmental samples onto nutrient media containing compounds that inhibit the growth of unwanted bacteria but not that of the target organism, by enriching environmental samples prior to selective isolation, or by pre-treating with either chemical or physical methods which favour the isolation of the target organism but not those of unwanted bacteria and fungi.

The incorporation of antibiotics into isolation media is very important in the selective isolation of actinomycetes (Orchard and Goodfellow, 1974; Goodfellow and Williams, 1986). The antifungal antibiotics cycloheximide and nystatin are routinely added to media for the selective isolation of actinomycetes to eliminate or control fungal growth on isolation plates. Media supplemented with antibacterial antibiotics are often used to reduce the number of Gram negative and fast growing bacteria, although the counts of the target organism may be reduced as well as those of the unwanted bacteria (Davies and Williams, 1970).

It is always difficult to select which antibiotic or combinations of antibiotics are likely to be most effective for the isolation of target organisms. One approach that has been applied successfully is to determine the antibiotic sensitivity patterns of representatives of a specific taxon and to supplement media with antibiotic(s) that inhibit unwanted bacteria but not that of the target organism. This has been done for streptomycetes (Vickers and Williams, 1987) but not for micromonosporae.

Figure 1.5. Stages of selective isolation



It is not difficult to isolate micromonosporae from natural habitats and several procedures have been proposed for this purpose (Vobis, 1991). Heat pre-treatment of environmental samples favour the isolation of micromonosporae (Rowbotham and Cross, 1977; Sandrak, 1977) as the spores of these organisms are resistant to high temperatures (Kawamoto, 1989). Micromonosporae are aerobic, mesophilic organisms but even so, their growth is often very slow, and on conventional isolation media they can be overgrown by fungi. However, media supplemented with cycloheximide limit the growth of fungi (Shearer, 1987).

The high micromonosporae counts associated with habitats such as soil need to be interpreted with care as most colonies growing on isolation plates originate from spores. The growth of micromonosporae in soils is similar to that of many other microorganisms in this habitat where supplies of nutrients are discontinuous. Micromonosporae exist in soils for long periods as spores that germinate in the presence of exogenous nutrients, the lack of which prevents germination of most or all spores added to sterile soil.

The major factor governing the distribution and activity of micromonosporae in soil are nutrient availability, moisture content, temperature and pH, although soil type and seasonal changes may also have an influence.

The dispersion and differential centrifugation (DDC) technique (Hopkins *et al.*, 1991) is claimed to be more effective in extracting actinomycetes propagules from environmental samples than the traditional method of shaking soil in saline prior to plating out on selective media (Manfio, 1995; Sembiring, 2000). Atalan *et al.* (2000) showed that the DDC procedure was between three and twelve times more effective in the extraction of streptomycetes propagules from non-heat pre-treated soil suspensions than a reciprocal shaking technique. There was also evidence from these preliminary experiments that different types of streptomycetes were isolated at different stages of the extraction procedure possibly reflecting the ease or otherwise of detaching specific types of actinomycete propagules from particulate matter. These observations suggest that the persistent association between soil particles and actinomycete propagules

may be major limitations to quantitative and representative sampling of actinomycete populations and that the DDC technique is effective in breaking down such interactions.

1.5.2. Pre-treatment of samples

The selectivity of isolation procedures can be promoted by pre-treatment of environmental samples prior to plating onto selective isolation media. Some examples of pre-treatment regimes used to selectively isolate actinomycetes are shown in Table 1.5. Micromonosporae propagules are relatively resistant to desiccation so the simple practice of air drying environmental samples can significantly increase counts on selective isolation media by reducing numbers of fast growing competitors. Resistance to desiccation is often accompanied by a degree of heat resistance. Actinomycete spores are more sensitive to wet than to dry heat so that lower temperatures are used to pre-treat aqueous soil suspensions (Goodfellow and Haynes, 1984). However, it should be noted that pre-treatment regimes can reduce the number of actinomycetes recovered (Williams *et al.*, 1972).

Table 1.5. Examples of pre-treatment regimes used for the selective isolation of actinomycetes

Pre-treatment	Environmental samples	Reference
Chemical:		
Phenol	Soil, water	Vipin Vyas *et al.* (1991)
Heating:		
Wet heat: 50°C for 10 minutes	Soil and sediment suspensions	Cross (1982); Goodfellow and Haynes (1984)
Heating:		
Dry heat: 100°C for 1 hour		Pisano *et al.* (1986)
Dry heat: 120°C for 1 hour		Nonomura and Ohara (1969)
Physical and physicochemical		
Dispersion and differential centrifugation	Soil and sediment	Hopkins *et al.* (1991)

1.5.3. Selective media

The selectivity of isolation media is influenced by nutrient composition, pH, and the presence of selective inhibitors, temperature and the incubation period. Many media have been recommended for the isolation of actinomycetes (Table 1.6) but as the various ingredients have usually been chosen empirically, the basis of their selection is unclear. Colloidal chitin (Hsu and Lockwood, 1975), starch-casein (Küster and Williams, 1964) and M3 agars (Rowbotham and Cross, 1977) have been widely used but little attempt has been made to evaluate the basis of their selectivity (Williams *et al*, 1984).

Table 1.6. Media used to selectively isolate actinomycetes from environmental samples

Major constituents	Antibiotics	Predominant isolates	References
Arginine-glycerol-mineral salts	None	*Streptomyces* spp.	El-Nakeeb and Lechevalier (1962)
Colloidal chitin mineral salts	None	*Streptomyces* and *Micromonospora* spp.	Hsu and Lockwood (1975)
Humic acid-vitamin	Tunicamycin	*Streptomyces*, *Micromonospora*, *Nocardia*	Hayakawa *et al.* (1991)
M3	None	*Rhodococcus* spp. *Micromonospora* spp.	Williams and Wellington (1982)
Starch-casein-mineral salts	Actidione, nystatin, penicillin and polymixin	*Streptomyces* *Micromonospora* spp.	Küster and Williams (1964)

Selective isolation media can be formulated in an objective way following advances in actinomycetes systematics (Williams *et al*, 1984; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992). Numerical phenetic databases, which contain extensive information on biochemical, nutritional and tolerance properties of the constituent taxa, are valuable resources for the formulation of taxon specific isolation media. However, the current status of such databases for the genus *Micromonospora* are somewhat sketchy and require coordination and additional studies.

1.6. Biodiversity and biogeographic distribution

Microbial diversity is a major resource in the development of biotechnological products and processes. Loss of biodiversity should be as ominous for microbiologists and biotechnologists as it is to conservationists (Bull *et al.*, 1992). Biodiversity is one of the Earth's most important but least utilised resources (Wilson, 1985). Biogeography has been defined as 'studying the global distribution of organisms' by participants at a 1996 American Academy of Microbiology Colloquium. By investigating biogeography, microbiologists can estimate systematically the extent of distribution and microbial diversity on the planet (Staley, 1999). Unlike plants and animals, little is known about threatened microorganisms. Indeed, no specific bacteria are considered naturally endangered. In contrast, pathogenic bacteria such as *Mycobacterium leprae* have been placed on the World Health Organisation's list for species to be eradicated. Understanding the role of specific organisms in their natural habitat, where they originated from and how they coevolved with their hosts may lead to new approaches to treating and preventing diseases.

There are two schools of thought regarding the biogeographic distribution of microorganisms. In 1934 Baas-Becking stated 'Everything is everywhere, the environment selects' implying that bacteria are wholly cosmopolitan in their distribution. In support of this hypothesis he pointed out that bacteria are readily dispersed from one area on Earth to another both by abiotic means such as wind and water currents and by biotic vectors such as birds that fly between continents and carry bacteria on their feathers or feet.

The alternative hypothesis, that species are endemic to specific areas, implies that a given organism has resided in an area long enough to have formed a cluster of phylogenetically related groups.

Recently developed molecular phylogenetic methods make it possible to test which of these two hypotheses better describes particular organisms (Staley and Gossink, 1999). Recent reports in the research literature on bacterial

biogeography tend to support Baas-Becking's hypothesis that many bacteria are 'cosmopolitan' in their distribution patterns. Garcia-Pichel *et al.*, (1996) showed that partial 16S rDNA sequences from two strains of *Microcoleus chthonoplastes* from Europe and two from North America are identical. Also, a strain of the archaeon *Archaeoglobus fulgidus* from the North Atlantic oil fields was shown to have 100% DNA:DNA reassociation with the type strain that was isolated from Italy by Beeder and workers (1994).

Search and discovery programmes are conveniently viewed as a succession of unit stages, beginning with the assembly of the appropriate biological material (sampling, isolation), and passing through screening (searching for the desired property among the assembly of microorganisms or genes), selection of the "best" organisms or property from among the subset of organisms which possess the desired attribute(s), to development and acceptance of the end product or process. Essentially screening is the process of bringing together genetic variation and screening criteria (Bull, *et al.*, 1992).

Chapter Two

Materials and Methods

2.0. Materials and Methods

2.1. Materials

All chemicals were obtained from Sigma-Aldrich Chemical Co. (Gillingham, UK) unless otherwise stated and were of reagent grade. Reagents and chemicals used in DNA experiments were of molecular biology grade.

2.1.1. Source and collection of samples

Environmental samples of soil, river and lake sediments were collected from 2-5cm below the top surface and stored in sterile plastic tubes or bags at 4°C. Marine sediments were collected from sites in the North West Pacific Ocean, at depths ranging from 1000m to 6500m, using the submersible fleet operated by the Japan Marine Science and Technology Center (JAMSTEC), Yokosuka, Japan. Wherever possible the samples were kept at 4°C, either in cool boxes or in fridges, until they reached the UK where they were stored in a cold room. Table 2.1 gives details of the sampling sites.

2.1.2. Physical characterisation of samples

The pH of the soil and sediment samples, with the exception of the marine sediments due to lack of material, was determined using a pH probe (Hanna Instruments, Italy). Each sample (1.0g) was placed into a beaker and 20ml of deionised water was added. The mixture was agitated and then left to stand to allow particulate matter to settle at the bottom of the beaker. Readings were taken three times and the average reading calculated.

Table 2.1. Source of environmental samples examined for the presence of micromonosporae.

Sample Code	Source	Sample Type	Date Collected
Aus-1	Cradle Mountain National Park, Tasmania, Australia. Southern end of Lake Dove	Temperate rainforest	13/08/96
Aus-2	Cradle Mountain National Park, Tasmania, Australia. Lake Dove opposite Ballroom Forest	Lake surface sediment. Temperate rainforest	14/08/96

Table 2.1. Continued.

Sample Code	Source	Sample Type	Date Collected
Aus-3	River Que (headwaters), Tasmania, Australia	Mixed soil sample from eucalypt and other root zones	15/08/96
Aus-4	Daintree River National Park, Queensland, Australia.	Soil sample from under Moreton Bay Ash. Tropical rainforest	17/08/96
Aus-5	Mount Lewis, Great Dividing Range, Queensland, Australia.	Rhizosphere soil from White Silky Oak. Tropical rainforest.	19/08/96
Aus-6	Mossman Gorge, Daintree River National Park, Queensland, Australia	Rhizosphere soil from Strangler Fig. Tropical rainforest.	20/08/96
Aus-7	Mossman Gorge, Daintree River National Park, Queensland, Australia	Soil from excavations of feral pig. Tropical rainforest.	20/08/96
Aus-8	Mossman Gorge, Daintree River National Park, Queensland, Australia	Humus collected in a tree bole. Tropical rainforest.	20/08/96
Aus-9	Lizard Island, Queensland, Australia	Mud from a mangrove swamp.	22/08/96
GBR-10	Dartmoor, England.	Shallow river sediment	26/01/97
GBR-11	Dartmoor, England.	Dry bog	26/01/97
GBR-12	Dartmoor, England.	Wet bog	26/01/97
Arg-13	San Martin Park, Mendoza, Argentina	Arid soil	12/10/96
Arg-14	Iguacu National Park, Puerto Iguacu, Argentina	Soil. Sub-tropical rain forest	19/10/96
Brz-15	Iguacu National Park, Foz do Iguacu, Brazil	Soil. Sub-tropical rain forest.	20/10/96
Czr-16	Chalvetice, North Bohemia, Czech Republic	Reed bed. Abandoned sedimentation pond from manganese mine	23/04/97
Czr-17	Tusimice, North Bohemia, Czech Republic	Soil from an active sedimentation pond near electricity generating station.	24/04/97
*Jpn-18	Pacific Ocean, Japan	Soft sediment from ~1000m.	08/91
Por-19	Febre River, Avintes, Portugal	River sediment	29/05/97
Por-20	Ave River, Spring, Portugal	River sediment	29/05/97
Por-21	Ave River, Nascente, Portugal	River sediment	29/05/97
Por-22	Leca River, Leca, Portugal	River sediment	29/05/97
Por-23	Cavado River, Cavado, Portugal	River sediment	29/05/97
Por-24	Landim, Portugal	Soil	29/05/97
Ant-25	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Col fellfield.	Soil from frost-sorted polygon fines.	08/02/95

Table 2.1. Continued.

Sample Code	Source	Sample Type	Date Collected
Ant-26	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Peak	Soil from alkaline area near marble outcrop	08/02/95
Ant-27	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Moraine Valley fellfield site	Soil from frost-sorted polygon fines	03/89
Ant-28	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (0-1cm core zone)	04/06/86
Ant-29	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (1-2cm core zone)	30/06/86
Ant-30	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (0-1cm core zone)	30/06/86
Ant-31	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (1-2cm core zone)	30/06/86
Ant-32	Edmonson Point, Wood Bay, Continental Antarctica (74 20'S, 160 08'E) Edmonson Point colonisation site.	Surface soil	06/01/96
Nzl-33	New Zealand	Soil	05/97
*Jpn-34	Hot volcanic spring, Hakone, Japan	Red sediment	16/07/97
*Jpn-35	Hot volcanic spring, Hakone, Japan	Sediment near to a spring at 100°C	16/07/97
*Jpn-36	Hot volcanic spring, Hakone, Japan	Sulphurous sediment	16/07/97
*Jpn-37	Hot volcanic spring, Hakone, Japan	Spring water and sediment	16/07/97
*Jpn-38	Hot volcanic spring, Hakone, Japan	Spring water and sediment	16/07/97
Gbr-39	Anglia Wreck, off the Dover Coast, England	Sediment from the sea floor (27m)	08/08/97
Atl-40	Sulphate: methane transition: Hole 995B, core 2H-04, Blake Ridge, Atlantic Ocean	Core sediment 21.0mbsf	25/11/95
Atl-41	Bottom-simulating reflector (BSR): Hole 995B, core 15X-02, Blake Ridge, Atlantic Ocean	Core sediment 449.06mbsf	26/11/95
GBR-42	Afon Mellte, West Glamorgan, Wales	Shallow river sediment	16/08/97
USA-43	St Louis, Missouri, USA.	Soil	06/09/97
Por-21	Ave River, Nascente, Portugal	River sediment	29/05/97
Por-22	Leca River, Leca, Portugal	River sediment	29/05/97
Por-23	Cavado River, Cavado, Portugal	River sediment	29/05/97
Por-24	Landim, Portugal	Soil	29/05/97
Ant-25	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Col fellfield.	Soil from frost-sorted polygon fines.	08/02/95

Table 2.1. Continued.

Sample Code	Source	Sample Type	Date Collected
Ant-26	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Peak	Soil from alkaline area near marble outcrop	08/02/95
Ant-27	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Moraine Valley fellfield site	Soil from frost-sorted polygon fines	03/89
Ant-28	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (0-1cm core zone)	04/06/86
Ant-29	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (1-2cm core zone)	30/06/86
Ant-30	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (0-1cm core zone)	30/06/86
Ant-31	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (1-2cm core zone)	30/06/86
Ant-32	Edmonson Point, Wood Bay, Continental Antarctica (74 20'S, 160 08'E) Edmonson Point colonisation site.	Surface soil	06/01/96
Nzl-33	New Zealand	Soil	05/97
*Jpn-34	Hot volcanic spring, Hakone, Japan	Red sediment	16/07/97
*Jpn-35	Hot volcanic spring, Hakone, Japan	Sediment near to a spring at 100°C	16/07/97
*Jpn-36	Hot volcanic spring, Hakone, Japan	Sulphurous sediment	16/07/97
*Jpn-37	Hot volcanic spring, Hakone, Japan	Spring water and sediment	16/07/97
*Jpn-38	Hot volcanic spring, Hakone, Japan	Spring water and sediment	16/07/97
Gbr-39	Anglia Wreck, off the Dover Coast, England	Sediment from the sea floor (27m)	08/08/97
Atl-40	Sulphate: methane transition: Hole 995B, core 2H-04, Blake Ridge, Atlantic Ocean	Core sediment 21.0mbsf	25/11/95
Atl-41	Bottom-simulating reflector (BSR): Hole 995B, core 15X-02, Blake Ridge, Atlantic Ocean	Core sediment 449.06mbsf	26/11/95
GBR-42	Afon Mellte, West Glamorgan, Wales	Shallow river sediment	16/08/97
USA-43	St Louis, Missouri, USA.	Soil	06/09/97
USA-44	Mammoth Cave National Park, Kentucky, USA	Soil	30/08/97
USA-45	Music Row, Nashville, Tennessee, USA.	Soil	31/08/97
USA-46	Laurel Falls, Blue Smoky Mountains National Park, Tennessee, USA	Soil	01/09/97
USA-47	Huntsville, Alabama, USA	Soil	02/09/97

Table 2.1. Continued.

Sample Code	Source	Sample Type	Date Collected
USA-48	Indy 500 Racetrack, Indianapolis, USA	Soil	N/A
USA-49	Central Park, New York City, New York, USA	Soil	11/09/97
Ind-50	Princess Cave, Selarong, Bantul, Java, Indonesia	Soil	29/10/97
Ind-51	Kiskendo Cave, Yogyakarta, Java, Indonesia	Soil, from outside the cave	29/10/97
Ind-52	Kiskendo Cave, Yogyakarta, Java, Indonesia	Soil from inside the cave	29/10/97
Ind-53	Kiskendo Cave, Yogyakarta, Java, Indonesia	Soil from 200m into the cave	29/10/97
Ind-54	Beach, Karang Bolong, Java, Indonesia	Sand from the beach	29/10/97
Ind-55	Cave, Karang Bolong, Java, Indonesia	Soil from inside the cave	29/10/97
Ind-56	Mangrove, Tritih Putih, Cilicap, Java, Indonesia	Mud from the mangrove	30/10/97
Ind-57	Mangrove, Tritih Putih, Cilicap, Java, Indonesia	Mud from a crab mound	30/10/97
Ind-58	Cave, Jatijajar, Gombong, Java, Indonesia	Soil from inside the cave	30/10/97
Ind-59	Cave, Jatijajar, Gombong, Java, Indonesia	Soil from outside the cave	30/10/97
Ind-60	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Lake sediment	01/11/97
Ind-61	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Organic litter	01/11/97
Ind-62	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Charred soil from a tree fire.	01/11/97
Ind-63	Rawa Pening, Java, Indonesia	Exposed mud flats	01/11/97
Ind-64	Rawa Pening, Java, Indonesia	Rice paddy field	01/11/97
Ind-65	Mt. Merapi, Yogyakarta, Java, Indonesia	Volcanic soil, 1994 deposition	02/11/97
Ind-66	Mt. Merapi, Yogyakarta, Java, Indonesia	Volcanic soil, 1997 deposition	02/11/97
Ind-67	Turgo, Abandoned village, Yogyakarta, Java, Indonesia	Organic litter	02/11/97
Ind-68	Turgo, Abandoned village, Yogyakarta, Java, Indonesia	Rhizosphere soil from Jack Fruit tree	02/11/97
Ind-69	Solo, Mt Merapi, Java, Yogyakarta, Indonesia	Soil from cultivated land	21/11/97
Ind-70	White crater lake, Bandung, Java, Indonesia	Soil from acidic volcanic crater	29/11/97
Ind-71	Tea plantation, Bandung, Java, Indonesia	Soil from tea plantation	29/11/97
Ind-72	Tea plantation, Bandung, Java, Indonesia	Soil from exposed mud flats	29/11/97

Table 2.1. Continued.

Sample Code	Source	Sample Type	Date Collected
Jpn-73	Dive 871-2, Suruga Bay, Pacific Ocean	Soft sediment, 1400m	6/1996
Jpn-74	Dive 869-2, Suruga Bay, Pacific Ocean	Soft sediment, 1400m	6/1996
Jpn-75	Dive 872-1, Suruga Bay, Pacific Ocean	Soft sediment, 1400m	6/1996
Bdh-76	Dhaka, Bangladesh	Soil	
Spn-77	Canulobes Caves, Costa Blanca, Spain	Soil from outside caves	19/09/97
Ind-78	Sumatra, Indonesia	Soil	N/A
Ind-79	Sulawesi, Indonesia	Soil	N/A
Ind-83	Irian Jaya, Indonesia	Soil	N/A
Nmb-84	Kavango region, N. Namibia	Cultivated sandy soil	3/4/98
Nmb-85	Tsuemb region, N. Central Namibia	Calcerous soil	3/4/98
Nmb-86	Uitkomst Research Station, Tsuemb, N. Central Namibia	Soil	3/4/98
Nmb-87	Waterberg Area, Central Namibia	Kalahari sand	3/4/98
Nmb-88	Mahenene Research Station, N. W. Namibia	Soil	3/4/98
Nmb-89	Katima Mulilo, N.E. Namibia	Soil	3/4/98
Nmb-90	Ogongo, N.W. Namibia	Soil from cultivated lake bottom	3/4/98
*JPN-91	Sagami Bay, Pacific Ocean	Soft sediment, 1168m	11/93
*JPN-93	Sagami Bay, Pacific Ocean	Soft sediment, 1168m	12/94
*JPN-94	Dive 6K#127 Japan Trench, Pacific Ocean	Soft sediment, 6142m	07/92
*JPN-95	Dive 6K#126 Japan Trench, Pacific Ocean	Soft sediment, 6300m	07/92
*JPN-98	Dive 6K#067 Japan Trench, Pacific Ocean	Soft sediment, 6475m	07/91
*JPN-99	Dive 6K#175 Izu Bonin Trench, Pacific Ocean	Soft sediment, 2679m	9/93
*JPN-100	Dive 2K#869 Suruga Bay, Pacific Ocean	Soft sediment, 1487m	06/96
*JPN-102	Dive 2K#870 Suruga Bay, Pacific Ocean	Soft sediment, 1151m	06/96
*JPN-103	Dive 89-1 Suruga Bay, Pacific Ocean	Soft sediment, 1948m	N/A
*JPN-104	Dive 6K#274 Japan Trench, Pacific Ocean	Soft sediment, 6048m	07/95
*JPN-105	Dive 6K#275 Japan Trench, Pacific Ocean	Soft sediment, 4418m	07/95

*supplied by JAMSTEC. The submersible employed in the dive is denoted as: **2K** Shinkai 2000; **6K** Shinkai 6500. NA, not available.

2.2. Pre-treatment and selective isolation of micromonosporae

2.2.1. Pre-treatment of samples

All terrestrial samples were subjected to the dispersion and differential centrifugation procedure of Hopkins *et al.*, (1991) (see Fig. 2.1.). Following this fractionation phenol was added to all terrestrial samples to give a 1.5% solution. All terrestrial samples were placed in a water bath at 30°C for 30 minutes. Ten-fold serial dilutions were then prepared using sterile phosphate buffered saline, PBS (Appendix B). Small amounts (0.3-0.5g) of the Japanese marine sediments, Antarctic and Atlantic sediment samples were serially diluted with sterile PBS as there were limited reserves of material and the numbers of fungi tended to be lower thus alleviating the need for phenol treatments.

2.2.2. Isolation and enumeration of micromonosporae

Two selective media were chosen for their repeated efficiency in the recovery of micromonosporae, Colloidal chitin (Hsu and Lockwood, 1975) and M3 agar (Rowbotham and Cross, 1977). All media were supplemented with 100µg ml^{-1} cycloheximide to inhibit fungal growth. The cycloheximide was filtered through a 0.2µm Nalgene filter and added to molten agar prior to pouring plates. The environmental samples (see Table 2.1) were subjected to dispersion and differential centrifugation (Hopkins *et al.*, 1991) prior to being plated out. Aliquots (200µl) of the appropriate dilutions were plated out onto the selective media plates in triplicate. Control plates inoculated with sterile Ringers solution were set up for each isolation medium. Alkaliphilic/alkalitolerant micromonosporae were isolated on the same selective media (colloidal chitina and M3) but the pH was adjusted to pH 10.0 using 2M NaOH. Plates were incubated at 30°C and examined for growth on a weekly basis for 4 weeks, as growth of micromonosporae on these types of media are typically very slow. Representatives of each morphologically distinct colony type were sub-cultured onto Micromonospora megalomiceum media (DSM). Putative micromonosporae were identified on the basis of their colony morphology: yellow/orange wrinkled colonies that produced black spores upon maturation. All media formulations are described in Appendix B.

Figure 2.1. Dispersion and differential centrifugation technique (Hopkins *et al.*, 1991)

Add 1.0g of sediment to 10ml of sodium cholate solution (0.1%w/v) and vortex for one minute. Add another 10ml of sodium cholate solution, 10ml of Chelex 100 (chelating resin, sodium form) and 30 sterile glass beads. Shake this suspension on a flatbed shaker for two hours at 5°C.

↓

Centrifuge the sample at 2,000rpm for 2 minutes. Decant supernatant 1A.

↓

Resuspend the pellet in 10ml 0.1M Tris Buffer (pH 7.4). Shake for one hour at 5°C.

↓

Centrifuge the sample at 2,000rpm for one minute. Add this supernatant (1B) to 1A.

↓

Resuspend the pellet in 10ml of sodium cholate solution and ultrasonicate for one minute. Add a further 10ml of sodium cholate solution and shake the suspension for one hour at 5°C.

↓

Centrifuge the sample at 2,000rpm for one minute. Decant supernatant 2A.

↓

Resuspend the pellet in 10ml of Tris buffer and shake for one minute.

↓

Centrifuge the sample at 2,000rpm for one minute. Add this supernatant (2B) to 2A.

↓

Resuspend the pellet in 20ml of Tris buffer and shake for one hour at 5°C.

↓

Centrifuge the sample at 2,000rpm one minute. Decant supernatant 3A.

↓

Resuspend the pellet in 20ml of Tris buffer and shake for one hour at 5°C.

↓

Centrifuge at 2,000rpm for one minute. Add this supernatant (3B) to 3A.

Take the three, pooled supernatants 1(1A+1B), 2(2A+2B) and 3(3A+3B) and centrifuge at 12,000rpm for 20 minutes. Resuspend the pellets in 10ml of sterile phosphate buffered saline. Store samples at 4°C until ready to apply pre-treatments.

2.2.3. Purification and maintenance of isolates

Over 700 putative micromonosporae strains were selected from the primary isolation plates. The strains were chosen to represent the numbers and diversity in individual samples. The selected strains were purified by streaking onto Micromonospora megalomiceum agar and incubation at 30°C for 5-7 days. Impure strains were streaked onto fresh plates and this procedure was repeated until pure cultures were obtained. Several loopfuls of biomass from pure cultures were suspended in duplicate volumes of 1.0ml of sterile 20% (w/v) glycerol and stored at –20°C and –80°C (Wellington and Williams, 1978).

2.3. Preliminary identification of micromonosporae

2.3.1. Morphological characteristics

Micromonosporae can be characterised by their colony growth form on agar media. Typically, their colonies are yellow/orange and as they mature, they produce brown/black or olive spores. Colonies often have a wrinkled walnut appearance, although some are smooth and shiny.

2.3.2. Scanning electron microscopy (SEM)

A $1cm^2$ sample was cut from an agar plate culture and placed in a universal bottle, covered with solution A (2% paraforamldehyde and 2% gluteraldehyde in PBS buffer) and left overnight at room temperature. Solution A was decanted and the sample covered with 25% acetone and left for 10-15 minutes, this procedure was repeated with 50%, 75% and 100% acetone, thus eliminating water from the sample. Care was taken not to let the surface of the sample dry out when using 100% acetone. The previous step was repeated twice with 100% acetone leaving for 30 minutes or longer. The samples were transferred to wire mesh pots and the vessel was filled with 100% acetone. The samples were dried by critical point drying with CO_2 using a Polaron Critical Point Drying Apparatus Model E3000 (Polaron Equipment Ltd., Watford, UK). The samples were subsequently coated with gold for 5 minutes using an SEM coating unit E5000. Samples were scanned on an Hitachi S-430 microscope.

Table 2.2. Strain histories of *Micromonospora* type strains included in the polyphasic taxonomic analyses

Collection Code	Name	Source and strain history
DSMZ	*Micromonospora chalcea* subsp. *chalcea*[√]	
DSMZ 1040	*Micromonospora purpurea*[√]	Schering Plough Corp.
NCIMB 12659	*Micromonospora olivaterospora*[√]	K.Collins. Soil, paddy field suburban Hiroshima, Japan.
NCIMB 12660	*Micromonospora echinospora* subsp. *pallida*[√]	K.Collins. Soil, Syracuse, New York, USA
NCIMB 12661	*Micromonospora purpureochromogenes*[√]	K.Collins. Adobe soil, California, USA.
NCIMB 12663	*Micromonospora carbonacea* subsp. *carbonacea*[√]	K.Collins. Soil, Olean, New York, USA.
NCIMB 12664	*Micromonospora carbonacea* subsp. *aurantiaca*[√]	K. Collins. Soil.
NCIMB 12665	*Micromonospora coerula*[√]	K.Collins. Soil, Mount Heleakula, Maui Island, Hawaii, USA.
NCIMB 12741	*Micromonospora rhodorangea*[√]	K.Collins. Soil.
NCIMB 12744	*Micromonospora echinospora* subsp. *echinospora*[√]	K.Collins. Soil, Jamesville, New York, USA.
NCIMB 12751	*Micromonospora inositola*[√]	K.Collins. Soil, Hokkaido, Japan.
NCIMB 12754	*Micromonospora aurantiaca*[√]	K. Collins. Soil, USSR.
NCIMB 12880	*Micromonospora chalcea*[√] subsp. *chalcea*	K.Collins. Mud from Powers Lake, Connecticut, USA.
NCIMB 12882	*Micromonospora purpurea*[√]	K.Collins. Soil, Syracuse, New York, USA.
NCIMB 12895	*Micromonospora chalcea* subsp. *chalcea*	K.Collins. Soil.
NCIMB 12896	*Micromonospora rosaria*[√]	K.Collins. Soil, San Jacimto Texas, USA.
NCIMB 2223	*Micromonospora halophytica* subsp. *halophytica*[√]	A.Woyciesjes. Salt pool mud, Syracuse, New York USA.
NCIMB 2225	*Micromonospora halophytica* subsp. *nigra*[√]	A.Woyciesjes. Salt pool mud, Syracuse, New York USA.
DSMZ 43026	*Micromonospora chalcea*[√]	Chas. Pfizer 1464-217L
DSMZ 43116	*Micromonospora chalcea*	
DSMZ 43141	*Micromonospora echinospora* subsp. *ferruginea*[√]	A. Woyciesjes. Soil
DSMZ 43814	*Micromonospora brunnea*[√]	Soil.
DSMZ 43895	*Micromonospora chalcea*	

Table 2.2. Continued.

Collection Code	Name	Source and strain history
DSMZ 44151	*Micromonospora chersina*[√]	Soil
DSMZ 46121	*Micromonospora coerula*[√]	

[T], type strain

2.5. Pyrolysis mass spectrometric analysis of micromonosporae

2.5.1. Selection of strains for pyrolysis mass spectrometric analysis

Of the 700 strains recovered from the environmental samples, approximately 350 micromonosporae were selected to represent all the major locations and habitats studied (see Table 2.3.). Eighteen type strains of micromonosporae from two major culture collections, Deutsche Sammlung von Mikroorganism Zellkulturen (DSMZ) and the National Collection for Industrial and Marine Bacteria (NCIMB) were included in the studies. This allowed a comparison to be made of known species with the potentially novel strains recovered from the environmental samples.

Table 2.3. Strains used in polyphasic taxonomic analyses

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
NCIMB 9599	*M. chalcea* subsp. *chalcea*					X	
DSMZ 1040	*M. purpurea*[√]					X	
NCIMB 12659	*M. olivaterospora*[√]	X	X	X	X		
NCIMB 12660	*M. echinospora* subsp. *pallida*[√]	X	X	X	X		
NCIMB 12661	*M. purpureochromogenes*[√]	X	X	X			
NCIMB 12663	*M. carbonacea* subsp. *carbonacea*[√]	X	X	X			
NCIMB 12664	*M. carbonacea* subsp. *aurantiaca*[√]	X	X	X	X		

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
NCIMB 12665	*M. coerula*√	X	X	X	X		
NCIMB 12741	*M. rhodorangea*√	X		X			
NCIMB 12744	*M. echinospora* subsp. *echinospora*√	X	X	X	X		
NCIMB 12751	*M. inositola*√	X	X	X	X		
NCIMB 12754	*M. aurantiaca*√	X	X	X	X		
NCIMB 12882	*M. chalcea* subsp. *chalcea*	X	X	X	X		
NCIMB 12895	*M. chalcea* subsp. *chalcea*					X	
NCIMB 12896	*M. rosaria*√	X		X			
NCIMB 2223	*M. halophytica* subsp. *halophytica*√	X		X			
NCIMB 2225	*M. halophytica* subsp. *nigra*√	X	X	X			
DSMZ 43026	*M. chalcea* **T**	X	X	X	X		
NCIMB 43116	*M. chalcea*					X	
DSMZ 43141	*M. echinospora* subsp. *ferruginea*√	X	X	X			
DSMZ 43814	*M. brunnea*√	X	X	X	X		
DSMZ 44151	*M. chersina*√	X	X	X	X		
DSMZ 46121	*M. coerula*√					X	
5-001	Australia	X	X	X	X	X	
6-001	Australia	X	X	X	X	X	
6-003	Australia	X	X	X	X	X	X
6-004	Australia	X	X	X	X		
7-001	Australia	X		X	X		
7-002	Australia	X					
7-003	Australia	X		X		X	
7-004	Australia	X	X	X		X	
7-005	Australia	X		X			
7-006	Australia	X	X	X			
7-007	Australia	X	X	X		X	X
7-008	Australia	X	X	X	X		
9-001	Australia	X	X	X		X	
9-002	Australia	X					
9-003	Australia	X	X	X	X		

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
9-005	Australia	X		X			
9-013	Australia	X		X			
9-016	Australia	X	X	X	X		
9-018	Australia	X	X	X	X	X	
9-019	Australia	X	X	X	X		
9-026	Australia	X	X	X	X		
9-030	Australia	X	X	X		X	
9-031	Australia	X	X	X	X	X	X
9-032	Australia	X	X	X		X	
9-033	Australia	X		X			
9-034	Australia	X	X	X	X	X	
9-035	Australia	X	X	X	X		
10-002	United Kingdom	X	X	X	X		
10-005	United Kingdom	X	X	X	X		
10-008	United Kingdom	X	X	X			
10-010	United Kingdom	X	X	X	X		
10-011	United Kingdom	X	X	X	X		
10-014B	United Kingdom	X	X	X	X		
10-015	United Kingdom	X	X	X	X		
10-016	United Kingdom	X	X	X	X		X
10-020	United Kingdom	X	X	X	X		
11-001	United Kingdom	X	X	X			
11-002	United Kingdom	X	X	X	X		
11-003	United Kingdom		X	X			
12-002	United Kingdom	X	X	X			
12-003	United Kingdom			X			
12-004	United Kingdom	X	X	X	X		
12-005	United Kingdom		X	X			
12-007	United Kingdom	X	X	X	X		
12-008	United Kingdom	X	X	X	X		
12-010	United Kingdom		X	X	X		
12-011	United Kingdom	X	X	X	X		
12-012	United Kingdom		X	X			
12-017	United Kingdom	X	X	X	X		
13-001	Argentina	X		X	X		
13-017	Argentina	X		X			
13-018	Argentina	X		X			
13-020	Argentina	X		X			
13-021	Argentina	X		X			
13-027	Argentina	X	X	X	X		
13-037	Argentina	X		X	X		
13-041	Argentina	X		X			
13-042	Argentina	X	X	X	X		X
13-047	Argentina	X	X	X	X		
13-050	Argentina	X		X			
14-003	Argentina	X	X	X			
14-023	Argentina	X		X			

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
14-026	Argentina	X	X	X	X		
14-046	Argentina	X	X	X	X		
14-049	Argentina	X	X	X	X		
14-054	Argentina	X		X	X		
14-064	Argentina	X	X	X	X		
14-065	Argentina	X	X	X	X		
14-067	Argentina	X	X	X			
14-071	Argentina	X	X	X	X		
14-099	Argentina	X		X			
15-005	Brazil	X	X	X	X		
15-010	Brazil	X	X	X	X		
15-013	Brazil	X	X	X	X		
15-017	Brazil	X		X			
15-021	Brazil	X		X			
15-027	Brazil	X		X			
16-002	Czech Republic	X	X	X	X		
16-004	Czech Republic	X	X	X	X		
16-005	Czech Republic	X	X	X	X		
16-006	Czech Republic	X					
16-007	Czech Republic		X	X	X		
16-008	Czech Republic			X			
16-009	Czech Republic		X	X	X		
17-001	Czech Republic	X	X	X	X		
17-002	Czech Republic	X	X	X	X		
17-003	Czech Republic	X	X	X	X		
17-004	Czech Republic	X	X	X	X		
18-019	Sagami Bay	X	X		X		
18-024	Sagami Bay	X					
18-031	Sagami Bay	X					
18-032	Sagami Bay	X	X		X		
18-037	Sagami Bay	X	X				
18-047	Sagami Bay	X	X		X		X
18-050	Sagami Bay	X	X		X		
18-057	Sagami Bay	X					
18-061	Sagami Bay	X					
18-063	Sagami Bay	X					
18-065	Sagami Bay	X	X				
18-070	Sagami Bay	X					
18-072	Sagami Bay	X	X		X		
18-080	Sagami Bay	X	X		X		
18-085	Sagami Bay	X	X		X		
18-251	Sagami Bay	X	X		X		
19-002	Portugal	X					
19-003	Portugal	X	X	X	X		
19-004	Portugal	X		X			
19-007	Portugal	X		X			
20-001	Portugal	X	X	X	X		

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
20-002	Portugal	X	X	X	X		
20-006	Portugal	X	X	X	X		
20-009	Portugal	X	X	X	X		
21-001	Portugal	X	X	X	X		X
21-002	Portugal	X		X			
21-003	Portugal	X	X	X	X		
22-001	Portugal	X	X	X	X		
22-003	Portugal	X		X			
23-005	Portugal	X		X			
23-006	Portugal	X		X			
23-008	Portugal	X		X			
23-009	Portugal	X	X	X	X		
23-011	Portugal	X		X			
23-012	Portugal	X	X	X	X		
24-001	Portugal	X					
24-002	Portugal	X	X	X	X		
24-003	Portugal	X		X			
24-005	Portugal	X		X			
24-006	Portugal		X		X		
24-009	Portugal	X	X	X	X		
24-013	Portugal	X	X	X	X		
25-001	Antarctica	X	X		X		X
27-001	Antarctica	X	X				X
28-001	Antarctica		X		X		
33-001	New Zealand	X		X	X		
33-002	New Zealand	X		X			
33-003	New Zealand	X	X	X			
33-004	New Zealand	X	X	X			
33-006	New Zealand	X		X			
33-007	New Zealand	X		X			
43-006	USA	X		X			
43-022	USA	X	X	X	X		
43-023	USA	X		X			
43-026	USA	X	X	X	X		
43-038	USA	X	X	X	X		
43-039	USA	X					
45-007	USA	X		X			
45-008	USA	X	X	X	X		
45-010	USA	X					
45-016	USA	X		X			
45-019	USA	X	X	X	X		
45-030	USA	X		X			
45-032	USA	X		X			
47-002	USA	X		X			
47-004	USA	X	X	X	X		
47-017	USA	X		X			
47-018	USA	X	X	X	X		

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
47-022	USA	X	X	X	X		
47-027	USA	X	X	X	X		
47-042	USA	X	X	X	X		X
48-007	USA	X		X			
48-016	USA	X	X	X	X		
48-019	USA	X		X			
48-022	USA	X	X	X	X		
48-025	USA	X		X			
48-028	USA	X		X			
48-033	USA	X	X	X	X		
49-009	USA	X		X	X		X
49-011	USA	X	X	X	X		
49-014	USA	X		X			
49-016	USA	X	X	X	X		
49-023	USA	X		X			
49-025	USA	X	X	X	X		
50-007	Indonesia	X	X	X	X		
53-002	Indonesia	X		X			
53-003	Indonesia	X	X	X	X		
55-003	Indonesia	X	X	X	X		
55-011	Indonesia	X	X	X	X		X
56-002	Indonesia	X		X	X		
56-012	Indonesia		X				
57-004	Indonesia	X	X	X	X		
57-005	Indonesia	X		X			
57-017	Indonesia	X		X			
57-020	Indonesia	X	X	X	X		
57-034	Indonesia	X		X			
57-039	Indonesia	X					
58-003	Indonesia	X					
58-009	Indonesia	X		X			
58-012	Indonesia	X		X			
58-013	Indonesia	X		X			
58-024	Indonesia	X		X			
61-002	Indonesia	X		X			
63-002	Indonesia	X	X	X	X		X
63-003	Indonesia	X					
63-004	Indonesia	X		X			
63-006	Indonesia	X		X			
63-009	Indonesia	X		X			
63-013	Indonesia	X	X	X	X		
63-018	Indonesia			X			
63-032	Indonesia	X		X			
63-034	Indonesia		X		X		
64-001	Indonesia	X		X			
64-006	Indonesia	X	X	X	X		
64-008	Indonesia	X	X				

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
64-022	Indonesia	X	X	X	X		
66-003	Indonesia	X		X			
66-007	Indonesia	X		X	X		
67-004	Indonesia			X			
73-001	Suruga Bay	X					
73-002	Suruga Bay	X					
73-007	Suruga Bay	X					
73-008	Suruga Bay	X	X		X		
73-009	Suruga Bay	X					
73-010	Suruga Bay	X					
73-011	Suruga Bay	X	X		X		
73-012	Suruga Bay	X					
73-013	Suruga Bay	X					
73-013	Suruga Bay	X					
74-002	Suruga Bay	X					
74-003	Suruga Bay	X					
74-005	Suruga Bay	X					
75-001	Suruga Bay	X					
75-002	Suruga Bay	X					
75-003	Suruga Bay	X					
76-002	Bangladesh	X					
76-003	Bangladesh	X	X	X	X		
76-004	Bangladesh	X		X			
76-006	Bangladesh	X		X			
76-007	Bangladesh	X	X	X	X		
76-010	Bangladesh	X		X			
76-013	Bangladesh	X	X	X			
76-016	Bangladesh	X		X			
76-019	Bangladesh	X	X	X	X		
76-023	Bangladesh	X		X			
76-028	Bangladesh	X	X	X	X		
76-035	Bangladesh	X	X	X			
76-041	Bangladesh	X	X	X	X		X
76-044	Bangladesh	X	X	X	X		
77-005	Spain	X	X	X	X		
77-006	Spain	X		X	X		
77-012	Spain	X	X	X	X		
77-018	Spain	X		X			
77-021	Spain	X					
77-022	Spain	X					
77-027	Spain	X		X			
77-028	Spain	X	X	X	X		
77-029	Spain	X	X	X	X		
77-032	Spain	X		X			
77-034	Spain	X		X			
81-002	Indonesia			X			
81-004	Indonesia	X	X	X	X		

Table 2.3. Continued.

Culture collection and reference number	Type strain or geographic location	PyMS	Numerical Taxonomy	Antimicrobial Profiling	Rapid Enzyme profiling	SSCP	16S Sequencing
81-015	Indonesia	X	X	X	X		
84-001	Namibia	X	X	X	X		
84-005	Namibia	X		X			
85-001	Namibia	X		X			
85-005	Namibia	X		X			
85-006	Namibia	X		X			
85-008	Namibia	X		X			
86-003	Namibia	X	X	X	X		
86-005	Namibia	X	X	X			X
86-010	Namibia	X	X	X	X		
87-002	Namibia	X		X			
87-004	Namibia	X	X	X	X		
87-008	Namibia	X	X	X	X		
89-006	Namibia	X	X	X	X		
89-008	Namibia	X	X	X	X		
89-009	Namibia		X	X	X		
89-010	Namibia	X		X			
90-002	Namibia	X	X	X			
90-004	Namibia	X		X			
90-011	Namibia	X	X	X	X		
91-001	Sagami Bay		X		X		
91-002	Sagami Bay		X		X		
91-003	Sagami Bay	X			X		
91-004	Sagami Bay	X	X		X		
91-005	Sagami Bay	X	X		X		
93-001	Sagami Bay	X					
93-002	Sagami Bay	X					
93-003	Sagami Bay	X					
93-004	Sagami Bay	X					
93-005	Sagami Bay	X					
93-006	Sagami Bay	X					
93-007	Sagami Bay	X					
93-008	Sagami Bay	X	X		X		
93-009	Sagami Bay	X					
93-010	Sagami Bay	X	X		X		
93-011	Sagami Bay	X	X				
94-001	Japan Trench	X					
95-001	Japan Trench	X					
98-001	Japan Trench	X					
99-001	Izu Bonin Trench	X					
100-001	Suruga Bay	X					
100-002	Suruga Bay	X	X				
102-001	Suruga Bay	X					
102-002	Suruga Bay	X			X		
102-003	Suruga Bay	X					

√, type strains; T, type genus.

2.5.2. Growth conditions

The selected strains were put into sub-groups of 32 strains according to the geographic location they originated from, i.e. strains from South America. Each sub-group was grown alongside the 18 type strains on Micromonospora megalomiceum agar in preparation for PyMS analysis. Growth conditions were standardised, this included all the media being from one batch and all plates being incubated in one incubator for the same period. A small loopful of biomass was spread over a sterile 0,45μm cellulose nitrate membrane, which had been placed on a Micromonospora megalomiceum plate. All strains were incubated at 30°C for 5 days.

2.5.3. Preparation of samples

Viton O-ring collars, used to seal the pyrolysis tubes at the inlet system to form a high vacuum, were positioned approximately 2 mm from the edge of the pyrolysis tubes. Ferro-nickel foils (Curie-point 530°C) were inserted into glass pyrolysis tubes (Horizon Instruments Ltd; Heathfield, West Sussex, England) to extend about two-thirds of their length out of the tubes. Biomass (ca. 50μg) was scraped from the membrane filters using a sterile disposable loop and smeared uniformly over the upper surface of foils. The size of the sample applied to each of the foils was adjusted initially to ensure that ion counts greater than one million were obtained. The tubes were placed in an incubator at 80°C for 10 minutes to ensure the biomass adhered to the foils and to dehydrate the sample thus preventing autolysis. The inoculated foils were then pushed inside the pyrolysis tubes using a stainless steel depth gauge, so that the tips of the foils lay 10 mm from the mouth of the tube. The tubes were loaded onto a sample carousel in a sequential series of replicates (A1, B1, C1,..A2, B2, C2,..A3, B3, C3,..etc) in order to facilitate the calculation of mass ion characteristicity ranks without interference from machine drift during the run.

Pyrolysis mass spectrometry (PyMS) was carried out using a Horizon RAPyD 400 instrument (Horizon Instruments Ltd; Heathfield, West Sussex, England). Liquid nitrogen was added to the cold trap reservoir of the RAPyD 400 prior to

running the samples. The temperature of the tube heater was set at 160°C and the expansion chamber at 170°C. The molecular beam was set to 180°C and the ion source to 190°C. The mass ion range was set to 50-150 as this usually gave the best discrimination. A set of 6 tubes prepared with randomly selected strains were pyrolysed prior to the full run in order to condition the system with a typical pyrolysate and to check the integrity of the machine. The rest of the analysis is carried out under the control of a PC.

2.5.4. Pyrolysis and mass spectrometric detection of fragments

Curie-point pyrolysis was carried out at 530°C for 2.4 seconds under vacuum with a temperature rise time of 0.6 seconds. The pyrolysate was ionised by collision with a low energy (30 eV) electron beam producing molecular ions. Any un-ionised products freeze on the cold trap, cooled with liquid nitrogen. The ions are accelerated out of the electron beam and then separated in a quadropole mass spectrometer, which delivered ions of a specific mass to the detector. The computer recorded the number of ions at each mass before altering the electromagnetic field in the quadropole, allowing ions of the next mass through. Ion counts were accumulated at scanning intervals of 0.35 seconds. Integrated ion counts for each sample were recorded as quantitative mass spectra and stored on a hard disk together with the total ion counts and pyrolysis sequence numbers.

2.5.5. Data analysis

The RAPyD software (Horizon Instruments) contains the statistical programs used for data analysis. Individual samples with very high ($> 6 \times 10^{15}$) or very low ($< 1 \times 10^{6}$) total mass ion counts were deleted from the analysis. Sample size variation between spectra was corrected in a normalisation procedure involving the calculation of the reproducibility of individual masses for the sample replicates (triplicate tubes), elimination of the masses which showed poor reproducibility and renormalization of the data based on the remaining masses. The ranked masses were the subject of principal component analyses (PCA) and canonical variate analyses (CVA). The results of the PCA and CVA analyses

were displayed as ordination plots which contained information on the relative position of the strains in multidimensional space. At this stage of the analysis, outliers from triplicate samples, if present, were removed from the dataset, which was then reanalysed. The PCA and CVA analyses were carried out using the GENSTAT statistical package (Numerical Algorithms Group, UK). Mahalanobis distances (Mahalanobis, 1936) calculated for each pair of strains were used to construct dendrograms using the unweighted pair group method with arithmetic averages algorithm (UPGMA; Sneath and Sokal, 1973).

2.6. Numerical taxonomic analysis of micromonosporae strains

2.6.1. Selection of strains for phenotypic tests

One hundred and ninety two test strains, including eighteen type strains of the genus *Micromonospora* and 164 micromonosporae isolates representative of the different regions and habitats studied and 15 duplicated cultures were examined (see Table 2.3).

2.6.2. Growth conditions

All of the strains were grown on Micromonospora megalomiceum agar for 5 days at 30°C. Large loopfuls of biomass was scraped from the plate and suspended in 2.0ml ¼ strength sterile Ringer's solution (Appendix B). If the colonies were hard or dry, a few sterile glass beads were added to help break up the biomass. These suspensions were stored at -20°C until they were required when they were thawed and vortexed prior to use.

2.6.3. Preparation of media

Basal medium was prepared according to Stevenson's method (1967) and was supplemented with carbon sources (see Table 2.4 for details). Agar A, for a final concentration of 10.0g/l (1%) 11.8g of agar (Oxoid No. 1) was added to 1 litre of distilled water. For agar B, a final concentration of 1.0g/l (0.1%) 11.2g of agar (Oxoid No. 1) was added to 1 litre of distilled water. Both agars were autoclaved at 121°C for 15 minutes.

Table 2.4. Characters examined in the numerical taxonomic study

Biochemical tests
Hydrogen sulphide production
Nitrate reduction

Substrate degradation tests
Arbutin
Casein
Cellulose
Chitin
Elastin
Gelatin
Starch
Tributyrin
Tween 20
Tween 40
Tween 60
Tween 80
Xylan

Nutritional tests
Sole carbon source (1% w/v)

Deoxy sugars
α-D (+) Fucose
α-L (-) Fucose

Disaccharides
D (+) Cellobiose
α-Lactose
Maltose
α-D (+) Melibiose
Sucrose
D (+) Trehalose
D (+) Turanose

Glycosides and related compounds
N-Acetyl-D-glucosamine
Arbutin
D-Gluconic acid
D-Glucosamine
Methyl-α-D-glucopyranoside
Methyl-β-D glucopyranoside
Methyl-α-D-mannopyranoside
Methyl-β-D-xylopyranoside

2-Keto-D-gluconic acid
Salicin
D (-) Mandelic acid

Carboxylic acids
Sodium acetate
Sodium-n-butyrate
Sodium propionate

Monosaccharides
Pentoses
L (+) Arabinose
D (-) Arabinose
D-Lyxose
D (-) Ribose
D (+) Xylose

Hexoses
D (-) Fructose
D (+) Galactose
D (+) Mannose
α-L-Rhamnose
L (-) Sorbose

Polysaccharides
Glycogen
Inulin
Starch
Xylan

Trisaccharides
D (+) Melizitose
D (+) Raffinose

Sole carbon source (0.1% w/v)
Aliphatic amino acids
DL-α-Alanine
L-Arginine
L-Ornithine monohydrochloride
L-Serine
L-Threonine
Amines
Spermine tetrahydrochloride

Aromatic amino acids
L-β-Phenylalanine
L-Proline
D-Tryptophan
L-Tryptophan
L-Tyrosine

Aromatic hydroxy acids
Ferulic acid

m-Hydroxybenzoic acid
p-Hydroxybenzoic acid

Tolerance to chemical inhibitors (%w/v)
Sodium chloride 1.0
3.0
5.0

Table 2.4. Continued.

Carboxylic acids	
Sodium pyruvate	
Dicarboxylic acids	
Sebacic acid	
Physiological tests	
Resistance to antibiotics (µg/ml)	
Aminoglycosides	
Gentamicin sulphate	5
Gentamicin sulphate	25
Neomycin sulphate	5
Neomycin sulphate	25
Streptomycin sulphate	5
Streptomycin sulphate	25
Coumarin	
Novobiocin	5
Novobiocin	25
β-Lactams	
Penicillin	
Penicillin V	5
Penicillin V	25
Rifamycin	
Rifampicin	5
Rifampicin	25
Tetracycline	
Tetracycline	5
Tetracycline	25
Miscellaneous	
Chloramphenicol	5
Chloramphenicol	25

Tolerance to chemical inhibitors (%w/v)	
Sodium chloride	7.0
Growth at pH	
	4.0
	5.0
	6.0
	7.0
	8.0
	9.0
	10.0

The nitrogen source was prepared by dissolving 67.0g of yeast nitrogen base in 1 litre of distilled water. Casamino acids (Difco) (100mg) were added to the basal medium and the solution was sterilised by filtration through a 0.2μm cellulose nitrate membrane. This gave a 10x nitrogen stock solution, 25ml of which was added to 212.5ml of agar A and 222.5ml of agar B. Negative control plates were prepared without an added carbon source, while positive control plates were supplemented with glucose (1%).

Carbon sources were prepared by dissolving either 2.5g in 12.5ml (for a 1% solution) or 0.25g in 2.5ml (for a 0.1% solution) in distilled water. All carbon sources were sterilised by tyndallisation, this involved steaming at 100°C for 30 minutes on three consecutive days. The carbon sources were added to bottles of molten agar A and agar B and plates were poured. The final composition of the media bottles was as follows:

1% CARBON SOURCES	**0.1% CARBON SOURCES**
212.5ml agar A	222.5ml agar B
25.0ml nitrogen source	25.0ml nitrogen source
12.5ml carbon source	2.5ml carbon source

2.6.4. Inoculation of test media

Inoculation of the test media was performed using a Denley multi-point inoculator (Denley Instruments Ltd., Billingshurst, West Sussex, UK), which allowed plates to be simultaneously inoculated with 10 test strains in a pre-set pattern. For the nutritional tests, negative control plates, lacking a carbon source and positive plates, containing glucose as the carbon source, were also inoculated. For the antibiotic sensitivity test, a negative control plate lacking antibiotic was set up.

Inoculated plates were incubated at 30°C and read at 7, 14 and 21 days for evidence of carbon source utilisation, substrate degradation or antibiotic sensitivity. For carbon source utilisation, a positive result was recorded if the growth in the presence of a carbon source was greater than that recorded in the positive control, similarly a negative result was recorded if the growth was equal

or less than that in the negative control. For degradation tests, if zones of degradation were visible around the point of inoculation then a positive result was recorded. For antibiotic sensitivity, a positive result was recorded if growth on the antibiotic plate was equal to or greater than that on the negative plate, i.e. a plate lacking antibiotic.

2.6.5. Biochemical tests

2.6.5.1. Nitrate reduction

Nitrate reduction was determined using the method described by Williams *et al.* (1983). Tubes of medium (4ml) were inoculated with 100µl of the strain suspension and incubated at 30°C for 14 days. One drop each of the Greiss-Ilovsvay reagents I and II were added to each tube (Appendix B). Each suspension was vortexed and the colour change observed. The development of a pink-red colour was indicative of a positive result caused by the reduction of nitrate to nitrite.

For any tubes where there was no visible reaction a small amount of zinc dust was added. These tubes were vortexed and examined for the development of a red colour that was indicative of the presence of nitrate, these tubes were scored negative. Tubes that still did not show any reaction were scored positive as this indicated that nitrate had been first reduced to nitrate and then to ammonia.

2.6.5.2. Hydrogen sulphide production

The production of hydrogen sulphide was examined according to the method of Küster and Williams (1964b) by inoculating tubes of media (4ml) with 100µl of strain suspension and inserting strips of sterile lead acetate paper (Merck) into the necks of the tubes, then incubating at 30°C. The strips were examined after 14 days for blackening which indicated the conversion of lead acetate to lead sulphide by hydrogen sulphide. Blackening of the strips was recorded as a positive result.

2.6.6. Data analysis

All data were recorded as positive or negative and converted to binary (1/0) format for the purposes of computer analysis. Data from the antimicrobial profiling and rapid enzyme profiling were added to the data generated from the numerical taxonomic analyses in an Excel spreadsheet. These data were then edited in a DOS program (Program Editor) to give a format acceptable for the MVSP 3.1 statistical program. The data file was imported into MVSP 3.1 for statistical analyses. The procedures used to analyse the data were the simple matching (S_{SM}; Sokal and Michener, 1958) and the Jaccard (S_j; Jaccard, 1908) coefficients. Clustering was achieved using the unweighted pair group method with arithmetic averages algorithm (UPGMA; Sneath and Sokal, 1973). The results of the analyses were presented as dendrograms in MVSP 3.1.

2.7. Antimicrobial profiling

2.7.1. Selection of strains for antimicrobial profiling

Two hundred and nineteen isolates and 18 type strains were selected for inclusion in the antimicrobial profiling (see Table 2.3.).

2.7.2. Selection of assay organisms

Six assay organisms were selected to demonstrate a wide spectrum of antimicrobial activity (see Table 2.5.) as previously used by Takizawa *et al.*, (1993) in a study of actinomycetes in Chesapeake Bay.

2.7.3. Growth conditions for assay and test strains

The six assay organisms were grown on ½ NA plates for 48 hours at 30°C. Selected test strains were grown on Micromonospora megalomicea medium at 30°C for 5-7 days.

2.7.4. Application of test strains to assay organism plates

Biomass of the assay organisms was suspended in 1.0ml of PBS (Appendix B). Aliquots (100µl) were pipetted onto ½ NA plates and spread with a sterile glass spreader. Agar plugs (11mm) were taken from areas of confluent growth on agar

plates of the selected test strains using a sterile core borer and placed onto the lawns of assay organisms. Four isolates were placed on each seeded plate of target organisms. The plates were incubated at 30°C and were examined daily for inhibition of growth of the assay organism.

Table 2.5. Target strains used in the antimicrobial profiling study

Strain	Source
Bacillus subtilis	UKC culture collection*
Candida albicans	UKC culture collection*
Enterococcus faecalis	NCIMB 775
Escherichia coli	UKC culture collection*
Pseudomonas aeruginosa	UKC culture collection*
Staphylococcus aureus	UKC culture collection*

* Research School of Biosciences (UKC) culture collection

2.7.5. Detection of antimicrobial activity

Antimicrobial activity was determined by observing the zones of inhibition surrounding the plugs of micromonosporae. A positive result was recorded wherever there was any evidence of inhibition. These results were used to calculate a diversity index (total number of antimicrobial patterns/ total number of isolates tested). These (+/-) results were also converted to a binary (1/0) format and added to the data generated in the numerical taxonomic analyses.

2.8. Rapid enzyme profiling

2.8.1. Selection of isolates for rapid enzyme profiling

Selection of the isolates was based on isolates included in the numerical taxonomic analyses. 140 isolates were chosen to represent the range of geographic locations and habitats assessed, these were analysed alongside 11 Type strains (see Table 2.3, Chapter Two). Fifteen duplicates were included in the study to test reproducibility of the results.

2.8.2. Growth conditions for test and type strains

All strains were grown on Micromonospora megalomiceum agar for 5 days at 30°C.

2.8.3. Preparation of strains for rapid enzyme profiling

Biomass was scraped from the plates, suspended in 2ml of sterile phosphate buffer (pH 7.4) and vortexed until an homogenous suspension was achieved. The density of each bacterial suspension was measured using a Densimat (bioMerieux, Marcy, L'Etoile, France), which is read in McFarland units. The McFarland index is a series of barium sulphate standards of known turbidity/absorbance. The turbidity for each standard is calculated to be equivalent to the absorbance attributable to a certain concentration of organisms. More biomass or phosphate buffer was added until the suspension was in the target range of 3.6 to 4.0. This gave a standardised concentration of cells for each test.

2.8.4. Preparation of enzyme test substrates and inoculation of strains

The substrates (see Table 2.6) were weighed into sterile universals and 11.0ml of sterile phosphate buffer (pH7.4) was added. Substrates that did not dissolve at room temperature were placed in a water bath (60°C) until the substrate had dissolved. Two micro-titre plates containing 96 wells were labelled for each substrate. Aliquots of the organisms (50µl) were pipetted into the wells. The substrates (100µl) were pipetted into the wells using a multi-pipette and the fluorescence was measured immediately using an automated Labtech Biolite F1 fluorescence microtitre plate reader (Labtech International Limited, Uckfield, UK) to give a time zero reading. Each micro-titre plate contained a control well, in which there was no organism, and this gave a reading for the background fluorescence. The micro-titre plates were incubated at 30°C and measurements of the fluorescence were taken after 18 hours. Data processing was performed using a combination of Biolite Software (Astroscan Ltd.) and Microsoft Excel 5.0 using a standard PC.

Table 2.6. Enzyme substrates used for rapid enzyme profiling

	Substrate	MW
1	4-methylumbelliferyl-beta-D-galactopyranoside	338.32
2	4-methylumbelliferyl-beta-D-glucopyranoside	338.32
3	4-methylumbelliferyl-N-acetyl-beta-D-glucosamide	379.4
4	4-methylumbelliferyl-beta-D-glucuronide	406.35
5	4-methylumbelliferyl-7-beta-D-xyloside	308.3
6	4-methylumbelliferyl-alpha-D-glucoside	338.32
7	4-methylumbelliferyl-alpha-D-galactoside	338.32
8	4-methylumbelliferyl-beta-D-fucoside	322.3
9	4-methylumbelliferyl-alpha-D-mannoside	338.32
10	4-methylumbelliferyl-phosphate	300.1
11	4-methylumbelliferyl-sulfate	294.3
12	L-analyl-7-amido-4-methylcoumarin	360.3
13	Leucyl-amido-4-methylcoumarin	293.8
14	Pyrolyl- amido-4-methylcoumarin	272.3
15	Pyroglutamyl- amido-4-methylcoumarin	286.29
16	4-methylumbelliferyl-beta-D-mannopyranoside	338.3
17	Glutamyl- amido-4-methylcoumarin	304.3
18	Lysyl- amido-4-methylcoumarin	303.4
19	Asparginyl- amido-4-methylcoumarin	410.9
20	Phenylalanyl- amido-4-methylcoumarin	322.4
21	Z-glycyl-prolyl- amido-4-methylcoumarin	463.5
22	Z-arginyl- amido-4-methylcoumarin	465.5
23	4-methylumbelliferyl-beta-cellobioside	500.5
24	4-methylumbelliferyl-butyrate	246.3
25	H-Ornithine- amido-4-methylcoumarin .2HCl	289.3
26	L-histidine-7-amido-4-methylcoumarin	312.3
27	4-methylumbelliferyl-beta-D-ribofuranoside	308.3
28	4-methylumbelliferyl-alpha-L-arabinoside	308
29	4-methylumbelliferyl-nonanoate	316.4
30	4-methylumbelliferyl-dodecanoate	351.42
31	4-methylumbelliferyl-palmitate	414.6

2.8.5. Manipulation of data

The data generated for the time zero and 18-hour readings were imported into an Excel spreadsheet. The time zero readings were subtracted from the 18-hour readings to give an overall fluorescence value. The background fluorescence value observed in the control wells was also subtracted from the test readings. Histograms were generated for each test substrate using the fluorescence data from each of the test organisms. The mid-point in the range of fluorescence values was set as the cut-off point; organisms were then attributed with either positive or negative responses depending on whether their fluorescence values

were above (positive) or below (negative) this fluorescence value (Rowlands, 1993). The cut-off values were used to produce +/- data for each test.

The results for the duplicated strains were analysed and information on tests shown to have a high-test error was omitted from the enzyme data matrix. The percentage positive for each of the tests was also calculated and any tests with identical results for all of the test strains were deleted from the database. The +/- results were converted to binary format (1/0) and were added to those already generated in the numerical taxonomic analysis.

2.9. Molecular systematics

2.9.1. Selection of strains for 16S rRNA gene sequencing

Representative putative micromonosporae were selected for sequencing by inspecting the clusters generated in the PyMS and numerical taxonomic analyses. Final decisions were based on the RFLP-SSCP profiles, which allowed the elimination of isolates with identical profiles.

2.9.2. Isolation of DNA

Genomic DNA from the bacterial strains was extracted and purified according to Pitcher *et al.*, (1989). This procedure was followed by a phenol-chloroform extraction to obtain DNA of the required purity to act as a template for the polymerase chain reaction.

The procedure was as follows:

1. Approximately 50 mg of biomass was taken from the surface of 5-day-old Micromonospora megalomiceum plate cultures incubated at 30°C, using a disposable plastic loop. The biomass was transferred to a 1.5 ml Eppendorf tube containing 100µl lysozyme solution (50 mg ml^{-1} in 10 mM Tris-HCl buffer, pH 8.0). The tubes were incubated for 12h at 37°C;
2. Complete cell lysis was achieved by the addition of 500 µl GES reagent (see Appendix B) and incubation at 37°C for 10 minutes;

3. Lysates were cooled on ice for 5 minutes and 250 µl of cold 7.5M ammonium acetate were added and the tubes mixed gently by inversion;
4. The lysates were left on ice for a further 10 minutes prior to the addition of 500 µl of chloroform/2-pentanol (24:1, v/v). The aqueous and organic phases were emulsified by shaking by hand and separated by centrifugation at 13000 rpm for 5 minutes in a microfuge;
5. The aqueous phase was transferred to a new Eppendorf tube, 0.54 volumes of cold iso-propanol (-20°C) were added to precipitate the DNA;
6. The tubes were inverted several times to mix the solutions until a white fibrous precipitate appeared. DNA was pelleted by centrifugation at 13 000 rpm for 15 minutes. The supernatant was decanted off, with care being taken to retain the pellet;
7. The pellets were washed twice with 500µl of 70% ethanol, and dried in an oven at 37°C;
8. The pellets were redissolved in 100µl of TE buffer (see Appendix B) and left to dissolve at 4°C.

2.9.3. Purification of genomic DNA (Pitcher, 1989)

1. Residual protein was removed by phenol chloroform extraction: 100 µl of phenol chloroform (1 phenol: 1 chloroform/isoamyl alcohol (24 chloroform: 1 isoamyl alcohol)) was added to each 100 µl DNA solution in an Eppendorf tube which was mixed by inversion for 20-30 seconds to give a milky suspension. The mixture was centrifuged at 13 000 rpm for 10 minutes and the upper aqueous phase was carefully removed, leaving the solid precipitate at the interface between the phases and put in a fresh Eppendorf tube. If the quantity of precipitate was substantial, this phenol chloroform extraction was repeated;
2. 100 µl of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase from the previous step. The mixture was inverted several times and centrifuged at 13 000 rpm for 10 minutes;
3. The aqueous phase was carefully removed and transferred to a clean Eppendorf tube. Two volumes of cold absolute ethanol were added, and the

mixture was inverted several times before leaving at -20°C for 1-2 hours to increase precipitation of the DNA from solution;

4. The DNA was pelleted by centrifuging at 13 000 rpm for 15 minutes, and the remaining liquid carefully decanted;
5. Ice-cold 70% ethanol (200 µl) was added to wash the pellet, and the tubes centrifuged for 10 minutes at 13 000 rpm;
6. The pellet of DNA was dried in an oven at 37°C, and then redissolved in 50 µl of TE buffer;
7. The concentration of DNA in the solution was determined by means of an UV spectrophotometer (BioRad, Laboratories; Hercules, California 94547), by measuring the absorbance of the solution at 260 nm. It was assumed that a solution of native DNA has an A_{260} of 1, taking the mass of one nucleotide pair as 660 Da (Chargaff, 1955).
8. The DNA extractions were stored at 4°C.

2.9.4. Amplification of 16S ribosomal DNA by the polymerase chain reaction (PCR)

The 16S rDNA was selectively amplified from genomic DNA templates by using PCR (Saiki *et al.*, 1988) with oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of eubacterial 16S rDNA genes. Primers were obtained from Oswel, (Southampton, UK). The primer sequences are shown below (DeLong *et al.*, 1992):

Eubac27F	AGA GTT TGA TCC TGG CTC AG
Eubac1492R	GGT TAC CTT GTT ACG ACT T

PCR amplification was performed with a DNA Thermal Cycler model PTC-200 (MJ Research, Massachusetts, USA) under the following conditions: Approximately 100 ng of template DNA, 10 µl of 10 x reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3 at 20°C), 6 µl $MgCl_2$ (Promega, UK), 2.5 U of Taq DNA polymerase (Boehringer Mannheim), 1 µM upstream primer, 1 µM downstream primer, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM

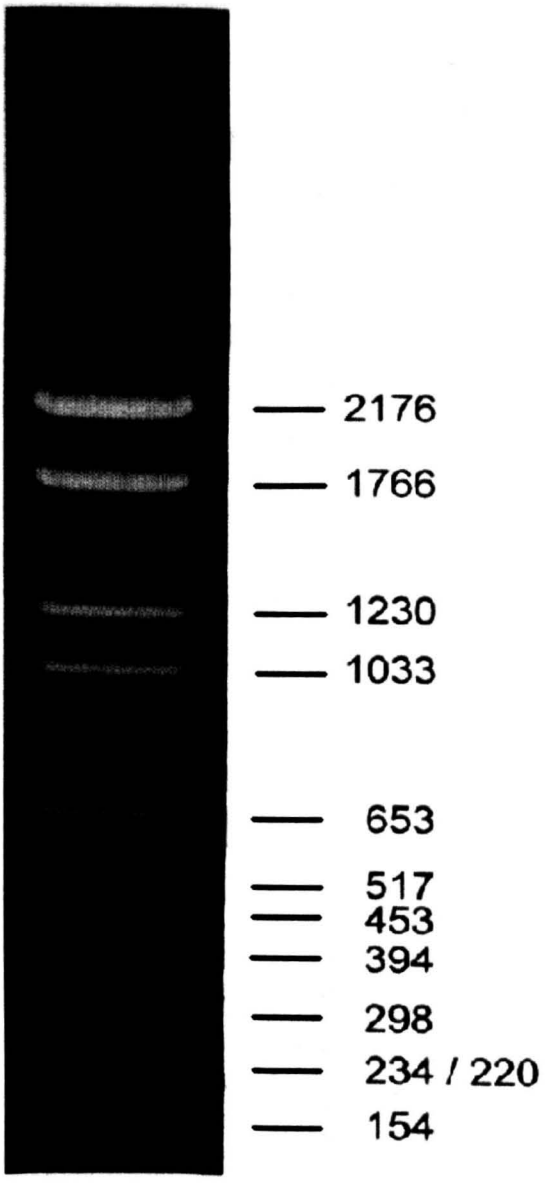
dTTP (dNTPs supplied as a 10 mM stock mix), distilled water combined in a total volume of 100 µl.

The tubes were incubated at 95°C for 5 minutes then the following amplification program: denaturation at 95°C for 45 seconds, primer annealing at 55°C for 45 seconds, chain extension at 72°C for 2 minutes with an additional extension time of 10 minutes on the final cycle, for 40 cycles. The tubes were then stored at 4°C.

2.9.5. Detection of PCR products by agarose gel electrophoresis

PCR products were detected by agarose gel electrophoresis. Gel loading buffer (2µl) was added to 3µl of the PCR reaction and loaded into the well of a 1.0% agarose gel made with TAE buffer. A set of reference markers (VI marker, Boehringer Mannheim) was also loaded to enable the identification of the size of the PCR products. The gel was prepared and run in 1x TAE buffer at 70 V for 1-2 hours, until the dye front had run across 75% of the length of the gel. The gel was stained for 20 minutes in an ethidium bromide solution (0.5µg/ml), followed by destaining in distilled water for 10-15 minutes. The bands on the gel were visualised by UV excitation. Amplified 16S rDNA bands are approximately 1.5 kb in size and can be identified with reference to a standard set of known molecular weight markers VI Boehringer Mannheim (Mixture of pBR328 DNA cleaved with *Bgl* 1 and pBR328 DNA cleaved with Hinf 1) see Figure 2.2) which is run alongside the PCR reactions on the gel. See Appendix B for reagent recipes.

Figure 2.2. *Bgl* 1/*Hinf* 1 digest of pBR328 DNA, used as a set of molecular weight markers



Molecular weight marker VI from Boehringer Mannheim ranging from 0.15-2.1 kbp.

2.9.6. Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) was detected following a modified version of the protocol described by Moyer *et al.* (1994). PCR products were digested using a tetrameric endonuclease (enzyme with a restriction target site of four base pairs) as follows: 0.1 µl of the restriction enzyme *Alu*I; 5 µl of the completed PCR reaction mixture, 3.8 µl dw; 1 µl Restriction Buffer (33mM Tris-acetate, pH7.9; 10mM Mg $(OA_C)_2$; 66mM KOA_C; 0.5mM diothreitol) to give a final reaction volume of 10 µl. Tubes were incubated in a waterbath at 37°C for two hours. The restriction fragments were separated by electrophoresis on a 2.5% agarose gel. The gel was stained for 20 minutes in an ethidium bromide solution (0.5µg/ml), followed by destaining in distilled water for 10-15 minutes. The bands on the gel were visualised by UV excitation. The size of the RFLP fragments was determined by comparison with molecular weight marker VI (see Fig. 2.2.).

2.9.7. Single strand conformation polymorphism analysis

Single strand conformation polymorphism (SSCP) was detected using a procedure described by Schweiger and Tebbe (1998). Representative isolates from the major PyMS and numerical taxonomic analyses, incorporating a wide range of geographic locations, were selected for inclusion in the SSCP analysis. Isolates were first subjected to RFLP, following which 15 µl of SSCP denaturing buffer was added to 5 µl of RFLP product. These were denatured using the Peltier DNA Thermocycler on a program set for 2 minutes at 94°C. The products were immediately put onto ice and 4.5µl of each product was loaded onto a 0.6X MDE gel (see Appendix B) with 0.6X TBE buffer. Empty gel lanes were loaded with denaturing buffer. The gel was run in a Hoefer$_{TM}$ SQ3 Sequencer at 4 Watts for approximately 8.5 Vh.

The staining procedure for the gel was as follows:

1. 20 minutes in 10% acetic acid.
2. Three washes of 2 minutes in distilled water,
3. 30 minutes in silver nitrate stain.
4. Wash with distilled water.

5. 6-12 minutes in sodium carbonate, stain until the bands become visible but remove when the gel turns yellow at the corners.
6. 15 minutes in 10% acetic acid.
7. 15 minutes in distilled water.
8. Dry for 1hour at 70°C.
9. Visualize on a light box.

See Appendix B for details of the solutions used.

2.9.8. Purification of amplified DNA from PCR reactions

Amplified DNA was purified from the reaction components by using a Qiaquick PCR Purification Kit (Qiagen, Crawley, W. Sussex, UK). The concentration of purified DNA was determined by UV spectrophotometry (see section 2.7.2) and adjusted to 40 ng μl^{-1} prior to sequencing.

Chapter Three

Selective Isolation

3.1. Introduction

Innumerable micromonosporae have been isolated from natural environments, including terrestrial, freshwater and marine habitats (Erikson, 1941; Rowlands, 1993; Umbreit and McCoy, 1941, Cross and Collins, 1966; Rowbotham and Cross, 1977; Weyland, 1969, 1981). Selective isolation involves a choice of extraction procedures, pre-treatment of samples and media upon which to grow the isolates. Procedures recommended for the selective isolation of actinomycetes have been extensively reviewed (Hayakawa and Nonomura, 1987; Nolan and Cross, 1988; Hsu and Lockwood, 1975; Jayashree *et al.*, 1991). This study sought to determine the most effective combination of extraction techniques, pre-treatments and selective media for the isolation of micromonosporae from environmental samples.

3.2. Characterisation of samples

3.2.1. Location and habitat of samples

Samples investigated during the course of this project came from locations encompassing all Continents and a variety of habitats: Africa, the Americas, Antarctica, Asia, Australasia, and Europe. Details of sample locations are given in Figure 1.3 Chapter One and Table 2.1 Chapter 2.

3.2.2. Collection, storage and characterisation of samples

Samples were collected and stored in plastic bags at 4°C. Aliquots of the samples were removed using aseptic technique to reduce potential contamination of the samples. The quantity of material available for many of the marine sediments was less than 0.5ml; therefore, it was deemed impracticable to use it for physical characterisation. Table 3.1 lists the pH values for the samples examined in this study.

Table 3.1. pH values of soil and sediment samples

Sample Code	Source	Sample Type	pH
Aus-1	Cradle Mountain National Park, Tasmania, Australia. Southern end of Lake Dove	Temperate rainforest.	4.4
Aus-2	Cradle Mountain National Park, Tasmania, Australia. Lake Dove opposite Ballroom Forest	Lake surface sediment. Temperate rainforest.	4.9
Aus-3	River Que (headwaters), Tasmania, Australia	Mixed soil sample from eucalypt and other root zones.	5.1
Aus-4	Daintree River National Park, Queensland, Australia.	Soil sample from under Moreton Bay Ash. Tropical rainforest.	5.7
Aus-5	Mount Lewis, Great Dividing Range, Queensland, Australia.	Rhizosphere soil from White Silky Oak. Tropical rainforest.	5.3
Aus-6	Mossman Gorge, Daintree River National Park, Queensland, Australia	Rhizosphere soil from Strangler Fig. Tropical rainforest	6.3
Aus-7	Mossman Gorge, Daintree River National Park, Queensland, Australia	Soil from excavations of feral pig. Tropical rainforest.	6.2
Aus-8	Mossman Gorge, Daintree River National Park, Queensland, Australia	Humus collected in a tree bole. Tropical rainforest.	6.7
Aus-9	Lizard Island, Queensland, Australia	Mud from a mangrove swamp.	6.4
GBR-10	Dartmoor, England.	Shallow river sediment.	7.9
GBR-11	Dartmoor, England.	Dry bog.	7.1
GBR-12	Dartmoor, England.	Wet bog.	6.6
Arg-13	San Martin Park, Mendoza, Argentina	Arid soil.	7.1
Arg-14	Iguacu National Park, Puerto Iguacu, Argentina	Soil. Sub-tropical rain forest.	7.7
Brz-15	Iguacu National Park, Foz do Iguacu, Brazil	Soil. Sub-tropical rain forest.	6.2
Czr-16	Chalvetice, North Bohemia, Czech Republic	Reed bed. Abandoned sedimentation pond from manganese mine.	7.8
Czr-17	Tusimice, North Bohemia, Czech Republic	Soil from an active sedimentation pond near electricity generating station.	8.7
Jpn-18	Pacific Ocean, Japan	Soft sediment from ~1000m.	8.8
Por-19	Febre River, Avintes, Portugal	River sediment.	7.6
Por-20	Ave River, Spring, Portugal	River sediment.	6.7
Por-21	Ave River, Nascente, Portugal	River sediment.	7.5
Por-22	Leca River, Leca, Portugal	River sediment.	6.8
Por-23	Cavado River, Cavado, Portugal	River sediment.	7.4
Por-24	Landim, Portugal	Soil.	7.5

Table 3.1. Continued.

Sample Code	Source	Sample Type	pH
Ant-25	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Col fellfield.	Soil from frost-sorted polygon fines.	8.3
Ant-26	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Jane Peak	Soil from alkaline area near marble outcrop.	8.2
Ant-27	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Moraine Valley fellfield site	Soil from frost-sorted polygon fines.	7.5
Ant-28	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (0-1cm core zone).	7.9
Ant-29	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Sombre Lake	Lake sediment at 10.5m depth (1-2cm core zone).	7.9
Ant-30	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (0-1cm core zone).	7.5
Ant-31	Signy Island, Maritime Antarctica (60 42'S, 45 35'W) Heywood Lake	Lake sediment at 6.2m depth (1-2cm core zone).	7.4
Ant-32	Edmonson Point, Wood Bay, Continental Antarctica (74 20'S, 160 08'E) Edmonson Point colonisation site.	Surface soil.	7.1
Nzl-33	New Zealand	Soil.	7.4
Jpn-34	Hot volcanic spring, Hakone, Japan	Red sediment.	4.2
Jpn-35	Hot volcanic spring, Hakone, Japan	Sediment near to a spring at 100°C.	3.7
Jpn-36	Hot volcanic spring, Hakone, Japan	Sulphurous sediment.	4.1
Jpn-37	Hot volcanic spring, Hakone, Japan	Spring water and sediment.	4.0
Jpn-38	Hot volcanic spring, Hakone, Japan	Spring water and sediment.	4.1
GBR-39	Anglia Wreck, off the Dover Coast, England	Sediment from the sea floor (27m).	8.4
Atl-40	Sulphate: methane transition: Hole 995B, core 2H-04, Blake Ridge, Atlantic Ocean	Core sediment 21.0mbsf.	N/D
Atl-41	Bottom-simulating reflector (BSR): Hole 995B, core 15X-02, Blake Ridge, Atlantic Ocean	Core sediment 449.06mbsf.	N/D
GBR-42	Afon Mettle, West Glamorgan, Wales	Shallow river sediment.	7.6
USA-43	St Louis, Missouri, USA.	Soil.	7.3
USA-44	Mammoth Cave National Park, Kentucky, USA	Soil.	7.1
USA-45	Music Row, Nashville, Tennessee, USA.	Soil.	7.6
USA-46	Laurel Falls, Blue Smoky Mountains National Park, Tennessee, USA	Soil.	7.4
USA-47	Huntsville, Alabama, USA	Soil.	6.8
USA-48	Indy 500 Racetrack, Indianapolis, USA	Soil.	6.9
USA-49	Central Park, New York City, New York, USA	Soil.	7.3
Ind-50	Princess Cave, Selarong, Bantul, Java, Indonesia	Soil.	7.0
Ind-51	Kiskendo Cave, Yogyakarta, Java, Indonesia.	Soil from the cave entrance.	7.2

Table 3.1. Continued.

Sample Code	Source	Sample Type	pH
Ind-52	Kiskendo Cave, Yogyakarta, Java, Indonesia	Soil from inside the cave.	7.2
Ind-53	Kiskendo Cave, Yogyakarta, Java, Indonesia	Soil from 200m into the cave.	8.3
Ind-54	Beach, Karang Bolong, Java, Indonesia	Sand from the beach.	7.8
Ind-55	Cave, Karang Bolong, Java, Indonesia	Soil from inside the cave.	8.2
Ind-56	Mangrove, Tritih Putih, Cilicap, Java, Indonesia	Mud from the mangrove.	7.4
Ind-57	Mangrove, Tritih Putih, Cilicap, Java, Indonesia	Mud from a crab soil deposit.	7.1
Ind-58	Cave, Jatijajar, Gombong, Java, Indonesia	Soil from inside the cave.	7.3
Ind-59	Cave, Jatijajar, Gombong, Java, Indonesia	Soil from outside the cave.	8.0
Ind-60	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Lake sediment.	2.4
Ind-61	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Organic litter.	3.1
Ind-62	Mirror Lake, Dieng Plateau, Wonosbo, Java, Indonesia	Charred soil from a tree fire.	4.9
Ind-63	Rawa Pening, Java, Indonesia	Exposed mud flats.	5.7
Ind-64	Rawa Pening, Java, Indonesia	Rice paddy field.	6.0
Ind-65	Mt. Merapi, Yogyakarta, Java, Indonesia	Volcanic soil, 1994 deposition.	6.2
Ind-66	Mt. Merapi, Yogyakarta, Java, Indonesia	Volcanic soil, 1997 deposition.	5.8
Ind-67	Turgo, Abandoned village, Yogyakarta, Java, Indonesia	Organic litter.	6.4
Ind-68	Turgo, Abandoned village, Yogyakarta, Java, Indonesia	Rhizosphere soil from Jack Fruit tree.	5.9
Ind-69	Solo, Mt Merapi, Java, Yogyakarta, Indonesia	Soil from cultivated land.	6.5
Ind-70	White crater lake, Bandung, Java, Indonesia	Soil from acidic volcanic crater.	3.2
Ind-71	Tea plantation, Bandung, Java, Indonesia	Soil from Tea Plantation.	7.2
Ind-72	Tea plantation, Bandung, Java, Indonesia	Soil from exposed mud flats.	7.8
Jpn-73	Dive 871-2, Suruga Bay, Pacific Ocean	Soft sediment, 1400m.	N/D
Jpn-74	Dive 869-2, Suruga Bay, Pacific Ocean	Soft sediment, 1400m.	N/D
Jpn-75	Dive 872-1, Suruga Bay, Pacific Ocean	Soft sediment, 1400m.	N/D
Spn-77	Canulobes Caves, Costa Blanca, Spain	Soil from outside caves.	7.1
Ind-78	Sumatra, Indonesia	Soil.	4.2
Ind-79	Sulawesi, Indonesia	Soil.	3.8
Ind-80	Irian Jaya, Indonesia	Soil.	4.6
Nmb-84	Kavango region, N. Namibia	Cultivated sandy soil.	6.9
Nmb-85	Tsuemb region, N. Central Namibia	Calcerous soil.	7.0
Nmb-86	Uitkomst Research Station, Tsuemb, N. Central Nambia	Soil.	7.2
Nmb-87	Waterberg Area, Central Namibia	Kalahari sand.	7.1

Table 3.1. Continued.

Sample Code	Source	Sample Type	pH
Nmb-88	Mahenene Research Station, N. W. Namibia	Soil.	7.0
Nmb-89	Katima Mulilo, N.E. Namibia	Soil.	7.4
Nmb-90	Ogongo, N.W. Namibia	Soil from cultivated lake bottom.	7.8
Jpn-91	Sagami Bay, Pacific Ocean	Soft sediment, 1168m.	N/D
Jpn-92	Sagami Bay, Pacific Ocean	Soft sediment, 1168m.	N/D
Jpn-93	Sagami Bay, Pacific Ocean	Soft sediment, 1168m.	N/D
Jpn-94	Dive 6K#127 Japan Trench, Pacific Ocean	Soft sediment, 6142m.	N/D
Jpn-95	Dive 6K#126 Japan Trench, Pacific Ocean	Soft sediment, 6300m.	N/D
Jpn-98	Dive 6K#067 Japan Trench, Pacific Ocean	Soft sediment, 6475m.	N/D
Jpn-99	Dive 6K#175 Izu Bonin Trench, Pacific Ocean	Soft sediment, 2679m.	N/D
Jpn-100	Dive 2K#869 Suruga Bay, Pacific Ocean	Soft sediment, 1487m.	N/D
Jpn-102	Dive 2K#870 Suruga Bay, Pacific Ocean	Soft sediment, 1151m.	N/D
Jpn-103	Dive 89-1 Suruga Bay, Pacific Ocean	Soft sediment, 1948m.	N/D
Jpn-104	Dive 6K#274 Japan Trench, Pacific Ocean	Soft sediment, 6048m.	N/D
Jpn-105	Dive 6K#275 Japan Trench, Pacific Ocean	Soft sediment, 4418m.	N/D

N/D pH not determined due to lack of material. pH determined by suspending 1.0g of a sample in 20ml dH_2O and measuring with a pH probe.

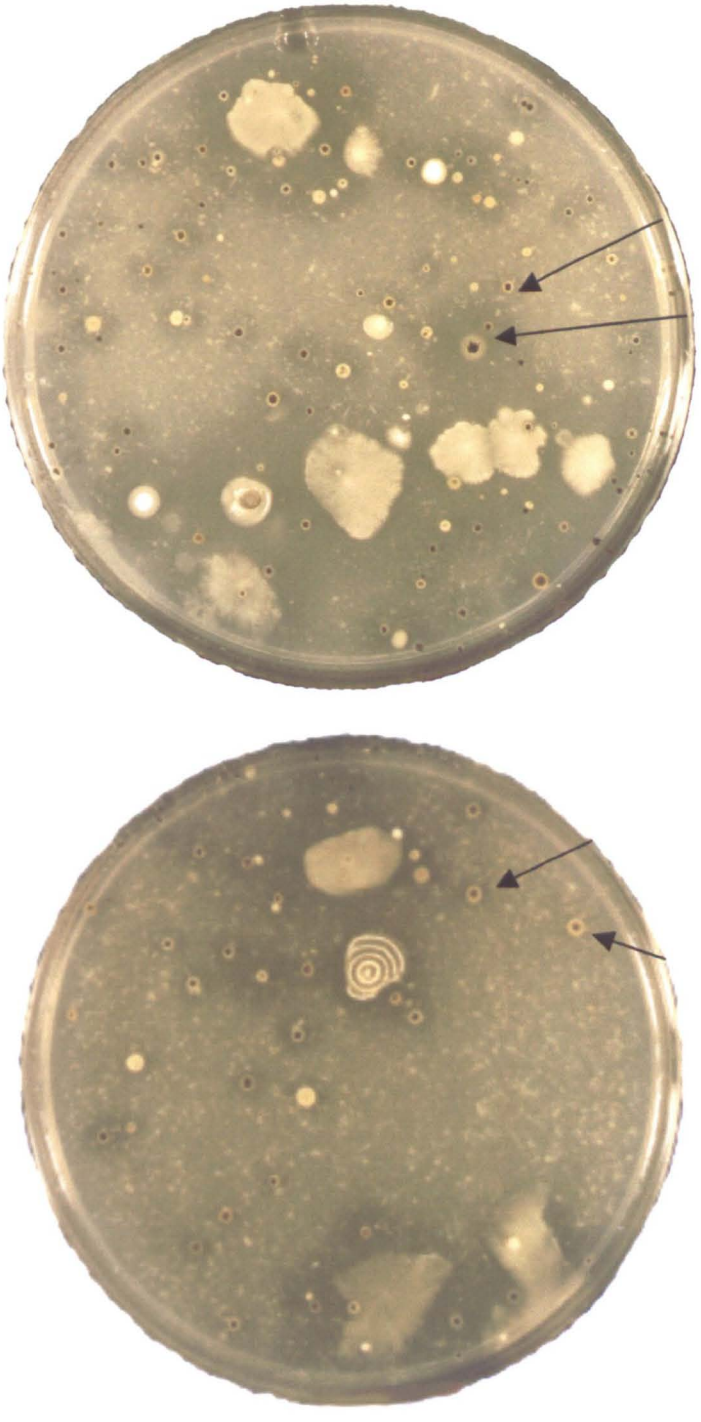
3.3. Selective isolation of micromonosporae

3.3.1. Recognition of *Micromonospora* strains on selective isolation plates

The recognition of *Micromonospora* isolates was based on their morphological characteristics on both selective isolation plates and on a rich medium. Colonies of *Micromonospora* species on agar medium are small and embedded in the medium. They are generally round and lichenoid or smooth, initially pale yellow/orange, becoming orange, red, brown, blue-green, khaki or purple. Mature colonies take on a progressively darker hue along with the production of brown-black, green-black, or black spores, and become shiny and mucoid (Kawamoto, 1989). The appearance of small orange colonies with black centres is the typical diagnostic appearance of micromonosporae on isolation plates (see Figures 3.1 and 3.2). Zones of clearing around *Micromonospora* colonies were

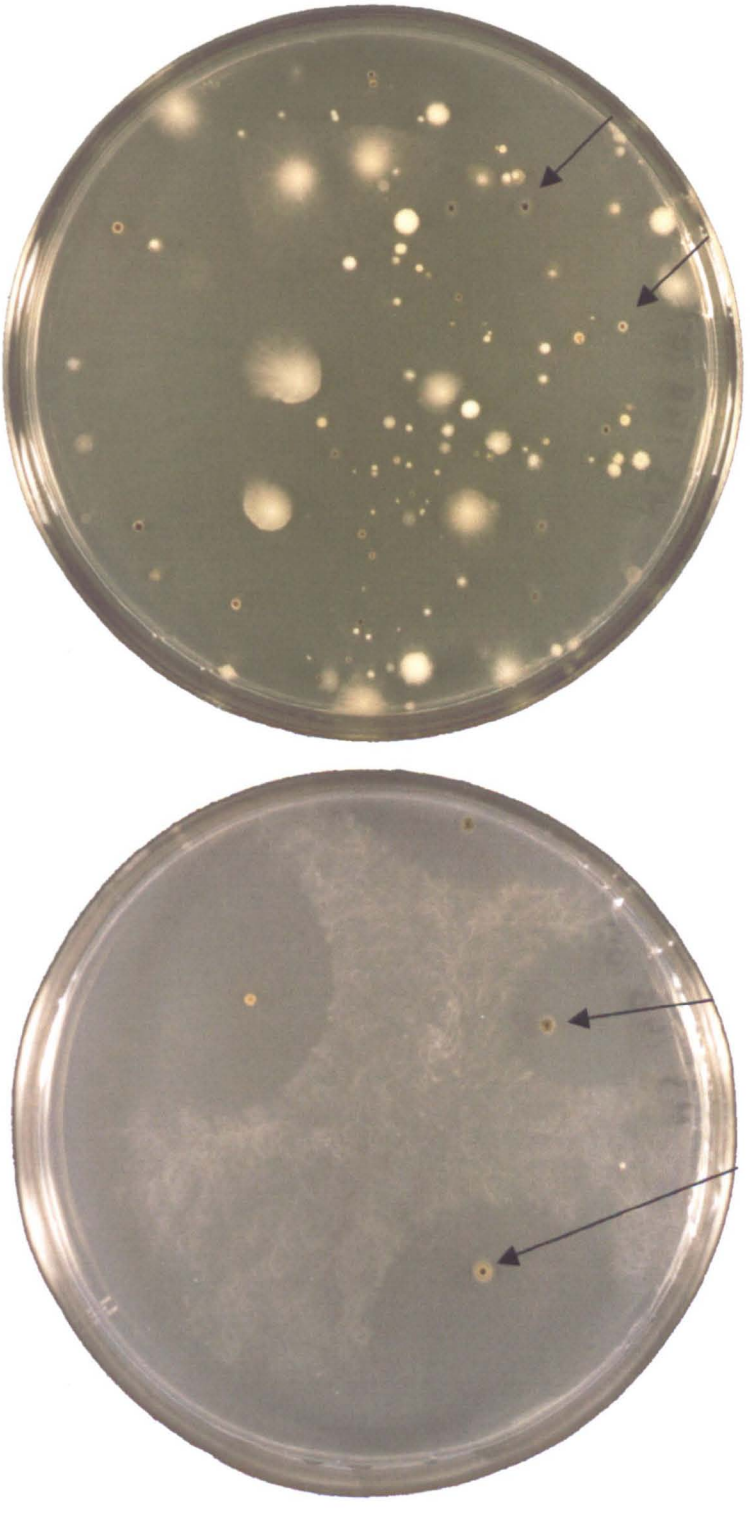
frequently observed on the colloidal chitin plates where the *Micromonospora* were degrading the chitin (see Figure 3.1). The colonies on M3 agar plates tended to be much smaller than those observed on the colloidal chitin plates and often produced concentric sporulation rings within the colony. Zones of inhibition were often observed around the *Micromonospora* colonies as can be seen in Figure 3.2. Colonies were picked from the selective isolation plates and sub-cultured onto a rich medium (Micromonospora megalomiceum agar). Figure 3.3 shows typical isolates of *Micromonospora* on Micromonospora megalomicea agar. Differentiation of the colonies is much more apparent on the rich medium as the more typical growth characteristics are exhibited making identification easier.

Figure 3.1. Colloidal chitin selective isolation plates showing putative *Micromonospora* strains. Plates incubated for 4 weeks at 30°C.



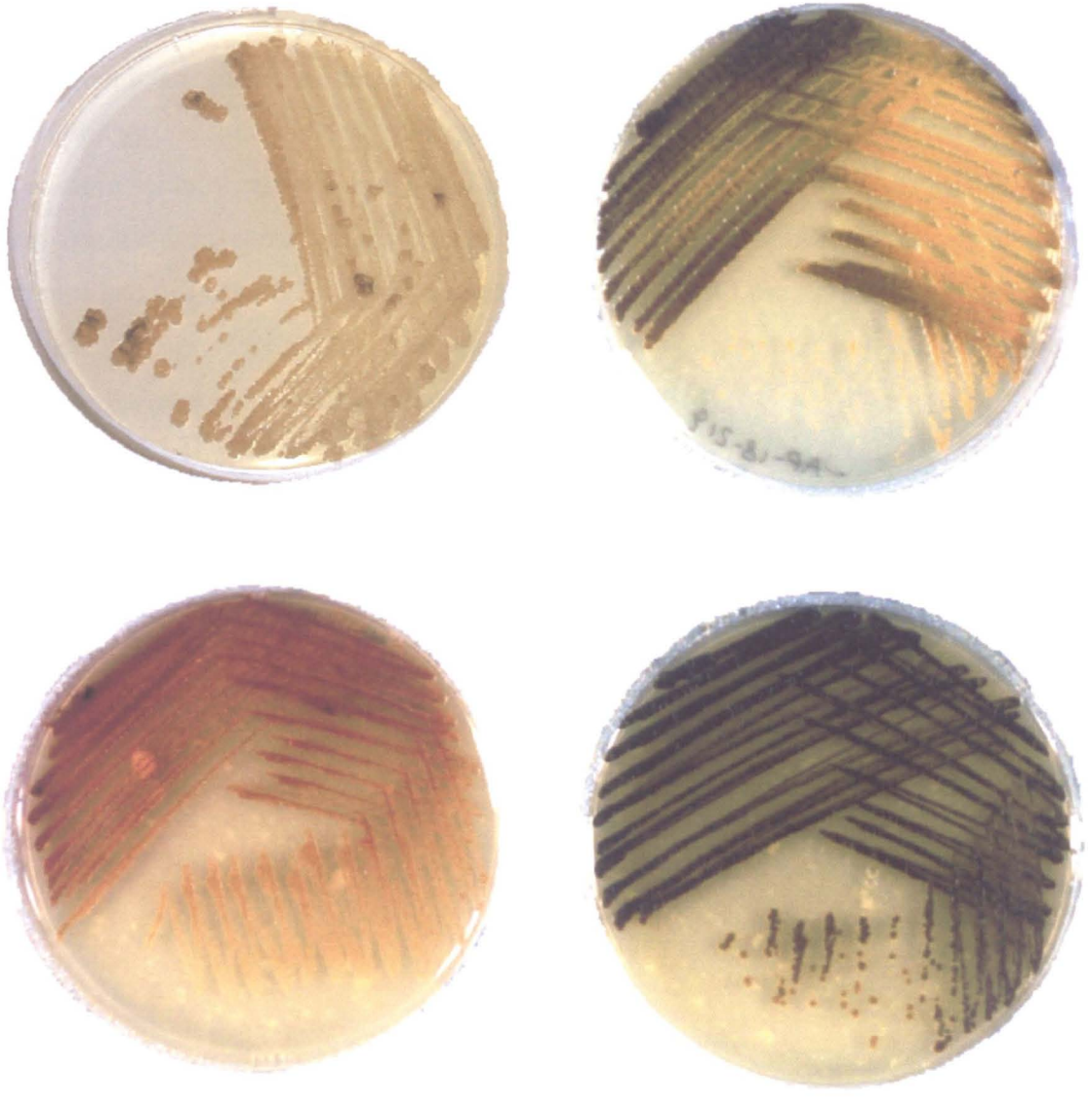
Putative *Micromonospora* isolates can be identified as the orange cultures with a black centre (as shown by the arrows).

Figure 3.2. M3 agar selective isolation plates showing putative *Micromonospora* strains. Plates incubated for 4 weeks at 30°C.



Putative *Micromononospora* isolates can be identified as the orange cultures with black centres (as shown by the arrows).

Figure 3.3. Subcultures of *Micromonospora* strains on Micromonospora megalomiceum agar representing four different strains, exhibiting characteristic morphologies, isolated from marine samples. Plates incubated for 7 days at 30°C.



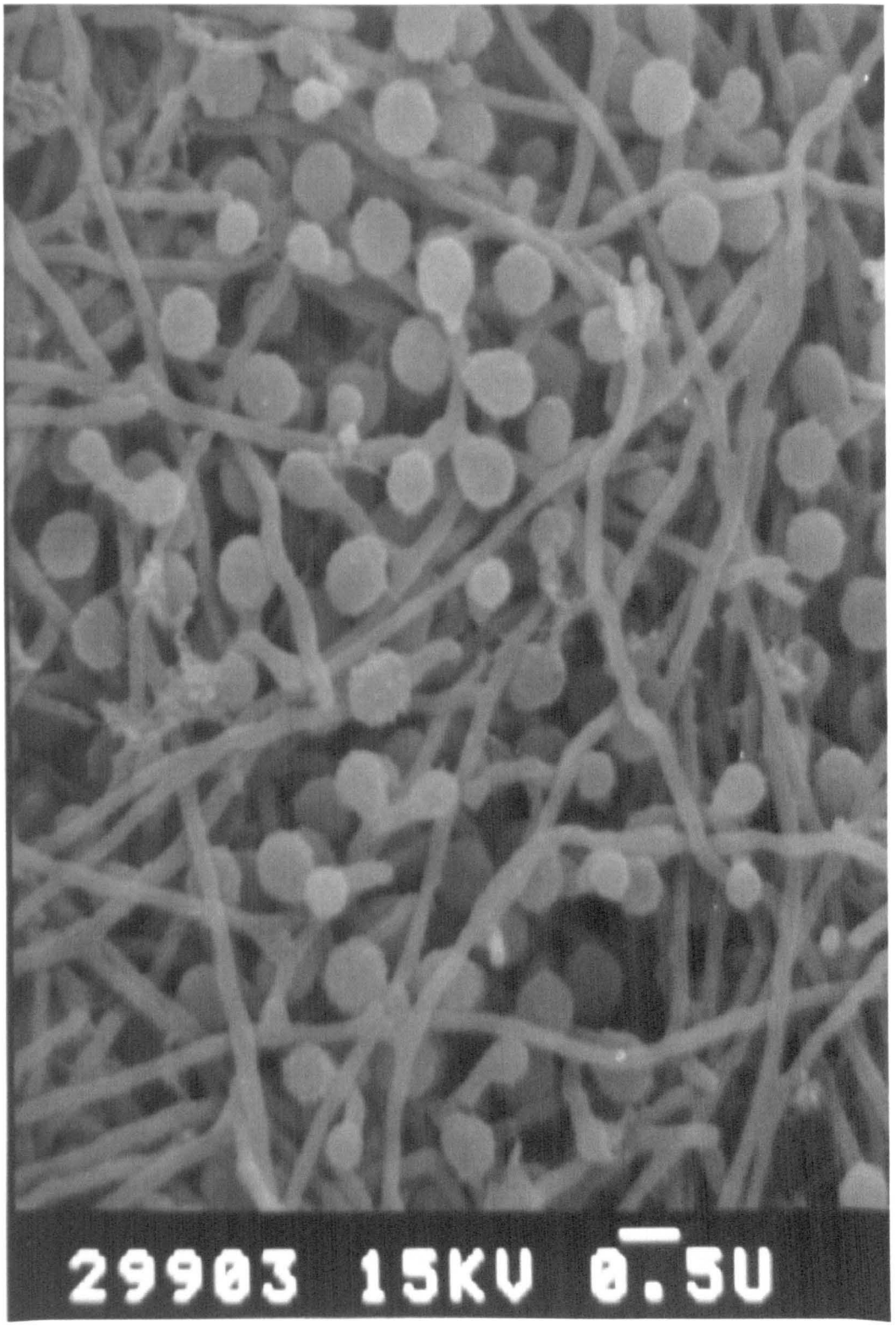
3.3.2. Gram staining of putative micromonosporae isolates

Representatives of isolates from the different geographic regions (see Figure 3.3 for typical morphological characteristics) stained Gram-positive. Some of these isolates were studied in further detail by scanning electron microscopy (see Section 3.3.3).

3.3.3. Scanning electron microscopy to identify micromonosporae

Selected putative micromonosporae were examined by scanning electron microscopy to make a detailed analysis of their spore arrangement and morphology. This revealed that the spores formed singly on the substrate mycelium, usually on short sporophores (<1μm). Branching of the sporophores was observed in some cases. Spore ornamentation was observed on some isolates and this usually took the form of blunt projections from the surface of the spore. Figure 3.4 shows a scanning electron micrograph typical of a *Micromonospora* isolate (Namibian isolate, Nmb-90-014).

Figure 3.4. Scanning electron micrograph of a typical *Micromonospora* strain isolated from a Namibian soil (isolate Nmb-90-014)



Micromonospora isolate Nmb-90-014 grown on Micromonospora megalomiceum agar at 30°C for 7 days.

3.3.4. Efficacy of pre-treatments and selective media, in the isolation of micromonosporae from environmental samples

A number of different pre-treatments and selective media commonly used for the isolation of actinomycetes and particularly micromonosporae were examined for their usefulness in the recovery of micromonosporae from an environmental sample (sample Jpn-18) known to contain micromonosporae (Colquhoun, 1999). Tables 3.2 and 3.3 give details of the numbers of micromonosporae recovered using the different methods and what percentage of the viable microbial population they formed.

Table 3.2. Numbers of micromonosporae recovered from a marine sediment sample (Jpn-18) using different pre-treatments and selective media

	Media					
Pre-treatment	**Cellulose asparagine (cfu/ml)**	**Starch casein nitrate (cfu/ml)**	**Colloidal chitin (cfu/ml)**	**Glycerol arginine salt (cfu/ml)**	**Humic acid vitamin (cfu/ml)**	**M3 (cfu/ml)**
1.5% Phenol at 30°C for 30 mins	$5 * 10^2$	$6 * 10^2$	$2 * 10^3$	$2 * 10^3$	$2 * 10^3$	$4 * 10^3$
0.01M NaOH	$8 * 10^2$	$2 * 10^3$	$7 * 10^3$	$1 * 10^3$	$3 * 10^3$	$2 * 10^3$
60°C for 10 mins	$3 * 10^2$	$1 * 10^3$	$1 * 10^4$	$2 * 10^3$	$1 * 10^3$	$1 * 10^4$
1.5% Phenol at 60°C for 10 mins	$5 * 10^2$	$6 * 10^2$	$2 * 10^3$	$1 * 10^3$	$6 * 10^3$	$4 * 10^3$
0.01M NaOH at 60°C for 10 mins	$6 * 10^3$	$1 * 10^3$	$6 * 10^3$	$1 * 10^4$	$5 * 10^3$	$1 * 10^3$
120°C for 60 mins	0	0	0	0	0	0
1.5% Phenol at 120°C for 60 mins	0	0	0	0	0	0
0.01M NaOH at120°C for 60 mins	0	0	0	0	0	0

All counts were made after incubation at 30°C for 3 to 4 weeks.

Table 3.3. Number of micromonosporae recovered as a percentage of the viable microbial population from a marine sediment sample (Jpn-18) on these selective media

	Selective media					
Pre-treatment	**Cellulose asparagine (%)**	**Starch casein nitrate (%)**	**Colloidal chitin (%)**	**Glycerol arginine salt (%)**	**Humic acid vitamin (%)**	**M3 (%)**
1.5% Phenol at 30°C for 30 mins	1	3	12	10	6	15
0.01M NaOH	1	3	11	1	3	3
60°C for 10 mins	1	2	15	5	2	10
1.5% Phenol at 60°C for 10 mins	1	2	7	5	20	11
0.01M NaOH at 60°C for 10 mins	3	0	3	3	2	1
120°C for 60 mins	0	0	0	0	0	0
1.5% Phenol at 120°C for 60 mins	0	0	0	0	0	0
0.01M NaOH 120°C for 60 mins	0	0	0	0	0	0

All counts were made after incubation at 30°C for 3 to 4 weeks.

There was a clear difference in the selectivity of the different pre-treatments and media for the recovery of micromonosporae. Some of the selective media (cellulose asparagine and starch casein nitrate) were not very selective towards micromonosporae, whereas colloidal chitin and M3 were highly selective and the majority of colonies recovered on these plates were micromonosporae. Most of the pre-treatments yielded between 10^2 and 10^4 micromonosporae/ml with the exception of the dry heat at 120°C for 60 min, which yielded no micromonosporae, suggesting that the spores were unable to withstand this temperature for this length of time. The 1.5% phenol pre-treatment yielded high numbers of micromonosporae at $5 * 10^3$ micromonosporae/ml. Pre-treatment with 0.01M NaOH generally yielded low numbers of micromonosporae except on the colloidal chitin agar. The heat pre-treatment of 60°C for 30 minutes in a

waterbath gave a good yield of micromonosporae (up to 1 * 10^4 micromonosporae/ml). Using combinations of phenol and 0.01M NaOH with different incubation temperatures and times resulted in little difference in the numbers of micromonosporae recovered.

The colloidal chitin, M3 and starch casein agars were highly effective for the recovery of micromonosporae with numbers recovered being of the order of 10^3 or 10^4. Whilst these were, in general, only one order of magnitude higher than the numbers recovered on the other selective media, the numbers of unwanted bacteria were much lower making the recoverey of micromonosporae much easier. The cellulose asparagine agar proved to be the least effective selective media as the numbers of micromonosporae recovered were generally low (10^2 micromonosporae/ml) with the exception of the combination of 0.01M NaOH and 60°C waterbath pre-treatment, which yielded a recovery of 10^3 micromonosporae/ml, and there were high numbers of unwanted bacteria which usually grew faster than the micromonosporae.

An assessment of the effectiveness of the pre-treatments and selective isolation media was made by comparing the recoveries of micromonosporae as a percentage of the viable population (see Table 3.3). This clearly showed colloidal chitin and M3 to be the most effective media with micromonosporae forming up to 15% of the viable population. Later rounds of isolation, in particular the South American samples, showed that micromonosporae could form up to 80% of the viable population, supporting the choice of these agars for efficient selective isolation of micromonosporae. High numbers of micromonosporae were also shown to be recovered using phenol as a pre-treatment; in general, the numbers were higher when the treatment was at 30°C, rather than 60°C with the exception of the humic acid vitamin agar.

3.3.5. Effectiveness of the dispersion and differential centrifugation technique for the recovery of micromonosporae

The dispersion and differential centrifugation technique (Hopkins *et. al.*, 1991) has been reported to be highly efficient in the recovery of actinomycetes from

environmental samples (Atalan *et al.*, 2000; Sembiring, 2000; Zulu, 1995). This study looked at how effective the protocol was for the recovery of micromonosporae and whether more target organisms were recovered in the first centrifugation fraction (A). The numbers of micromonosporae recovered in the different fractions from the marine sediment Jpn-18, using the different pre-treatments and selective media are detailed in Tables 3.4 to 3.6.

Table 3.4. Micromonosporae recovered per ml of sample from centrifugation fraction A

	Media					
Pre-treatment	**Cellulose Asparagine**	**Starch Casein Nitrate**	**Colloidal Chitin**	**Glycerol Arginine Salt**	**Humic Acid Vitamin**	**M3**
None	$1 * 10^4$	$1 * 10^4$	$6 * 10^4$	0	$5 * 10^4$	$3 * 10^4$
1.5% Phenol at30°C for 30 mins	$5 * 10^4$	$1 * 10^4$	$1 * 10^4$	$1 * 10^5$	$5 * 10^4$	$5 * 10^4$
0.01M NaOH	$1 * 10^4$	$5 * 10^4$	$6 * 10^4$	$3 * 10^4$	$1 * 10^4$	$3 * 10^4$
60°C for 10 mins	0	0	$1 * 10^5$	0	$3 * 10^4$	$6 * 10^4$
120°C for 60 mins	0	0	0	0	0	0

All counts were made after incubation at 30°C for 3 to 4 weeks.

Table 3.5. Micromonosporae recovered per ml of sample from centrifugation fraction B

	Media					
Pre-treatment	**Cellulose Asparagine**	**Starch Casein Nitrate**	**Colloidal Chitin**	**Glycerol Arginine Salt**	**Humic Acid Vitamin**	**M3**
None	0	0	0	0	0	$3 * 10^4$
1.5% Phenol at 30°C for 30 mins	$5 * 10^4$	$1 * 10^4$	$1 * 10^4$	$1 * 10^4$	$1 * 10^4$	$6 * 10^4$
0.01M NaOH	$5 * 10^4$	$1 * 10^4$	$8 * 10^3$	$1 * 10^4$	$6 * 10^4$	$1 * 10^5$
60°C for 10 mins	0	0	$1 * 10^5$	$3 * 10^4$	$1 * 10^4$	$1 * 10^5$
120°C for 60 mins	0	0	0	0	$1 * 10^4$	0

All counts were made after incubation at 30°C for 3 to 4 weeks.

Table 3.6. Micromonosporae recovered per ml of sample from centrifugation fraction C

	Media					
Pre-treatment	**Cellulose Asparagine**	**Starch Casein Nitrate**	**Colloidal Chitin**	**Glycerol Arginine Salt**	**Humic Acid Vitamin**	**M3**
None	0	0	$3 * 10^3$	0	0	$1 * 10^3$
1.5% Phenol at 30°C for 30 mins	81	45	$1 * 10^3$	$3 * 10^3$	71	$6 * 10^3$
0.01M NaOH	27	$2 * 10^2$	$8 * 10^3$	39	28	$1 * 10^3$
60°C for 10 mins	0	0	$1 * 10^3$	$3 * 10^3$	79	$2 * 10^3$
120°C for 60 mins	0	0	0	0	52	0

All counts were made after incubation at 30°C for 3 to 4 weeks.

The numbers recovered from the centrifugation fractions (A and B) were very similar, with both yielding counts in the order of 10^4 and 10^5 micromonosporae per ml of sample. However, fraction C contained much lower numbers of micromonosporae. This suggests that it is valuable to isolate from both fractions A and B as there is a high recovery of micromonosporae in each and different portions of the population may be represented in the two fractions.

The highest numbers of micromonosporae were recovered on the colloidal chitin and M3 agars and the phenol pre-treatment consistently showed efficient recovery of micromonosporae. This was in agreement with the trends observed in section 3.2.1. However, higher numbers of micromonosporae were recovered using the dispersion and differential centrifugation technique. The dispersion and differential centrifugation technique also proved to be more selective towards micromonosporae than just diluting and vortexing the samples. Table 3.7 shows that micromonosporae formed a higher percentage of the viable population using the dispersion and differential centrifugation technique than the traditional vortex technique (Table 3.3). The colloidal chitin and M3 agars showed a recovery of 52 and 46% in combination with a 1.5% phenol pre-treatment using the dispersion and differential centrifugation technique compared with 12 and 15% using the traditional vortex technique.

Table 3.7. Number of micromonosporae recovered as a percentage of the viable microbial population of the marine sediment sample (Jpn-18) using the dispersion and differential centrifugation technique*

	Media					
Pre-treatment	**Cellulose Asparagine (%)**	**Starch Casein Nitrate (%)**	**Colloidal Chitin (%)**	**Glycerol Arginine Salt (%)**	**Humic Acid Vitamin (%)**	**M3 (%)**
1.5% Phenol at 30°C for 30 mins	5	9	52	15	7	46
0.01M NaOH	3	7	35	10	5	29
60°C for 10 mins	4	11	39	12	7	32
120°C for 60 mins	0	0	0	0	0	0

* Results are based on centrifugation fraction A

Based on the above results, the differential and dispersion technique coupled with a 1.5% phenol treatment at 30°C for 30 minutes was selected as the most effective pre-treatment for samples, along with colloidal chitin and M3 agar as the selective media for the recovery of micromonosporae from environmental samples.

The marine samples were not subjected to the dispersion and differential centrifugation technique, as the material available was limited (often less than 1.0g). Instead, these samples were serially diluted using small volumes of phosphate buffered saline.

Tables 3.8a. to c. Percentage increase in the recovery of micromonosporae from soils and sediments using the dispersion and differential centrifugation technique over traditional vortexing

Table 3.8a. Centrifugation fraction A (*100)

	Media					
Pre-treatment	**Cellulose Asparagine (%)**	**Starch Casein Nitrate (%)**	**Colloidal Chitin (%)**	**Glycerol Arginine Salt (%)**	**Humic Acid Vitamin (%)**	**M3 (%)**
1.5% Phenol at 30°C for 30 mins	99	24	6.7	76	22	9.7
0.01M NaOH	19	24	7.9	24	4.2	11
60°C for 10 mins	+	+	11	+	24	5.7
120°C for 60 mins	+	+	+	+	++	+

+, no isolates on the DDC plates; ++, no isolates on the vortex plates

Table 3.8b. Centrifugation fraction B (*100)

	Media					
Pre-treatment	**Cellulose Asparagine (%)**	**Starch Casein Nitrate (%)**	**Colloidal Chitin (%)**	**Glycerol Arginine Salt (%)**	**Humic Acid Vitamin (%)**	**M3 (%)**
1.5% Phenol at 30°C for 30 mins	9.9	24	6.7	6.7	4.4	13
0.01M NaOH	59	7.4	0.1	6.5	20	46
60°C for 10 mins	+	+	12	11	9	12
120°C for 60 mins	+	+	+	+	+	+

+, no isolates on the DDC plates

Tables 3.8a. to c. Continued.

Table 3.8c. Centrifugation fraction C (*100)

	Media					
Pre-treatment	**Cellulose Asparagine (%)**	**Starch Casein Nitrate (%)**	**Colloidal Chitin (%)**	**Glycerol Arginine Salt (%)**	**Humic Acid Vitamin (%)**	**M3 (%)**
1.5% Phenol at 30°C for 30 mins	-	-	-	6	-	4
0.01M NaOH	-	-	0.1	-	-	-
60°C for 10 mins	+	+	-	0.2	-	-
120°C for 60 mins	+	+	+	+	+	+

+, no isolates on the DDC plates; -, isolates on DDC plates lower than on the vortex plates

3.4. Isolation of micromonosporae from terrestrial samples

All of the terrestrial samples collected were subjected to dispersion and differential centrifugation and pre-treated with 1.5% phenol at 30°C for 30 minutes before being plated onto colloidal chitin and M3 agars. A summary of the abundance of micromonosporae in the terrestrial samples is given in Table 3.9.

Table 3.9. Isolation of micromonosporae from terrestrial samples

	Number of micromonosporae (cfu/g)			
	Colloidal chitin agar		M3 agar	
Sample Code	Centrifugation Fraction A	Centrifugation Fraction B	Centrifugation Fraction A	Centrifugation Fraction B
Aus-1	0	0	0	0
Aus-2	0	0	0	0
Aus-3	0	0	0	0
Aus-4	0	0	$2 * 10^4$	0
Aus-5	0	$2 * 10^3$	0	0
Aus-6	$2 * 10^3$	0	0	0
Aus-7	0	$5 * 10^3$	$2 * 10^3$	0
Aus-8	0	0	0	0
Aus-9	0	$1 * 10^4$	0	$1 * 10^4$
GBR-10	10	15	42	30
GBR-11	$2 * 10^2$	0	$2 * 10^2$	$2 * 10^2$
GBR-12	$3 * 10^2$	$5 * 10^2$	$1 * 10^3$	$2 * 10^2$
Arg-13	$1 * 10^4$	$2 * 10^3$	$5 * 10^4$	$2 * 10^4$
Arg-14	$7 * 10^4$	$2 * 10^4$	$6 * 10^4$	$2 * 10^4$
Brz-15	$1 * 10^4$	$3 * 10^2$	$3 * 10^4$	0
Czr-16	0	$2 * 10^2$	$7 * 10^2$	$3 * 10^2$
Czr-17	0	$6 * 10^2$	$2 * 10^2$	$2 * 10^2$
Por-19	$8 * 10^2$	$2 * 10^2$	$2 * 10^2$	$3 * 10^2$
Por-20	$3 * 10^2$	2	$3 * 10^2$	5.0
Por-21	0	$5 * 10^2$	0	$3 * 10^2$
Por-22	0	$5 * 10^2$	0	0
Por-23	$2 * 10^3$	$2 * 10^3$	$1 * 10^3$	$5 * 10^3$
Por-24	$1 * 10^3$	$3 * 10^2$	$2 * 10^3$	$2 * 10^2$
Ant-25	1	0	0	0
Ant-26	0	0	0	0
Ant-27	0	0	0	0
Ant-28	1	0	0	0
Ant-29	0	0	0	0
Ant-30	0	0	0	0
Ant-31	0	0	0	0
Ant-32	0	0	0	0

Table 3.9. Continued.

	Number of micromonosporae (cfu/g)			
	Colloidal chitin agar		M3 agar	
Sample Code	Centrifugation Fraction A	Centrifugation Fraction B	Centrifugation Fraction A	Centrifugation Fraction B
Nzl-33	$2 * 10^2$	$7 * 10^2$	$2 * 10^3$	$2 * 10^2$
Jpn-34	0	0	0	0
Jpn-35	0	0	0	0
Jpn-36	0	0	0	0
Jpn-37	0	0	0	0
Jpn-38	0	0	0	0
GBR-39	0	0	0	0
Atl-40	0	0	0	0
Atl-41	0	0	0	0
GBR-42	0	0	0	0
USA-43	$7 * 10^2$	$2 * 10^3$	0	$2 * 10^2$
USA-44	0	$3 * 10^2$	0	$2 * 10^2$
USA-45	$2 * 10^4$	$3 * 10^4$	$8 * 10^2$	$1 * 10^4$
USA-46	0	$3 * 10^2$	0	$2 * 10^2$
USA-47	$7 * 10^2$	$8 * 10^3$	$2 * 10^2$	$5 * 10^3$
USA-48	$3 * 10^3$	$5 * 10^2$	$2 * 10^2$	$2 * 10^3$
USA-49	$3 * 10^3$	$1 * 10^3$	$4 * 10^3$	$2 * 10^3$
Ind-50	$1 * 10^2$	50	$1 * 10^2$	0
Ind-51	0	0	0	0
Ind-52	0	25	0	100
Ind-53	25	0	75	0
Ind-54	0	0	0	0
Ind-55	50	75	0	25
Ind-56	50	0	25	0
Ind-57	$2 * 10^2$	$1 * 10^2$	75	$1 * 10^2$
Ind-58	25	0	0	$3 * 10^2$
Ind-59	0	0	0	0
Ind-60	0	0	0	0
Ind-61	25	25	0	0
Ind-62	0	0	0	0
Ind-63	$4 * 10^2$	0	$6 * 10^2$	0

Table 3.9. Continued.

	Number of micromonosporae (cfu/g)			
	Colloidal chitin agar		M3 agar	
Sample Code	Centrifugation Fraction A	Centrifugation Fraction B	Centrifugation Fraction A	Centrifugation Fraction B
Ind-64	$2 * 10^3$	$5 * 10^3$	0	0
Ind-65	25	0	0	0
Ind-66	0	50	0	0
Ind-67	0	0	0	0
Ind-68	0	0	0	0
Ind-69	25	50	25	25
Ind-70	0	$3 * 10^2$	0	0
Ind-71	0	$7 * 10^3$	0	0
Ind-72	$4 * 10^3$	$1 * 10^4$	0	0
Bdh-76	$2 * 10^4$	$9 * 10^3$	$2 * 10^4$	$7 * 10^3$
Spn-77	$2 * 10^2$	$2 * 10^3$	0	$2 * 10^2$
Ind-78	0	0	0	0
Ind-79	0	0	0	0
Ind-80	0	0	0	0
Nmb-84	$2 * 10^3$	0	0	$5 * 10^3$
Nmb-85	0	$2 * 10^3$	0	$1 * 10^4$
Nmb-86	0	$3 * 10^3$	$2 * 10^3$	$1 * 10^4$
Nmb-87	0	$2 * 10^3$	$3 * 10^3$	$2 * 10^3$
Nmb-88	0	0	0	0
Nmb-89	0	$7 * 10^3$	$7 * 10^3$	$1 * 10^3$
Nmb-90	$7 * 10^3$	$9 * 10^4$	$7 * 10^3$	$2 * 10^4$

Samples were subjected to dispersion and differential centrifugation, serially diluted, pre-treated with 1.5% phenol for 30 minutes at 30°C, aliquots were inoculated onto colloidal chitin and M3 agar and the plates were incubated at 30°C for 3 to 4 weeks.

3.5. Isolation of micromonosporae from marine samples

The marine samples were serially diluted in phosphate buffered saline before being plated onto colloidal chitin and M3 agars. The abundance of micromonosporae in the marine samples is summarised in Table 3.10.

Table 3.10. Isolation of micromonosporae from marine samples

Sample Code	Number of micromonosporae (cfu/ml)	
	Colloidal chitin agar	M3 agar
Jpn-18	$5 * 10^2$	$2 * 10^3$
Jpn-73	73	103
Jpn-74	79	82
Jpn-75	61	87
Jpn-91	58	65
Jpn-92	29	43
Jpn-93	42	38
Jpn-94	7	12
Jpn-95	10	16
Jpn-98	17	24
Jpn-99	26	29
Jpn-100	15	23
Jpn-102	21	24
Jpn-103	22	22
Jpn-104	6	9
Jpn-105	13	19
Jpn-106	16	19
Jpn-107	19	16

Samples were serially diluted, vortexed and aliquots inoculated onto colloidal chitin and M3 agar. Plates were incubated for 3 to 4 weeks at 30°C.

High numbers of micromonosporae were recovered from many of the terrestrial samples. In many cases, the recovery was in the order of 10^3 to 10^4 micromonosporae per gram of sample. Samples containing particularly high numbers of micromonosporae were; Aus-9 from Lizard Island, the Argentinean samples Arg-13 and Arg-14 from Mendoza and Iguacu National Park respectively; the American samples USA-45 and USA-49 from Tennessee and

Central Park, New York; an Indonesian sample (Ind-72) from the mud flats adjacent to a tea plantation and a Namibian sample (Nmb-90) from a cultivated lake bottom in Ogongo. There is little in common between the types of samples (i.e. similarity of habitat) containing high numbers of micromonosporae but all had pH values above neutral (pH 7.1 to 7.8) with the exception of the Australian sample (Aus-9) that had a pH value of 6.4. Some of the samples that contained high numbers of micromonosporae were from aquatic environments or where there has been a high incidence of water, such as the Indonesian mud flats and the cultivated lake bottom sample from Namibia.

Many of the samples contained few or no micromonosporae, including four of the Australian samples, most of the Antarctic samples, all of the Japanese hot spring samples (Jpn-34-38), the marine sediment off the coast of Great Britain, the cores from the mid-Atlantic Ridge and some of the Indonesian samples. The link between most of these samples is low pH (3.7 to 6.7). The Antarctic samples generally had higher pH values (>7.0) but contained very few microbes. Some of the Indonesian samples containing no *Micromonospora* isolates (Ind-51 and Ind-54) had pH values above 7.0 but were from similar habitats (i.e. caves). From these results, it would appear that micromonosporae are rarely isolated from habitats with low pH values (<6.5) and from the Antarctic region.

The recovery of micromonosporae from marine soft sediment samples was generally much lower than from terrestrial samples. The highest recovery came from the shallowest sediment off the Pacific coast of Japan (~1000m), whilst the lowest numbers were observed in samples from depths in excess of 6,000m (Jpn-94, Jpn-95, Jpn-98 and Jpn-104). These results suggest that micromonosporae are not indigenous in marine sediments but are more likely to be a result of terrestrial wash in of spores. These spores probably remain dormant until the samples are cultivated in the laboratory.

3.6. Isolation of alkaliphilic/alkalitolerant and thermophilic micromonosporae from selected terrestrial samples

There are many reports dating back to the early work of Jensen (1930; 1932) of *Micromonospora* strains being isolated from alkaline soils. Therefore, it seemed

a natural progression to examine whether the samples contained any alkaliphilic or alkalitolerant *Micromonospora* strains. The Indonesian samples were selected for inclusion in this study, see Chapter 2, Section 2.2.2 for details of the isolation methods. Table 3.11 shows the abundance of alkaliphilic/alkalitolerant micromonosporae in the samples tested.

Table 3.11. Abundance of alkaliphilic/alkalitolerant micromonosporae in Indonesian soils

	Micromonosporae (cfu/g)			
	Centrifugation Fraction A		Centrifugation Fraction B	
Sample Code	Colloidal Chitin	M3	Colloidal Chitin	M3
Ind-50	$2*10^2$	50	0	0
Ind-51	0	0	0	0
Ind-52	0	0	0	0
Ind-53	0	0	0	0
Ind-54	0	0	0	0
Ind-55	50	0	0	0
Ind-56	$3*10^2$	0	0	0
Ind-57	$2*10^2$	$1*10^2$	$2*10^2$	0
Ind-58	$1*10^2$	$8*10^2$	50	$1*10^2$
Ind-59	0	0	0	0
Ind-60	0	0	0	0
Ind-61	0	0	0	0
Ind-62	0	$6*10^2$	0	0
Ind-63	$1*10^3$	0	0	0
Ind-64	$5*10^3$	$1*10^3$	$4*10^3$	$6*10^2$
Ind-65	25	25	0	0
Ind-66	50	25	25	0
Ind-67	0	$2*10^2$	0	0
Ind-68	0	0	0	0
Ind-69	0	0	0	0

Samples were subjected to dispersion and differential centrifugation, pre-treatment with 1.5% phenol at 30°C for 30 minutes and aliquots were plated onto colloidal chitin and M3 agar at pH 10.0 and incubated at 30°C for 3 to 4 weeks.

Over half of the samples tested contained micromonosporae that were either alkaliphilic or alkalitolerant. The highest number ($5.4*10^3$ cfu/g) of micromonosporae was recovered from the rice paddy field sample (Ind-64, pH 6.4). This figure was higher than the number of alkaliphilic or alkalitolerant micromonosporae recovered from samples of a more neutral or slightly alkaline pH (i.e. pH 7-8). The numbers of micromonosporae recovered in this study

suggest that alkaliphilic or alkalitolerant micromonosporae are common in the natural environment.

To date there have been no reports of workers looking for thermophilic *Micromonospora* strains. However, there are actinomycetes which are known to be thermophilic i.e. *Saccharomonospora viridis* (Cross, 1981). Based on this information it was considered appropriate to analyse some of the samples for potential thermophilic *Micromonospora* strains.

The same Indonesian samples were analysed for the presence of thermophilic micromonosporae by incubating selective isolation plates at 55°C. These samples were found to contain no *Micromonospora* strains. In view of the reasonably large numbers (>1000 in some cases) of micromonosporae known to be in these samples, it is probable that this genus is primarily mesophilic.

3.7. Discussion

3.7.1. Recovery of micromonosporae from environmental samples

Innumerable media formulations have been proposed for the selective isolation of actinomycetes. However, no medium is entirely selective for actinomycetes or micromonosporae. Selective media may be selective for different micromonosporae populations, in which case it is important to employ more than one selective medium to gain a more accurate picture of micromonosporae populations in soils and sediments. M3 and colloidal chitin agar proved to be the most efficient of the media employed in this study, for the recovery of micromonosporae and gave rise to different portions of the micromonosporae population. Pre-treatments play an important role in the selective isolation of micromonosporae. Micromonosporae are slow growing organisms, especially on selective agars where nutrients are often limited or require further breakdown before being metabolised. As a result, they are often out-competed by faster growing organisms, for example gram-negative bacteria. Therefore, it is advantageous to reduce the number of gram-negative bacteria; this can be achieved by pre-treating the samples with chemicals, heat or a combination of both. For the different pre-treatments tested during this study, a 30-minute

incubation with 1.5% phenol at 30°C proved the most effective at reducing the numbers of gram negatives and other non-target bacteria.

An important observation of this study was the demonstration that the modified dispersion and differential centrifugation method was more effective in extracting micromonosporae propagules from environmental samples than the classical tumble shaking procedure. These results confirm observations made in previous studies that showed that the modified DDC method was more effective in extracting actinomycetes propagules from environmental samples than the simple classical shaking procedures (Manfio, 1995; Atalan, 2000; Sembiring, 2000).

The observations outlined above suggest that there are strong associations between micromonosporae and particulate matter in soils and sediment and these may be limiting the recovery of micromonosporae and distorting the study of micromonosporae communities in natural habitats. The modified DDC procedure, which involves the use of mild detergent (sodium cholate), buffering (Tris buffer) and attenuated physical disruption (glass beads and mild ultrasonication), may be effective in disrupting such associations.

Large numbers of micromonosporae were recovered from many of the environmental samples examined, with the highest number ($9*10^4$cfu/g) being recovered from Ogongo Namibia (Nmb-90), soil from a cultivated lake bottom. Most of the colonies on the selective isolation plates (M3 and colloidal chitin) inoculated with this sample were putative micromonosporae, in some cases over 100 putative micromonosporae were isolated on each agar plate.

This study suggests that two factors concerning the nature of the habitat are very influential on the micromonosporae population. These are pH and moisture content of the habitat. In general, habitats with pH values of seven or above favoured the presence of micromonosporae. Conversely, habitats with low pH (<6.5) generally contained low numbers of micromonosporae and if pH values dropped below 5.0, no micromonosporae were isolated. The second factor to

play a role in micromonosporae populations was the moisture content of the sample. Micromonosporae were readily isolated from most of the aquatic samples examined, including most of the marine samples. It would appear that micromonosporae found in marine sediments are likely to be there as a result of terrestrial wash in of spores as the numbers recovered decreased as the depth of the samples increased. The numbers of micromonosporae isolated from the marine sediments are likely to be an underestimation of the population as these samples were subject to traditional shaking and not the DDC technique due to the limited amount of material available. As has been previously mentioned the DDC technique liberates more micromonosporae from soil and sediment samples than the traditional shaking technique. It was also found that habitats frequently exposed to water (i.e. mud flats) generally contained high numbers of micromonosporae.

It is generally acknowledged that perhaps only 0.1 to 5% of the microorganisms in most natural samples can be cultivated, thus only a small fraction of the extant microbial diversity in the natural environment can be described by such a technique (Tiedje, 1995). Advances in molecular and chemical techniques provide exciting alternatives for evaluating microbial diversity but were not employed at this point in the present project. A molecular approach, whereby microbial DNA present in the sediment could be amplified and sequenced would give a more complete and accurate picture of the diversity present as well identifying the major inhabitants of the community. However, such a method relies on known conserved DNA sequences as primer annealing sites, and the total microbial diversity may still not be fully revealed.

With many studies focussing on assessing diversity using molecular techniques in order to include the non-culturable portions of the microbial population, it is easy to forget why studies such as this one using culturable bacteria are so important. Bacteria are of prime importance in many industries including biotechnological, pharmaceutical and environmental companies. The main reasons for their importance is their ability to produce compounds of commercial value or their role in biotransformation of a low value compound to one of a greater value or degradation of a toxic compound to an inert one. The one thing

that all of these roles demands is that these bacteria can be cultivated in the laboratory. Therefore, whilst understanding the true diversity of a microbial population is important, the culturability of individual organisms is crucial to the continuance and expansion of many industries.

Chapter Four

Pyrolysis Mass Spectrometry

4.1. Assessment of diversity amongst isolated micromonosporae by pyrolysis mass spectrometry

4.1.1. Type strains of micromonosporae used as markers of known bacterial diversity

Eighteen type strains from the NCIMB and DSMZ culture collections were analysed alongside isolates of putative micromonosporae from geographic locations all over the world (see Table 4.1.).

Table 4.1. Details of the *Micromonospora* type strains used in the PyMS studies.

Collection Code	Valid species name	Source and strain history	Reference
NCIMB 12659	*Micromonospora olivaterospora*	K.Collins. Soil, paddy field suburban Hiroshima, Japan.	Kawamoto, *et al.*, 1983
NCIMB 12660	*Micromonospora echinospora* subsp. *pallida*	K. Collins. Soil, Syracuse, New York, USA.	Luedemann & Brodsky, 1964
NCIMB 12661	*Micromonospora purpureochromogenes*	K. Collins. Adobe soil, California, USA.	Waksman & Curtis, 1916
NCIMB 12663	*Micromonospora carbonacea* subsp. *carbonacea*	K. Collins. Soil, Olean, New York, USA.	Luedemann & Brodsky, 1965
NCIMB 12664	*Micromonospora carbonacea* subsp. *aurantiaca*	K. Collins. Soil.	Luedemann & Brodsky, 1965
NCIMB 12665	*Micromonospora coerula*	K. Collins. Soil, Mount Heleakula, Maui Island, Hawaii, USA.	Jensen, 1932
NCIMB 12741	*Micromonospora rhodorangea*	K. Collins. Soil.	Wagman *et al.*, 1974
NCIMB 12744	*Micromonospora echinospora* subsp. *echinospora*	K. Collins. Soil, Jamesville, New York, USA.	Luedemann & Brodsky, 1964
NCIMB 12751	*Micromonospora inositola*	K. Collins. Soil, Hokkaido, Japan.	Kawamoto *et al.*, 1974
NCIMB 12754	*Micromonospora aurantiaca*	K. Collins. Soil, USSR.	Sveshnikova *et al.*, 1969
NCIMB 12882	*Micromonospora purpurea*	K. Collins. Soil, Syracuse, New York, USA.	Luedemann & Brodsky, 1964
NCIMB 12896	*Micromonospora rosaria*	K. Collins. Soil, San Jacimto, Texas, USA.	Horan & Brodsky, 1986
NCIMB 2223	*Micromonospora halophytica* subsp. *halophytica*	A. Woyciesjes. Salt pool mud, Syracuse, New York, USA.	Weinstein *et al*, 1969
NCIMB	*Micromonospora*	A. Woyciesjes. Salt pool	Weinstein *et al*,

2225	*halophytica* subsp. *nigra*	mud, Syracuse, New York, USA.	1969
DSMZ 43026	*Micromonospora chalcea* **T**	Chas. Pfizer 1464-217L	(Foulerton, 1905) Ørskov, 1923
DSMZ 43141	*Micromonospora echinospora* subsp. *ferruginea*	A. Woyciesjes. Soil	Luedemann & Brodsky, 1964
DSMZ 43814	*Micromonospora brunnea*	Soil.	Sveshnikova *et al.*, 1969
DSMZ 44151	*Micromonospora chersina*	Soil.	Tomita *et al.* (1992)

4.1.2. Examining the effect of incubation time on the reproducibility of PyMS results

An initial experiment looked at the effect of the length of incubation of cultures prior to the PyMS run on the subsequent clustering of strains in the data analysis. A small number of type strains: *M. brunnea*, *M. carbonacea* subsp. *aurantiaca*, *M. echinospora* subsp. *ferruginea*, *M. aurantiaca* were used in this analysis. The cultures were incubated for periods between 5 and 10 days at 30°C on Micromonospora megalomiceum medium, all other conditions were kept as standardised as possible.

4.1.3. Examining the reproducibility of PyMS

The reproducibility of PyMS analyses were investigated by having two technicians (J. Mexson and S. Heald) prepare individual sets of PyMS samples. The effect of membrane filters, different batches of medium and source of culture were also studied. By growing cultures on membrane filters, carryover of agar onto the PyMS foils can be minimised. Eighteen type strains (see Table 4.1.) were grown on membrane filters on Micromonospora megalomiceum medium at 30°c for 5 days by two technicians. Samples were also taken from cultures with no membrane filter, from an alternative batch of medium, from a single colony and from confluent growth.

An analysis was also made of two sets of type strains that had been grown for two different PyMS experiments. The files were concatenated and the isolates

were deleted leaving two sets of type strains. These were analysed in the usual way (see Section 2.5.5.).

4.1.4. Investigating the diversity of isolates from a wide range of geographic locations and environmental habitats

Two hundred and seventy seven strains were subjected to PyMS analysis alongside the eighteen type strains described in Table 4.1. The isolates were divided into groups of 32 or less according to their geographic location, i.e. all Indonesian isolates were examined in one experiment and all South American isolates in another. Once the type strains were incorporated into the experiment this gave a full run of 50 strains, all of which were run in triplicate. The different geographic groups of isolates were run alongside a set of *Micromonospora* type strains in order to study their relationship to established reference strains of the genus. The analysis sought to answer two questions. Firstly, was there any clustering of samples according to their geographic location or habitat? Secondly, were any pyro-groups comprised entirely of isolates? These clusters may contain potentially novel species of *Micromonospora*. All isolates and type strains were cultured on Micromonospora megalomiceum agar at 30°C for 5 days prior to analysis. Isolates examined in these experiments are listed in Tables 4.3a-4.3i.

4.1.5. Analysis of pyrolysis mass spectrometric data

The data were analysed by PCA and CVA as described in Section 2.5.5. Determination of the relationships between PyMS test strains was based on analysis of the fifty most characteristic mass peaks. The triplicate samples for each strain showed reproducibility within the 95% confidence limit. The results of the experiments are shown as dendrograms, constructed using the UPGMA algorithm with the relationships being expressed as percentage similarities. An example of a full sequential PyMS data analysis is given in Appendix D.

4.1.6. Results of analysis of PyMS data

4.1.6.1. Examining the effect of incubation time on the reproducibility of PyMS data

The results of the initial PyMS analyses are shown as dendrograms in Figures 4.1 and 4.2. With reference to Figure 4.1 it appears that the length of incubation has had little effect on the clustering of the *M. aurantiaca* and *M. echinospora* subsp. *ferruginea* cultures incubated for 5-10 days as they have both formed discreet independent pyro-groups. The *M. carbonacea* subsp. *carbonacea* culture has fallen into two pyro-groups, one for 6, 7 and 9 days and the other for 5, 8 and 10 days. The pyro-group for the 5, 8 and 10 day cultures appears to be more consistent as the groupings are tighter, i.e. 100% similarity. The culture *M. carbonacea* subsp. *aurantiaca* has formed one pyro-group, although the *M. brunnea* cultures are interspersed amongst the former. The analysis shows the results for *M. brunnea* to be very inconsistent with different incubation periods as it groups with 3 out of the 4 other type strains tested. This may be an effect of the production of secondary metabolites, as examination of the plate cultures of this species revealed colouring of the medium on all plates with the exception of the five-day plate culture. It was noted that none of the other species used in the investigation were visibly secreting secondary metabolites into the medium. As micromonosporae are known for their production of secondary metabolites it was concluded that it would be prudent to use a short incubation time in order to minimise their production and henceforth their influence on the PyMS analyses. Therefore, since the other strains had exhibited little or no effect from the length of incubation it was decided that a 5-day incubation period would give sufficient growth of the culture with a minimum amount of secondary metabolite production and would be suitable for assessing the diversity of putative micromonosporae.

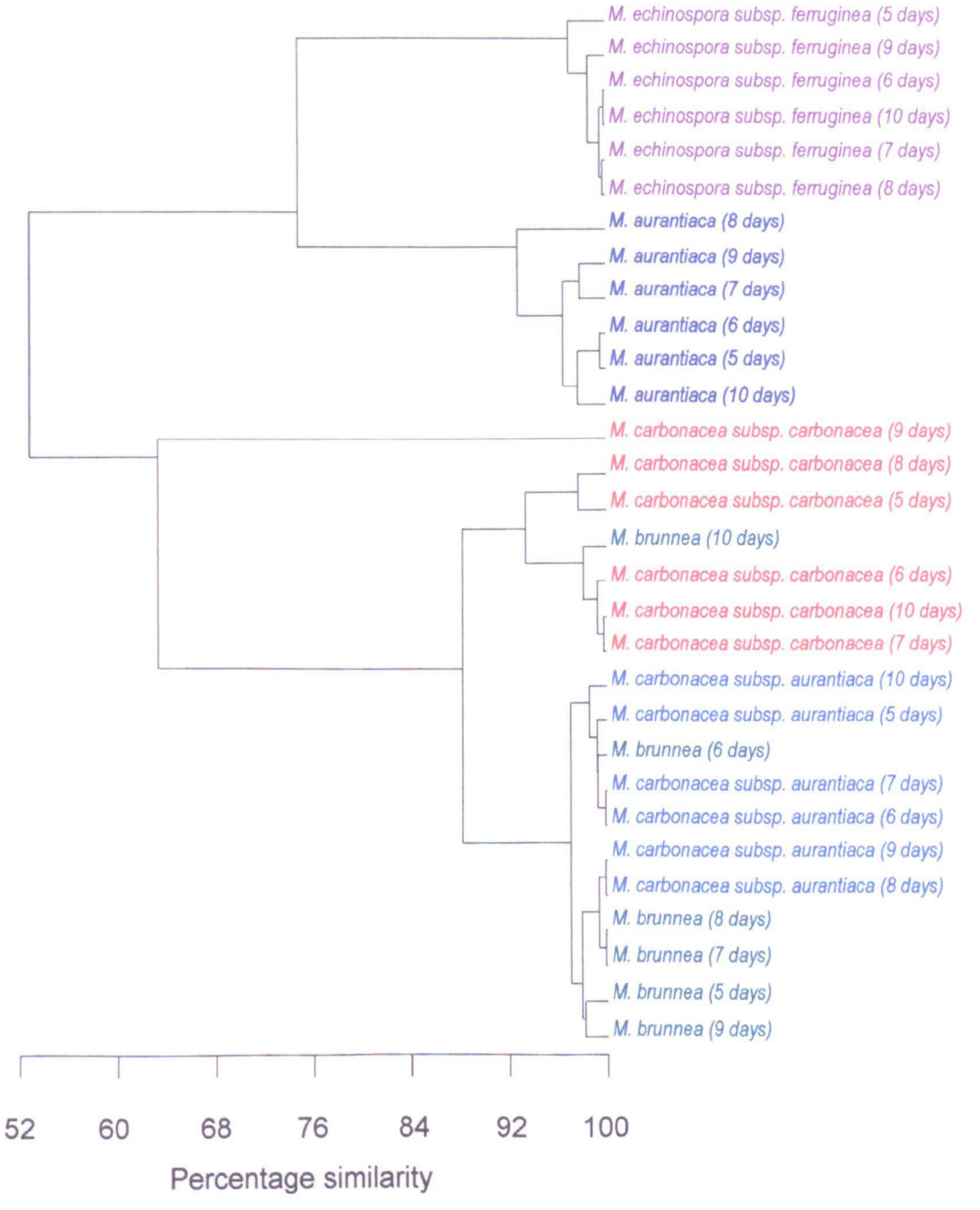
4.1.6.2. Examining the reproducibility of PyMS data

The second experiment looked at the effect of different technicians preparing the same samples on the grouping of micromonosporae type strains. Most type strains grouped together regardless of which technician prepared the sample. *M.*

inositola clustered with *M. carbonacea* subsp. *carbonacea* but the two individual strains were separated at the 99% similarity level. The two *M. carbonacea* subsp. *aurantiaca* samples fell into two pyro-groups; one sample formed a single membered pyro-group whilst the second sample grouped with *M. brunnea*. *M. halophytica* subsp. *halophytica* grouped in the same group but were on different branches. A large pyro-group of 5 replicates of *M. chalcea* all grouped together with one replicate sitting in a sub pyro-group. The effect of the membrane filters, different batches of media, use of single colonies and old biomass were all examined using *M. chalcea*. The grouping of these samples showed that the aforementioned variables did have an effect on the outcome of the PyMS results with samples from plates with no filters, or single colonies, or from separate plates forming groups distinct from the replicates of *M. chalcea*. There were minor differences when a different batch of media was used.

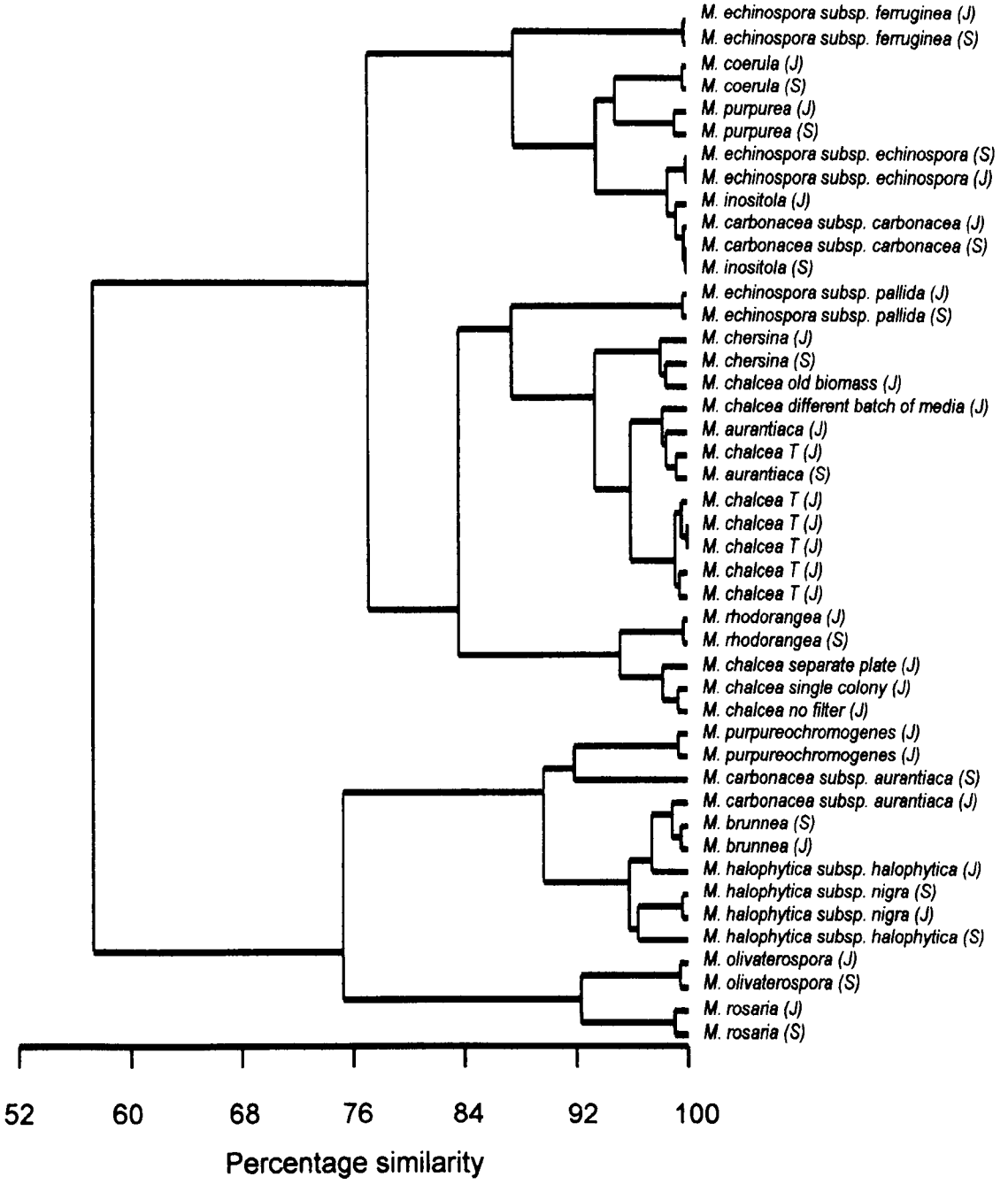
The third experiment examined the integrity of the pyro-groups of micromonosporae type strains that had been analysed on two separate occasions. Figure 4.2 clearly shows that the grouping of the type strains remains very stable between separate PyMS analyses. With the exception of *M. brunnea* all of the Type strains form tight individual pyro-groups at or above the 99% similarity level. *M. brunnea* falls into two pyro-groups, with *M. halophytica* subsp. *halophytica* and *M. purpureochromogenes*, which are separated at the 96% similarity level, this type strain also fell into multiple pyro-groups in the analysis of incubation periods. There is also one pyro-group containing four type strains, *M. inositola*, *M. carbonacea* subsp. *carbonacea*, *M. echinospora* subsp. *echinospora* and *M. coerula*, all grouping at the 99.7% similarity level.

Figure 4.1. Dendrogram showing the effect of incubation time on the clustering of selected *Micromonospora* type strains



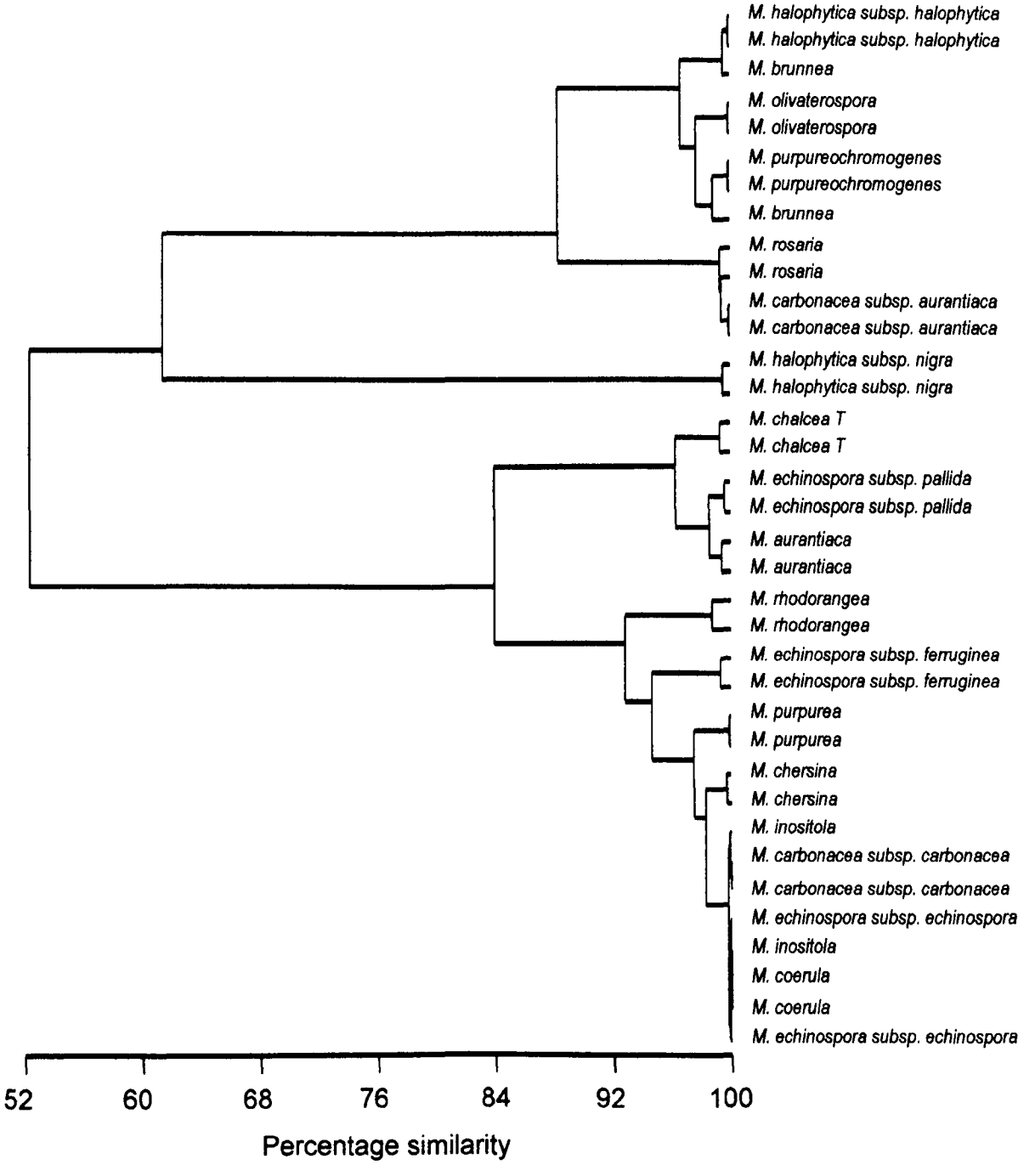
Dendrogram based on PyMS data analysed using GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

Figure 4.2. Dendrogram showing the reproducibility of PyMS analyses when two technicians prepare samples



Dendrogram based on PyMS data using GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm
J=J. Mexson, S=S. Heald

Figure 4.3. Dendrogram showing the relationship between type strains of the genus *Micromonospora* analysed on different days



Dendrogram based on PyMS data analysed using GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm

4.1.7. Results of PyMS analysis of geographic isolates

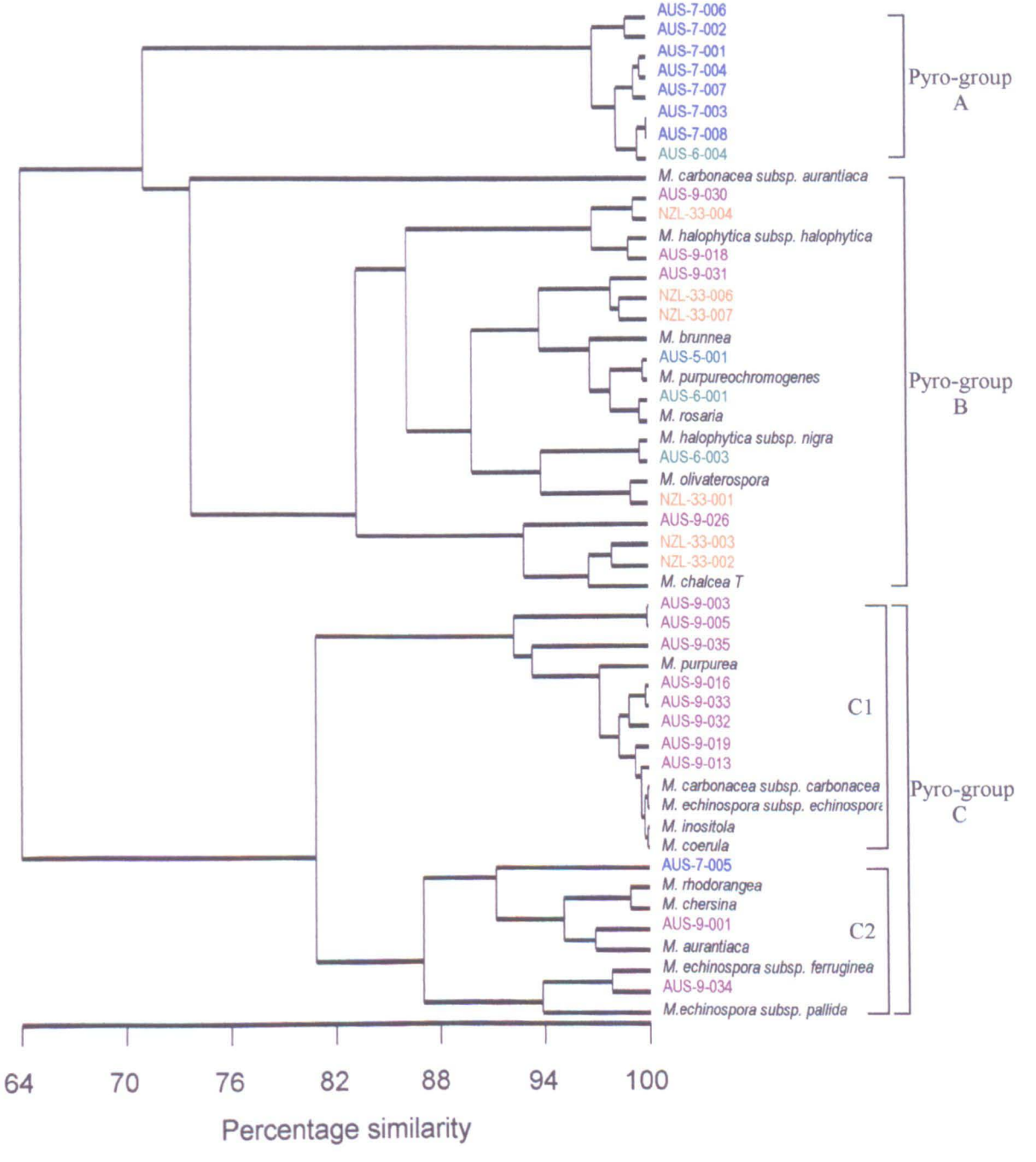
4.1.7.1. Analysis of isolates from Australia and New Zealand

The results of this analysis are shown as a dendrogram in Figure 4.4. Isolates from a number of Australian sites and one New Zealand site were examined alongside the type strains. Three major pyro-groups were formed in this analysis (A, B and C), C contains 2 sub pyro-groups (C1 and C2). All of the Aus-7 isolates with the exception of Aus-7-005 form a tight pyro-group (A) with no type strains defined at the 70% similarity level. Pyro-group B contains a number of type strains and all of the New Zealand strains as well as isolates from Aus-5, Aus-6 and Aus-9. Pyro-group C contains the majority of the Aus-9 isolates, one Aus-7 isolate and a number of type strains. Pyro-group C1 contains eight Aus-9 isolates and five type strains, four of which; *M. carbonacea* subsp. *carbonacea*, *M. echinospora* subsp. *echinospora*, *M. inositola* and *M. coerula*, are grouped very tightly together at 99.5% similarity. Pyro-group C2 contains only three isolates and five type strains.

Table 4.2a. Isolates from Australia and New Zealand examined by PyMS

Isolate	Sample type and geographic location
AUS-5-001	Rhizosphere soil. Mount Lewis, Great Dividing Range, Queensland, Australia.
AUS-6-001 AUS-6-003 AUS-6-004	Rhizosphere soil. Mossman Gorge, Daintree River National Park, Queensland, Australia.
AUS-7-001 AUS-7-002 AUS-7-003 AUS-7-004 AUS-7-005 AUS-7-006 AUS-7-007 AUS-7-008	Rhizosphere soil. Mossman Gorge, Daintree River National Park, Queensland, Australia.
AUS-9-001 AUS-9-003 AUS-9-005 AUS-9-013 AUS-9-016 AUS-9-018 AUS-9-019 AUS-9-026 AUS-9-030 AUS-9-031 AUS-9-032 AUS-9-033 AUS-9-034 AUS-9-035	Mud from a mangrove swamp. Lizard Island, Queensland, Australia.
NZL-33-001 NZL-33-002 NZL-33-003 NZL-33-004 NZL-33-006 NZL-33-007	Soil from New Zealand.

Figure 4.4. Dendrogram representing the relationships between isolates from Australia, New Zealand and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

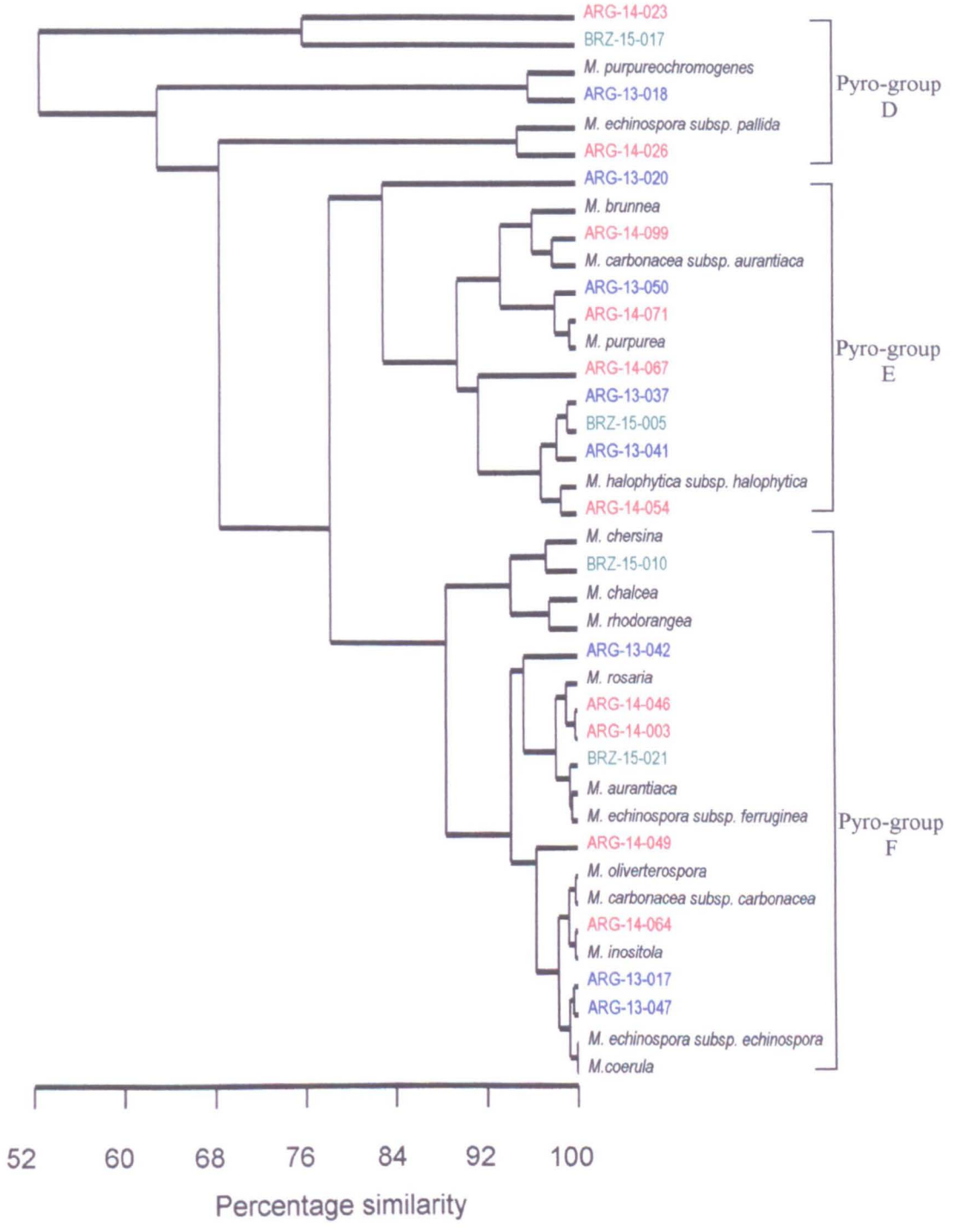
4.1.7.2. Analysis of isolates from South America

The result of this analysis is shown as a dendrogram in Figure 4.5. Twenty-four isolates from three sample sites in South America were examined alongside 18 type strains of *Micromonospora*. Three major pyro-groups were formed (D, E and F). Representatives from all three sample sites were found in each of the pyro-groups. Pyro-group D was defined at the 52% similarity level and contained two type strains. Pyro-group E contained four type strains. Pyro-group F was the largest of the three pyro-groups; the type strains were interspersed amongst the isolates and were present in all of the sub pyro-groups. The groupings were tighter in this pyro-group than the others with some of isolates such as Arg-14-046 and Arg-14-003 showing almost 100% similarity. Arg-14-064 was closely related to *M. inositola* with the branching showing almost 100% similarity. There appeared to be little evidence of isolates grouping according to their sample sites but the diversity of the isolates was high as shown by the percentage similarity values the pyro-groups and sub pyro-groups branched at.

Table 4.2b. Isolates from South America examined by PyMS

Isolates	Sample type and geographic location
Arg-13-001 Arg-13-017 Arg-13-018 Arg-13-020 Arg-13-027 Arg-13-037 Arg-13-041 Arg-13-042 Arg-13-047 Arg-13-050	Soil from arid land. St Martins Park, Mendoza, Argentina.
Arg-14-003 Arg-14-023 Arg-14-026 Arg-14-046 Arg-14-049 Arg-14-054 Arg-14-064 Arg-14-067 Arg-14-071 Arg-14-099	Sub-tropical rain forest soil. Iguacu National Park, Puerto Iguacu, Argentina.
Brz-15-005 Brz-15-010 Brz-15-017 Brz-15-021	Sub-tropical rain forest soil. Iguacu National Park, Foz do Iguacu, Brazil.

Figure 4.5. Dendrogram representing the relationships between isolates from South America and type strains of the genus *Micromonospora*.



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

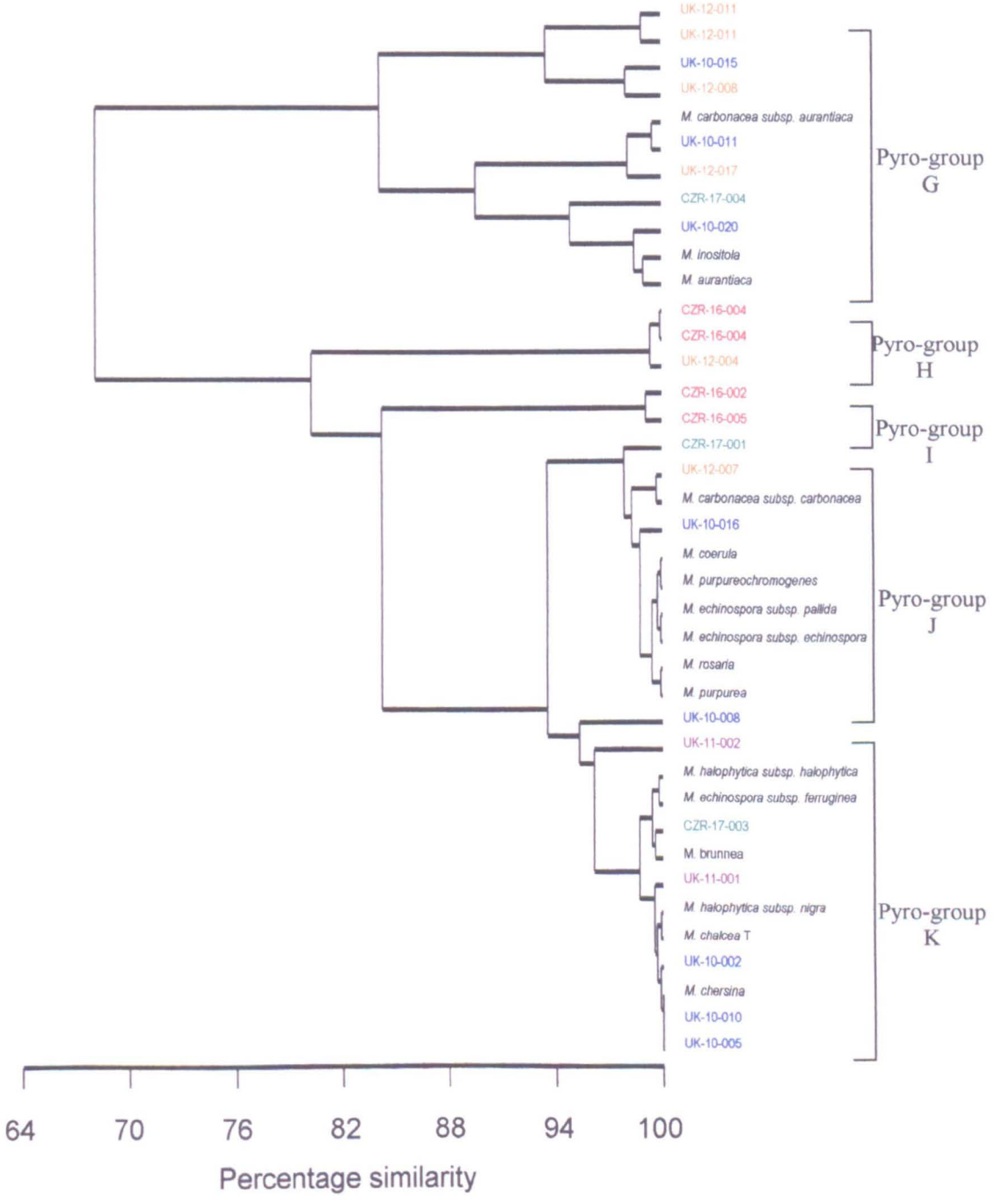
4.1.7.3. Analysis of isolates from the Czech Republic and the United Kingdom

Twenty-two isolates from the Czech Republic and the United Kingdom were analysed alongside 18 type strains of the genus *Micromonospora*, the results are presented as a dendrogram in figure 4.6. Three major pyro-groups (G, J and K) and two minor pyro-groups (H and I) were formed. Pyro-group G contained 3 type strains and 7 isolates; 3 of which were from UK-10, 3 from UK-12 and 1 from CZR-17. UK-12-011, UK-10-015 and UK-11-003 formed a sub pyro-group defined at the 94% similarity level. Pyro-group J consisted almost entirely of type strains with only 3 isolates present; 1 from CZR-17 and 2 from UK-10. Six out of the 7 type strains in this pyro-group formed their own sub pyro-group. Pyro-group K contained 6 type strains, 4 isolates from UK-10, 1 from UK-11 and 1 from CZR-17. The minor pyro-groups H and I contained no type strains and were defined at the 80% and 83% similarity level respectively. Both pyro-groups contained isolates that were very similar to one another with their pyro-groups being defined at or above the 98% similarity level.

Table 4.2c. Isolates from the Czech Republic and the United Kingdom examined by PyMS

Isolates	Sample type and geographic location
UK-10-002 UK-10-005 UK-10-008 UK-10-010 UK-10-011 UK-10-014 UK-10-015 UK-10-016 UK-10-020	Shallow river sediment. Dartmoor, United Kingdom.
UK-11-001 UK-11-002	Dry bog. Dartmoor, United Kingdom.
UK-12-004 UK-12-007 UK-12-008 UK-12-011 UK-12-017	Wet bog. Dartmoor, United Kingdom.
CZR-16-002 CZR-16-004 CZR-16-005	Sediment from a reed bed. Chalvetice, North Bohemia, Czech Republic.
CZR-17-001 CZR-17-003 CZR-17-004	Sediment from a flyash dump. Tusimice, North Bohemia, Czech Republic.

Figure 4.6. Dendrogram representing the relationships between isolates from the United Kingdom, Czech Republic and type strains of the genus *Micromonospora*.



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

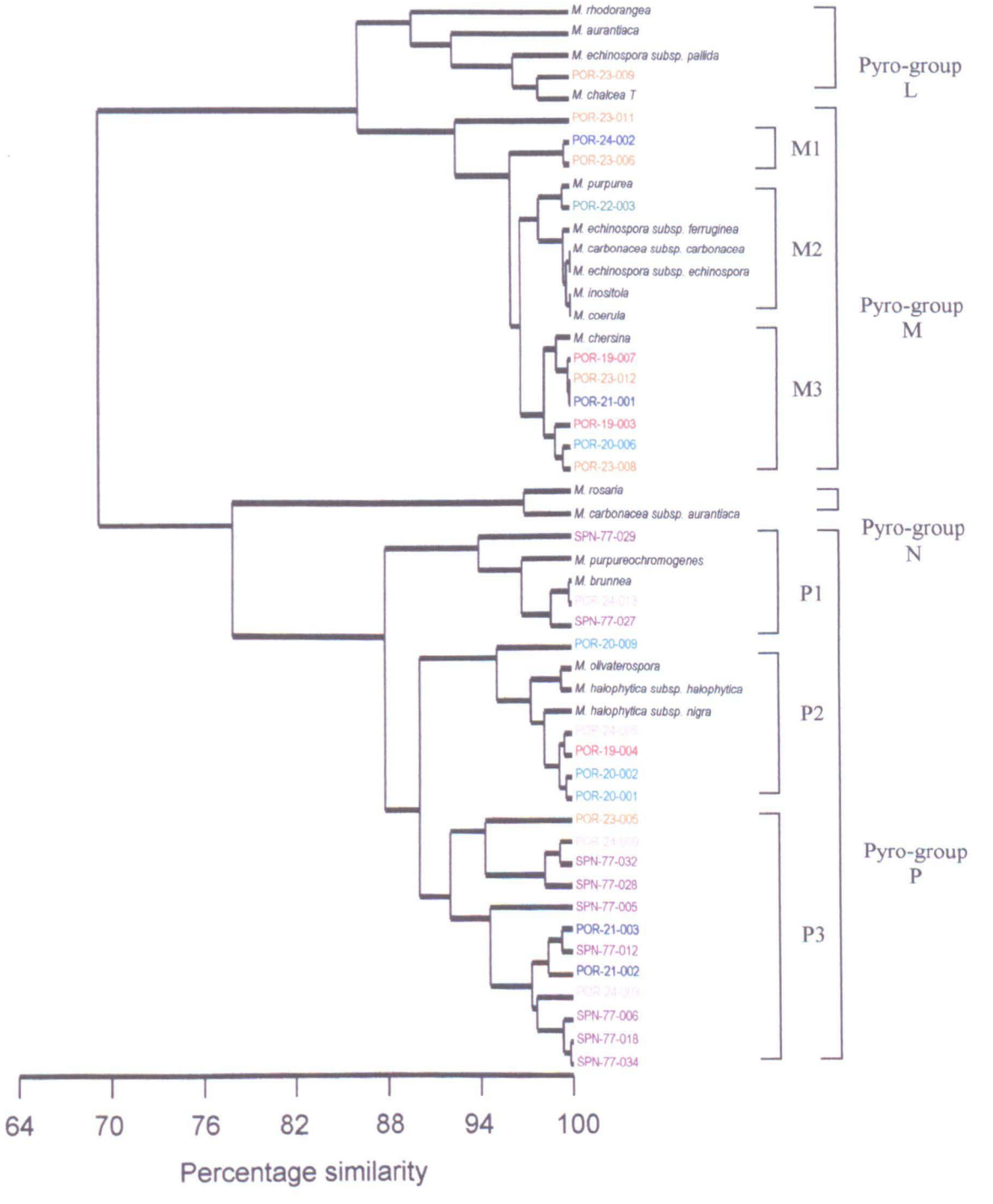
4.1.7.4. Analysis of isolates from Portugal and Spain

Thirty-one isolates from Portugal and Spain were analysed using PyMS, the results are shown in a dendrogram in Figure 4.7. Two major pyro-groups (M and P) and two minor pyro-groups (L and N) were formed. Pyro-group L defined at the 84% similarity level contained 4 type strains and 1 isolate Por-23-009 that was most closely related to *M. chalcea*. Pyro-group M contained 10 isolates from Por-19, Por-21, Por-22, Por-23 and Por-24. Por-23-011 formed a single membered sub pyro-group at the 92% similarity level. Por-24-002 and Por-23-006 were very closely related at almost 100% similarity. Por-22-003 was very closely related to *M. purpurea*. Five type strains: *M. echinospora* subsp. *ferruginea*, *M. carbonacea* subsp. *carbonacea*, *M. echinospora* subsp. *echinospora* and *M. coerula* formed a sub pyro-group (M1) containing no isolates. The sub pyro-group M2 contained only 1 type strain (*M. chersina*) and 6 isolates from: Por-19, Por-20, Por-21 and Por-23. Pyro-group N was a minor one, comprised of 2 type strains: *M. rosaria* and *M. carbonacea* subsp. *aurantiaca*. Pyro-group P was the largest and was comprised of 20 isolates from both Portugal and Spain: Por-19, Por-20, Por-23, Por-24, Spn-77 and 5 Type strains. This pyro-group could be divided into 3 sub pyro-groups (P1, P2 and P3). P1 was defined at the 95% similarity level and contained 2 type strains and 3 isolates: Por-24-013, Spn-77-027 and Spn-77-029. Sub pyro-groups P2 contained 3 type strains all of which were grouped closely together and 5 isolates all from Portugal. P3 was the largest sub pyro-group, defined at the 92% similarity level and contained no type strains. It was mainly composed of isolates from Spain (7) and some from Portugal (5).

Table 4.2d. Isolates from Portugal and Spain examined by PyMS analysis

Isolates	Sample type and geographic location
Por-19-003 Por-19-004 Por-19-007	Sediment. River Febre, Avintes, Portugal.
Por-20-001 Por-20-002 Por-20-006 Por-20-009	Sediment. River Ave, Spring, Portugal.
Por-21-001 Por-21-002 Por-21-003	Sediment. River Ave, Nascente, Portugal.
Por-22-001 Por-22-003	Sediment. River Leca, Portugal.
Por-23-005 Por-23-006 Por-23-008 Por-23-009 Por-23-011 Por-23-012	Sediment. River Cavado, Portugal.
Por-24-003 Por-24-005 Por-24-009 Por-24-013	Soil. Landim, Portugal.
Spn-77-005 Spn-77-006 Spn-77-012 Spn-77-018 Spn-77-027 Spn-77-028 Spn-77-029 Spn-77-032 Spn-77-034	Alkaline soil. Canulobes caves, Costa Brava, Spain.

Figure 4.7. Dendrogram representing the relationships between isolates from Portugal, Spain, and type strains of the genus *Micromonospora*.



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

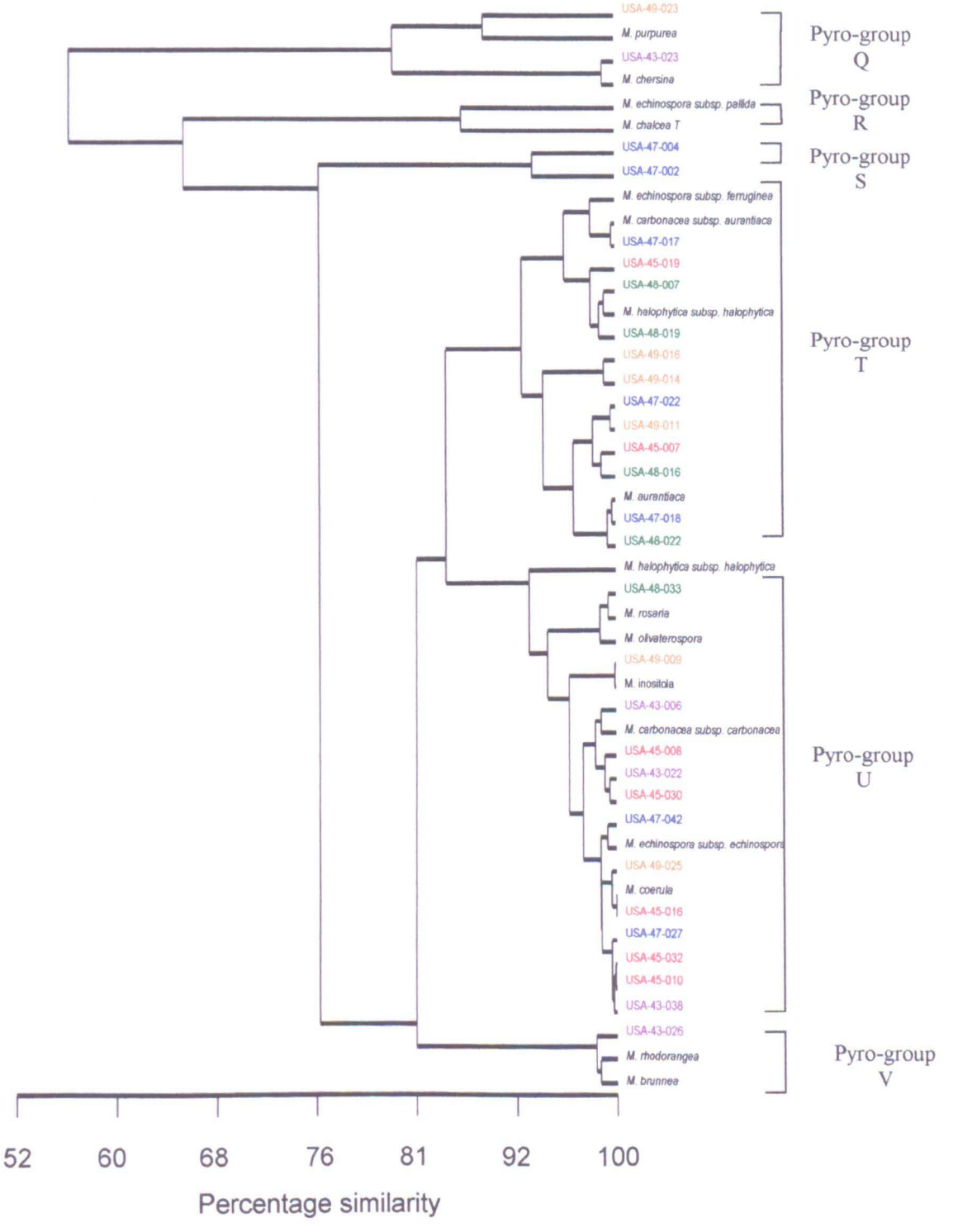
4.1.7.5. Analysis of isolates from the USA

Thirty isolates from five USA samples were examined by PyMS the results are shown as a dendrogram (Figure 4.8). Two major pyro-groups (T and U) and four minor pyro-groups (Q, R, S and V) were formed. Pyro-group Q (2 isolates and 2 type strains) was defined at the 84% similarity level, USA-49-023 and *M. purpurea* were grouped at the 91% similarity level, whilst USA-43-023 and *M. chersina* were shown to be closely related at the 99% similarity level. Pyro-group R consisted of 2 type strains: *M. echinospora* subsp. *pallida* and *M. chalcea*. Two strains from USA-47 made up pyro-group S and were defined at the 94% similarity level. Pyro-group T comprised of 4 type strains and 12 isolates from: USA-45, USA-47, USA-48 and USA-49. Two small sub pyro-groups (T1 and T2) only contained isolates, T1: USA-49-015 and USA-49-014 and T2: USA-47-022, USA-49-011, USA-45-007 and USA-48-016. The largest pyro-group was U and this contained 7 type strains and 14 isolates from all five sample sites. USA-49-009 showed 100% similarity with *M. inositola*. The type strains were scattered throughout the pyro-group although there was one small sub pyro-group containing four isolates, USA-47-027, USA-45-032, USA-45-010 and USA-43-038, which were closely related at 99.5% or above similarity. Pyro-group V contained two type strains and one isolate, USA-43-026.

Table 4.2e. Isolates from the USA examined by PyMS analysis

Isolates	Sample type and geographic location
USA-43-006	Soil. St. Louis, Missouri, USA.
USA-43-022	
USA-43-023	
USA-43-026	
USA-43-038	
USA-45-007	Soil. Music Row, Nashville, Tennessee, USA.
USA-45-008	
USA-45-010	
USA-45-016	
USA-45-019	
USA-45-030	
USA-45-032	
USA-47-002	Soil. Huntsville, Alabama, USA.
USA-47-017	
USA-47-018	
USA-47-022	
USA-47-027	
USA-47-042	
USA-48-007	Soil. Indy 500 Racetrack, Indianapolis, USA.
USA-48-016	
USA-48-019	
USA-48-022	
USA-48-033	
USA-49-009	Soil. Central Park, New York City, New York, USA.
USA-49-011	
USA-49-014	
USA-49-016	
USA-49-023	
USA-49-025	

Figure 4.8. Dendrogram representing the relationships between isolates from the USA and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

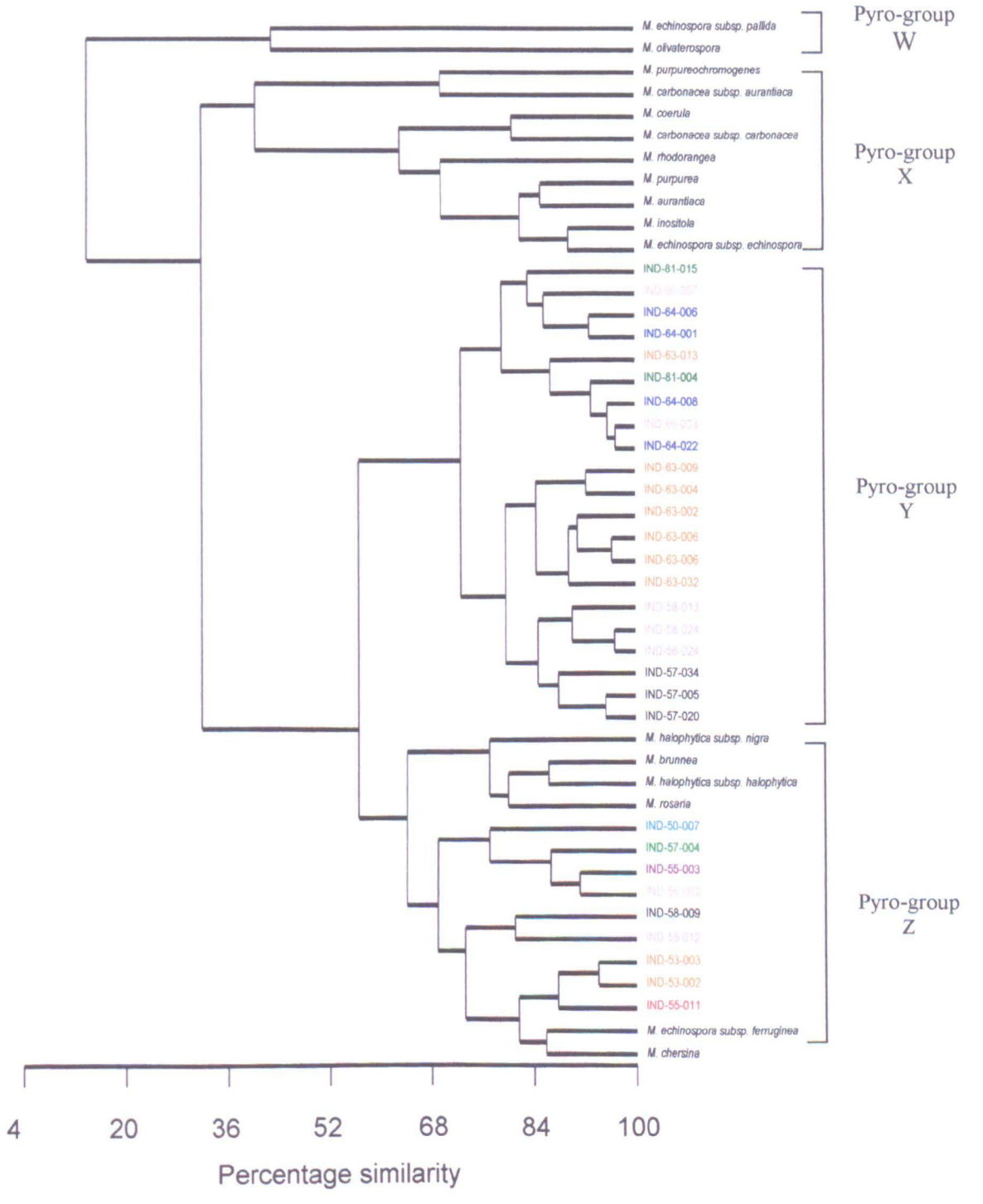
4.1.7.6. Analysis of isolates from Indonesia

Thirty isolates from Indonesia were analysed alongside 18 type strains of the genus *Micromonospora*, the results are presented as a dendrogram in Figure 4.9. Three major pyro-groups (X, Y, and Z) and one minor pyro-group (W) were formed. Minor pyro-group W consisted of 2 type strains; *M. echinospora* subsp. *pallida* and *M. olivaterospora*, separated at the 44% similarity level. Pyro-group X, defined at the 44% similarity level, was a larger pyro-group and contained 9 type strains and no isolates. Pyro-group Y was the largest pyro-group and was composed entirely of isolates. There were 21 isolates in this pyro-group and they represented 6 different sample sites; Ind-56, Ind-57, Ind-63, Ind-64, Ind-66 and Ind-81. Within this pyro-group there were 2 sub pyro-groups (Y1 and Y2) containing 9 and 12 isolates respectively. Sub pyro-group Y1 was defined at the 80% similarity level and contained isolates from; Ind-81, Ind-66, Ind-64 and Ind-63. Whilst sub pyro-group Y2, also defined at the 80% similarity level, contained isolates from; Ind-63, Ind-56 and Ind-57. Pyro-group Z contained 6 type strains and 9 isolates from: Ind-50, Ind-55, Ind-56, Ind-57 and Ind-58.

Table 4.2f. Isolates from Indonesia examined by PyMS analysis.

Isolates	Sample type and geographic location
Ind-50-007	Alkaline soil. Princess Cave, Yogyakarta, Java, Indonesia.
Ind-53-002 Ind-53-003	Soil. Kiskendo Cave, Yogyakarta, Java, Indonesia.
Ind-55-003 Ind-55-011	Soil. Cave, Karang Bolong, Java, Indonesia.
Ind-56-002	Soil. Mangrove, Tritih Putih, Cilicap, Java, Indonesia.
Ind-57-004 Ind-57-005 Ind-57-017 Ind-57-020 Ind-57-034	Soil from a crab mound. Mangrove, Tritih Putih, Cilicap, Java, Indonesia.
Ind-58-009 Ind-58-012 Ind-58-013 Ind-58-024	Soil. Cave Jatijajar, Gombong, Java, Indonesia.
Ind-61-002	Organic litter. Dieng Plateau, Wonosbo, Java, Indonesia.
Ind-63-002 Ind-63-004 Ind-63-006 Ind-63-009 Ind-63-013 Ind-63-032	Soil from exposed mud flats. Rawa Pening, Java, Indonesia.
Ind-64-001 Ind-64-006 Ind-64-008 Ind-64-022	Soil from a rice paddy field. Rawa Pening, Java, Indonesia.
Ind-66-003 Ind-66-007	Soil from a volcanic eruption (1997). Mt. Merapi, Yogyakarta, Java, Indonesia.
Ind-81-004 Ind-81-015	Soil from a tea plantation. Bandung, Java, Indonesia.

Figure 4.9. Dendrogram representing the relationships between isolates from Indonesia and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

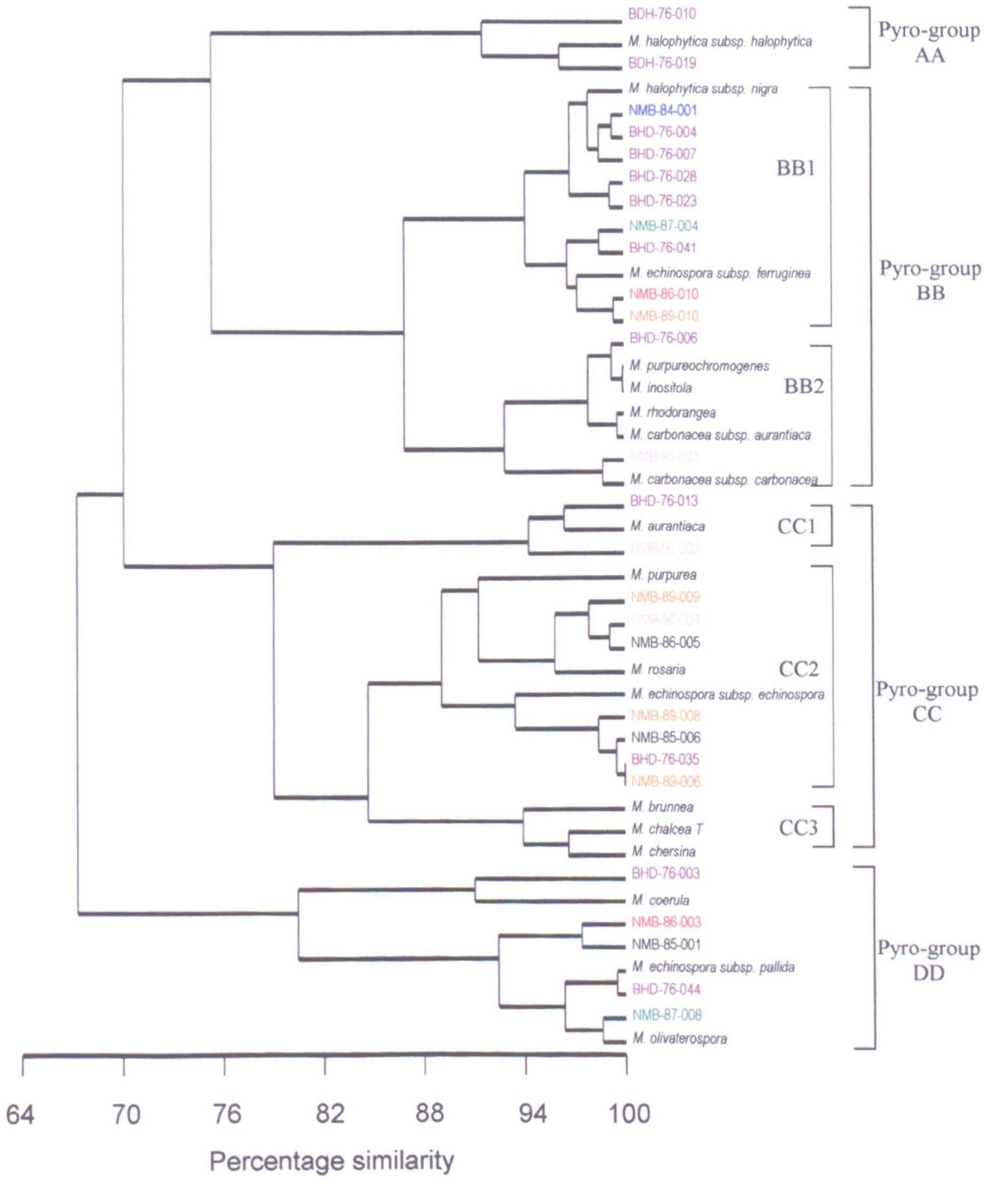
4.1.7.7. Isolates from Bangladesh and Namibia examined by PyMS

Twenty-seven isolates from Bangladesh and Namibia (see Table 4.2g.) were examined alongside 18 type strains (see Table 4.1.) by PyMS. One minor pyro-group (AA) and three major pyro-groups (BB, CC and DD) were formed. Pyro-group AA, defined at the 93% similarity level, comprised of two isolates from the Bangladesh sample and one type strain, *M. halophytica* subsp. *halophytica*. Pyro-group BB was defined at the 87% similarity level and was composed of 7 type strains and 11 isolates from; Bdh-76, Nmb-84, Nmb-87, Nmb-86, Nmb-89 and Nmb-90. Pyro-group BB could be split into 2 sub-clusters, BB1 and BB2. Sub pyro-group BB1 contained mainly isolates (9) and 2 type strains; *M. halophytica* subsp. *nigra* and *M. echinospora* subsp. *ferruginea*. Pyro-group BB2, defined at the 92% similarity level, contained only 2 isolates (Bdh-76-006 and Nmb-90-011) and 5 type strains. Pyro-group CC, defined at the 79% similarity level, contained 9 isolates and 4 type strains. Pyro-group CC could be split into 3 sub pyro-groups (CC1, CC2 and CC3). Sub pyro-group CC1 was defined at the 95% similarity level and was composed of 1 type strain (*M. aurantiaca*) and 2 isolates (Bdh-76-013 and Nmb-90-002). Pyro-group CC2, defined at the 90% similarity level, contained 3 type strains and 7 isolates, one from the Bangladesh sample and the rest from the Namibian samples; Nmb-89, Nmb-90, Nmb-86 and Nmb-85. Isolates Bdh-76-035 and Nmb-89-006 grouped at the 100% similarity level and were closely related to Nmb-85-006. Pyro-group CC3, defined at the 94% similarity level, contained 3 type strains. Pyro-group DD, defined at the 80% similarity level, contained 3 type strains and 5 isolates from Bdh-76, Nmb-85, Nmb-86 and Nmb-87. Isolate Bdh-76-044 was closely related to *M. echinospora* subsp. *pallida*, grouping at the 99% similarity level.

Table 4.2g. Isolates from Bangladesh and Namibia examined by PyMS analysis

Isolates	Sample type and geographic location
Bdh-76-003 Bdh-76-004 Bdh-76-006 Bdh-76-007 Bdh-76-010 Bdh-76-013 Bdh-76-016 Bdh-76-019 Bdh-76-023 Bdh-76-028 Bdh-76-035 Bdh-76-041 Bdh-76-044	Soil. Dhaka. Bangladesh.
Nmb-84-001 Nmb-84-005	Cultivated sandy soil. Kavango region, North Namibia.
Nmb-85-001 Nmb-85-005 Nmb-85-008 Nmb-85-008	Calcerous soil. Tsuemb region, North Central Namibia.
Nmb-86-003 Nmb-86-005 Nmb-86-010	Soil. Uitkomst Research Station, Tsuemb, North Central Namibia.
Nmb-87-002 Nmb-87-004 Nmb-87-008	Kalahari sand. Waterberg, Central Namibia.
Nmb-89-006 Nmb-89-009 Nmb-89-010	Soil. Katima Mulilo, North East Namibia.
Nmb-90-002 Nmb-90-004 Nmb-90-011	Soil from cultivated lake bottom. Oshana, Ogongo, North West Namibia.

Figure 4.10. Dendrogram representing the relationships between isolates from Bangladesh and Namibia and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

4.1.7.8. Isolates from the Pacific Ocean examined by PyMS

Sixty-five isolates (see Table 4.2h) and 18 type strains (see Table 4.1) were examined by PyMS. The results are shown as dendrograms in Figures 4.11a and b. Three major pyro-groups (EE, FF and GG) were defined in the initial analysis (Figure 4.11a.) and two minor (HH and II) and two major (JJ and KK) in the second analysis (Figure 4.11b.). In Figure 4.11a, pyro-group EE, defined at the 82% similarity level, contains 6 type strains and 7 isolates from; Jpn-18, Jpn-93, Jpn-95 and Jpn-102. Pyro-group FF, defined at the 86% similarity level, is composed entirely of isolates from a variety of samples sites; Jpn-18, Jpn-73, Jpn-74, Jpn-75, Jpn-91 and Jpn-93. A number of the isolates are very closely related (>99% similarity); Jpn-75-002, Jpn-93-001, Jpn-73-009 and Jpn-91-002. Cluster GG, defined at the 79% similarity level, is the largest pyro-group and contains 11 type strains and 12 isolates from Jpn-18, Jpn-94, Jpn-102, Jpn-75, Jpn-73, Jpn-93 and Jpn-91. The type strains; *M. carbonacea* subsp. *aurantiaca*, *M. echinospora* subsp. *echinospora*. *M. aurantiaca* and *M. coerula* are grouped in a tight pyro-group with isolates; Jpn-93-005, Jpn-93-004, Jpn-91-001 and Jpn-93-006, all of these strains are closely related at greater than 99% similarity. Figure 4.11b shows a dendrogram of the second group of marine isolates, for which there were two minor (HH and II) and two major (JJ and KK) pyro-groups. Pyro-group HH, defined at the 84% similarity level, contained 8 isolates from; Jpn-18, Jpn-74, Jpn-102, Jpn-91, Jpn-73 and Jpn-91 and no type strains. Pyro-groups II, defined at the 90% similarity level, was comprised of 5 isolates; Jpn-73-013, Jpn-91-001, Jpn-74-003, Jpn95-001, Jpn-91-005 and Jpn-74-003, and 1 type strain *M. olivaterospora*. Cluster JJ, defined at the 85% similarity level contained a high number of type strains (9) and only a small number of isolates; Jpn-18-251, Jpn-73-009, Jpn-91-003, Jpn-100-002 and Jpn-18-070. Pyro-group KK, defined at the 87% similarity level, contained a higher number of isolates (13) and 7 type strains. The isolates Jpn-75-005 and Jpn-18-072 were very closely related at > 99% similarity.

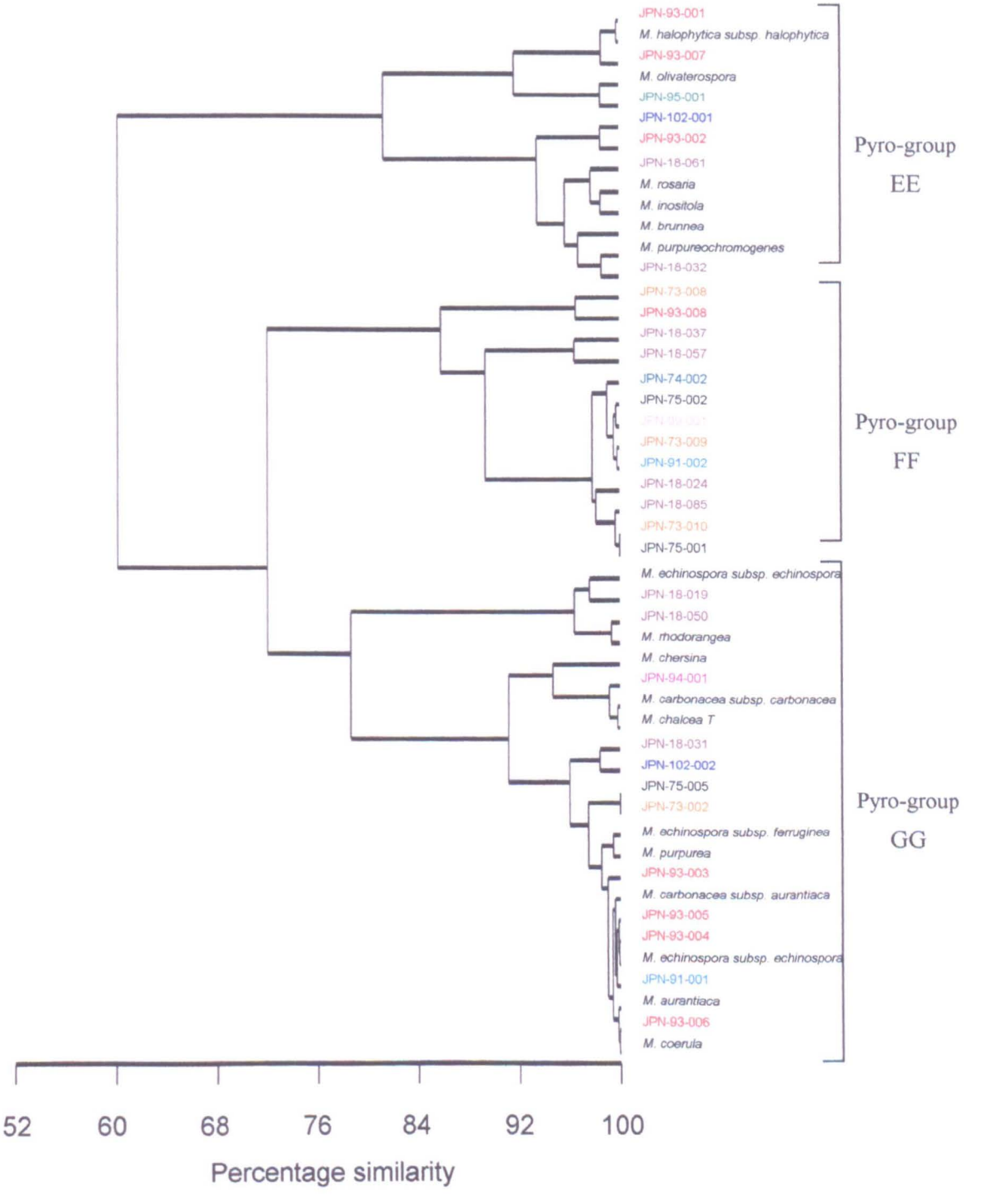
Table 4.2h. Isolates from the Pacific Ocean examined in PyMS analysis 4.11a and b.

Isolates	Sample type and geographic location
Jpn-18-019	Soft sediment, ~1000m. Off the coast of Japan, Pacific Ocean.
Jpn-18-024	
Jpn-18-031	
Jpn-18-032	
Jpn-18-037	
Jpn-18-047	
Jpn-18-050	
Jpn-18-057	
Jpn-18-061	
Jpn-18-063	
Jpn-18-065	
Jpn-18-070	
Jpn-18-072	
Jpn-18-080	
Jpn-18-085	
Jpn-18-251	
Jpn-73-002	Soft sediment, 1400m, Suruga Bay, Pacific Ocean.
Jpn-73-007	
Jpn-73-008	
Jpn-73-009	
Jpn-73-010	
Jpn-73-011	
Jpn-73-012	
Jpn-73-013	
Jpn-74-001	Soft sediment, 1400m, Suruga Bay, Pacific Ocean.
Jpn-74-002	
Jpn-74-003	
Jpn-74-005	Soft sediment, 1400m, Suruga Bay, Pacific Ocean.
Jpn-75-001	
Jpn-75-002	
Jpn-75-003	
Jpn-91-001	Soft sediment, 1168m. Sagami Bay, Pacific Ocean.
Jpn-91-002	
Jpn-91-003	
Jpn-91-004	
Jpn-91-005	
Jpn-93-001	Soft sediment, 1168m. Sagami Bay, Pacific Ocean.
Jpn-93-002	
Jpn-93-003	
Jpn-93-004	
Jpn-93-005	
Jpn-93-006	
Jpn-93-007	
Jpn-93-008	
Jpn-93-009	
Jpn-93-010	
Jpn-93-011	
Jpn-94-001	Soft sediment, 6142m. Japan Trench, Pacific Ocean.

Table 4.2h. Continued.

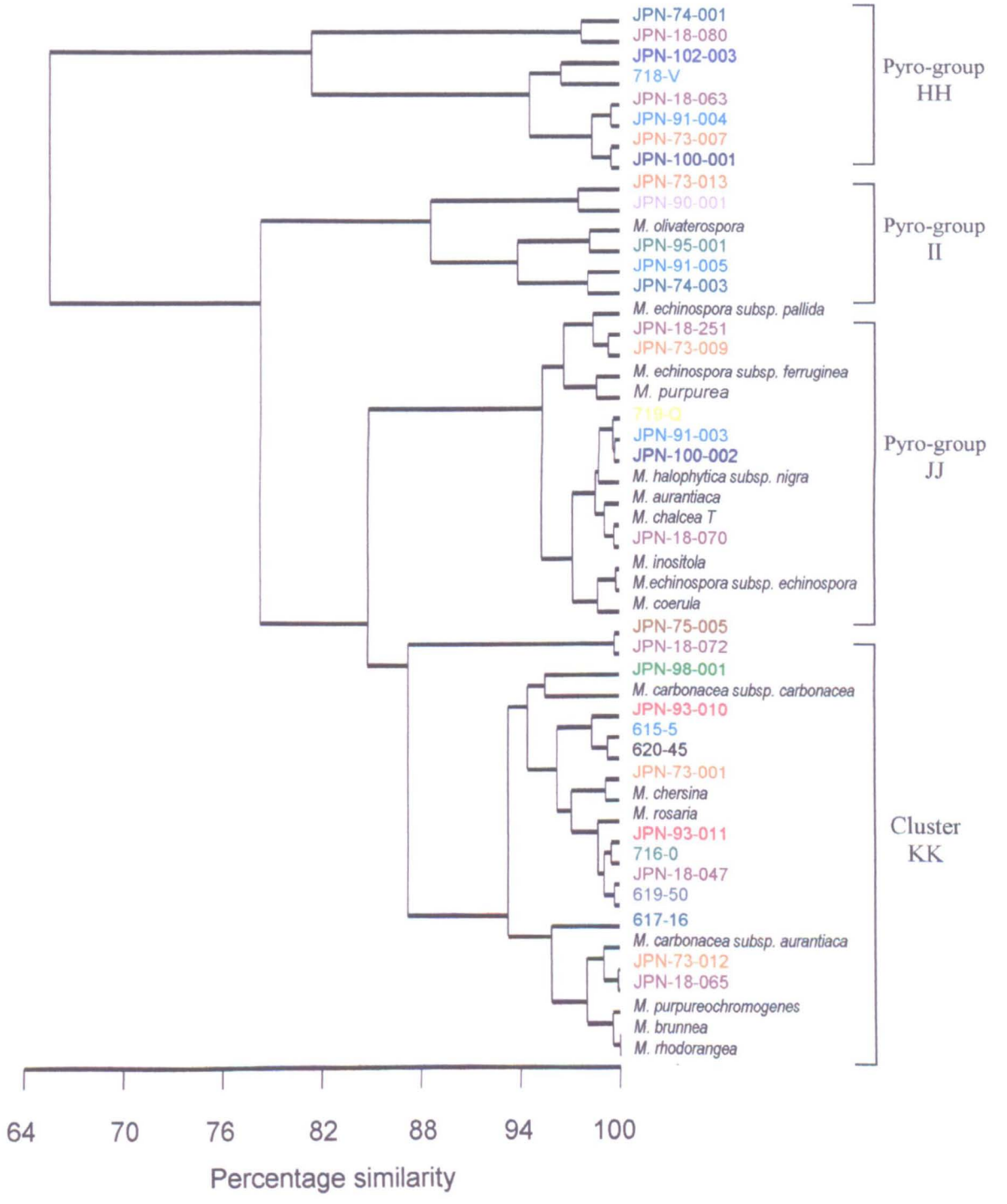
Isolates	Sample type and geographic location
Jpn-95-001	Soft sediment, 6300m. Japan Trench, Pacific Ocean.
Jpn-98-001	Soft sediment, 6475m. Japan Trench, Pacific Ocean.
Jpn-99-001	Soft sediment, 2679m. Izu Bonin Trench, Pacific Ocean.
Jpn-102-001 Jpn-102-002 Jpn-102-003	Soft sediment, 1151m. Suruga Bay, Pacific Ocean.

Figure 4.11a. Dendrogram representing the relationships between isolates from marine sediments from the Pacific Ocean and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

Figure 4.11b. Dendrogram representing the relationships between isolates from marine sediments from the Pacific Ocean and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

4.1.7.9. Isolates from all geographic locations previously studied, examined by PyMS

Representatives from all the major clusters previously defined by PyMS analysis (see Table 4.2i) were analysed alongside eighteen type strains of the genus *Micromonospora* (see Table 4.1). The results are shown as a dendrogram in Figure 4.12. Seven pyro-groups were formed, of which 5 were minor pyro-groups (LL, MM, NN, PP and QQ) and 2 were major pyro-groups (RR and SS). Pyro-group LL, defined at the 70% similarity level, consisted of 1 isolate (Jpn-100-002) and 1 type strain (*M. rhodorangea*). Pyro-group MM, defined at the 83% similarity level, contained 2 type strains (*M. halophytica* subsp. *nigra* and *M. purpurea*) and 8 isolates from; USA-43, Aus-9, Ind-57, USA-48, Arg-13 and Aus-7. Pyro-group NN, defined at the 93% similarity level, contained 3 type strains and 7 isolates from; Por-20, Czr-16, Czr-17, Bdh-76, Por-19, UK-12 and UK-11. Pyro-group PP, defined at the 94% similarity level, contained 3 type strains and 3 isolates from; USA-47, Ant-27 and Ant-28. Pyro-group QQ was comprised entirely of isolates and was defined at the 96% similarity level. Most of the isolates were representatives from marine samples; Jpn-18, Jpn-73 and Jpn-91 but there were also representatives from Bdh-76, Arg-14, Jpn-93 and Arg-13. Pyro-group RR, defined at the 94% similarity level, was the largest pyro-group and contained a high number of isolates (37) and a small number of type strains (7). The isolates included representatives from USA-45, Aus-9, Nzd-33, Ind-57, Jpn-18, Arg-14, Bdh-76, Por-23, Czr-16, UK-10, Por-24, Ant-25, USA-48, Spn-77, Ind-64, USA-47, Nmb-86, Ind-55, Por-21, Ind-64, Nmb-86, Jpn-93, Czr-16, UK-10, Arg-13, USA-47, Brz-15, Jpn-93, Ind-50 and Aus-6. Pyro-group SS, defined at the 93% similarity level, contained 2 type strains (*M. halophytica* and *M. rosaria*) and 17 isolates. The isolates included representatives from Aus-7, Ind-55, Ind-63, USA-49, Brz-15, Spn-77, Bdh-76, Nmb-86, Aus-9, Ind-53, Arg-13, Ind-63, Jpn-91 and UK-10.

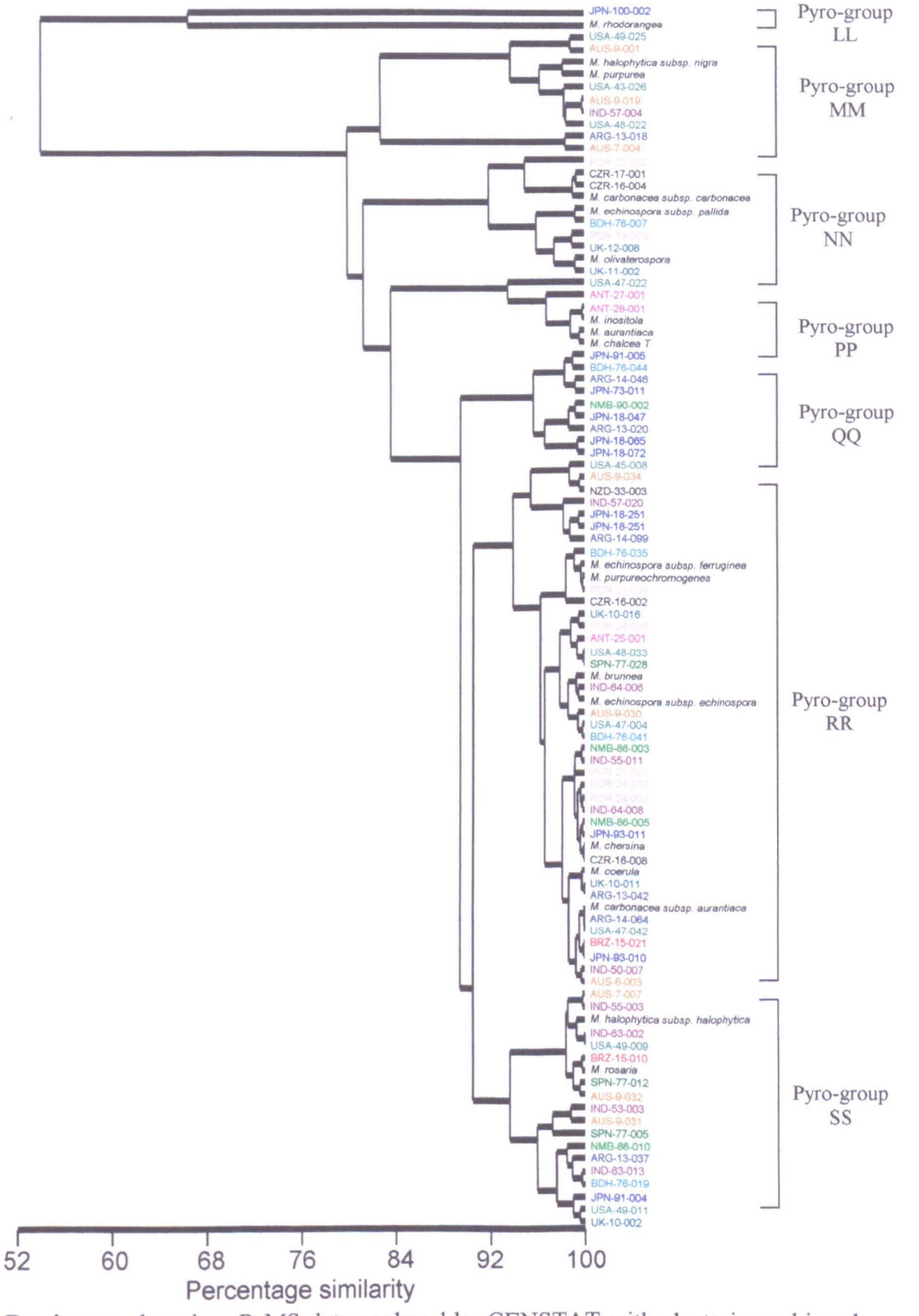
Table 4.2i. Isolates from all geographic locations examined by PyMS analysis

Isolates	Sample type and geographic location
Aus-6-003	Rhizosphere soil. Mossman Gorge, Daintree River National Park, Queensland, Australia.
Aus-9-001 Aus-9-019 Aus-9-030 Aus-9-031 Aus-9-032 Aus-9-034	Mud from a mangrove swamp. Lizard Island, Queensland, Australia.
UK-10-002 UK-10-011 UK-10-016	Shallow river sediment. Dartmoor, England.
UK-11-002	Dry bog. Dartmoor, England.
UK-12-008	Wet bog. Dartmoor, England.
Arg-13-018 Arg-13-020 Arg-13-037 Arg-13-037 Arg-13-042	Arid soil. St. Martin Park, Mendoza, Argentina.
Arg-14-046 Arg-14-064 Arg-14-099	Soil from sub-tropical rain forest. Iguacu National Park, Puerto Iguacu, Argentina.
Brz-15-010 Brz-15-021	Soil from sub-tropical rain forest. Iguacu National Park, Foz do Iguacu, Brazil.
Czr-16-002 Czr-16-004	Sediment from reed bed. Chvaletice, North Bohemia, Czech Republic.
Czr-17-001 Czr-17-008	Sediment from an active sedimentation pond. Tusimice, North Bohemia, Czech Republic.
Jpn-18-047 Jpn-18-065 Jpn-18-072 Jpn-18-251	Soft sediment ~1000m. Pacific Ocean, Japan.
Por-19-003	River sediment. Febre river, Avintes, Portugal.
Por-20-002	River sediment. Ave river, Spring, Portugal.
Por-21-001	River sediment. Ave river, Nascente, Portugal.
Por-23-009	River sediment. Cavado river, Portugal.
Por-24-002 Por-24-009 Por-24-013	Soil. Landim, Portugal.
Ant-25-001	Soil from frost-sorted polygon fines. Jane Col fellfield, Signy Island, Maritime Antarctica.
Ant-27-001	Soil from frost-sorted polygon fines. Moraine Valley fellfield site, Signy Island, Maritime Antarctica.
Ant-28-001	Sombre lake sediment at 10.5m depth (1-2cm zone). Signy Island, Maritime Antarctica.
Nzl-33-003	Soil sample. New Zealand.
USA-43-026	Soil. St Louis, Missouri, USA.
USA-45-008	Soil. Music row, Nashville, Tennessee, USA.

Table 4.2i. Continued.

Isolates	Sample type and geographic location
USA-47-004 USA-47-022 USA-47-042	Soil. Huntsville, Alabama, USA.
USA-48-022 USA-48-033	Soil. Indy 500 racetrack, Indianapolis, USA.
USA-49-009 USA-49-011 USA-49-025	Soil. Central Park, New York City, New York, USA.
Ind-50-007	Soil. Princess cave, Yogyakarta, Java, Indonesia.
Ind-53-003	Soil. Kiskendo cave, Yogyakarta, Java, Indonesia.
Ind-55-003 Ind-55-011	Soil. Beach cave, Karang Bolong, Yogyakarta, Java, Indonesia.
Ind-57-004 Ind-57-020	Soil from a crab mound. Tritih Putih, Cilicap, Java, Indonesia.
Ind-63-002 Ind-63-013	Soil from exposed mud flats. Rawa Pening, Java, Indonesia.
Ind-64-006 Ind-64-008	Soil from a rice paddy field. Rawa Pening, Java, Indonesia.
Jpn-73-011	Soft sediment, 1400m, Suruga Bay, Pacific Ocean.
Bdh-76-007 Bdh-76-019 Bdh-76-035 Bdh-76-041 Bdh-76-044	Soil. Dhaka, Bangladesh.
Spn-77-005 Spn-77-012 Spn-77-028	Soil. Canulobes caves. Costa Brava, Spain.
Nmb-86-003 Nmb-86-005 Nmb-86-010	Soil. Uitkomst Research Station, Tsuemb, North Central Namibia.
Nmb-90-002	Soil from a cultivated lake bottom. Oshana, Ogongo, North West Namibia.
Jpn-91-004 Jpn-91-005 Jpn-93-010 Jpn-93-011	Soft sediment, 1168m. Sagami Bay, Pacific Ocean.
Jpn-100-002	Soft sediment, 1151m. Suruga Bay, Pacific Ocean.

Figure 4.12. Dendrogram representing the relationships between isolates from all geographic locations and type strains of the genus *Micromonospora*



Dendrogram based on PyMS data analysed by GENSTAT with clustering achieved using the unweighted-pair group method with arithmetic averages algorithm.

4.1.8. Discussion of PyMS data

The PyMS analyses yielded a vast amount of information about the environmental isolates, their relationship to established species of *Micromonospora* and the significance of their geographic location. Initial experiments (see Figure 4.1) showed that for most strains, with the exception of *M. brunnea*, the period of incubation had little effect on the grouping of the strains in the PyMS analysis. A 5-day incubation period was selected as a standard incubation period for the subsequent analyses as this limited the effect that production of secondary metabolites may have on the profile of the organism being examined. The inclusion of mass ion peaks produced by the production of these metabolites may result in a shift from one pyro-group to another. A duplicate set of isolates prepared by two technicians showed excellent reproducibility of PyMS data (see Figure 4.2). Analysis of two subsets of type strains taken from different PyMS analyses revealed consistent groupings with the exception of *M. brunnea*. The separation of the *M. brunnea* strains may be a result of a difference in growth rate of the strains and hence a difference in the rate of production of secondary metabolites.

The pyro-groups generated by the PyMS analyses revealed the relationships between isolates and type strains of the genus *Micromonospora*. In a number of cases it revealed pyro-groups composed entirely of isolates, suggesting that these strains are distinct from the type strains and may be novel species of *Micromonospora* (pyro-group A in Figure 4.4, pyro-group Y in Figure 4.9). The dendrogram (Figure 4.12.) representing the relationship between isolates from all geographic locations and type strains of the genus *Micromonospora* revealed little grouping of isolates according to their location for example isolates from USA samples were found in pyro-groups MM, RR and SS. Whilst this initially indicates that there is little evidence for the biogeographic distribution of geovars of this genus, the high level of diversity amongst the isolates may be distorting the overall picture when this genus is looked at on a global level. When isolates are looked at on a more localised level, i.e. samples from just one continent, there is some evidence for biogeographic distribution of geovars. Figure 4.4 shows three major pyro-groups, one of which (A) contains isolates only from sample

Aus-6, with the exception of one isolate from Aus-7. Pyro-group B contains all the New Zealand strains and a few from Australia and sub pyro-group C1 contains strains solely from Aus-9.

The percentage similarity values on the dendrograms representing the PyMS data are only relevant to the individual analyses, where a particular set of test strains are compared. For this reason the same set of type strains were included in all analyses in order to give a base with which to compare the isolates. The presence of outlying strains in a data set tends to exaggerate any similarities between related strains, resulting in apparently homogenous clusters of actually different strains. Removing outliers form the dataset and reanalysing the data can resolve this effect. As the outliers are removed, different sets of characteristic mass peaks are used to determine the relationships between the test strains in the data set; as a result the relationships of the test strains can be more clearly resolved. This technique is based on whole cell profiling and directly reflects the phenotypic properties of the isolate, which is why the standardisation of conditions for the preparation of isolates is critical, as the profile generated from the PyMS analysis is dependant upon which genes are being expressed at that time. The true meaning of the relationships between the unknown strains can only be determined by subsequent phenotypic or molecular profiling. The technique's main advantage is to allow the screening of large numbers of isolates in a short time and to provide information on the grouping of test strains, from which representatives can be selected for inclusion in additional studies.

PyMS has been shown to be of value for the separation of actinomycetes at and below the species level: as an initial step in the detection and circumscription of novel actinomycetes and for the detection of identical strains (Sanglier *et al.*, 1992). A number of studies have shown that PyMS is able to distinguish isolates with distinct phenotypic profiles (Sanglier *et al.*, 1992; Goodacre and Kell, 1996). PyMS has excellent potential for industrial microbiology in the application to biotechnological screening programmes with high throughputs of unknown organisms, where rapid, automated and cost effective procedures are highly desired.

Chapter Five

Numerical Taxonomy

5.1. Introduction

This analysis sought to test the validity of the pyrogroups defined by pyrolysis mass spectrometry by comparing them to the clusters generated from a numerical taxonomic study, and to examine the isolates for evidence of biogeographic distribution. Previous investigations have demonstrated that clusters generated by PyMS analysis show good congruence with clusters generated from numerical taxonomic studies (Atalan *et al.*, 2000; Bull e*t al.*, 2000).

5.2. Preparation of final data matrix and calculation of test error

Two hundred and seven strains including fifteen duplicated cultures, were examined for 134 unit characters. Sixteen unit characters were excluded from the final data matrix prior to analysis as they provided little differential value (Table 5.1). Tests with all positive or negative results provide little discrimination between the strains tested and whilst they may be useful for identification purposes, they are of little value for numerical taxonomic studies. Twenty-seven strains were removed from the matrix due to either poor growth on the positive control plates or contamination of test plates.

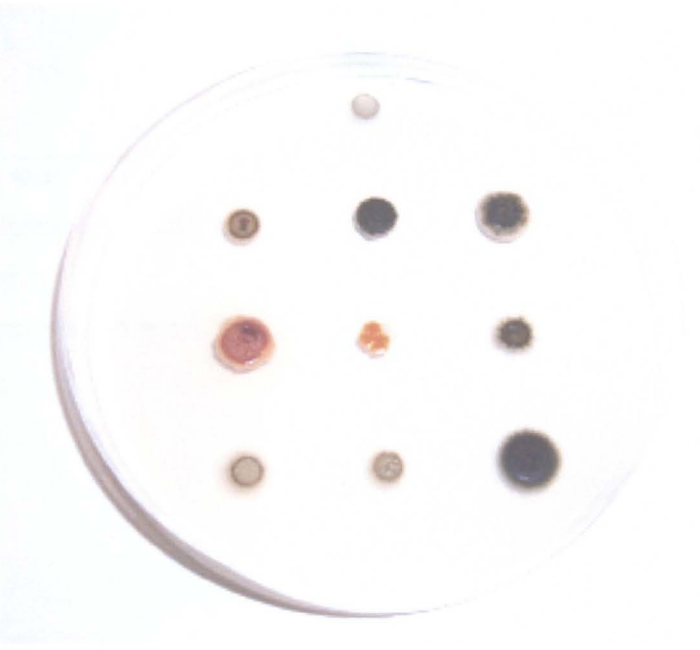
The percentage agreement between the fifteen duplicated strains and test variance (Si^2) were used to assess the intra-operator reproducibility (Table 5.1). Tests found to have an Si^2 value greater than 0.2 were deleted from the final data matrix. (See Table 5.1.). The average probability of an erroneous test result (p) for a single operator calculated from the pooled variance (S^2) for the remaining tests was 4.37%. The final data matrix was composed of 180 strains and 116 unit characters.

The majority of the *Micromonospora* strains studied were able to degrade, casein, chitin, elastin, starch and xylan; utilise a variety of monosaccharides, disaccharides and polysaccharides as sole carbon sources for energy and growth; tolerate levels of sodium chloride of 1% w/v; grew at pH 7.0 or 8.0, and possessed glucosidase enzymes. In contrast, very few strains degrade tributyrin

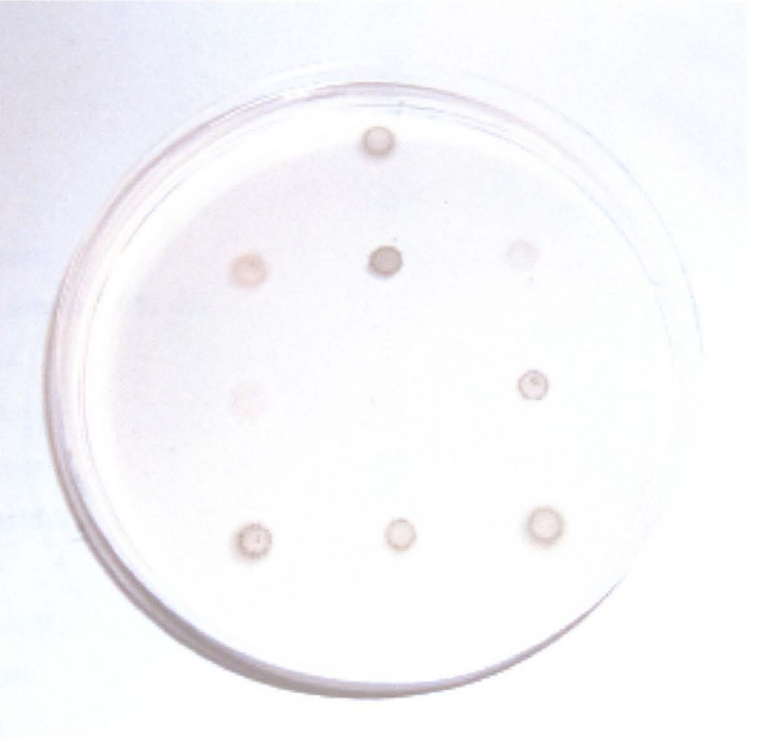
or Tween 20; utilise aromatic hydroxy acids or inulin as a sole carbon source; tolerate sodium chloride levels of 7% w/v or grow at pH 4.0; are resistant to penicillin V concentrations of 5μg or higher. Examples of positive growth of *Micromonospora* isolates on xylan plates and negative growth on sorbose plates are shown in Figure 5.1.

Figure 5.1 Examples of growth of *Micromonospora* strains on agar supplemented with different carbon sources. Plates incubated for 21 days at 30°C

Growth on sodium pyruvate



Growth on sorbose



The growth of *Micromonospora* strains on these two carbon sources were compared with growth of the organisms on a negative control plate (no added carbon source).

Table 5.1. Intra-operator test error calculated from comparison of data from fifteen duplicated strains and percentage frequency of positive results for all tests included in the numerical taxonomic study

Tests	Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
Biochemical tests:			
Hydrogen sulphide production	100	0	83
Nitrate reduction	100	0	60
Substrate degradation tests:			
Arbutin	60	0.2	60
Casein	100	0	95
Cellulose	60	0.2	38
Chitin	100	0	73
Elastin	100	0	95
Gelatin	94	0.03	32
Starch	94	0.03	75
Tributyrin	100	0	0
Tween 20	100	0	0
Tween 40	94	0.03	62
Tween 60	88	0.07	22
Tween 80	94	0.03	27
Xylan	94	0.03	91
Nutritional tests			
Sole carbon source 1.0% w/v			
Deoxy sugars			
* α-D (+) Fucose	53	0.23	27
* α-L (-) Fucose	60	0.2	38
Monosaccharides			
Pentoses:			
*L (+) Arabinose	60	0.2	56
D (-) Arabinose	66	0.17	42
D-Lyxose	100	0	16
*D (-) Ribose	53	0.23	33
D (+) Xylose	100	0	78

Table 5.1. Continued.

Tests	Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
Hexoses:			
D (-) Fructose	100	0	81
D (+) Galactose	100	0	78
D (+) Mannose	100	0	80
* α-L-Rhamnose	47	0.27	37
L (-) Sorbose	100	0	1
Disaccharides:			
D (+) Cellobiose	88	0.07	74
α-Lactose	94	0.03	22
* Maltose	60	0.2	34
* α-D (+) Melibiose	53	0.23	29
D-Sucrose	100	0	79
* D (+) Trehalose	60	0.2	27
* D (+) Turanose	53	0.23	19
Trisaccharides:			
D (+) Melezitose	100	0	8
* Raffinose	53	0.23	19
Polysaccharides:			
Glycogen	100	0	86
Inulin	94	0.03	10
Starch	100	0	97
Xylan	94	0.03	91
Sole carbon sources (0.1% w/v)			
Aliphatic amino acids:			
DL-α-Alanine	100	0	32
L-Arginine	100	0	28
L-Ornithine monohydrochloride	100	0	15
L-Serine	94	0.03	26
* L-Threonine	53	0.23	22
Glycosidases and related compounds:			
N-Acetyl-D-glucosamine	94	0.03	37

Table 5.1. Continued.

Tests	Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
* Arbutin	47	0.27	2
D-Gluconic acid	94	0.03	71
* D-Glucosamine	53	0.23	41
Methyl-α-D-glucopyranoside	94	0.03	15
Methyl-β-D-glucopyranoside	100	0	61
* Methyl-α-D-mannopyranoside	60	0.2	28
* Methyl-β-D-xylopyranoside	60	0.2	33
* 2-keto-D-gluconic acid	53	0.23	27
D (-) Mandelic acid	94	0.03	19
* Salicin	60	0.2	8
Carboxylic acids:			
Sodium acetate	100	0	5
Sodium-n-butyrate	94	0.03	33
Sodium propionate	100	0	48
Sodium pyruvate	94	0.03	81
Dicarboxylic acids:			
Sebacic acid	100	0	0
Amines:			
Spermine tetrahydrochloride	100	0	47
Aromatic amino acids:			
L-β-Phenylalanine	100	0	28
L-Proline	100	0	48
* D-Tryptophan	60	0.2	16
L-Tryptophan	100	0	33
L-Tyrosine	100	0	8
Aromatic hydroxy acids:			
Ferulic acid	100	0	1
m-Hydroxybenzoic acids	100	0	0
p-Hydroxybenzoic acids	100	0	0
Physiological tests			
Resistance to antibiotics (μg/ml)			
Aminoglycosides:			

Table 5.1. Continued.

Tests		Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
Gentamicin sulphate	5	100	0	44
Gentamicin sulphate	25	100	0	39
Neomycin sulphate	5	100	0	23
Neomycin sulphate	25	100	0	7
Streptomycin sulphate	5	100	0	37
Streptomycin sulphate	25	100	0	33
Coumarin:				
Novobiocin	5	100	0	19
Novobiocin	25	100	0	10
β-Lactams				
Penicillin:				
Penicillin V	5	100	0	11
Penicillin V	25	100	0	11
Rifamycin:				
Rifampicin	5	100	0	21
Rifampicin	25	94	0.03	14
Tetracyline:				
Tetracyline hydrochloride	5	100	0	23
Tetracyline hydrochloride	25	100	0	20
Miscellaneous:				
Chloramphenicol	5	100	0	22
Chloramphenicol	25	100	0	14
Tolerance to chemical inhibitors				
(% w/v):				
Sodium chloride	1.0	100	0	100
Sodium chloride	3.0	100	0	54
Sodium chloride	5.0	100	0	10
Sodium chloride	7.0	100	0	7
Growth at pH:				
pH 4.0		100	0	8
pH 5.0		88	0.07	19
pH 6.0		94	0.03	46

Table 5.1. Continued.

Tests	Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
pH 7.0	94	0.03	57
pH 8.0	100	0	87
pH 9.0	88	0.07	65
pH 10.0	82	0.1	57
Antibiosis tests			
Inhibition of:			
Escherichia coli	100	0	27
Staphylococcus aureus	100	0	13
Enterococcus faecalis	100	0	10
Bacillus subtilis	100	0	25
Pseudomonas aeruginosa	100	0	0
Candida albicans	100	0	7
Enzyme tests			
Cleavage of 4-methylumbelliferone substrates:			
4-methylumbelliferyl-β-D-galactopyranoside	100	0	50
4-methylumbelliferyl-β-D-glucopyranoside	100	0	41
4-methylumbelliferyl-N-acetyl-β-D-glucosamide	100	0	40
4-methylumbelliferyl-β-D-glucuronide	100	0	69
4-methylumbelliferyl-7-β-D-xyloside	94	0.03	42
4-methylumbelliferyl-α-D-glucoside	100	0	78
4-methylumbelliferyl-α-D-galactoside	94	0.03	45
4-methylumbelliferyl-β-D-fucoside	100	0	49
4-methylumbelliferyl-α-D-mannoside	94	0.03	31
4-methylumbelliferyl-phosphate	94	0.03	30
4-methylumbelliferyl-sulfate	100	0	23
4-methylumbelliferyl-β-cellobioside	94	0.03	34
4-methylumbelliferyl-butyrate	94	0.03	54
4-methylumbelliferyl-β-D-ribofuranoside	94	0.03	10

Table 5.1. Continued.

Tests	Percentage agreement between duplicated strains	Variance Si^2	Percentage frequency of positive results
4-methylumbelliferyl-alpha-L-arabinoside	94	0.03	40
4-methylumbelliferyl-β-D-mannopyranoside	94	0.03	38
4-methylumbelliferyl-nonanoate	94	0.03	72
4-methylumbelliferyl-dodecanoate	100	0	17
4-methylumbelliferyl-palmitate	100	0	31
Cleavage of 7-amido-methyl coumarin substrates:			
Leucyl-amido-4-methylcoumarin	100	0	63
Prolyl-amido-4-methylcoumarin	94	0.03	50
Pyroglutamyl-amido-4-methylcoumarin	100	0	8
Glutamyl-amido-4-methylcoumarin	100	0	33
Lysyl-amido-4-methylcoumarin	100	0	8
Asparginyl-amido-4-methylcoumarin	94	0.03	34
Phenylalanyl-amido-4-methylcoumarin	100	0	63
Z-glycyl-prolyl-amido-4-methylcoumarin	100	0	21
Z-arginyl-amido-4-methylcoumarin	100	0	39
H-Ornithine-amido-4-methylcoumarin.2HCl	94	0.03	29
L-analyl-7-amido-4-methylcoumarin	100	0	53
L-histidine-7-amido-4-methylcoumarin	94	0.03	33

* Denotes tests eliminated from the final data matrix
Z, the N-terminal carbobenzoxy group of endopeptidase substrate.

5.3. Cophenetic correlation coefficients

The cophenetic correlation coefficients were calculated for the dendrograms based on the S_J and S_{SM} coefficients and the UPGMA clustering algorithm. The values for the S_J and S_{SM} cophenetic correlation coefficients were 0.81 and 0.62 respectively. The classification based on the S_J and the UPGMA algorithm was chosen for further analysis as it had the highest cophenetic correlation value and several well-defined clusters.

5.4. Numerical classification based on the Jaccard coefficient and the UPGMA clustering algorithm

5.4.1. Distribution of test strains to major, minor and single membered clusters

The 166 test strains and 14 type strains were recovered in 5 major, (4 or more strains), 15 minor (2-3 strains) and 124 single membered clusters defined at the 69% similarity level (Figure 5.2.). These phena accounted for 11.7%, 19.4% and 68.8% of the test strains, respectively. The number of strains that constitute a major or minor cluster varies according to the papers you read. The numbers used here were decided upon after personal communication with A. C. Ward. The percentage similarity level at which the clusters are defined is subjective. However, the general practise is to select a level that cuts through the major branches on the dendrograms and provides good aggregate clusters (M, Goodfellow, personal communication). Where possible the clusters were named after the type strains they contained. Clusters containing more than one type strain were given multiple names. The composition of the clusters is detailed in Table 5.2.

Five (*M. carbonacea* subsp. *carbonacea*, *M. inositola*, *M. coerula*, *M. echinospora* subsp. e*chinospora* and *M. carbonacea* subsp. a*urantiaca*) of the fourteen type strains examined formed one major cluster (Cluster 1a) with

isolates. Two type strains (*M. purpurea* and *M. chersina*) formed minor clusters (Cluster 2a and Cluster 3a) with isolates Jpn-18-085 and Nmb-89-009 respectively. The remaining type strains all formed single membered clusters. Of the major clusters, Cluster 8a was composed of one isolate from Antarctica, two from the NW Pacific Ocean and one from the USA; Cluster 14a was comprised of three UK isolates and one from the Czech Republic; Cluster 19a was composed entirely of marine isolates with three from Sagami Bay and one from the Japan Sea; Cluster 20a contained two isolates from the USA, one marine and one Brazilian isolate. Of the minor clusters; Cluster 4a contained three isolates from Lizard Island, Australia; Cluster 6a contained isolates from Bangladesh; Cluster 7a was composed entirely of Argentinean isolates; Cluster 9a contained Portuguese isolates; Cluster 12a contained Brazilian isolates; Cluster 15a was comprised of three Indonesian isolates; Cluster 16a contained Namibian isolates; Cluster 18a contained isolates from the UK.

A comparison of the composition of the clusters generated by S_J and S_{SM} is made in Table 5.3. The percentage positive frequency data for strains assigned to the major clusters is shown in Table 5.4. The intra-cluster similarity values and centrotype strains of the major and minor clusters are shown in Table 5.5. Centrotypes are only meaningful for clusters with three or more strains, as with clusters of only two strains, the first strain will always be selected as the centrotype.

5.4.2. Characteristics of *Micromonospora* strains

Over 80% of the strains in the major clusters were positive for hydrogen sulphide production; degradation of casein, chitin, elastin and xylan; growth on the sole carbon source (1% w/v) D (-) fructose, D (+) galactose, D (+) xylose, sucrose, mannose, glycogen, starch, xylan; growth on sole carbon source (0.1% w/v) sodium pyruvate; tolerance to (1%w/v) NaCl and pH 8.0.

Conversely the strains in the major clusters were negative (<20% positive) for growth on the sole carbon source (1% w/v) tributyrin, tween 20, lyxose, L (-) sorbose, D (+) melezitose, inulin; growth on the sole carbon source (0.1% w/v)

L-ornithine monohydrochloride, methyl-α-D-glucopyranoside, D (-) mandelic acid, sodium acetate, sebacic acid, L-tyrosine, ferulic acid, *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid; resistance to (μg/ml) neomycin sulphate 5 and 25, penicillin V 5 and 25, rifampicin 25, tetracycline 25, chloramphenicol 25; tolerance to (% w/v) NaCl 5 and 7, pH 4 and 5; resistance to *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Candida albicans* and did not contain the enzymes to cleave pyroglutamyl-amido-4-methylcoumarin, lysyl-amido-4-methylcoumarin, 4-methylumbelliferyl-β-D-ribofuranoside and 4-methylumbelliferyl-dodecanoate.

Table 5.2. Designation and source of strains assigned to clusters defined at the 69% similarity level in the analysis based on the Jaccard coefficient and the unweighted pair group arithmetic averages algorithm

Strain code	Description	Source
Major clusters		
Cluster 1a		
12663	*M. carbonacea* subsp. *carbonacea*	NCIMB. New York, USA *
12751	*M. inositola*	NCIMB. Hokkaido, Japan *
12665	*M. coerula*	NCIMB. Hawaii, USA *
12744	*M. echinospora* subsp. *echinospora*	NCIMB. New York, USA *
12664	*M. carbonacea* subsp. *aurantiaca*	NCIMB. Soil. *
Cluster 8a (*Micromonospora* sp.)		
Ant-28-001	*Micromonospora* sp.	Signy Island, Antarctica ♠
Jpn-73-008	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
USA-49-016	*Micromonospora* sp.	New York, USA ♠
Jpn-73-011	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
Cluster 14a (*Micromonospora* sp.)		
UK-10-015	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-12-011	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-10-020	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Czr-17-002	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Cluster 19a (*Micromonospora* sp.)		
Jpn-18-072	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Jpn-91-002	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Jpn-91-005	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Jpn-91-004	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Cluster 20a (*Micromonospora* sp.)		
Brz-15-005	*Micromonospora* sp.	Iguacu National Park, Brazil♠
USA-43-022	*Micromonospora* sp	Missouri, USA ♠

Table 5.2. Continued

Strain code	Description	Source
Major clusters		
Cluster 20a (***Micromonospora* sp.**)		
USA-47-018	*Micromonospora* sp	Alabama, USA ♠
Jpn-18-047	*Micromonospora* sp	Japan Sea, Pacific Ocean ♠
Minor clusters		
Cluster 2a (*M. purpurea)*		
12882	*M. purpurea*	NCIMB. New York, USA ♠
Jpn-18-085		Japan Sea, Pacific Ocean ♠
Cluster 3a (***M. chersina)***		
44151	*M. chersina*	DSMZ. Soil *
Nmb-89-009		Katima Mulilo, Namibia ♠
Cluster 4a (*Micromonospora* sp.)		
Aus-9-016	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-9-019	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-9-034	*Micromonospora* sp.	Lizard Island, Australia ♠
Cluster 5a (*Micromonospora* sp.)		
Aus-9-035	*Micromonospora* sp.	Lizard Island, Australia ♠
UK-11-002	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Nmb-84-001	*Micromonospora* sp.	Kavango, Namibia ♠
Cluster 6a (*Micromonospora* sp.)		
Bdh-76-019	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-028	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Cluster 7a (*Micromonospora* sp.)		
Arg-14-003	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Arg-14-026	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Arg-14-064	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Cluster 9a (*Micromonospora* sp.)		
Por-23-012	*Micromonospora* sp.	Cavado River, Portugal ♠
Por-24-002	*Micromonospora* sp.	Landim, Portugal ♠
Cluster 10a (*Micromonospora* sp.)		
Arg-14-049	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
USA-49-011	*Micromonospora* sp.	New York, USA ♠
Ind-81-004	*Micromonospora* sp.	Bandung, Java, Indonesia ♠
Cluster 11a (*Micromonospora* sp.)		
Nzl-33-003	*Micromonospora* sp.	New Zealand ♠
Ind-56-012	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
Cluster 12a (*Micromonospora* sp.)		
Brz-15-010	*Micromonospora* sp.	Iguacu National Park, Brazil♠

Table 5.2. Continued

Strain code	Description	Source
Brz-15-013	*Micromonospora* sp.	Iguacu National Park, Brazil♠
Cluster 13a (*Micromonospora* sp.)		
Jpn-18-251	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Por-24-009	*Micromonospora* sp.	Landim, Portugal ♠
Cluster 15a (*Micromonospora* sp.)		
Ind-63-013	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Ind-63-034	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Ind-64-022	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Cluster 16a (*Micromonospora* sp.)		
Nmb-89-006	*Micromonospora* sp.	Katima Mulilo, Namibia ♠
Nmb-89-008	*Micromonospora* sp.	Katima Mulilo, Namibia ♠
Cluster 17a (*Micromonospora* sp.)		
Nmb-90-002	*Micromonospora* sp.	Ogongo, Namibia ♠
Nmb-90-011	*Micromonospora* sp.	Ogongo, Namibia ♠
Cluster 18a (*Micromonospora* sp.)		
UK-10-005	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-12-012	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Single membered clusters		
2223	*M. halophytica* subsp. *halophytica*	NCIMB. New York, USA *
2225	*M. halophytica* subsp. *nigra*	NCIMB. New York, USA *
Aus-5-001	*Micromonospora* sp.	Mount Lewis, Australia ♠
12659	*M. olivaterospora*	NCIMB. Hiroshima, Japan *
43141	*M. echinospora* subsp. *ferruginea*	DSMZ. Soil *
12660	*M. echinospora* subsp. *pallida*	NCIMB. New York, USA *
UK-10-002	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-10-010	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Bdh-76-003	*Micromonospora* sp.	Dhaka, Bangladesh ♠
UK-10-008	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Jpn-18-032	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Aus-9-032	*Micromonospora* sp.	Lizard Island, Australia ♠
Bdh-76-007	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-013	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-035	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Czr-16-002	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Por-20-002	*Micromonospora* sp.	Ave River, Spring, Portugal♠
Por-21-001	*Micromonospora* sp.	Ave River, Nascente, Portugal ♠
Por-20-006	*Micromonospora* sp.	Ave River, Spring, Portugal♠
Nzl-33-004	*Micromonospora* sp.	New Zealand ♠
USA-47-022	*Micromonospora* sp.	Alabama, USA ♠
USA-48-016	*Micromonospora* sp.	Indianapolis, USA ♠

Table 5.2. Continued

Strain code	Description	Source
Ind-53-003	*Micromonospora* sp.	Yogyakarta, Java, Indonesia♠
Bdh-76-041	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-044	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Aus-9-003	*Micromonospora* sp.	Lizard Island, Australia ♠
Czr-16-004	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Por-20-009	*Micromonospora* sp.	Ave River, Spring, Portugal♠
UK-10-011	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Arg-14-065	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Por-19-003	*Micromonospora* sp.	Febre River, Avintes, Portugal ♠
Ind-81-015	*Micromonospora* sp.	Bandung, Java, Indonesia ♠
USA-43-026	*Micromonospora* sp.	Missouri, USA ♠
Jpn-91-001	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Ant-25-001	*Micromonospora* sp.	Signy Island, Antarctica ♠
USA-45-019	*Micromonospora* sp.	Tennessee, USA ♠
Ind-57-020	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
UK-10-014	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Spn-77-028	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Por-21-008	*Micromonospora* sp.	Ave River, Nascente, Portugal ♠
Ind-64-006	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Aus-6-001	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-6-003	*Micromonospora* sp.	Mossman Gorge, Australia ♠
USA-48-033	*Micromonospora* sp.	Indianapolis, USA ♠
Aus-9-031	*Micromonospora* sp.	Lizard Island, Australia ♠
Czr-17-001	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Czr-17-003	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Por-20-001	*Micromonospora* sp.	Ave River, Spring, Portugal♠
UK-12-017	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Jpn-100-002	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
Aus-7-006	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Nmb-87-008	*Micromonospora* sp.	Waterberg, Namibia ♠
Aus-9-001	*Micromonospora* sp.	Lizard Island, Australia ♠
USA-47-004	*Micromonospora* sp.	Alabama, USA ♠
Aus-9-030	*Micromonospora* sp.	Lizard Island, Australia ♠
Czr-16-005	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Arg-14-046	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
UK-10-016	*Micromonospora* sp.	Dartmoor, United Kingdom♠
USA-48-022	*Micromonospora* sp.	Indianapolis, USA ♠
USA-49-009	*Micromonospora* sp.	New York, USA ♠
Spn-77-005	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Spn-77-029	*Micromonospora* sp.	Canulobes Caves, Spain ♠
USA-47-027	*Micromonospora* sp.	Alabama, USA ♠
Jpn-93-001	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
12661	*M. purpureochromogenes*	NCIMB. California, USA *

Table 5.2. Continued

Strain code	Description	Source
Ind-55-011	*Micromonospora* sp.	Karang Bolong, Java, Indonesia ♠
Ind-57-004	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
Czr-16-007	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Czr-16-009	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Por-24-006	*Micromonospora* sp.	Landim, Portugal ♠
USA-47-047	*Micromonospora* sp.	Alabama, USA ♠
USA-43-038	*Micromonospora* sp.	Missouri, USA ♠
USA-47-042	*Micromonospora* sp.	Alabama, USA ♠
Spn-77-012	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Jpn-93-008	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
UK-12-002	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-12-004	*Micromonospora* sp.	Dartmoor, United Kingdom♠
43026	*M. chalcea*^{T}	DSMZ. Pfizer. *
Por-21-003	*Micromonospora* sp.	Ave River, Nascente, Portugal ♠
Por-24-013	*Micromonospora* sp.	Landim, Portugal ♠
Por-23-009	*Micromonospora* sp.	Cavado River, Portugal ♠
UK-12-007	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Por-22-001	*Micromonospora* sp.	Leca River, Portugal ♠
USA-45-008	*Micromonospora* sp.	Tennessee, USA ♠
Nmb-87-004	*Micromonospora* sp.	Waterberg, Namibia ♠
Aus-6-004	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-7-004	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-7-007	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-7-008	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Jpn-18-030	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Jpn-18-065	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Aus-9-018	*Micromonospora* sp.	Lizard Island, Australia ♠
Ind-50-007	*Micromonospora* sp.	Bantul, Java, Indonesia ♠
Ind-63-002	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Arg-14-071	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
UK-12-010	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Ind-55-003	*Micromonospora* sp.	Karang Bolong, Java, Indonesia ♠
Aus-9-026	*Micromonospora* sp.	Lizard Island, Australia ♠
Nmb-86-003	*Micromonospora* sp.	Tsuemb, Namibia ♠
Nmb-86-005	*Micromonospora* sp.	Tsuemb, Namibia ♠
Czr-17-004	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Nmb-86-010	*Micromonospora* sp.	Tsuemb, Namibia ♠
Jpn-93-010	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Arg-13-027	*Micromonospora* sp.	Mendoza, Argentina ♠
Arg-13-042	*Micromonospora* sp.	Mendoza, Argentina ♠
Arg-13-047	*Micromonospora* sp.	Mendoza, Argentina ♠

♠ See Chapter Two, Table 2.1 for full details of sample locations
* See Chapter Two, Table 2.2 for complete histories of the type strains
Abbreviations; NCIMB, National Collection of Industrial and Marine Bacteria, UK; DSMZ, Deutsche Sammlung Mikroorganism und Zelfulturen, Germany.

Table 5.3. Composition of clusters defined in the S_J, UPGMA analysis compared with those obtained in the S_{SM} UPGMA analysis

S_J UPGMA		S_{SM} UPGMA Cluster
Major clusters		
Cluster 1 a (*Micromonospora* sp.)		
12663	*M. carbonacea* subsp. carbonacea	12b
12751	*M. inositola*	12b
12665	*M. coerula*	12b
12744	*M. echinospora* subsp. *echinospora*	12b
12664	*M. carbonacea* subsp. *aurantiaca*	12b
Cluster 2 a (*M. purpurea*)		
12882	*M. purpurea*	13b
Jpn-18-085		13b
Cluster 3 a (*M. chersina*)		
44151	*M. chersina*	SMC
Nmb-89-009		SMC
Cluster 4 a (*Micromonospora* sp.)		
Aus-9-016		14b
Aus-9-019		14b
Aus-9-034		14b
Minor clusters		
Cluster 5 a (*Micromonospora* sp.)		
Aus-9-035		14b
UK-11-002		14b
Nmb-84-001		14b
Cluster 6 a (*Micromonospora* sp.)		
Bdh-76-019		21b
Bdh-76-028		21b
Cluster 7 a (*Micromonospora* sp.)		
Arg-14-003		16b
Arg-14-026		16b
Arg-14-064		16b
Cluster 8 a (*Micromonospora* sp.)		
Ant-28-001		16b
Jpn-73-008		16b
USA-49-016		16b
Jpn-73-011		16b
Cluster 9 a (*Micromonospora* sp.)		
Por-23-012		16b
Por-24-002		16b +Nzl-33-004

Table 5.3. Continued.

S_J UPGMA	S_{SM} UPGMA Cluster
Cluster 10 a (*Micromonospora* sp.)	
Arg-14-049	18b
USA-49-011	18b
Ind-81-004	18b
Cluster 11 a (*Micromonospora* sp.)	
Nzl-33-003	17b
Ind-56-012	17b
Cluster 12 a (*Micromonospora* sp.)	
Brz-15-010	SMC
Brz-15-013	SMC
Cluster 13 a (*Micromonospora* sp.)	
Jpn-18-251	19b
Por-24-009	19b
Cluster 14 a (*Micromonospora* sp.)	
UK-10-015	29b
UK-12-011	SMC
UK-10-020	SMC
Czr-17-002	SMC
Cluster 15 a (*Micromonospora* sp.)	
Ind-63-013	SMC
Ind-63-034	SMC
Ind-64-022	SMC
Cluster 16 a (*Micromonospora* sp.)	
Nmb-89-006	23b
Nmb-89-008	23b
Cluster 17 a (*Micromonospora* sp.)	
Nmb-90-002	24b
Nmb-90-011	24b
Cluster 18 a (*Micromonospora* sp.)	
UK-10-005	26b
UK-12-012	26b
Cluster 19 a (*Micromonospora* sp.)	
Jpn-18-072	28b
Jpn-91-002	28b
Jpn-91-005	28b
Jpn-91-004	28b
Cluster 20 a (*Micromonospora* sp.)	
Brz-15-005	27b
USA-43-022	27b
USA-47-018	27b
Jpn-18-047	27b

Table 5.3. Continued.

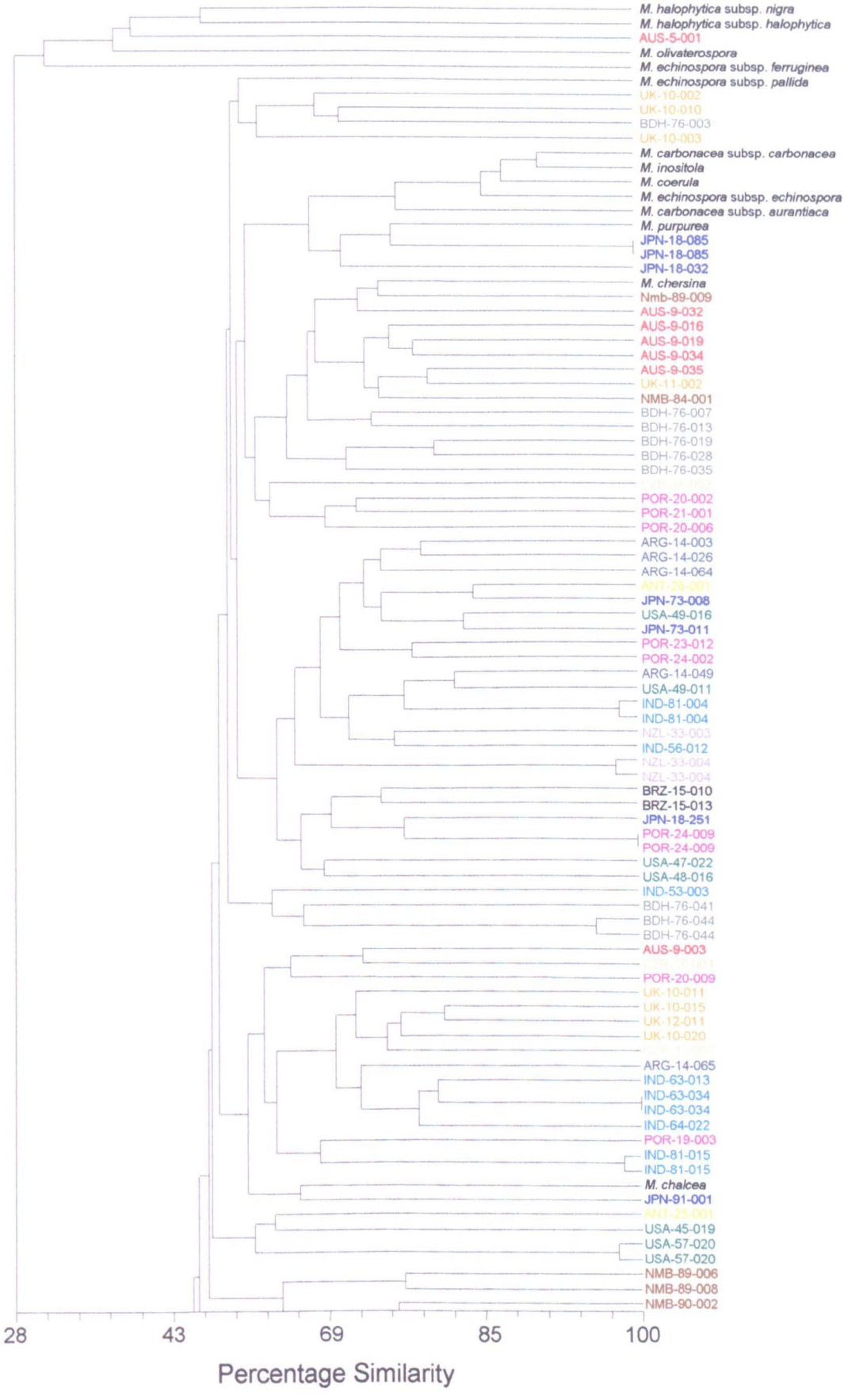
S_J UPGMA	S_{SM} UPGMA Cluster
	New clusters
	Cluster 1b
	2223 *M. halophytica* Subsp. *halophytica*
	2225 *M. halophytica* subsp. *nigra*
	Aus-5-001
	12659 *M. olivat-erospora*
	Cluster 2b
	Por-21-003
	Por-24-013
	Cluster 3b
	USA-45-008
	Nmb-87-004
	Cluster 4b
	Aus-6-004
	Aus-7-008
	Aus-7-007
	Aus-7-004
	Cluster 5b
	Aus-7-006
	Aus-9-030
	UK-12-004
	Spn-77-028
	Cluster 6b
	Jpn-18-030
	Jpn-18-085
	Cluster 7b
	USA-47-027
	Jpn-93-008
	Spn-77-012
	Cluster 8b
	Aus-9-018
	Ind-50-007
	Ind-63-002
	Arg-14-071
	Cluster 9b
	Aus-9-026
	Nmb-86-010
	Nmb-86-005

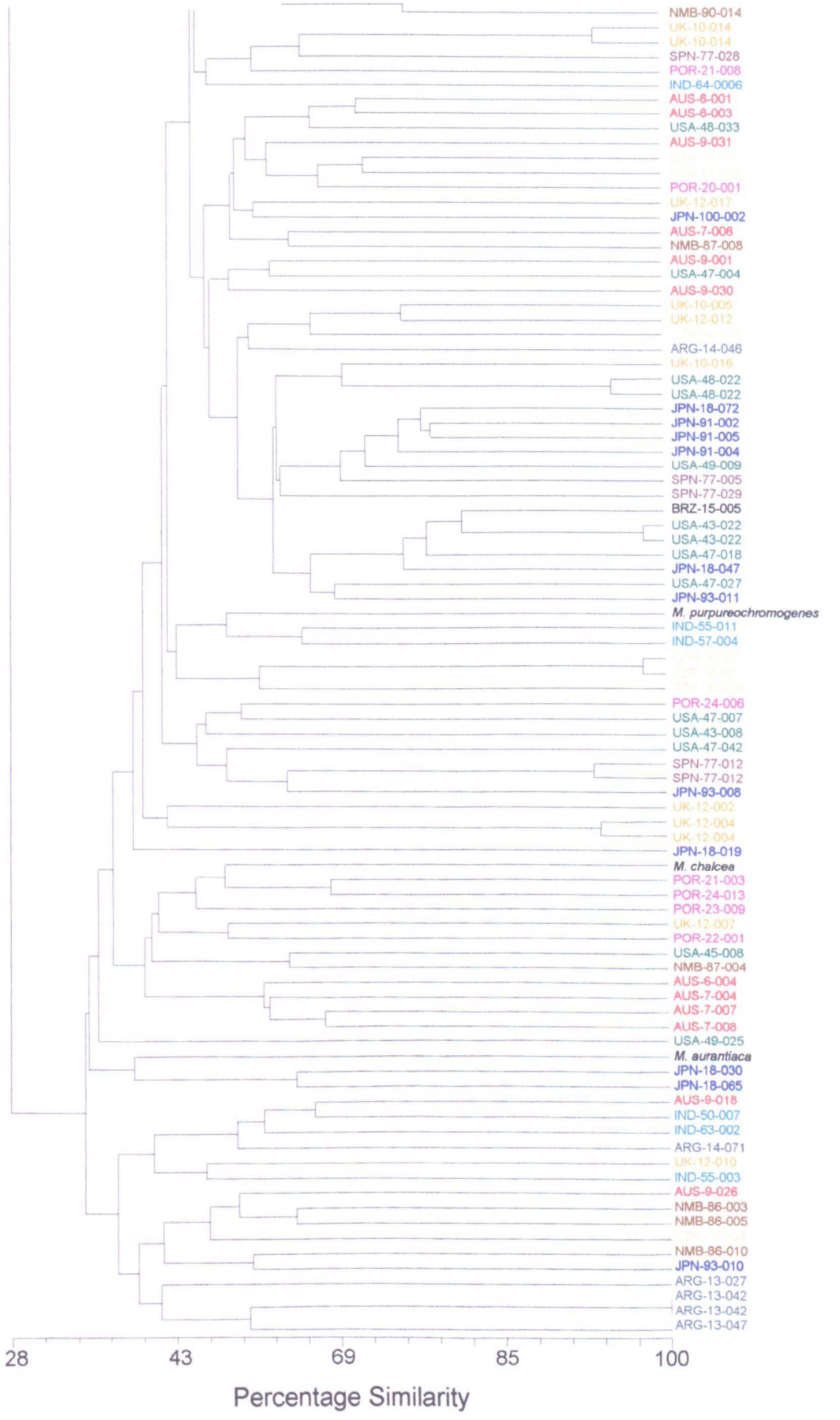
Table 5.3. Continued.

S_J UPGMA	S_{SM} UPGMA Cluster
	Cluster 10b
	Aus-6-001
	Aus-6-003
	Cluster 11b
	UK-10-002
	UK-10-010
	Bdh-76-003
	Cluster 20b
	Czr-17-001
	Czr-17-003
	Cluster 22b
	Por-20-002
	Por-20-006
	Cluster 25b
	Ind-55-011
	Ind-57-004

SMC; single membered cluster

Figure 5.2. Dendrogram showing major, minor and single membered clusters defined in the S_J UPGMA analysis


M. halophytica subsp. nigra
M. halophytica subsp. halophytica
AUS-5-001
M. olivaterospora
M. echinospora subsp. ferruginea
M. echinospora subsp. pallida
UK-10-002
UK-10-010
BDH-76-003
UK-10-003
M. carbonacea subsp. carbonacea
M. inositola
M. coerula
M. echinospora subsp. echinospora
M. carbonacea subsp. aurantiaca
M. purpurea
JPN-18-085
JPN-18-085
JPN-18-032
M. chersina
Nmb-89-009
AUS-9-032
AUS-9-016
AUS-9-019
AUS-9-034
AUS-9-035
UK-11-002
NMB-84-001
BDH-76-007
BDH-76-013
BDH-76-019
BDH-76-028
BDH-76-035
POR-20-002
POR-21-001
POR-20-006
ARG-14-003
ARG-14-026
ARG-14-064
JPN-73-008
USA-49-016
JPN-73-011
POR-23-012
POR-24-002
ARG-14-049
USA-49-011
IND-81-004
IND-81-004
IND-56-012
BRZ-15-010
BRZ-15-013
JPN-18-251
POR-24-009
POR-24-009
USA-47-022
USA-48-016
IND-53-003
BDH-76-041
BDH-76-044
BDH-76-044
AUS-9-003
POR-20-009
UK-10-011
UK-10-015
UK-12-011
UK-10-020
ARG-14-065
IND-63-013
IND-63-034
IND-63-034
IND-64-022
POR-19-003
IND-81-015
IND-81-015
M. chalcea
JPN-91-001
USA-45-019
USA-57-020
USA-57-020
NMB-89-006
NMB-89-008
NMB-90-002
28
43
69
85
100
Percentage Similarity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UK-10-014
UK-10-014
SPN-77-028
POR-21-008
IND-64-0006
AUS-6-001
AUS-6-003
USA-48-033
AUS-9-031
POR-20-001
UK-12-017
JPN-100-002
AUS-7-006
NMB-87-008
AUS-9-001
USA-47-004
AUS-9-030
UK-10-005
UK-12-012
ARG-14-046
UK-10-016
USA-48-022
USA-48-022
JPN-18-072
JPN-91-002
JPN-91-005
JPN-91-004
USA-49-009
SPN-77-005
SPN-77-029
BRZ-15-005
USA-43-022
USA-43-022
USA-47-018
JPN-18-047
USA-47-027
JPN-93-011
M. purpureochromogenes
IND-55-011
IND-57-004
POR-24-006
USA-47-007
USA-43-008
USA-47-042
SPN-77-012
SPN-77-012
JPN-93-008
UK-12-002
UK-12-004
UK-12-004
JPN-18-019
M. chalcea
POR-21-003
POR-24-013
POR-23-009
UK-12-007
POR-22-001
USA-45-008
NMB-87-004
AUS-6-004
AUS-7-004
AUS-7-007
AUS-7-008
USA-49-025
M. aurantiaca
JPN-18-030
JPN-18-065
AUS-9-018
IND-50-007
IND-63-002
ARG-14-071
UK-12-010
IND-55-003
AUS-9-026
NMB-86-003
NMB-86-005
NMB-86-010
JPN-93-010
ARG-13-027
ARG-13-042
ARG-13-042
ARG-13-047
28
43
69
85
100
Percentage Similarity

Table 5.4. Frequency of positive characters found in the five major *Micromonospora* clusters defined at the 69% similarity level based on the Jaccard coefficient and the unweighted pair group method with arithmetic averages algorithm

	Micromonospora sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.
Character Cluster	**1a**	**8a**	**14a**	**19a**	**20a**
Number of strains	**5**	**4**	**4**	**4**	**4**
Biochemical tests:					
Hydrogen sulphide production	5	3	4	3	1
Nitrate reduction	5	4	4	1	0
Substrate degradation tests:					
Casein	5	4	4	4	4
Chitin	5	4	4	4	4
Elastin	5	4	4	4	4
Gelatin	0	0	0	3	2
Starch	5	4	4	4	4
Tributyrin	0	0	0	0	0
Tween 20	0	0	0	0	0
Tween 40	4	3	3	0	0
Tween 60	1	0	4	1	0
Tween 80	5	4	1	1	0
Xylan	5	4	4	4	4
Nutritional tests **Sole carbon source 1.0% w/v**					
Monosaccharides **Pentoses:**					
D-Lyxose	1	0	4	1	3
D (+) Xylose	5	4	4	4	4
Hexoses:					
D (-) Fructose	5	4	4	4	4
D (+) Galactose	5	4	4	4	4
D (+) Mannose	5	4	4	4	4

Table 5.4. Continued

Character	Cluster	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.
	Number of strains	**5**	**4**	**4**	**4**	**4**
Hexoses:						
L (-) Sorbose		0	0	0	0	0
D (+) Cellobiose		5	3	3	4	4
Disaccharides:						
D-Sucrose		5	4	4	4	4
Trisaccharides:						
D (+) Melezitose		0	0	0	0	0
Polysaccharides:						
Glycogen		5	4	4	4	4
Inulin		0	0	0	0	0
Starch		5	4	4	4	4
Xylan		5	4	4	4	4
Sole carbon sources (0.1% w/v)						
Aliphatic amino acids:						
DL-α-Alanine		0	0	1	0	1
L-Arginine		0	3	2	3	0
L-Ornithine monohydrochloride		0	0	0	1	0
L-Serine		5	0	1	0	0
Glycosidases and related compounds:						
N-Acetyl-D-glucosamine		0	0	3	1	0
D-Gluconic acid		0	3	4	2	2
Methyl-α-D-glucopyranoside		0	0	2	1	3
Methyl-β-D-glucopyranoside		0	3	4	4	4
Salicin		5	4	4	4	4
D (-) Mandelic acid		1	0	0	0	0
Carboxylic acids:						
Sodium acetate		0	4	1	1	1
Sodium-n-butyrate		5	0	0	0	0
Sodium propionate		5	4	3	2	2
Sodium pyruvate		5	3	4	4	4

Table 5.4. Continued.

		Micromonospora sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.
Character	**Cluster**	**1a**	**8a**	**14a**	**19a**	**20a**
	Number of strains	**5**	**4**	**4**	**4**	**4**
Dicarboxylic acids:						
Sebacic acid		0	0	0	0	0
Amines:						
Spermine tetrahydrochloride		0	4	3	0	0
Aromatic amino acids:						
L-β-Phenylalanine		0	4	0	4	0
L-Proline		5	3	1	2	1
L-Tryptophan		0	3	0	4	3
L-Tyrosine		5	0	0	0	0
Aromatic hydroxy acids:						
Ferulic acid		0	0	0	0	0
m-Hydroxybenzoic acids		0	0	0	0	0
p-Hydroxybenzoic acids		0	0	0	0	0
Physiological tests **Resistance to antibiotics (μg/ml)**						
Aminoglycosides:						
Gentamicin sulphate	5	5	0	4	2	0
Gentamicin sulphate	25	5	2	3	3	0
Neomycin sulphate	5	1	0	4	0	0
Neomycin sulphate	25	0	0	1	0	0
Streptomycin sulphate	5	5	0	4	0	0
Streptomycin sulphate	25	5	0	4	0	0
Coumarin:						
Novobiocin	5	5	0	3	0	0
Novobiocin	25	5	0	1	0	0
β-Lactams **Penicillin:**						
Penicillin V	5	1	0	3	0	0
Penicillin V	25	1	0	2	1	2
Rifamycin:						
Rifampicin	5	3	0	3	0	0

Table 5.4. Continued.

		Micromonospora sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.
Character	**Cluster**	**1a**	**8a**	**14a**	**19a**	**20a**
	Number of strains	**5**	**4**	**4**	**4**	**4**
Rifamycin:						
Rifampicin	25	2	0	1	0	0
Tetracylines:						
Tetracyline hydrochloride	5	3	0	4	1	1
Tetracyline hydrochloride	25	0	0	3	0	0
Miscellaneous:						
Chloramphenicol	5	2	0	2	3	0
Chloramphenicol	25	2	0	1	3	0
Tolerance to chemical inhibitors (% w/v):						
Sodium chloride	1.0	5	4	4	4	4
Sodium chloride	3.0	2	0	2	1	0
Sodium chloride	5.0	1	0	3	0	0
Sodium chloride	7.0	1	0	1	0	0
Growth at pH:						
pH 4.0		5	0	4	0	0
pH 5.0		5	0	4	1	0
pH 6.0		5	0	4	1	1
pH 7.0		5	0	4	2	1
pH 8.0		5	4	3	3	3
pH 9.0		5	0	4	0	1
pH 10.0		5	2	3	3	2
Antibiosis tests **Inhibition of:**						
Escherichia coli		0	1	0	0	0
Staphylococcus aureus		0	0	0	0	0
Enterococcus faecalis		0	1	0	0	0
Bacillus subtilis		2	0	0	0	2
Pseudomonas aeruginosa		0	0	0	0	0
Candida albicans		0	0	0	0	0
Enzyme tests **Cleavage of 4-methylumbelliferone substrates:**						
4-methylumbelliferyl-β-D-galactopyranoside		1	3	4	4	2
4-methylumbelliferyl-β-D-glucopyranoside		1	0	3	4	4
4-methylumbelliferyl-N-acetyl-β-D-glucosamide		0	0	4	0	3
4-methylumbelliferyl-β-D-glucuronide		5	4	2	3	3

Table 5.4. Continued.

	Micromonospora sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.	*Micromonospora* sp.
Character Cluster	**1a**	**8a**	**14a**	**19a**	**20a**
Number of strains	**5**	**4**	**4**	**4**	**4**
4-methylumbelliferyl-7-β-D-xyloside	0	0	2	4	4
4-methylumbelliferyl-α-D-glucoside	5	4	4	3	3
4-methylumbelliferyl-α-D-galactoside	0	2	3	4	1
4-methylumbelliferyl-β-D-fucoside	0	0	3	3	4
4-methylumbelliferyl-α-D-mannoside	0	0	0	0	1
4-methylumbelliferyl-phosphate	0	0	2	0	0
4-methylumbelliferyl-sulfate	4	0	0	0	0
4-methylumbelliferyl-β-D-mannopyranoside	1	0	3	1	4
4-methylumbelliferyl-β-cellobioside	0	0	4	1	4
4-methylumbelliferyl-butyrate	0	1	4	4	1
4-methylumbelliferyl-β-D-ribofuranoside	1	0	0	0	0
4-methylumbelliferyl-alpha-L-arabinoside	1	4	2	3	4
4-methylumbelliferyl-nonanoate	5	4	4	0	4
4-methylumbelliferyl-dodecanoate	0	1	0	1	0
4-methylumbelliferyl-palmitate	0	1	1	3	0
Cleavage of 7-amido-methyl coumarin substrates:					
L-analyl-7-amido-4-methylcoumarin	0	4	4	4	4
Leucyl-amido-4-methylcoumarin	1	4	4	3	4
Prolyl-amido-4-methylcoumarin	0	0	4	3	4
Pyroglutamyl-amido-4-methylcoumarin	1	0	0	0	1
Glutamyl-amido-4-methylcoumarin	0	0	4	4	4
Lysyl-amido-4-methylcoumarin	0	0	0	1	1
Asparginyl-amido-4-methylcoumarin	0	0	3	4	3
Phenylalanyl-amido-4-methylcoumarin	4	4	3	3	3
Z-glycyl-prolyl-amido-4-methylcoumarin	1	0	0	0	0
Z-arginyl-amido-4-methylcoumarin	4	0	0	3	3
H-ornithine-amido-4-methylcoumarin.2HCl	0	1	3	4	4
L-histidine-7-amido-4-methylcoumarin	0	0	3	2	4

Table 5.5. Centrotype strains and mean intra-cluster similarity values for the *Micromonospora* major clusters defined using the Jaccard coefficient and the unweighted pair group method with arithmetic averages algorithm

Cluster	Centrotype Strain	Mean intra-cluster similarity (%)
Cluster 1a	12665 *M. coerula*	85
Cluster 8a	USA-49-016	77
Cluster 14a	UK-12-011	76
Cluster 19a	Jpn-91-005	69
Cluster 20a	USA-43-022	72

5.4.3. Identification of *Micromonospora* strains using selected tests from the numerical taxonomic study

The computer program Taxon (A. C. Ward, unpublished data; Appendix D) was used to identify which tests would be discriminatory for *Micromonospora* strains based on the data generated in the numerical taxonomic study. These tests could be of use for the rapid identification of micromonosporae. The program generated a computer identification matrix from which tests could be selected for the identification of micromonosporae. The tests detailed below (Table 5.6.) gave results of high numbers of either positive or negative results, which are useful for discrimination purposes. The validity of using these tests for identification purposes was tested by using them to assign strains to the correct cluster, it was found that in all the cases tested the strain was assigned correctly.

Table 5.6. Tests for the identification of *Micromonospora*

Test	Result
Methyl-β-D-glucopyranoside	Negative
L-Tyrosine	Negative
Ferulic acid	Negative
Sodium pyruvate	Positive
Tween 20	Negative
D (+) Melezitose	Negative
pH 4.0	Negative
pH 8.0	Positive
1% NaCl	Positive
m-hydroxybenzoic acid	Negative
Casein	Positive
Xylan	Positive
Glycogen	Positive
Tributyrin	Negative

5.5. Numerical classification based on the Simple Matching coefficient

5.5.1. Distribution of the test strains to major, minor and single membered clusters

The 166 test strains and 14 type strains were recovered into 8 major clusters, 21 minor clusters and 73 single membered clusters defined at the 82% similarity level (Figure 5.2.). These phena account for 23%, 37% and 40% of the test and type strains respectively. The composition of the clusters is detailed in Table 5.7.

Ten of the fourteen type strains analysed fell into 2 major and 2 minor clusters with the remaining four, forming single membered clusters. Cluster 12b was composed entirely of type strains (*M. carbonacea* subsp. *carbonacea*, *M. inositola*, *M. coerula*, *M. echinospora* subsp. *echinospora* and *M. carbonacea* subsp. *aurantiaca*). Cluster 1b contained 3 type strains (*M. halophytica* subsp. *halophytica*, *M. halophytica* subsp. *nigra* and *M. olivaterospora*) and 1 isolate (Aus-5-001). Two Portuguese isolates were clustered with *M. chalcea* (Cluster 2b) and two marine isolates clustered with *M. purpurea* (Cluster 13b). All of the remaining major and minor clusters were composed of isolates. Cluster 4b

Table 5.7. Designation and source of strains assigned to clusters defined at the 82% similarity level in the analysis based on the Simple Matching coefficient and UPGMA

Strain code	Description	Source
Major clusters		
Cluster 1b		
2223	*M. halophytica subsp. halophytica*	NCIMB. New York, USA *
2225	*M halophytica subsp. nigra*	NCIMB. New York, USA *
Aus-5-001	*Micromonospora* sp.	Mount Lewis, Australia ♠
12659	*M. olivaterospora*	NCIMB. Hiroshima, Japan *
Cluster 4b (*Micromonospora* sp.)		
Aus-6-004	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-7-008	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-7-007	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus7-004	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Cluster 7b (*Micromonospora* sp.)		
Aus-9-018	*Micromonospora* sp.	Lizard Island, Australia ♠
Ind-50-007	*Micromonospora* sp.	Bantul, Java, Indonesia ♠
Ind-63-002	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Arg-14-071	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Cluster 12b		
12663	*M. carbonacea* subsp. *carbonacea*	NCIMB. New York, USA *
12751	*M. inositola*	NCIMB. Hokkaido, Japan *
12665	*M. coerula*	NCIMB. Hawaii, USA *
12744	*M. echinospora* subsp. *echinospora*	NCIMB. New York, USA *
12664	*M. carbonacea* subsp. *aurantiaca*	NCIMB. Soil *
Cluster 14b (*Micromonospora* sp.)		
Aus-9-016	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-9-019	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-9-034	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-9-035	*Micromonospora* sp.	Lizard Island, Australia ♠
UK-11-002	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Nmb-84-001	*Micromonospora* sp.	Kavango, Namibia ♠
Cluster 16b (*Micromonospora* sp.)		
Arg-14-003	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Arg-14-026	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Ant-28-001	*Micromonospora* sp.	Signy Island, Antarctica ♠
Jpn-73-008	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
USA-49-016	*Micromonospora* sp.	New York, USA ♠
Jpn-73-011	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
Arg-14-064	*Micromonospora* sp.	Iguacu National Park, Argentina ♠

Table 5.7. Continued

Strain code	Description	Source
Cluster 27b (*Micromonospora* sp.)		
Brz-15-005	*Micromonospora* sp.	Iguacu National Park, Brazil♠
USA-43-022	*Micromonospora* sp.	Missouri, USA ♠
USA-47-018	*Micromonospora* sp.	Alabama, USA ♠
Jpn-18-047	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Cluster 28b (*Micromonospora* sp.)		
Jpn-18-072	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Jpn-91-002	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Jpn-91-005	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Jpn-91-004	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Minor clusters		
Cluster 2b (*M. chalcea)*		
43026	*M. chalcea*	DSMZ. Pfizer *
Por-21-003		Ave River, Nascente, Portugal ♠
Por-24-013		Landim, Portugal ♠
Cluster 3b (*Micromonospora* sp.)		
Por-22-001	*Micromonospora* sp.	Leca River, Portugal ♠
USA-45-008	*Micromonospora* sp.	Tennessee, USA ♠
Nmb-87-004	*Micromonospora* sp.	Waterberg, Namibia ♠
Cluster 5b (*Micromonospora* sp.)		
Jpn-18-030	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Jpn-18-065	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Cluster 6b (*Micromonospora* sp.)		
USA-47-027	*Micromonospora* sp.	Alabama, USA ♠
Jpn-93-008	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Spn-77-012	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Cluster 8b (*Micromonospora* sp.)		
Aus-9-026	*Micromonospora* sp.	Lizard Island, Australia ♠
Nmb-86-010	*Micromonospora* sp.	Tsuemb, Namibia ♠
Nmb-86-005	*Micromonospora* sp.	Tsuemb, Namibia ♠
Cluster 9b (*Micromonospora* sp.)		
Arg-13-042	*Micromonospora* sp.	Mendoza, Argentina ♠
Arg-13-047	*Micromonospora* sp.	Mendoza, Argentina ♠
Cluster 10b (*Micromonospora* sp.)		
Aus-6-001	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-6-003	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Cluster 11b (*Micromonospora* sp.)		
UK-10-002	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-10-010	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Bdh-76-003	*Micromonospora* sp.	Dhaka, Bangladesh ♠

Table 5.7. Continued

Strain code	Description	Source
Cluster 13b (*M. purpurea)*		
12882	*M. purpurea*	NCIMB. New York, USA*
Jpn-18-032		Japan Sea, Pacific Ocean ♠
Jpn-18-085		Japan Sea, Pacific Ocean ♠
Cluster 15b (*Micromonospora* sp.)		
Bdh-76-007	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-013	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Cluster 17b (*Micromonospora* sp.)		
Nzl-33-003	*Micromonospora* sp.	New Zealand ♠
Ind-56-012	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
Cluster 18b (*Micromonospora* sp.)		
Arg-14-049	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
USA-49-011	*Micromonospora* sp.	New York, USA ♠
Ind-81-004	*Micromonospora* sp.	Bandung, Java, Indonesia ♠
Cluster 19b (*Micromonospora* sp.)		
Jpn-18-251	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Por-24-009	*Micromonospora* sp.	Landim, Portugal ♠
Cluster 20b (*Micromonospora* sp.)		
Czr-17-001	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Czr-17-003	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Cluster 21b (*Micromonospora* sp.)		
Bdh-76-019	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-028	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Cluster 22b (*Micromonospora* sp.)		
Por-20-002	*Micromonospora* sp.	Ave River, Spring, Portugal♠
Por-20-006	*Micromonospora* sp.	Ave River, Spring, Portugal♠
Cluster 23b (*Micromonospora* sp.)		
Nmb-89-006	*Micromonospora* sp.	Katima Mulilo, Namibia ♠
Nmb-89-008	*Micromonospora* sp.	Katima Mulilo, Namibia ♠
Cluster 24b (*Micromonospora* sp.)		
Nmb-90-002	*Micromonospora* sp.	Ogongo, Namibia ♠
Nmb-90-011	*Micromonospora* sp.	Ogongo, Namibia ♠
Cluster 25b (*Micromonospora* sp.)		
Ind-55-011	*Micromonospora* sp.	Karang Bolong, Java, Indonesia ♠
Ind-57-004	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
Cluster 26b (*Micromonospora* sp.)		
UK-10-005	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-12-012	*Micromonospora* sp.	Dartmoor, United Kingdom♠

Table 5.7. Continued

Strain code	Description	Source
Cluster 29b (*Micromonospora* sp.)		
UK-10-011	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-10-015	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Single membered clusters		
43141	*M. echinospora* subsp. *ferruginea*	DSMZ. Soil *
12754	*M. aurantiaca*	NCIMB. *
Aus-9-001	*Micromonospora* sp.	Lizard Island, Australia ♠
Aus-7-006	*Micromonospora* sp.	Mossman Gorge, Australia ♠
Aus-9-030	*Micromonospora* sp.	Lizard Island, Australia ♠
UK-12-004	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Spn-77-028	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Jpn-18-019	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Ind-64-006	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Spn-77-028	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Jpn-18-019	*Micromonospora* sp.	Japan Sea, Pacific Ocean ♠
Ind-64-006	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Arg-13-027	*Micromonospora* sp.	Mendoza, Argentina ♠
Jpn-93-010	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Czr-17-004	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Nmb-86-003	*Micromonospora* sp.	Tsuemb, Namibia ♠
USA-48-033	*Micromonospora* sp.	Indianapolis, USA ♠
Nmb-87-008	*Micromonospora* sp.	Waterberg, Namibia ♠
UK-12-010	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Ind-55-003	*Micromonospora* sp.	Karang, Bolong, Java, Indonesia ♠
Jpn-100-002	*Micromonospora* sp.	Suruga Bay, Pacific Ocean ♠
Aus-9-031	*Micromonospora* sp.	Lizard Island, Australia ♠
UK-12-017	*Micromonospora* sp.	Dartmoor, United Kingdom♠
12660	*M. echinospora* subsp. *pallida*	NCIMB. New York, USA *
12661	*M. purpureochromogenes*	NCIMB. California, USA *
Por-20-001	*Micromonospora* sp.	Ave River, Spring, Portugal ♠
UK-12-007	*Micromonospora* sp.	Dartmoor, United Kingdom♠
UK-10-008	*Micromonospora* sp.	Dartmoor, United Kingdom♠
USA-45-019	*Micromonospora* sp.	Tennessee, USA ♠
UK-10-014	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Por-21-008	*Micromonospora* sp.	Ave River, Nascente, Portugal ♠
Ind-53-003	*Micromonospora* sp.	Yogyakarta, Java Indonesia♠
Bdh-76-041	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-044	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Bdh-76-035	*Micromonospora* sp.	Dhaka, Bangladesh ♠
Por-21-001	*Micromonospora* sp.	Ave River, Nascente, Portugal ♠
Por-23-009	*Micromonospora* sp.	Cavado River, Portugal ♠
Czr-16-002	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Czr-16-007	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Czr-16-009	*Micromonospora* sp.	Chvaletice, Czech Republic♠

Table 5.7. Continued

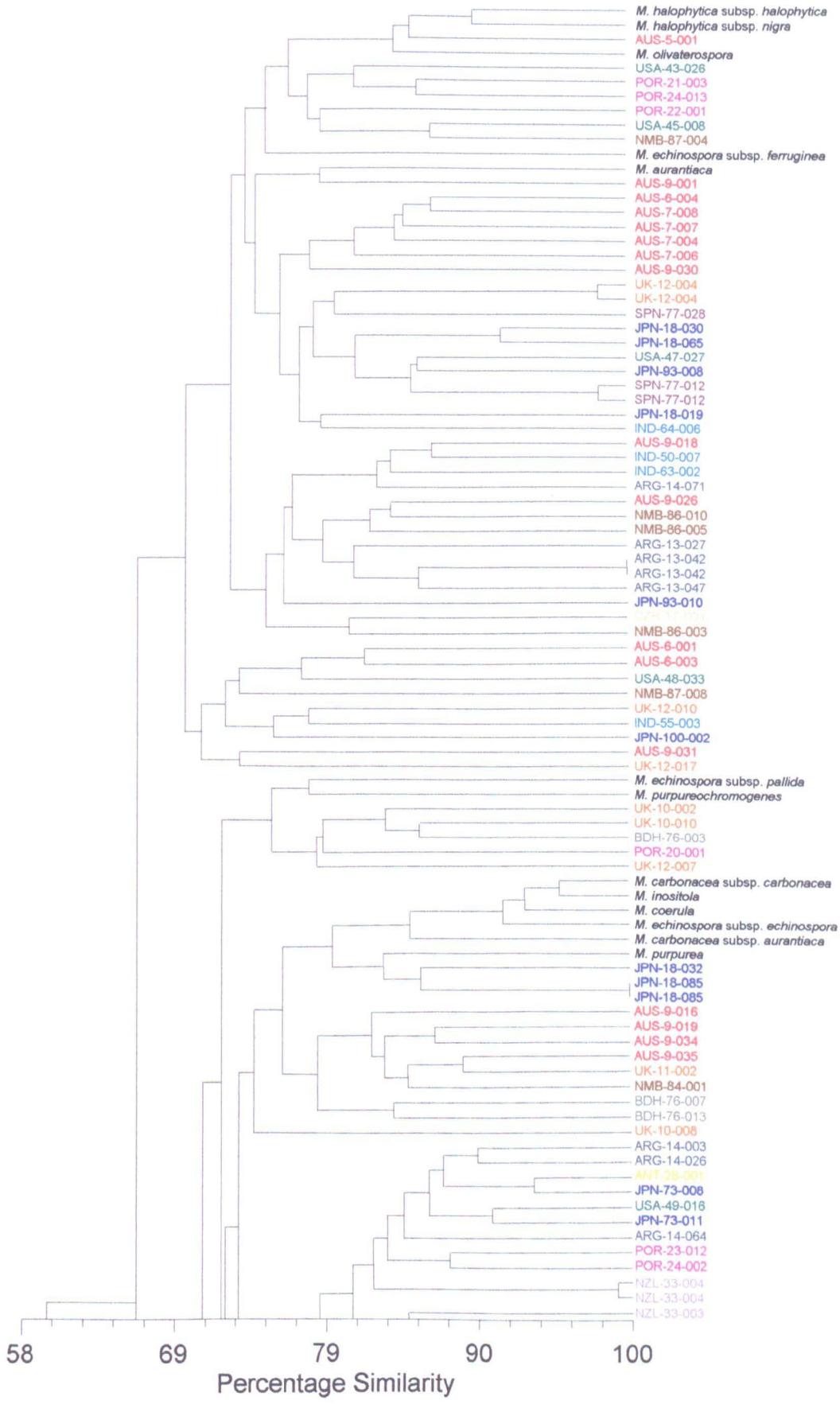
Strain code	Description	Source
USA-48-016	*Micromonospora* sp.	Indianapolis, USA ♠
44151	*M. chersina*	DSMZ. Soil *
Aus-9-032	*Micromonospora* sp.	Lizard Island, Australia ♠
Nmb-89-009	*Micromonospora* sp.	Katima Mulilo, Namibia ♠
Brz-15-010	*Micromonospora* sp.	Iguacu National Park, Brazil ♠
Brz-15-013	*Micromonospora* sp.	Iguacu National Park, Brazil♠
Por-19-003	*Micromonospora* sp.	Febre River, Avintes, Portugal ♠
Ind-81-015	*Micromonospora* sp.	Bandung, Java, Indonesia ♠
Ant-25-001	*Micromonospora* sp.	Signy Island, Antarctica ♠
USA-47-022	*Micromonospora* sp.	Alabama, USA ♠
CZR-16-005	*Micromonospora* sp.	Chvaletice, Czech Republic♠
UK-10-016	*Micromonospora* sp.	Dartmoor, United Kingdom♠
ARG-14-046	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
UK-10-020	*Micromonospora* sp.	Dartmoor, United Kingdom♠
USA-48-022	*Micromonospora* sp.	Indianapolis, USA ♠
Jpn-93-011	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Spn-77-005	*Micromonospora* sp.	Canulobes Caves, Spain ♠
USA-49-009	*Micromonospora* sp.	New York, USA ♠
USA-47-004	*Micromonospora* sp.	Alabama, USA ♠
UK-12-002	*Micromonospora* sp	Dartmoor, United Kingdom♠
USA-47-042	*Micromonospora* sp.	Alabama, USA ♠
Ind-57-020	*Micromonospora* sp.	Cilicap, Java, Indonesia ♠
Por-24-006	*Micromonospora* sp.	Landim, Portugal ♠
USA-47-047	*Micromonospora* sp.	Alabama, USA ♠
USA-43-038	*Micromonospora* sp.	Missouri, USA ♠
Spn-77-029	*Micromonospora* sp.	Canulobes Caves, Spain ♠
Jpn-91-001	*Micromonospora* sp.	Sagami Bay, Pacific Ocean♠
Aus-9-003	*Micromonospora* sp.	Lizard Island, Australia ♠
CZR-16-004	*Micromonospora* sp.	Chvaletice, Czech Republic♠
Arg-14-065	*Micromonospora* sp.	Iguacu National Park, Argentina ♠
Por-20-009	*Micromonospora* sp.	Ave River, Spring, Portugal ♠
UK-12-011	*Micromonospora* sp.	Dartmoor, United Kingdom♠
Czr-17-002	*Micromonospora* sp.	Tusimice, Czech Republic ♠
Ind-63-013	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Ind-63-034	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠
Ind-64-022	*Micromonospora* sp.	Rawa Pening, Java, Indonesia ♠

♠ See Chapter Two, Table 2.1 for full details of sample locations
* See Chapter Two, Table 2.2 for complete histories of the type strains
Abbreviations; NCIMB, National Collection of Industrial and Marine Bacteria, UK; DSMZ, Deutsche Sammlung Mikroorganism und Zelfulturen, Germany.

contained four Australian isolates from Mossman Gorge; Cluster 7b contained two Indonesian isolates, one Australian and one Argentinean; Cluster 14b was composed mainly of isolates from Lizard Island, Australia (four out of six isolates), one UK isolate and one from Namibia. Cluster 17b contained isolates from Argentina (3), Antarctica (1), Japan (2) and the USA (1). Cluster 27b contained two isolates from the USA, one from Brazil and one from Japan. Cluster 28b was composed entirely of marine isolates with one from Sagami Bay and one from the Japan Sea.

Of the minor clusters; Cluster 5b contained marine isolates from the Japan Sea; Cluster 8b contained two Namibian isolates and one isolate from the USA; Cluster 9b was comprised of isolates from Iguacu National Park, Argentina; Cluster 10b contained isolates from Mossman Gorge, Australia; Cluster 11b was comprised of two isolates from the UK and one from Bangladesh; Cluster 20b contained isolates from the Czech Republic; Cluster 21b contained isolates from Bangladesh; Cluster 22b contained Portuguese isolates; Clusters 23b and 24b were composed of Namibian isolates; Cluster 25b was composed of Indonesian isolates; Cluster 26b and 29b contained isolates from the UK.

Figure 5.3. Dendrogram showing major, minor and single membered clusters defined in the S_{SM} UPGMA analysis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M. halophytica subsp. nigra
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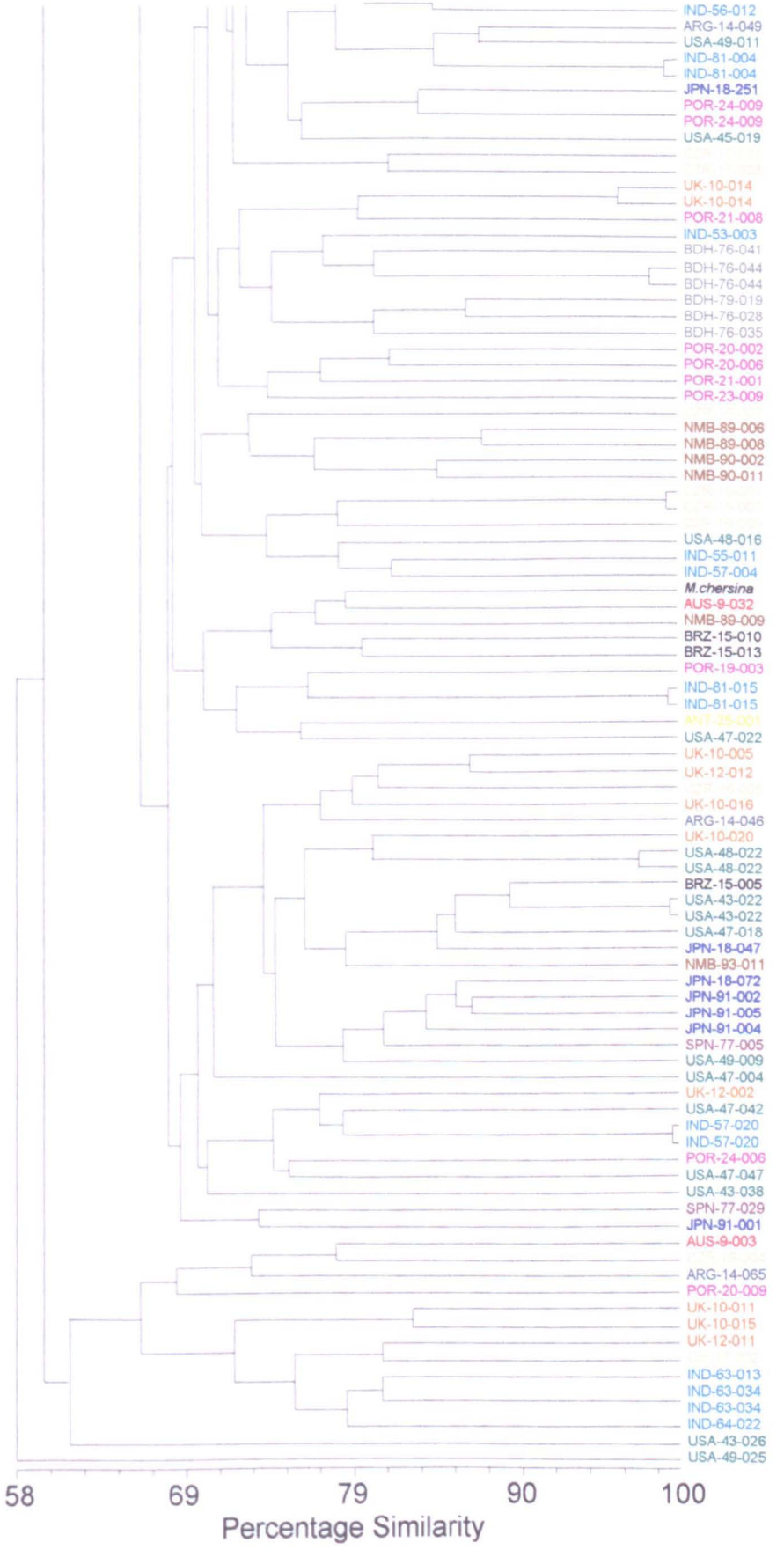
M. olivaterospora
USA-43-026
POR-21-003
POR-24-013
POR-22-001
USA-45-008
NMB-87-004
M. echinospora subsp. ferruginea
M. aurantiaca
AUS-9-001
AUS-6-004
AUS-7-008
AUS-7-007
AUS-7-004
AUS-7-006
AUS-9-030
UK-12-004
UK-12-004
SPN-77-028
JPN-18-030
JPN-18-065
USA-47-027
JPN-93-008
SPN-77-012
SPN-77-012
JPN-18-019
IND-64-006
AUS-9-018
IND-50-007
IND-63-002
ARG-14-071
AUS-9-026
NMB-86-010
NMB-86-005
ARG-13-027
ARG-13-042
ARG-13-042
ARG-13-047
JPN-93-010
NMB-86-003
AUS-6-001
AUS-6-003
USA-48-033
NMB-87-008
UK-12-010
IND-55-003
JPN-100-002
AUS-9-031
UK-12-017
M. echinospora subsp. pallida
M. purpureochromogenes
UK-10-002
UK-10-010
BDH-76-003
POR-20-001
UK-12-007
M. carbonacea subsp. carbonacea
M. inositola
M. coerula
M. echinospora subsp. echinospora
M. carbonacea subsp. aurantiaca
M. purpurea
JPN-18-032
JPN-18-085
JPN-18-085
AUS-9-016
AUS-9-019
AUS-9-034
AUS-9-035
UK-11-002
NMB-84-001
BDH-76-007
BDH-76-013
UK-10-008
ARG-14-003
ARG-14-026
ANT-28-001
JPN-73-008
USA-49-016
JPN-73-011
ARG-14-064
POR-23-012
POR-24-002
NZL-33-004
NZL-33-004
NZL-33-003
58
69
79
90
100
Percentage Similarity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POR-24-009
USA-45-019
UK-10-014
UK-10-014
POR-21-008
IND-53-003
BDH-76-041
BDH-76-044
BDH-76-044
BDH-79-019
BDH-76-028
BDH-76-035
POR-20-002
POR-20-006
POR-21-001
POR-23-009
NMB-89-006
NMB-89-008
NMB-90-002
NMB-90-011
USA-48-016
IND-55-011
IND-57-004
M.chersina
AUS-9-032
NMB-89-009
BRZ-15-010
BRZ-15-013
POR-19-003
IND-81-015
IND-81-015
USA-47-022
UK-10-005
UK-12-012
UK-10-016
ARG-14-046
UK-10-020
USA-48-022
USA-48-022
BRZ-15-005
USA-43-022
USA-43-022
USA-47-018
JPN-18-047
NMB-93-011
JPN-18-072
JPN-91-002
JPN-91-005
JPN-91-004
SPN-77-005
USA-49-009
USA-47-004
UK-12-002
USA-47-042
IND-57-020
IND-57-020
POR-24-006
USA-47-047
USA-43-038
SPN-77-029
JPN-91-001
AUS-9-003
ARG-14-065
POR-20-009
UK-10-011
UK-10-015
UK-12-011
IND-63-013
IND-63-034
IND-63-034
IND-64-022
USA-43-026
USA-49-025
58
69
79
90
100
Percentage Similarity

5.6. Discussion of numerical classification

The data generated by the numerical classification yielded a great deal of information on the *Micromonospora* isolates tested and their relationships with valid type strains of this genus. Despite the analysis containing 164 test isolates there was only a small number of isolates from each sample site which probably led to an under emphasis of the clustering resulting from location and habitat. By analysing only a small number of isolates from a large number of sample sites small differences in the isolates from particular locations may be masked as the isolates are forced into artificial clusters due to the large heterogeneity of the sample set. If larger numbers of isolates from each sample site were examined, the differences between isolates from each sample site may become more apparent as, based on the observations of this study, the isolates would cluster more tightly. However, there was evidence for biogeographic distribution in the taxonomic structure as a number of discrete clusters were formed with isolates from a particular region (for example Clusters 4a, 7a, 14a, 15a and 16a). This suggests that micromonosporae are not very cosmopolitan (particular strains scattered throughout the world) but tend to be endemic. Clustering of isolates due to their location appeared to be more evident than clustering because of similarities in habitat. As detailed above, there were a number of clusters containing isolates from a particular region but there was little evidence of separation according to the individual sample sites, particularly with isolates from similar habitats but from different locations.

There was good agreement between the classification using the Jaccard coefficient and the Simple Matching coefficient, this suggests that the classification is a natural one and the clusters can be relied upon to be a good representation of the relationships between the isolates tested. In the present study, clusters were defined at the 69% (S_J, UPGMA) and 82% (S_{SM}, UPGMA) similarity levels. This choice of cut-off points, although somewhat arbitrary, is influenced by the clustering behaviour of the type strains and isolates. The number of single membered clusters recovered in both analyses was very high (S_J, 69%; S_{SM}, 40%). This trend was also observed in an earlier numerical study

on over two hundred micromonosporae isolated from a number of different locations and habitats (Rowlands, 1993). The large number of single membered clusters found in both these studies is unusual given the results of comparable studies (0% *Gordona* and *Tsukamurella*, Goodfellow *et al.*, 1991; 23% *Rhodococcus*, Goodfellow *et al.*, 1990a; 0.2 % *Streptomyces*, Williams *et al.*, 1983). Priest and Barbour (1985), in a similar numerical taxonomic analysis of *Lactobacilli* from whiskey distilleries, concluded that their test strains formed the nuclei of novel taxa and were phenotypically similar but genetically different. The high incidence of minor clusters and single membered clusters recovered in this study suggests that the genus *Micromonospora* may contain many undescribed species, genetically unstable strains or organisms of established taxa lacking plasmids.

In a numerical taxonomic study of micromonosporae, Bibikova and colleagues (1989) found that this genus exhibited a tendency to divide into two subgenera. The strains assigned to these two subgenera displayed very different properties; with one cluster containing strains rarely isolated from soil substrates, are more sensitive to antibiotics and exhibit virtually no antibiotic properties; conversely, the second cluster contained strains widespread in soil, are resistant to antibiotics and are active antibiotic producers. The heterogeneity exhibited in the isolates examined during the course of this study would support the theory that the genus *Micromonospora* is comprised of two or more genera at this present time.

In the present investigation the test error (p) was 4.37%. This value, is well within the 10% guideline defined by Sneath and Johnson (1972) although it is higher than the experimental error recorded for members of the *Actinomadura* (p=4.5%, Goodfellow *et al.*, 1979), *Actinomyces* (p=3.12%, Holmberg and Nord, 1975), *Corynebacterium* (p=2.4%, Jones, 1975), *Rhodococcus*, (p=3.7%, Goodfellow *et al.*, 1982a), *Streptomyces* (p=3.4%, Williams *et al.*, 1983) and *Thermomonospora* (p=1.1%, McCarthy and Cross, 1984). The lower the test error the more confidence there is in the value of the results.

It has been suggested that confidence can be placed in numerical taxonomies where cophenetic correlation values of 0.8 or greater are found (Jones and

Sackin, 1980; Sackin and Jones, 1993). The cophenetic correlation value for the S_J analysis based on UPGMA was 0.81. It can be concluded that the dendrogram generated in the S_J UPGMA analysis is a good representation of the taxonomic structure inherent in the corresponding similarity matrix. Intra-cluster similarity values for the S_J, UPGMA analysis ranged from 67 to 88%, indicating that the clusters were fairly tight and homogenous.

Numerical taxonomic studies provide a wide range of information on the nutritional, physiological, biochemical and antibiotic resistance properties of the isolates tested. The information generated in such studies can be subjected to cluster analysis, which will reveal the taxonomic structure and result in the formation of a dendrograms (or more precisely a phenogram, because it expresses phenetic relationships). Using the data generated from numerical studies, diagnostic characters can be selected for the identification of additional strains. This could be useful for future identification of *Micromonospora* strains from isolation plates as putative *Micromonospora* could be subjected to a small number of tests in order to confirm they belong to this genus. The numerical data also provided information that could be incorporated into the choice of selective isolation media, or could be used to compose new selective media for the isolation of *Micromonospora*.

5.7. Comparison of clusters generated by PyMS and numerical taxonomic studies

5.7.1. Congruence of numerical taxonomic clusters and PyMS pyrogroups

Table 5.8 compares the pyrogroups generated in the PyMS analyses with those produced from the numerical taxonomic analysis using the Jaccard coefficient and the simple matching coefficient.

Table 5.8. Numerical taxonomy clusters and corresponding PyMS pyrogroups

Numerical Taxonomy Cluster	Members	Corresponding PyMS pyrogroup
4a	Aus-9-016, Aus-9-019	C1
7a	Arg-14-003, Arg-14-064	F
14a	UK-10-015, UK-12-011	G
15a	Ind-63-013, Ind-64-022	R
16a	Nmb-89-008, Nmb-89-006	V2
4b	Aus-6-004, Aus-7-008, Aus-7-007, Aus-7-004	A
10b	Aus-6-001, Aus-6-003	B
11b	UK-10-002, UK-10-010	K
25b	Ind-55-011, Ind-57-004	S

The clusters generated in the numerical taxonomic study tended to be much smaller than those defined by PyMS analysis, this was partly due to there being a reduced number of isolates examined in the numerical taxonomy study. The way in which the isolates were analysed in the PyMS study was different to the numerical taxonomic study as the PyMS study was a progressive study of the different sampling locations. In the PyMS analysis, the isolates were examined in small geographical groups (~35 isolates) whereas in the numerical taxonomy study, isolates from all geographic locations were examined together.

Good agreement was found with the Australian samples both in the Jaccards and simple matching analyses. Cluster 4a (Aus-9-016 and Aus-9-019) formed part of a larger pyrogroup, C1, which also contained other isolates from Lizard Island. Whilst cluster 4b (Aus-6-004, Aus-7-008, Aus-7-007 and Aus-7-004) formed part of a larger pyrogroup, A, which contained additional isolates from the Mossman Gorge sample. Cluster 10b (Aus-6-001 and Aus-6-003) clustered in a larger more heterogeneous pyrogroup, B. Cluster 7a (Arg-14-003 and Arg-14-064) formed part of a larger pyrogroup F that contained Argentinean isolates from Mendoza and Iguacu National Park and isolates from Iguacu National Park, Brazil. Two isolates (UK-10-015 and UK-10-011) from cluster 14a were clustered in a pyrogroup (G) containing isolates from other Dartmoor samples and one from the Czech Republic. The isolates in cluster 11b (UK-10-002 and UK-10-010) formed a very tight pyrogroup with another shallow river sediment

isolate (UK-10-005), a dry river sediment isolate (UK-11-001) and several type strains; *M. chalcea*, *M. halophytica* subsp. *nigra* and *M. brunnea*. Isolates (Ind-63-013 and Ind-64-022) from cluster 15a clustered in a sub-cluster of heterogeneous pyrogroup (C), which only contained isolates and no type strains. Isolates from cluster 25b (Ind-55-011 and Ind-57-004) formed part of a heterogeneous pyrogroup (S), which contained isolates from 5 other Indonesian sample sites and a number of type strains. Two isolates (Nmb-89-008 and Nmb-89-006) from cluster 16a were recovered in a large sub-pyrogroup V2, which contained isolates from 2 other Namibian sample sites and one from the Bangladeshi site, along with 3 type strains; *M. purpurea*, *M. rosaria* and *M. echinospora* subsp. *echinospora*.

5.7.2. Discussion of congruence between PyMS and numerical taxonomic data

Generally, the pyrogroups formed in the PyMS analyses were reflected in the numerical taxonomy clusters. Agreement between the two techniques may have been improved if the numerical study had included all of the strains examined by PyMS and vice versa. Also, as the PyMS analyses were performed as discrete studies on particular regions, analysis of the numerical data in the same manner may have revealed a higher level of agreement between the two techniques. However, on the basis of the information generated from the two studies there appears to be preliminary evidence for the biogeographic distribution of the genus *Micromonospora*, with a large number of clusters in the numerical study being composed of isolates from a single region or sample site and a number of sub-pyrogroups in the PyMS studies exhibiting clustering of isolates from individual sample sites.

It would be interesting to test this hypothesis further by looking at the genotypes for isolates and determining if the clusters containing isolates from one particular region contained the same genotype, which would suggest that these genotypes are endemic to particular regions. This could be done by either analysis of the 16S rDNA gene sequences or by DNA-DNA hybridisation studies.

The data suggest that the *Micromonospora* isolates studied form a heterogeneous group as the percentage similarity levels at which the clusters are formed in both techniques are all relatively low, often less than 80% similar. Another trend observed throughout both sets of analyses is the formation of clusters or pyrogroups predominantly containing type strains, often with the total exclusion of any isolates. This observation suggests that the genus either may be over classified, or the study contains a large number of novel isolates. It is possible that because of poor definition, the genus *Micromonospora* may be comprised of more than one genus and additional classification using a plethora of numerical, chemical and molecular analyses would be required to resolve this.

Chapter Six

Antibiosis and

Rapid Enzyme Profiling

6.1. Antibiosis

6.1.1. Introduction

The ability of organisms to cause antibiosis for example, Takizawa *et al.*, 1993, can be used to investigate the diversity of strains by distinguishing isolates on the basis of their individual antimicrobial profiles. This technique can distinguish between strains that may belong to a single species, as production of bioactive agents is often strain-specific and not species-specific (Okami, 1986).

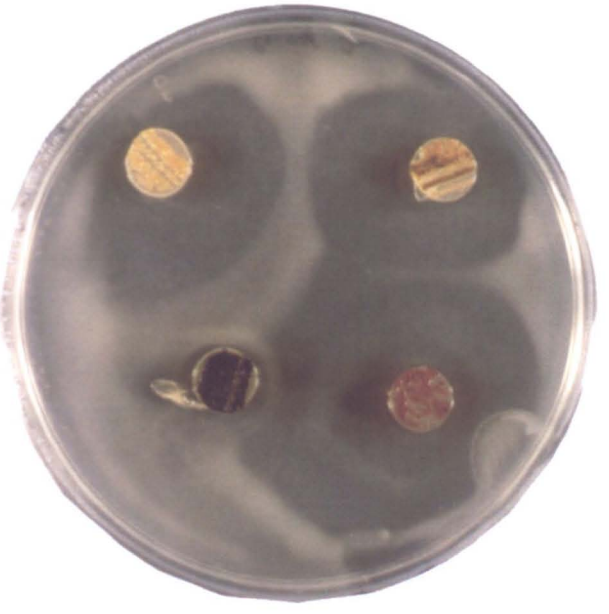
This study aimed to determine the diversity index for isolates recovered from the different geographic regions investigated and establish if any particular regions are a potential source of novel *Micromonospora* strains. The data generated from this work was also included in the numerical taxonomy data set.

6.1.2. Antimicrobial profiling

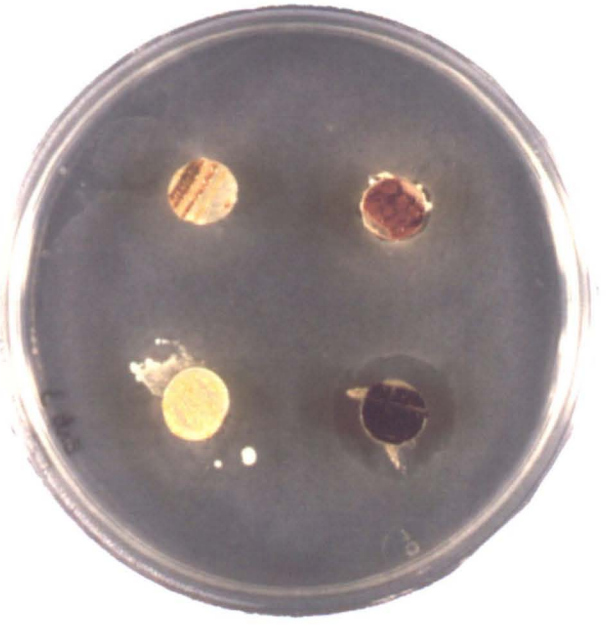
Two hundred and one strains were selected to represent all geographic regions and types of habitat investigated. These strains, alongside eighteen type strains, were tested for their ability to inhibit the growth of three Gram-positive, two Gram-negative bacteria and a yeast strain (*Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* respectively). The antimicrobial profile of each isolate (characteristic pattern of resistance or sensitivity of test organisms to the isolate) was determined. The antimicrobial profiles were used to compute a diversity index (diversity = number of antimicrobial patterns/ total number of isolates tested) for each geographical region. The diversity index indicates the likelihood of a particular strain representing a novel isolate from that region (see Table 6.1).

Figure 6.1. Examples of *Micromonospora* strains showing antimicrobial activity towards target organisms in the antibiosis study

Staphylococcus aureus



Escherichia coli



Plugs of mature micromonosporae colonies are placed on an agar plate seeded with a target organism. Plates are incubated for 3 days at 30°C.

Table 6.1. Diversity of micromonosporae from different geographic regions based on antimicrobial profiles of isolates

Geographical region	Number of isolates	Total number of antimicrobial profiles	Diversity index[a]
Africa	19	4	0.21
The Americas	62	14	0.23
Asia	43	8	0.19
Australasia	31	8	0.26
Europe	64	11	0.17

[a] Diversity index = total number of antimicrobial patterns/total number of isolates tested

6.1.3. Antimicrobial profiles and diversity indices

Inhibition of target organisms by *Micromonospora* strains can be seen in Figure 6.1. A zone of inhibition around the agar plug containing the *Micromonospora* strain demonstrates sensitivity of the target organism to *Micromonospora.* The zone of inhibition varied from 1-2mm to over 10mm around the plug, depending upon the level of sensitivity of the target organism. Table 6.1 shows the number of antimicrobial patterns observed from each geographic region and the corresponding diversity index. The diversity indices ranged from 0.17 to 0.26. These numbers are low compared to those observed at different stations in the Chesapeake Bay, 0.22 to 1.0 (Takizawa *et al.*, 1993). The number of antimicrobial profiles increases as the number of target organisms used to determine these profiles is increased, therefore, the actual diversity may be higher than indicated, as only six target organisms were tested in this study. The highest diversity was observed from the Australasian region, which included 10 environmental samples from Australia (9) and New Zealand (1).

6.1.4. Discussion

A more comprehensive study using a larger number of target organisms would provide more information than this present study. Despite the large number of isolates recovered from the different geographical regions the diversity indices for all regions were relatively low. These results are in contrast to the

observations made in Chapters four and five where both the PyMS and numerical taxonomic data suggested that environmental samples from the regions studied are highly diverse in the micromonosporae they contain. However, the antimicrobial profiling technique is valuable as a tool for identifying which strains are producing antimicrobial products. This information would be worth exploring further but was not done so during this study.

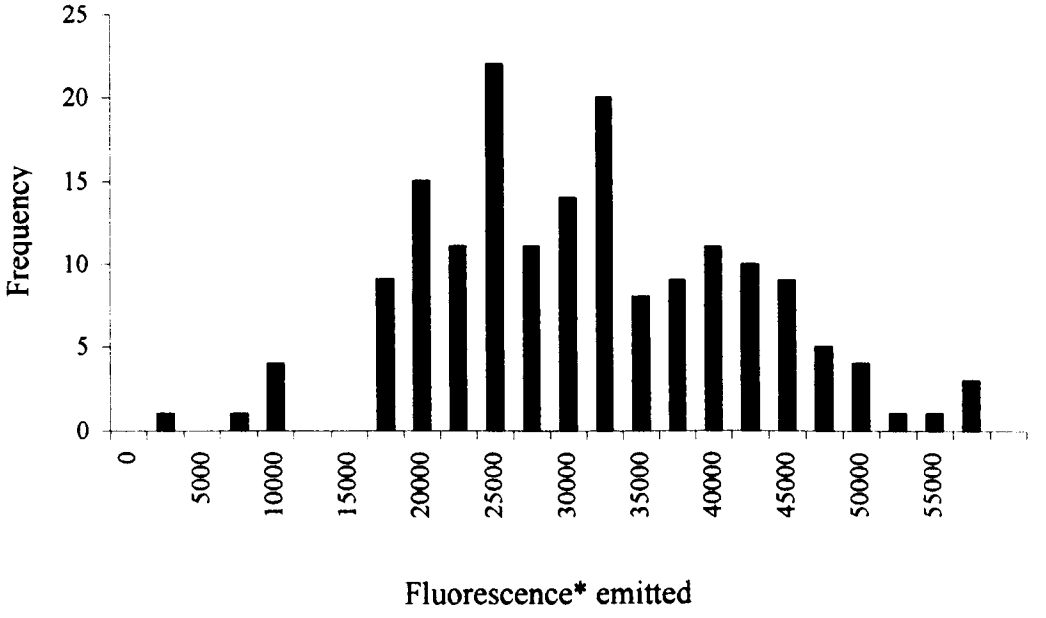
6.2. Rapid enzyme profiling

6.2.1. Introduction

Little attempt has been made to determine the enzymic profiles of *Micromonospora* strains. The most comprehensive studies to date have been carried out by Kawamoto *et al.* (1983) and Rowlands (1993). These studies have shown that a number of enzymes may be discriminatory for *Micromonospora* species. Kawamoto and co-workers (1983) found that a number of enzymes (β-N-acetylgluco-amidine, β-galactosidase, α-glucosidase, β-glucosidase, and *p*-nitrophenylglycosidase) were nearly always detected in *Micromonospora.* In contrast α-fucosidase, β-fucosidase, β-glucoronidase and α-xylosidase were rarely detected. However, the *Micromonospora* strains were found to have variable patterns of α-galactosidase, α-mannosidase, and β-xylosidase activity indicating the potential value of enzymic tests for the classification and identification of *Micromonospora* species.

One of the major advantages of the enzyme screening is the simplicity and rapidity of the technique. Once cultures have been grown, the tests can be completed within 18 hours. The frequency of fluorescence levels can be depicted graphically to show the distribution of numbers of micromonosporae emitting different fluorescence levels (see Figure 6.2). The technique also lends itself to handling large numbers of organisms, making it ideal for high throughput screening. The data generated from this work was also incorporated into the numerical taxonomy data set.

Figure 6.2. Graph showing frequency of fluorescence levels emitted by *Micromonospora* strains for the β-fucosidase enzyme

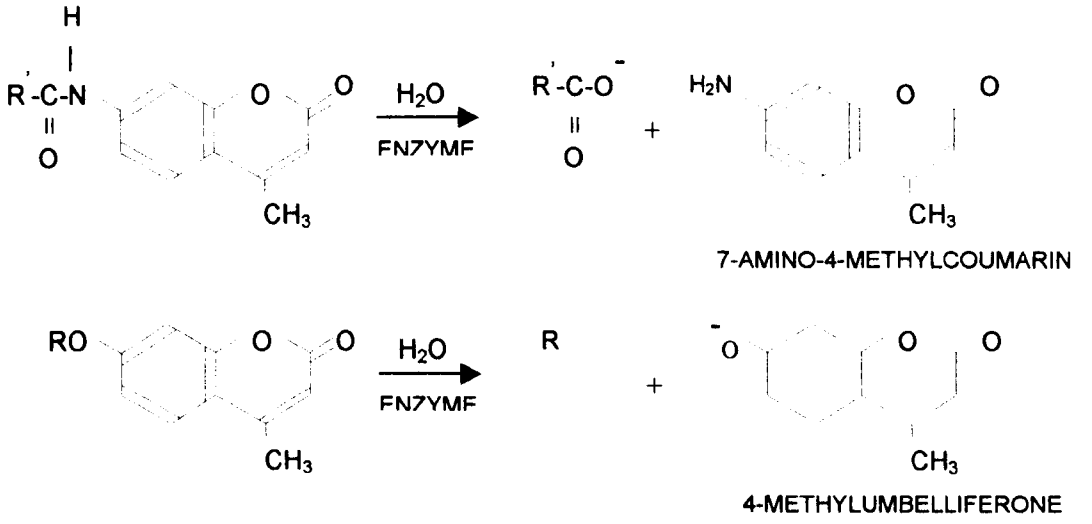


*Fluorescence measured at 365nm excitation and 440nm emission wavelengths.

6.2.2. *Micromonospora* strains selected for rapid enzyme profiling

Two hundred and seven strains, including 18 type strains were examined for their ability to enzymatically transform 4-methylumbelliferone and 7-amino-4-methylcoumarin substrates. The strains selected had previously been included in the numerical taxonomic study. In all cases, the relevant hydrolytic enzyme cleaves the conjugated substrates and the parent molecule is released.

The parent molecules are highly fluorescent in the visible region of the electromagnetic spectrum; in contrast, the corresponding derivatives are only weakly fluorescent in this region. For the purposes of this part of the study, the strains were considered positive for an enzyme if the fluorescence level was five times greater than the control (A. C. Ward, personal communication).



R, organic acid, inorganic acid or sugar; R', amino acid or peptide

6.2.3. Detection of enzyme activities in the genus *Micromonospora*

Micromonospora strains were found to contain many of the enzymes tested in this study. The percentage agreement between duplicated strains was very high with all but one of the enzymes showing 100% agreement (see Table 6.2). The genus was considered positive for an enzyme when >90% of the strains examined, tested positive. Table 6.3 shows which enzymes can be assigned to the micromonosporae examined. There were 12 enzymes testing positive, of which, six were found in all the micromonosporae studied: 4-methylumbelliferyl-β-D-galactopyranoside, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-fucoside, 4-methylumbelliferyl-α-D-mannoside, prolyl-amido-4-methylcoumarin, L-alanyl-7-amido-4-methylcoumarin. Of the 19 enzymes testing negative, five were not found in any of the micromonosporae studied: 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-butyrate, 4-methylumbelliferyl-nonanoate, 4-methylumbelliferyl-laurate, H-Ornithine-amido-4-methylcoumarin.2HCl.

Table 6.2. Percentage agreement between duplicated strains

Test	Percentage agreement between duplicated strains
Cleavage of substrates based on 4-methylumbelliferone:	
4-methylumbelliferyl-β-D-galactopyranoside	100
4-methylumbelliferyl-β-D-glucopyranoside	100
4-methylumbelliferyl-N-acetyl-β-D-glucosamide	100
4-methylumbelliferyl-β-D-glucuronide	100
4-methylumbelliferyl-α-D-glucoside	100
4-methylumbelliferyl-α-D-galactoside	100
4-methylumbelliferyl-β-D-fucoside	100
4-methylumbelliferyl-α-D-mannoside	100
4-methylumbelliferyl-phosphate	92
4-methylumbelliferyl-sulfate	100
4-methylumbelliferyl-β-cellobioside	100
4-methylumbelliferyl-butyrate	100
4-methylumbelliferyl-β-D-ribofuranoside	100
4-methylumbelliferyl-alpha-L-arabinoside	100
4-methylumbelliferyl-β-D-mannopyranoside	100
4-methylumbelliferyl-nonanoate	100
4-methylumbelliferyl-laurate	100
4-methylumbelliferyl-palmitate	100
Cleavage of substrates based on 7-amino-4-methyl coumarin:	
Leucyl-amino-4-methylcoumarin	100
Prolyl-amino-4-methylcoumarin	100
Pyroglutamyl-amino-4-methylcoumarin	100
Glutamyl-amino-4-methylcoumarin	100
Lysyl-amino-4-methylcoumarin	100
Asparginyl-amino-4-methylcoumarin	100
Phenylalanyl-amino-4-methylcoumarin	100
Z-glycyl-prolyl-amino-4-methylcoumarin	100
Z-arginyl-amino-4-methylcoumarin	100

Z, N-terminal carbobenzoxy group of the endopeptidase substrates

Table 6.3. Percentage of strains testing positive for the enzymes examined

Cleavage of 4-methylumbelliferone substrates:	Percentage of positive strains
4-methylumbelliferyl-β-D-galactopyranoside	**100**
4-methylumbelliferyl-β-D-glucopyranoside	**100**
4-methylumbelliferyl-N-acetyl-β-D-glucosamide	**98**
4-methylumbelliferyl-β-D-glucuronide	0
4-methylumbelliferyl-α-D-glucoside	**95**
4-methylumbelliferyl-α-D-galactoside	**96**
4-methylumbelliferyl-β-D-fucoside	**100**
4-methylumbelliferyl-α-D-mannoside	**100**
4-methylumbelliferyl-phosphate	27
4-methylumbelliferyl-sulfate	56

Table 6.3. Continued.

Cleavage of 4-methylumbelliferone substrates:	**Percentage of positive strains**
4-methylumbelliferyl-β-cellobioside	56
4-methylumbelliferyl-butyrate	0
4-methylumbelliferyl-β-D-ribofuranoside	4
4-methylumbelliferyl-alpha-L-arabinoside	20
4-methylumbelliferyl-β-D-mannopyranoside	26
4-methylumbelliferyl-nonanoate	0
4-methylumbelliferyl-laurate	0
4-methylumbelliferyl-palmitate	4
Cleavage of 7-amino-4-methyl coumarin substrates:	
Leucyl-amino-4-methylcoumarin	**99**
Prolyl-amino-4-methylcoumarin	**100**
Pyroglutamyl-amino-4-methylcoumarin	87
Glutamyl-amino-4-methylcoumarin	25
Lysyl-amino-4-methylcoumarin	**91**
H-Ornithine-amino-4-methylcoumarin.2HCl	0
L-alanyl-7-amino-4-methylcoumarin	**100**
L-histidine-7-amino-4-methylcoumarin	25
Asparginyl-amino-4-methylcoumarin	3
Phenylalanyl-amino-4-methylcoumarin	**92**
Z-glycyl-prolyl-amino-4-methylcoumarin	23
Z-arginyl-amino-4-methylcoumarin	3

Z, N-terminal carbobenzoxy group of the endopeptidase substrates

6.2.4. Discussion

The rapid enzyme profiling proved to be a reliable and efficient technique for screening large numbers of organisms. Several enzymes were identified as being common to micromonosporae (see section 6.2.3). These enzymes could be used as a rapid means of identifying putative micromonosporae strains. Conversely, enzymes for which micromonosporae were consistently negative could also be used for identification purposes. Used singly, this data is not comprehensive enough to identify micromonosporae but when considered in conjunction with other properties such as morphological and nutritional requirements it can provide valuable information.

Chapter Seven

Molecular Systematics

7.1. Introduction

The use of 16S rRNA gene sequences to detect prokaryotic phylogenies is well documented (Olsen *et al.*, 1998; Olsen and Woese, 1993; Woese *et al.*, 1990; Woese, 1987, 1994) and is regularly used to determine the homogeneity of groups of bacteria (Fuhrman and Davies, 1997). Therefore, sequencing of 16S rDNA of representative isolates from pyro-groups defined in the PyMS analysis and clusters in the numerical taxonomic analysis and comparison to sequences of type strains of the genus *Micromonospora* is a logical step in determining the novelty of the isolates.

7.2. 16S ribosomal gene sequencing

7.2.1. Selection of isolates for 16S ribosomal gene sequencing

Sixteen isolates were selected for 16S ribosomal gene sequencing. The selection was based on choosing representatives of pyro-groups and clusters from the previous PyMS and numerical taxonomic analysis. Table 7.1 lists these isolates, along with the pyro-group and clusters to which they have been assigned.

Table 7.1. Isolates selected for 16S ribosomal RNA gene sequencing

Isolate	PyMS pyro-group*	Numerical Taxonomic Cluster ♠
Aus-6-003	B	SMC
Aus-7-007	A	SMC
Aus-9-031	B	SMC
GBR-10-016	J	ND
Arg-13-042	F	SMC
Jpn-18-047	GG	20a
Por-21-001	M	SMC
Ant-25-001	MM	SMC
Ant-27-001	KK	8a
USA-47-042	T	ND

Table 7.1. Continued.

Isolate	PyMS pyro-group*	Numerical Taxonomic Cluster ♠
USA-49-009	T	SMC
Ind-55-011	Y	SMC
Ind-63-002	X	SMC
Bdh-76-041	BB	SMC
Nmb-86-005	CC	SMC

SMC, single membered cluster; ND, not determined.
* Pyro-group defined in PyMS analyses as described in Chapter Four.
♠ Numerical taxonomic cluster defined in Chapter Five.

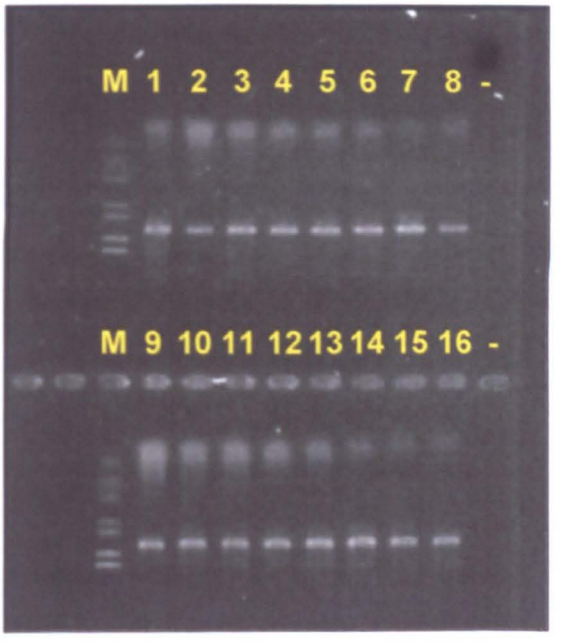
7.2.2. Amplification of 16S rDNA gene sequences

The 16S regions of isolates were amplified from genomic DNA, extracted according to the methods of Pitcher (1989), using universal primers (see Chapter Two, section 2.9.4 for full details).

7.2.3. Detection of amplified DNA following the polymerase chain reaction

The amplified DNA was visualised by running the reaction mixtures on a 1% (w/v) agarose gel after the polymerase chain reaction. Gels were stained in ethidium bromide solution and visualised under UV light. Figure 7.1 shows the products of 16S ribosomal DNA amplification by PCR. Molecular markers of known size were used to determine the size of the DNA fragments. The expected size of amplified 16S ribosomal RNA genes was approximately 1.5 kb, after purification (see Chapter Two, section 2.9.8) the concentration of DNA template was adjusted to 150-200ng μl^{-1} prior to sequencing.

Figure 7.1. Gel electrophoresis of DNA fragments generated by PCR using primers specific for 16S ribosomal RNA-encoding regions



M, DNA molecular weight marker VI;
1. Aus-6-003
2. Aus-7-007
3. Aus-9-031
4. GBR-10-016
5. Arg-13-042
6. Jpn-18-047
7. Por-21-001
8. Ant-25-001
9. Ant-27-001
10. USA-47-042
11. USA-47-042
12. USA-49-009
13. Ind-55-011
14. Ind-63-002
15. Bdh-76-041
16. Nmb-86-005

7.2.4. Sequencing of 16S genes of *Micromonospora* isolates

Amplified 16S rDNA gene products were sent to ABC (Imperial College School of Medicine, London, UK) for sequencing. Unfortunately, ABC were unable to sequence the isolates and did not give reasons as to why the sequence attempts were unsuccessful.

Following repeated failures to sequence *Micromonospora* isolates with ABC attempts were made to sequence the isolates using the facilities of Dr. Murphy at Wye College. These attempts were also unsuccessful. There were two problems with the sequencing, firstly, obtaining high quantities of pure amplified DNA was difficult and secondly the sequence data showed more than one sequence.

7.3. Single strand conformation polymorphism analysis of isolates

7.3.1. Introduction

In view of the difficulties experienced whilst trying to sequence the 16S genes of *Micromonospora* isolates, an alternative molecular method was sought for establishing relationships between the isolates. Single strand conformation polymorphism (SSCP) is a highly sensitive technique and has been used to detect minor sequence changes in PCR-amplified DNA (Shefffield *et al.*, 1993) which made it a good choice detecting differences in the 16S gene sequences of *Micromonospora* isolates. Another positive point for this technique was that small amounts of amplified DNA are easily detected (~50ng/ul).

In order to detect differences in the 16S gene sequences of *Micromonospora* isolates using SSCP it was decided to treat the amplified 16S PCR products with restriction enzymes to yield multiple bands of digested PCR products.

7.3.2. Selection of isolates and type strains for RFLP and SSCP

Twenty-two *Micromonospora* isolates, fourteen type strains and six reference strains were selected for inclusion in the SSCP study. The *Micromonospora* isolates were selected as representatives of pyro-groups and clusters formed in

the PyMS and numerical taxonomic studies respectively (see Table7.2 and 7.3). A detailed study was made of the Australian isolates as these had been shown to be distinct from the type strains in the PyMS analysis (see Table 7.2). A more general analysis was made of other *Micromonospora* isolates (see Table 7.3). Type strains previously included in the PyMS and numerical taxonomic studies were examined alongside reference strains of the same name but from different culture collections (see Table 7.4).

7.3.3. Restriction enzyme digestion of amplified DNA

The restriction enzyme *Alu*I was chosen for the digestion of the 16S regions of the *Micromonospora* isolates as it cuts at 214, 524, 810 and 1015 base pairs, producing fragments of approximately 205bp, 214bp, 286bp, 310bp and >400bp.

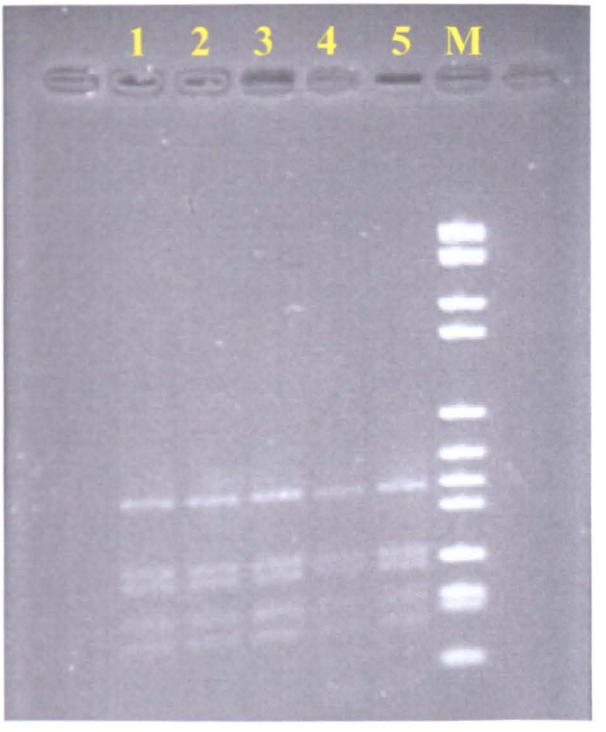
7.3.4. Detection of restriction fragment length polymorphism amongst amplified 16S rDNA

Digested DNA was viewed by running the reaction mixtures on a 2.5% (w/v) agarose gel after incubation with the appropriate restriction enzymes. Gels were stained in ethidium bromide solution and visualised under UV light. Figure 7.2 shows bands of digested 16S DNA fluorescing under UV light after restriction digestion by *Alu*I for five *Micromonospora* isolates. Molecular markers of known size were used to determine the size of DNA fragments generated by restriction digestion.

7.3.5. Detection of isolates with different 16S ribosomal RNA gene sequences by RFLP analysis

16S DNA digestion by the enzyme *Alu*I produced banding patterns of low molecular weight DNA fragments on agarose gels. These results gave little resolution in identifying *Micromonospora* isolates, as the RFLP profiles (DNA banding patterns) appeared to be identical. Figure 7.2 shows typical RFLP profiles for five *Micromonospora* isolates.

Figure 7.2. RFLP profiles for *Micromonospora* isolates on a 2.5% agarose gel with DNA molecular weight markers



1. Aus-9-001
2. Aus-9-031
3. Ant-25-001
4. USA-49-009
5. Bdh-76-041

M, DNA molecular weight marker VI

7.3.6. Detection of differences in 16S rDNA sequences by SSCP analysis

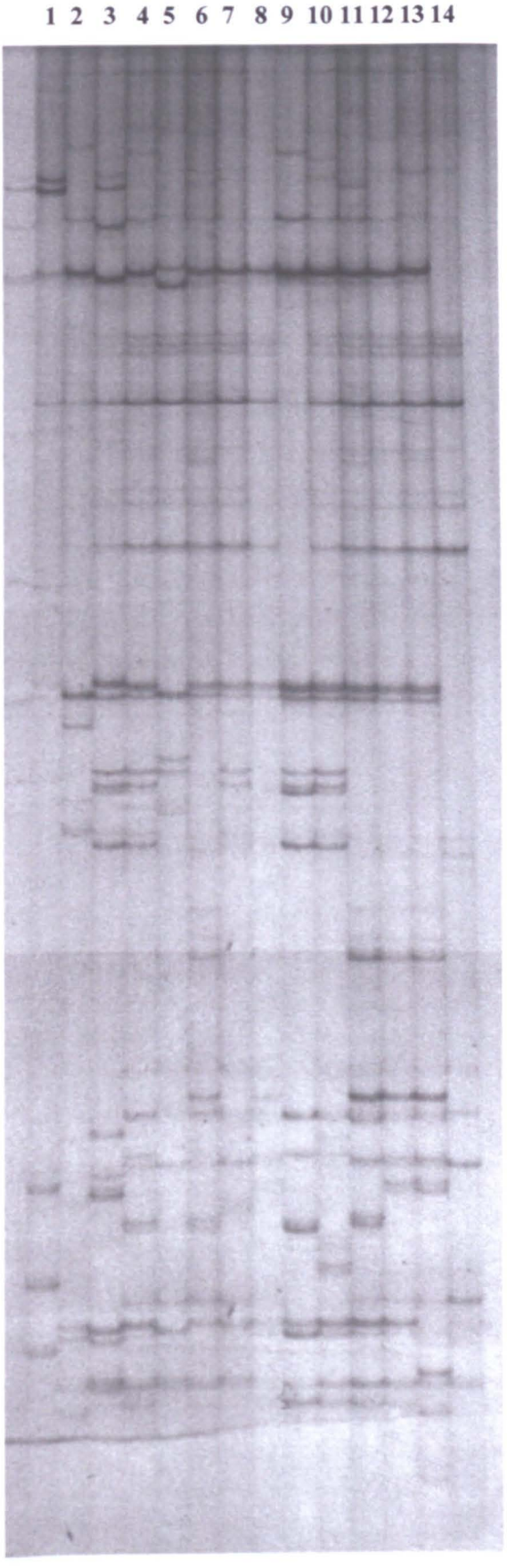
Although the RFLP profiles were identical for the *Micromonospora* isolates tested, they may have different 16S sequences. These differences are not highlighted by the RFLP technique, as it is more sensitive to changes in length of DNA and not changes in base composition. So to test if the *Micromonospora* isolates did have identical 16S rDNA sequences aliquots of the RFLP suspensions were run on high-resolution gels (see Chapter Two section 2.9.7. for details).

Table 7.2. Australian *Micromonospora* isolates examined by SSCP analysis

Lane No.	*Micromonospora* isolates	PyMS pyro-group
1	Aus-5-001	B
2	Aus-6-001	B
3	Aus-6-003	B
4	Aus-6-004	N/D
5	Aus-7-003	A
6	Aus-7-004	A
7	Aus-7-007	A
8	Aus-7-008	N/D
9	Aus-9-001	N/D
10	Aus-9-018	B
11	Aus-9-030	B
12	Aus-9-031	B
13	Aus-9-032	C1
14	Aus-9-034	C2

N/D, not determined

Figure 7.3. SSCP profile of Australian *Micromonospora* isolates. The 16S region was amplified by PCR, followed by restriction using *Alu*1.



1. Aus-5-001
2. Aus-6-001
3. Aus-6-003
4. Aus-6-004
5. Aus-7-003
6. Aus-7-004
7. Aus-7-007
8. Aus-7-008
9. Aus-9-001
10. Aus-9-018
11. Aus-9-030
12. Aus-9-031
13. Aus-9-032
14. Aus-9-034

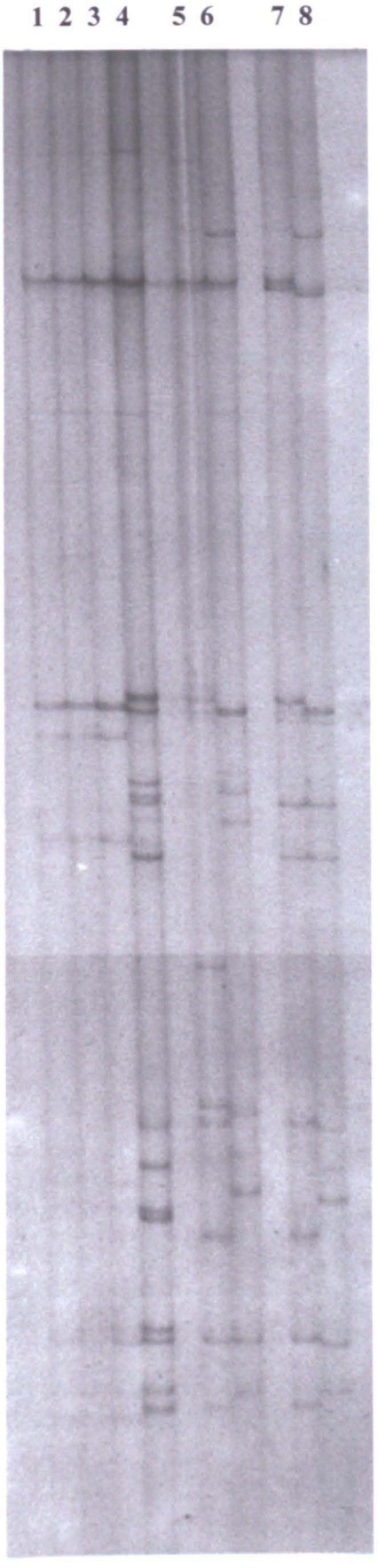
7.3.7. SSCP analysis of Australian *Micromonospora* isolates

Figure 7.3 shows the SSCP profiles for a selection of Australian *Micromonospora* isolates. There are clear differences in the banding patterns of isolates from the same pyro-groups for example the isolates from Mossman Gorge Aus-7-003, Aus-7-004 and Aus-7-007 are all in pyro-group A which suggest they are closely related but the SSCP profile of the 16S PCR-RFLP products show distinct differences in the banding patterns.

Table 7.3. *Micromonospora* isolates examined by SSCP analysis

Lane No.	*Micromonospora* isolates	PyMS pyro-group
1	Arg-13-041	E
2	Arg-13-042	F
3	Ant-25-001	RR
4	Ant-27-001	PP
5	Ant-28-001	PP
6	USA-49-009	U
7	Bdh-76-041	BB1
8	Nmb-86-005	CC2

Figure 7.4. SSCP profile of *Micromonospora* isolates. The 16S region was amplified by PCR, followed by restriction using *Alu*1.



1. Arg-13-041
2. Arg-13-042
3. Ant-25-001
4. Ant-27-001
5. Ant-28-001
6. USA-49-009
7. Bdh-76-041
8. Nmb-86-005

7.3.8. SSCP analysis of some *Micromonospora* isolates

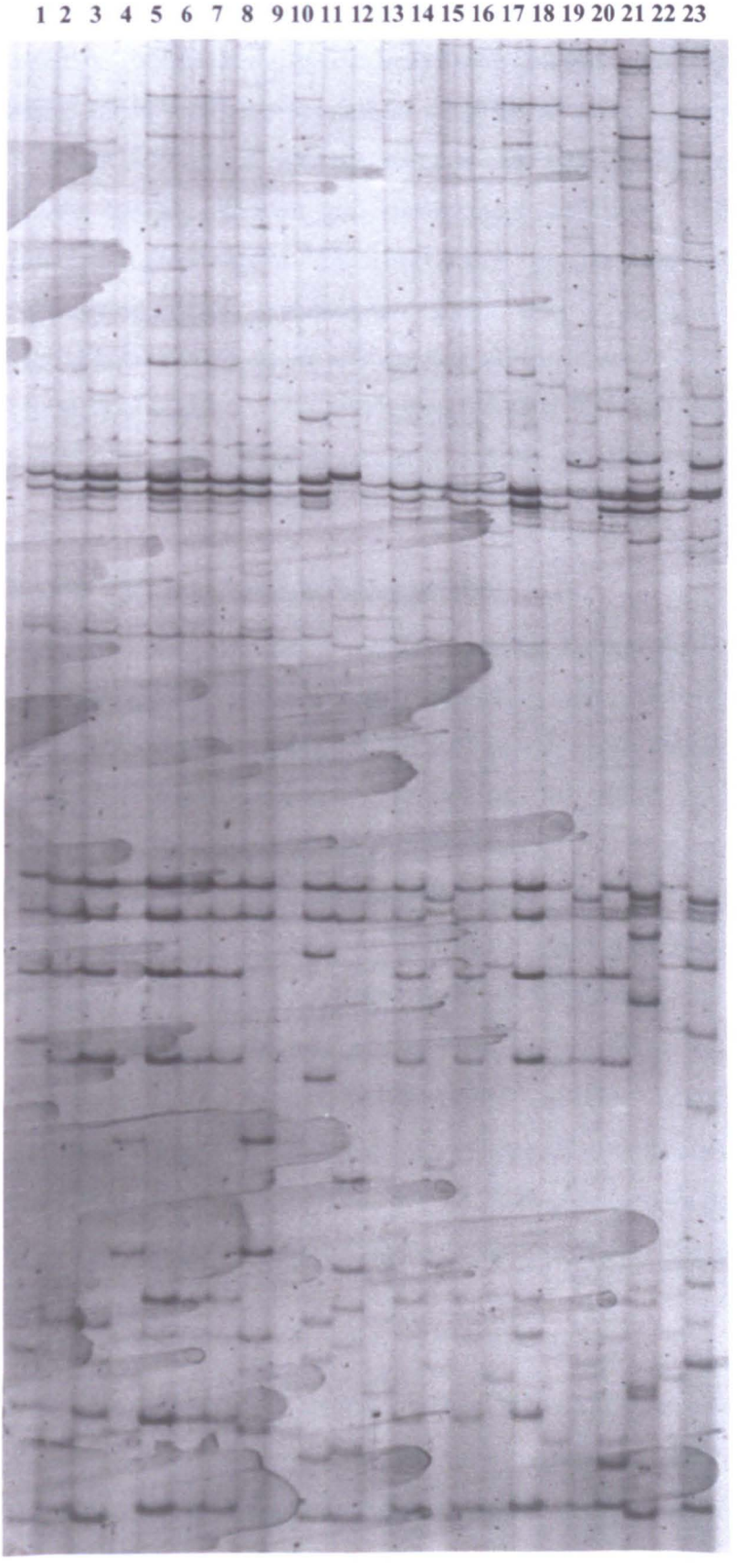
Figure 7.4 shows the SSCP profiles of eight *Micromonospora* isolates. Lanes 1-3 contain isolates with identical PCR-RFLP-SSCP profiles, two of these isolates are from the same environmental sample (Arg-13) but the third isolate is from Antarctica. All three isolates are from different pyro-groups. The remaining five isolates all have different banding patterns suggesting that they have different 16S rRNA gene sequences and probably belong to different species.

Table 7.4. *Micromonospora* type and reference strains examined by SSCP analysis

Lane No.	*Micromonospora* type strains	Source
1	*M. olivaterospora*^{T}	NCIMB 12659
2	*M. echinospora* subsp. *pallida*^{T}	NCIMB 12660
3	*M. purpureochromogenes*^{T}	NCIMB 12661
4	*M. rhodorangea*^{T}	NCIMB 12741
5	*M echinospora* subsp. *echinospora*^{T}	NCIMB 12744
6	*M. inositola*^{T}	NCIMB 12751
7	*M. aurantiaca*^{T}	NCIMB 12754
8	*M. purpurea*	DSMZ 1040
9	*M. echinospora* subsp. *ferruginea*^{T}	DSMZ 43141
10	*M. brunnea*^{T}	DSMZ 43814
11	*M. chersina*^{T}	DSMZ 44151
12	Aus-9-018	-
13	Aus-9-034	-
14	Arg-13-041	-
15	*M. carbonacea* subsp. *carbonacea*^{T}	NCIMB 12663
16	*M. carbonacea* subsp. *aurantiaca*^{T}	NCIMB 12664
17	*M. coerula*^{T}	NCIMB 12665
18	*M. chalcea*^{T}	DSMZ 43026
19	*M. chalcea* subsp. *chalcea*	NCIMB 9599
20	*M. chalcea* subsp. *chalcea*	NCIMB 12882
21	*M. chalcea* subsp. *chalcea*	NCIMB 12895
22	*M. coerula*	DSMZ 46121
23	*M. chalcea*	NCIMB 43116

NCIMB, National Collection of Industrial and Marine Bacteria; DSMZ Deutsche Sammlung von Mikroorganism Zelfulturen, Germany. ^{T}, type strain.

Figure 7.5. SSCP profile of *Micromonospora* type and reference strains. The 16S region was amplified by PCR, followed by restriction using *Alu*1.



1. NCIMB 12659
2. NCIMB 12660
3. NCIMB 12661
4. NCIMB 12741
5. NCIMB 12744
6. NCIMB 12751
7. NCIMB 12754
8. DSMZ 1040
9. DSMZ 43141
10. DSMZ 43814
11. DSMZ 44151
12. Aus-9-018
13. Aus-9-034
14. Arg-13-041
15. NCIMB 12663
16. NCIMB 12664
17. NCIMB 12665
18. DSMZ 43026
19. NCIMB 9599
20. NCIMB 12882
21. NCIMB 12895
22. DSMZ 46121
23. NCIMB 43116

7.3.9. SSCP analysis of some *Micromonospora* type and reference strains

Fourteen *Micromonospora* type strains and six reference strains from DSMZ and NCIMB culture collections were examined using SSCP analysis. The strains tested included several species with more than one accession number. The PCR-RFLP-SSCP profiles generated for the type and reference strains showed there to be clear differences between many of the type strains suggesting that these strains contain different 16S rRNA gene sequences. The *Micromonospora* isolate Aus-9-034 had a PCR-RFLP-SSCP profile identical to *Micromonospora carbonacea* subsp. *carbonacea.* There were a number of type strains, *M. purpureochromogenes, M echinospora* subsp. *echinospora, M. inositola* and *M. aurantiaca* (Lanes 3, 5, 6, 7) showing identical PCR-RFLP-SSCP profiles, which would suggest that these strains have identical 16S rRNA gene sequences.

7.3.10. Discussion of SSCP analysis data

The differences observed in the SSCP profiles amongst *Micromonospora* isolates from the same pyro-groups suggest that there is heterogeneity amongst the pyro-groups. Given that many of the pyro-groups were distinct from pyro-groups containing *Micromonospora* type strains, the SSCP data suggests that the diversity amongst the *Micromonospora* isolates may be greater than first thought. The large number of *Micromonospora* type strains with identical PCR-RFLP-SSCP profiles suggests that the 16S rRNA gene sequences are not sufficiently divergent in this genus to distinguish different species (Kasai *et al.*, 2000). Koch *et al.*, 1996, also observed homogeneity in the 16S rRNA gene sequences of *Micromonospora.* This study also raised questions concerning the validity of type strains in culture collections as a number of supposedly identical type strains were obtained from the DSMZ and NCIMB culture collections and these were found to have different 16S rRNA gene sequences according to their PCR-RFLP-SSCP profiles.

SSCP proved to be a rapid and effective method for analysing large numbers of *Micromonospora* isolates. The number of isolates in this study was limited due to problems obtaining high quantities of good quality PCR products for

Micromonospora strains. It showed great potential for distinguishing novel isolates and would be useful as a screen for organisms prior to more in depth studies involving DNA:DNA hybridisation.

Chapter Eight

Discussion

8.1. Discussion

8.1.1. Introduction

There are numerous citations in the literature of new products being discovered when rare or novel organisms, often actinomycetes, are examined in new or existing screens (Nolan and Cross, 1988; Okami and Hotta, 1988; Bull *et al.*, 1992, 2000). With the media and public hostility towards genetically engineered or modified products, there is increasing demand for natural products (Bull *et al.*, 1992, 2000).

The conservation and managed exploitation of microbial diversity requires urgent consideration and has rightly become a focus of international concern (Hawskworth, 1991; Bull, 1996; Constanza *et al*, 1997). Microorganisms are a major resource for biotechnology companies so the conservation of the microbial gene pool is essential for future biotechnological development (Bull *et al.*, 2000). It is common knowledge that tropical rainforests are rich in plant and animal species (Ho *et al.*, 2000). In contrast, little is known about the diversity of microbial flora in tropical rainforests, although it is anticipated that they will have a rich microbiota. The conservation of locations with high levels of endemism to protect the gene pool is imperative for the future of microbial diversity and new natural products (Bull *et al.*, 2000).). This justifies the continued efforts in research of these organisms.

Estimations of microbial diversity are notoriously subjective, as the number of microbial taxa known and described represents a small fraction of the total global diversity as it is thought that the majority of microorganisms are as yet uncultured (~90%) (O'Donnell *et al.*, 1994). This inability to culture representative portions of the microbial population has been referred to as 'the great plate count anomaly' (Staley and Konopka, 1983). A number of molecular methods have been developed to analyse the diversity of microbial taxa in the natural environment; genus-specific probes (Giovanni *et al.*, 1988), PCR based DNA fingerprinting techniques such as randomly amplified polymorphic DNA fragments (RAPD), (Micheli *et al.*, 1994) and single strand conformation

polymorphism (SSCP), (Schwieger and Tebbe, 1998). Analysis of DNA extracted from environmental samples has shown that molecular genetic diversity is much greater in natural habitats than was previously thought (Olsen *et al.*, 1986; Pace *et al.*, 1986; Ward *et al.*, 1990: Embley and Stackebrandt, 1997: Head *et al.*, 1998).

Taxonomy has a crucial role in the representation of microbial diversity. Methods used in taxonomy are continually being developed to take into account new technologies, with the aim of providing more complete and robust taxonomies. Polyphasic taxonomy is now widely practiced in circumscribing new species.

8.1.2. Selective isolation and diversity of the genus *Micromonospora*

The discovery that some micromonosporae produce commercially significant bioactive compounds (Welsch, 1942; Waksman *et al.*, 1947) led to an increased interest in these organisms and many attempts were made to isolate a greater number of these organisms from the environment. Micromonosporae are relatively easy to isolate from natural habitats and several selective isolation procedures are available for this purpose (Hopkins *et al.*, 1991; Vobis, 1991). Most of these isolation procedures involve heat or chemical pre-treatment of environmental samples as *Micromonospora* spores are resistant to relatively high temperatures and chemical treatments (Suarez *et al.*, 1985; Kawamoto, 1989).

Micromonosporae are common in freshwater (Erikson, 1941; Umbreit and McCoy, 1941; Colmer and McCoy, 1943; Cross and Collins, 1966; Willoughby, 1969; Johnston and Cross, 1976; Rowbotham and Cross, 1977; Kawamoto, 1989; Rowlands, 1993), marine (Weyland, 1969, 1970, 1981; Hunter *et al.*, 1981; Takizawa *et al.*, 1983; Goodfellow and Haynes, 1984), and terrestrial habitats (Jensen, 1930, 1932; Kriss, 1939; Nonomura and Ohara, 1957; Ishizawa *et al.*, 1969; Orchard, 1978; Nonomura and Hayakawa 1988; Rowlands, 1993).

The origin of *Micromonospora* species in aquatic environments is unclear, they could be indigenous to these environments or they may be components of

terrestrial wash-in. It is also uncertain to what extent either they or other actinomycetes represent physiologically active components in such environments (Goodfellow and Haynes, 1984; Jensen *et al.*, 1991). On the balance of information available, it is likely, that micromonosporae recovered from aquatic environments originated from terrestrial sources but have adapted to this environment. One of the features of micromonosporae is their ability to grow on resistant carbon compounds such as cellulose, chitin, lignin and starch, many of which are found in lake mud or aquatic sediments (Erikson, 1941).

In this present investigation, micromonosporae were isolated from aquatic and terrestrial samples by using the dispersion and differential centrifugation technique combined with a phenol pre-treatment and two selective media (colloidal chitin and M3). The dispersion and differential centrifugation (DDC) technique proved far more effective in the recovery of micromonosporae than the traditional vortexing technique with an almost 10,000 fold increase in the numbers of micromonosporae recovered. These findings are strongly supported in the literature (Manfio, 1995; Atalan *et al.*, 2000; Sembiring, 2000) and suggests that micromonosporae are closely associated with particulate matter in soils, so to achieve efficient recoveries of these organisms from environmental samples it is important to apply procedures that will facilitate the disassociation of the organisms from the particles present in the sample. These observations suggest that the DDC technique can be used to break down these interactions between soil particles and actinomycete propagules.

A number of sample pre-treatments were tested for their ability to decrease the number of fungi, Gram-negative and other non-target Gram-positive bacteria whilst encouraging the isolation of the target genus *Micromonospora*. A 1.5% phenol pre-treatment of samples for 30 minutes at 30°C was found to be most effective for the recovery of micromonosporae from natural habitats. Phenol is commonly used as a pre-treatment for samples during the recovery of actinomycetes (Vipin Vyas *et al.*, 1991).

In the present investigation, the counts of micromonosporae ranged from 0 to 6.67 * 10^4 cfu/g for the terrestrial samples and 6 to 4.65 * 10^2 cfu/g for the marine samples. The highest counts of 6.67 * 10^4 cfu/g were recovered from a terrestrial Argentinean sample whilst the highest marine counts 4.65 * 10^2 cfu/g were recovered from a shallow marine sediment. Low or zero counts of micromonosporae were continually found in environmental samples with low pH values. The micromonosporae counts recorded for the terrestrial samples are similar to those reported in previous studies (Rowlands, 1993)

There have been a number of reports in the literature that there is no correlation between pH and other physical characteristics of samples and the number of actinomycetes recovered (Goodfellow and Haynes, 1984; Jensen *et al.*, 1991). However, this investigation showed a distinct relationship between pH and moisture content of the sample and the number of micromonosporae recovered. Higher numbers of micromonosporae were generally recovered from habitats with pH values in excess of seven. From the terrestrial samples examined higher numbers of micromonosporae were recovered from those with a high water content for example 1.35 * 10^4 cfu/g (Ind-72) mud flats adjacent to a tea plantation and 9.0 *10^4 cfu/g (Nmb-90) cultivated lake bottom.

One of the many constraints on quantitative and representative sampling of microorganisms from natural habitats is the lack of appropriate selective isolation procedures. The selectivity of isolation media is influenced by a number of factors; nutrient composition, pH and the presence of selective inhibitors such as antibiotics and salt concentrations, as well as other incubation conditions (Bull *et al.*, 2000). This study initially focused on a number of commonly used selective media for actinomycetes; colloidal chitin, cellulose asparagine, glycerol arginine and salts, humic acid vitamin, M3 and starch casein nitrate agars. Two of these, colloidal chitin and M3 were found to be highly efficient for the recovery of micromonosporae. In view of what is already known about the ability of micromonosporae to grow on resistant carbon compounds, it is no surprise that the colloidal chitin agar proved to be an effective medium for the selective isolation of this genus.

Traditionally, the ingredients used in selective isolation media have been chosen empirically, hence the basis of selectivity is unknown (Williams *et al.*, 1984). With the advent of computer-assisted procedures, it is now possible to objectively formulate selective isolation media (Bull *et al.*, 1992, Ferguson, 1997).

A search of the literature on micromonosporae reveals that the only report of isolations of alkalitolerant or alkaliphilic micromonosporae has been by Rowlands (1993). Rowlands isolated alkalitolerant micromonosporae from diverse soils by plating heat-treated soil suspensions onto isolation media adjusted to pH 10.0. She also observed that higher counts of alkalitolerant micromonosporae were recovered from soils with a slightly acid, as opposed to alkaline, pH. The presence of alkaliphilic microorganisms occurring in acidic environments is well documented (Horikoshi and Akiba, 1982). The existence of alkalitolerant micromonosporae in acidic soils may be explained by the periodic occurrence of microhabitats of higher pH. There is evidence that such micro sites are produced by ammonification of substrates such as amino acids or chitin through the activities of acidophilic soil microorganisms (Williams and Mayfield, 1971; Williams and Robinson, 1981). Isolations using the Indonesian samples were made at pH 10.0 and a number of micromonosporae were recovered, suggesting that some micromonosporae are alkalitolerant or alkaliphilic. These organisms would be worth further investigation as they may possess novel metabolic pathways as a result of their adaptation to such harsh conditions.

There are numerous reports of thermophilic actinomycetes in the literature (Attwell and Colwell, 1981; Haynes, 1982; Goodfellow *et al.*, 1987a; O'Donnell *et al.*, 1993b). The study of thermophilic actinomycetes is much less comprehensive than that of their mesophilic counterparts. This is surprising given the biotechnological potential of these bacteria as sources of thermostable enzymes and other products of industrial interest (Sharp and Munster, 1986). Attempts were made to isolate thermophilic micromonosporae from the Indonesian samples examined by plating onto colloidal chitin and M3 agar and

incubating at 50°C. No thermophilic micromonosporae were isolated. This suggests that thermophilic micromonosporae are rare, if they exist at all.

It can be concluded that micromonosporae, including alkalitolerant or alkaliphilic strains, are common and widely distributed throughout natural ecosystems. It has already been pointed out that micromonosporae are relatively easy to isolate and recognise on selective isolation plates but it is far from clear, given the current inadequate systematics on this genus, to what extent such strains are representative of the micromonosporae community found in natural habitats and whether they are cosmopolitan in their distribution. It was for these reasons that representative isolates and validly described type strains of *Micromonospora* were the subject of comprehensive chemo and numerical taxonomic procedures.

8.1.3. Chemotaxonomic analysis of micromonosporae isolates

Pyrolysis mass spectrometry (PyMS) is widely used for microbial characterisation of whole organisms. It involves generating a fingerprint of an organism that can be compared to fingerprints of other organisms using statistical methods. Some of the main features of PyMS are: measurement of large numbers of characters that together reflect overall cell composition, small quantities of biomass are required for analysis, pre-analysis processing is minimal, no specialized reagents are required, analysis is rapid, and the analysis costs are low. The procedure is applicable to all microorganisms with minimal taxon-specific modification and is useful for discrimination at the sub-species as well as the species level.

It has been acknowledged that there is a need to ensure that organisms growing on selective isolation plates represent novel, or previously uninvestigated, centres of taxonomic variation (Goodfellow and O'Donnell, 1989: Bull et al., 2000). PyMS provides a useful tool in the search and discovery of new bioactive compounds as it has been shown to highlight subtle phenotypic differences between strains of the same species (Goodacre *et al.*, 1998) and to group commercially significant actinomycetes (Sanglier *et al.*, 1992). This technique has also been widely used for the rapid grouping of environmental isolates

(Ferguson, 1997; Colquhoun, 1999; Colquhoun *et al*, 1998, 2000; Sembiring, 2000).

In this project, PyMS was used for the preliminary discrimination of large numbers of micromonosporae isolates. Initial PyMS experiments determined the most suitable incubation period for *Micromonospora* strains prior to PyMS analysis and the effect of intra-operator reproducibility (see Figures 4.1 and 4.2). The reproducibility of PyMS analyses was assessed by comparing two sets of type strains analysed on separate days; there was excellent agreement between the two data sets with the exception of *Micromonospora brunnea* which had given irreproducible results in the two initial experiments. The isolates were examined in geographic batches alongside validly described type strains. For example, Figure 4.5 shows the PyMS analysis for South American isolates from Argentinean and Brazilian samples and type strains of the genus *Micromonospora.*

The pyro-groups generated by the PyMS analyses revealed the relationships between the isolates and type strains of the genus *Micromonospora.* In several cases, PyMS analysis revealed pyro-groups composed entirely of isolates, suggesting that these isolates are distinct from the type strains and may be novel species of *Micromonospora* (for example; pyro-group A in Figure 4.4, which was comprised entirely of Australian isolates and pyro-group X in Figure 4.9, which was comprised entirely of Indonesian isolates). Groups such as these are very interesting as they may represent previously uncultured strains of *Micromonospora* and should be investigated in further detail as they may have novel metabolic pathways with the capacity for synthesis of novel bioactive compounds.

In general, there was little evidence of the isolates grouping according to their sample sites. What was more commonly observed was a general distribution of isolates from different sample sites throughout the pyro-groups. There were a few exceptions to this, most notable were the sample sets from Australia and New Zealand (see Figure 4.4). In this analysis pyro-group A was distinct from the other pyro-groups, at the 72% similarity level, and contained seven isolates

from the Mossman Gorge sample (Aus-7) and one isolate from a second Mossman Gorge sample (Aus-6). This pyro-group contains isolates that are clearly different from the other Australian and New Zealand isolates, and *Micromonospora* Type strains. All the New Zealand (Nzl-33) strains were grouped in pyro-group B, along with a few Australian isolates, whilst sub pyro-group C1 contained isolates solely from Lizard Island, Australia (Aus-9).

An assessment, using PyMS analysis, of the biogeographic distribution of the genus *Micromonospora* was carried out (see Figure 4.12). This analysis revealed little evidence of grouping of isolates according to their location. This indicates that *Micromonospora* probably have a cosmopolitan distribution. These results need to be interpreted with caution as the high level of diversity amongst the isolates may be masking smaller differences in local populations of micromonosporae. Plus, it must be remembered that the different sample sites were only represented by a small number of isolates (1 or 2) so were not a true reflection of the total diversity found at the different locations but gave a general indication. It is highly likely that if the different locations were studied in greater depth then evidence of geovars may come to light.

It is unsurprising to find that micromonosporae are cosmopolitan in their distribution, as we have previously discussed how hardy the spores are; being able to survive desiccation, chemical treatments and high temperatures. These properties of micromonosporae spores all help to promote the survival and distribution of this genus.

The present study demonstrated the potential of pyrolysis mass spectrometry as a rapid method for the circumscription of novel taxospecies, discriminating organisms at the infraspecies level, for the rationalisation of large industrial screening programmes and for grouping large numbers of environmental isolates, from which representatives can be selected for further taxonomic evaluation, notably small subunit rRNA sequencing.

8.1.4. A comprehensive numerical taxonomic analysis of micromonosporae isolates

The application of numerical taxonomic methods has led to significant improvements in the development of sound and stable bacterial classifications (Goodfellow and Dickinson, 1985; MacDonnell and Colwell, 1985). One of the main contributions has been the determination of homogeneous groups that can be equated with taxospecies (Goodfellow and O'Donnell, 1993).

Numerical taxonomic databases are essentially information storage and retrieval systems. They contain essential information on the biochemical, morphological, nutritional, physiological, enzyme and tolerance profiles of the constituent taxa. Improvements in the classification and identification of bacteria derived from the application of numerical taxonomic procedures are the basis of a new taxonomic approach to the selective isolation of industrially important organisms, especially actinomycetes (Goodfellow and O'Donnell, 1989)

Numerical analysis of a genus makes it possible to determine its degree of homogeneity, to identify groups of similar species and in some cases to distinguish very different clusters or individual species within the genus under consideration (Bibikova *et al.*, 1989). The circumscription and definition of the genus *Micromonospora* is highly subjective with the majority of taxa separated by only a few phenotypic properties. Originally, the genus *Micromonospora* was considered to belong to the family Micromonosporaceae (Muchanan and Gibons, 1974). A radical reorganization of traditional actinomycete systematics on the basis of broad biochemical and mathematical methods led a number of researchers to conclude that it would be best to abolish the family Micromonosporaceae and move the genus to the family Actinoplanaceae (Goodfellow and Pirouz, 1982).

Representatives of several poorly studied actinomycete taxa have been assigned to well circumscribed clusters (taxospecies) on the basis of overall similarity (Williams *et al.*, 1983; Goodfellow *et al.*, 1991). This approach to actinomycete classification is in complete contrast to the traditional practice of assigning

strains to taxa using a small number of subjectively chosen characters, notably morphological and pigmentation features. The application of the numerical taxonomic procedure has gone a long way towards fulfilling the criteria required for a good "taxonomy", namely that the classifications should be stable, objective and predictive (Sneath and Sokal, 1973).

The application of numerical taxonomic procedures has led to marked improvements in the classification of several actinomycete genera (Goodfellow and Cross, 1984; Goodfellow, 1989). Extensive numerical phenetic surveys have provided a framework for the improved taxonomy of *Actinomyces* (Holmberg and Nord, 1975; Schofield and Schaal, 1981), *Corynebacterium* (Jones, 1975; Goodfellow *et al.*, 1982a), *Mycobacterium* (Goodfellow and Wayne, 1982), *Nocardia* (Orchard and Goodfellow, 1980), *Rhodococcus* (Goodfellow and Alderson, 1977; Goodfellow *et al.*, 1982; 1990b) and *Tsukamurella* (Goodfellow *et al.*, 1991). Significantly less attention has been paid, with the exception of streptomycetes, towards the sporoactinomycetes, including *Micromonospora*. However, improved classifications have been generated for *Actinomadura* (Athalye *et al.*, 1985), *Saccharopolyspora* (Goodfellow *et al.*, 1989), and *Thermomonospora* (McCarthy and Cross, 1984).

Numerical taxonomy has mainly been applied to the revision of known taxa but is also well suited for the initial delineation and characterisation of populations of poorly described organisms or environmental isolates (Goodfellow and O'Donnell, 1993). The application of numerical taxonomic methods has been successfully used to detect variation amongst bacteria isolated from both aquatic and terrestrial habitats (Aznar *et al.*, 1992; White *et al.*, 1993).

Numerical taxonomies are only as good as the data upon which they are based. It is imperative that the quality of the test data be critically examined using appropriate measures (Jones and Sackin, 1980). Quality can be assessed by determining the stability of classifications using different statistics and by estimating test error and test reproducibility.

In the present investigation the test error (p) was 4.37%. This value is well within the 10% guideline Sneath and Johnson proposed in 1972 but is higher than experimental error recorded for members of the genera *Actinomyces* (p=3.12%, Holmberg and Nord, 1975; p=1.6%, Schofield and Schaal, 1981), *Corynebacterium* (p=2.4%, Jones, 1975), *Rhodococcus* (p=3.7%, Goodfellow and Alderson, 1977) and *Tsukamurella* (p=1.9%, Goodfellow *et al.*, 1991).

It has been suggested that confidence can be placed in numerical taxonomies where cophenetic correlation values of 0.8 or above are found (Jones and Sackin, 1980; Sackin and Jones, 1993). The cophenetic correlation values recorded for the Jaccards and simple matching coefficient analyses based on UPGMA were 0.81 and 0.62 respectively. It can be concluded that the dendrograms generated in the S_J UPGMA analysis are good representations of the taxonomic structure inherent in the corresponding similarity matrix.

Relatively good congruence was found between the classifications based on the S_{SM} and S_J UPGMA analyses, which suggests that the classification is a natural one and the clusters can be relied upon to give a good representation of the relationships between the isolates under examination. In the present study, clusters were defined at the 69% (S_J, UPGMA), 82% (S_{SM}, UPGMA) similarity levels. This choice of cut-off points, although somewhat arbitrary was influenced by the clustering nature of type and representative strains. The cut-off points are within the range of values commonly applied in numerical taxonomic surveys of actinomycetes (Williams *et al.*, 1983; Athlaye *et al.*, 1985; Goodfellow *et al.*, 1989, 1991; Rowlands, 1993).

In the S_J analysis the 166 test strains and 14 type strains were recovered into 5 major (4 or more strains), 15 minor (2-3 strains) and 124 single member clusters. In the corresponding S_{SM} analysis the 166 test strains and 14 type strains were recovered into 8 major clusters, 21 minor clusters and 73 single member clusters. In both analyses, a high number of single member clusters were recovered, this is indicative of a high degree of diversity amongst the isolates under examination.

A notable feature of this investigation was the high number of test strains assigned to single member clusters. The recovery of 69% in the S_J and 40% of the test strains in the S_{SM} analysis to single member clusters is unusual given the results of other studies on actinomycetes which show a range of 3 to 21% of test strains in single member clusters (Goodfellow *et al.*, 1979; Orchard and Goodfellow, 1980; Goodfellow *et al.*, 1982b; Athlaye *et al.*, 1985; Goodfellow *et al.*, 1990a). Rowlands (1993) in a similar numerical taxonomic analysis of micromonosporae also found a high number of isolates assigned to single member clusters, 58% in the S_{SM} UPGMA analysis. Priest and Barbour (1985) also found a high number of single member clusters during a numerical taxonomic analysis of lactobacilli from whisky distilleries and they concluded that their test strains formed the nuclei of novel taxa and were phenotypically similar but genetically different. It is also possible that the single member clusters recovered in the present study represent nuclei of novel taxa, genetically unstable strains or organisms of established taxa lacking plasmids.

Bibikova *et al.*, (1989) carried out a numerical analysis of thirty-two *Micromonospora* strains belonging to nine different groups based on cultural, morphological and physiological properties, the ability to produce antibiotics of several chemical classes and sensitivity to different antibiotics. Most of the strains in a given group were recovered in distinct clusters, although two strains were recovered as single member clusters. Their results demonstrated a tendency for the genus to divide into two subgenera at a similarity level of 61%. The strains in each subgenus had very different properties. Cluster A contained strains rarely isolated from soils, were sensitive to many antibiotics and exhibit virtually no antibiotic properties. Conversely, the strains of cluster B were widespread in soil, are highly resistant to antibiotics and are active antibiotic producers.

Although it is difficult to interpret the numerical phenetic data, a measure of confidence can be placed in the numerical classification based on the S_J, UPGMA analysis given the acceptable cophenetic correlation and test error values and the good congruence found with the S_{SM}, UPGMA analysis. It is highly encouraging that the integrity of most of the major, minor and single

member clusters defined in the S_J, UPGMA analysis were recovered in the corresponding S_{SM}, UPGMA analysis.

8.1.5. Comparison of PyMS pyro-groups and numerical taxonomic clusters

The comparisons between isolates recovered in the PyMS pyro-groups and those recovered in the numerical taxonomic clusters are limited because of the way in which isolates were examined for each technique. The PyMS study was a progressive analysis of the different sampling locations where the isolates were examined in small geographical groups (~35 isolates), whilst in the numerical taxonomic study, isolates from all geographic locations were examined together (~150). However, there are a number of comparisons that can be drawn between the two techniques (see Chapter 5, Table 5.8). In a number of cases, good agreement was found between numerical taxonomic clusters and PyMS pyro-groups, although the tendency was for minor numerical taxonomic clusters to form part of larger PyMS pyro-groups. For example, numerical taxonomic cluster 4b (Aus-6-004, Aus-7-008, Aus-7-007, Aus-7-004) formed part of a larger pyro-group A, which contained additional isolates from the Aus-7 Mossman Gorge sample. Other actinomycete studies have shown excellent agreement between numerical taxonomic clusters and PyMS pyro-groups (Colquhoun *et al.*, 2000).

The results of this present study affirm the value of pyrolysis mass spectrometry in characterising microorganisms and screening large numbers of environmental isolates prior to screening. PyMS can yield valuable information on the groupings of organisms in just one day, compared to several weeks required for similar information to be gained from a numerical taxonomic analysis. These attributes coupled with the high speed of analysis (approximately 2 minutes per sample), small sample size required (~50-100μg), high reproducibility and the high automated throughput all contribute to making PyMS an ideal method for industrial screening of microorganisms.

8.1.6. Antibiosis and rapid enzyme profiling as tools in bacterial systematics

The ability of organisms to cause antibiosis can be used to investigate the diversity of strains by distinguishing isolates on the basis of their individual antimicrobial profiles (Takizawa *et al.*, 1993). This technique can distinguish between strains that may belong to a single species, as production of bioactive agents is often strain-specific and not species-specific (Okami, 1986).

Despite the large number of isolates recovered from the different geographical regions the diversity indices, calculated by their antibiosis patterns, for each region were relatively low. These results were in contrast to the observations made in Chapters four and five, where both the PyMS and numerical taxonomic data suggested that environmental samples from the regions studied were highly diverse in the micromonosporae they contained. The diversity indices may have revealed more diversity if a larger number of target organisms had been included in the study, for example filamentous fungi (Takizawa *et al.*, 1993).

This technique is quick, simple and cheap to use. It is a valuable tool for identifying which strains are producing anti-microbial products and yields information that can be incorporated into numerical taxonomic studies. As a means of identifying diversity of environmental isolates it was not very successful in this project but as previously discussed that may due to the limited number of target organisms used.

Rapid enzyme tests have been used in a number of taxonomic studies of actinomycetes (Goodfellow *et al.*, 1987a, 1990b, 1991; Whitehead, 1990). The rapid enzyme profiling proved to be a reliable and efficient technique for screening large numbers of organisms. There was evidence that the enzyme tests may be of value as a tool in the taxonomy of the genus *Micromonospora* as a number of enzymes were identified as being common to this genus. Excellent reproducibility of results was shown with this technique, as the percentage agreement between duplicated strains for each substrate was 100% with the exception of one, 4-methylumbelliferyl-phosphate. All of the strains cleaved 4-methylumbelliferyl-β-D-galactopyranoside, 4-methylumbelliferyl-β-D-

glucopyranoside, 4-methylumbelliferyl-β-D-fucoside, 4-methylumbelliferyl-α-D-mannoside, pyrolyl-amido-4-methylcoumarin and L-analyl-7-amido-4-methylcoumarin. Over 90% of the strains cleaved another six substrates. Conversely, there were five substrates for which none of the micromonosporae examined cleaved, these were; 4-methylumbelliferyl-β-D-glucoronide, 4-methylumbelliferyl-butyrate, 4-methylumbelliferyl-nonanoate, 4-methylumbelliferyl-laurate and H-Ornithine-amido-4-methylcoumarin.2HCl.

A search of the literature reveals that there is only one other reference to a study of rapid enzyme profiling of the genus *Micromonospora* and Rowlands undertook this in 1993. Unfortunately, there is very little information that can be drawn from a comparison of this present study with that of Rowlands as the substrates being examined were different. What can be said of this former study is that it too proved that enzymes could be used as a rapid means of identifying micromonosporae.

Results from this study suggest that micromonosporae may have species-specific biochemical profiles. However, additional studies on representative strains and further conjugated substrates are needed to prove the point. There is considerable scope for the design and synthesis of additional 7-amino-4-methylcoumarin and 4-methylumbelliferone derivatives in order to extend the range of detectable enzymatic activities (James, 1993).

8.1.7. Molecular systematics

It has become clear that classifications derived from the application of numerical and chemo-taxonomic need to be interpreted with care as similarity values between strains can be distorted by factors such test and sampling error, failure to standardise conditions and the statistics used (Sneath and Johnston, 1972; Sackin and Jones, 1993). It is, therefore, important to evaluate numerical and chemotaxonomies in the light of information derived from the application of other taxonomic methods, notably molecular systematics (Goodfellow and O'Donnell, 1993). Such studies should be carried out using strains that represent numerically and chemotaxonomically circumscribed clusters as taxonomic

structure can be distorted when using unrepresentative strains (Hartford and Sneath, 1988).

The past decade has witnessed an extraordinary increase in molecular approaches towards bacterial systematics including the introduction of rapid protocols for the analysis of RNA and DNA. The various nucleic acid approaches have been used to not only to establish a bacterial phylogeny but also to unravel the evolution of biochemical, ecological and physiological relationships.

In the present study the attempt to evaluate the taxonomic status of carefully chosen representatives of selected pyro-groups and numerical taxonomic clusters by 16S rRNA gene sequencing was thwarted by a series of technical problems. Initially, it proved difficult to amplify sufficient quantities of high quality DNA from many of the strains. For many of the strains being examined, no 16S sequences were obtained and for the remaining strains, 16S sequences of poor quality were acquired. The poor quality of these 16S sequences was largely due to there being more than one sequence recovered for an individual strain. This could be a result of micromonosporae containing more than one 16S rRNA operon, which would result in multiple 16S gene sequences.

Koch *et al.*, (1996b) performed a 16S rDNA sequence based study on the taxonomy of the genus *Micromonospora.* Their proposals for a 16S rDNA sequence based phylogeny of this genus did not always agree with other taxonomic characteristics such as numerical and chemotaxonomic characters. Kasai *et al.*, (2000) proposed that the 16S rDNA sequences are not sufficiently divergent in the genus *Micromonospora* to distinguish different species. The problems associated with sequencing genes from micromonosporae may account for why so little molecular systematic work has been carried out on representatives of the genus *Micromonospora* (Koch *et al.*, 1996a,b; Kasai *et al.*, 2000).

The use of SSCP as a taxonomic tool for studying large numbers of environmental isolates is a relatively new idea. A search of the literature only reveals a few reports on the application of the SSCP technique to the study of

bacterial diversity in natural environments (Lee *et al.*, 1996; Schwieger and Tebbe, 1998).

The PCR-RFLP-SSCP of 16S genes of *Micromonospora* proved highly successful in distinguishing between different isolates. It also revealed that the PyMS pyro-groups were probably quite heterogeneous and that there may be more diversity amongst the isolates studied than the PyMS results suggest. The SSCP data highlighted the fact that there were differences in the 16S rRNA gene sequences of type strains held under more than one accession number in a culture collection. In this study three strains of *Micromonospora chalcea* subsp. *chalcea* were examined and all three had different PCR-RFLP-SSCP profiles, this raises questions over the validity of culture collections and the way in which microorganisms are preserved and stored. It would be worthwhile to sequence the 16S rRNA gene sequences of these strains and to perform DNA:DNA hybridisation to determine if the strains are different.

This study has clearly generated several areas where further work is required to make a comprehensive review of the classification of the genus *Micromonospora* but it has also highlighted areas that are worth further investigation from a biotechnological point of view. These ideas are discussed below:

8.2. Future work

- Additional investigation of the alkalitolerant or alkaliphilic micromonosporae recovered from the Indonesian samples, as these organisms could be a potential source of novel bioactive compounds.

- Characterisation of representatives of pyro-groups and numerical taxonomic clusters using 16S rRNA gene sequencing

- DNA:DNA pairing should be used to assess the novelty of *Micromonospora* isolates, the resulting information may reveal any relationships between novel isolates and biogeographic distribution.

- Gene probes (Giovanni *et al.*, 1988; Pace *et al.*, 1986) should be used to probe environmental samples to identify non-culturable *Micromonospora*; this would help to reveal the true diversity of *Micromonospora* populations and may assist with the choice of selective isolation procedures to target rare *Micromonospora*.

- Given the history of commercially important metabolites synthesised by the genus *Micromonospora*, the organisms isolated during this study ought to be incorporated into biotechnological screens for bioactive compounds. This would be of particular interest for the isolates that showed antimicrobial activity in the antibiosis investigation.

- Definition of minimal standards for the delineation of *Micromonospora* species.

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Appendix A

Useful World Wide Web sites

Actinomycetes – *Streptomyces* Internet Resource Center
http://biosci.cbs.umn.edu/asirc/index.html

American Society for Microbiology
http://www.asmusa.org/

List of bacterial names with standing in nomenclature
http://bacterio.cict.fr/

NCBI Taxonomy
http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/

Society for Actinomycetes Japan
http://www.nih.go.jp/khotta/saj/

Society for General Microbiology
http://www.socgenmicrobiol.org.uk/

Appendix B

Media and reagents

M3 agar (Rowbotham and Cross, 1974)

Potassium nitrate 0.1g; sodium chloride 0.29g; magnesium sulphate heptahydrate 0.1g; calcium carbonate 0.02g; sodium propionate 0.2g; iron sulphate heptahydrate 200μg; zinc sulphate heptahydrate 180μg; manganese quadrahydrate 20μg; Lab M agar (No.1) 18g; distilled water 1 litre. pH 7.2; autoclave at 121°C for 20 minutes.

The molten agar was supplemented with filter-sterilised thiamine hydrochloride solution to give a final concentration of 4.0μg per litre. 20ml of phosphate solution containing 0.186g of potassium dihydrogen orthophosphate and 0.74g of disodium hydrogen phosphatedodecahydrate, autoclaved separately was also added before the plates were poured.

Glucose/yeast extract agar

Yeast extract 10g; glucose 10g; Lab M agar (No. 1) 15g; distilled water 1 litre, pH 7.2; autoclave at 121°C for 20 minutes.

Micromonosporae maintenance medium (Luedemann, 1971)

Yeast extract 5g; casamino acids (Difco) 5g; glucose 10g; soluble starch (Fisons) 20g; calcium carbonate 4g; Lab M agar (No.1) 15g; distilled water 1 litre, pH 7.2; autoclave at 121°C for 20 minutes.

Colloidal chitin agar (Hsu & Lockwood, 1975)

Powdered chitin (40g) was dissolved in 400ml of concentrated hydrochloric acid and stirred for one hour. The chitin was precipitated as a colloidal suspension by adding it to 2 litres of cold distilled water. The suspension was allowed to settle and the supernatant was decanted. An additional 2 litres was added to the suspension. The suspension was collected by filtration on a coarse filter (Whatman No.1) and then washed by resuspending it in an additional 2 litres of

distilled water. This washing procedure was repeated until the pH of the suspension was approximately 3.5. The water content of the chitin was determined by drying a sample at 70°C. The remaining chitin suspension was stored at 4°C until required.

The colloidal chitin medium was prepared by using a volume of the colloidal chitin suspension, which gave 4g of dry weight chitin per litre; potassium dihydrogen orthophosphate 0.7g; potassium dihydrogen orthophosphate 0.3g; magnesium sulphate 0.5g; iron sulphate 0.01g; zinc sulphate 0.001g; manganese chloride 0.001g; 20g Lab M agar (N0. 1); distilled water 1 litre; pH 8.0; autoclave at 121°C for 20 minutes.

½ Strength Nutrient agar

Nutrient agar (Oxoid); 14g; distilled water 1 litre; pH 7.2; autoclave at 121°C for 20 minutes.

¼ Strength sterile Ringer's solution:

Dissolve half a Ringer's tablet in 1 litre distilled water; autoclave at 121°C for 20 minutes.

50mM phosphate buffered saline (pH 7.0)

Prepare 0.2M solutions of Na_2HP0_4 and NaH_2P0_4. Take 30.5ml of 0.2M Na_2HP0_4 and 19.5ml of NaH_2P0_4 and dilute to 100ml with distilled water. Make up a solution of 50mM $MgS0_4$ and 0.4M NaCl. Autoclave both solutions at 121°C for 20 minutes. Mix the $MgS0_4$/NaCl and phosphate solutions in the ratio of 4:1.

TE buffer (pH 8.0)

10mM Tris hydrochloride; 10mM EDTA; autoclave at 121°C for 20 minutes.

TAE buffer (50X stock solution)

Tris Base, 242g; glacial acetic acid, 57.1ml; 0.5M EDTA (pH 8.0), 100ml; distilled water, 1 litre.

TBE buffer (50X stock solution)

Tris Base, 54g; Boric acid, 27.5g; 0.5M EDTA (pH 8.0), 20ml; distilled water, 1 litre.

Gel loading buffer IV (Manniatis)

0.25% bromophenol blue, 40% sucrose in water (w/v). Store at 4°C.

SSCP loading buffer

95% formamide (v/v)

10mM NaOH

0.25% Bromophenol blue

0.25% Xylene cyanol

MDE gel

2X MDE gel solution (Flowgen, Staffordshire, UK), 12.5ml; Ultra pure TBE (National diagnostics, UK), 3.0ml; Milli Q water, 34.28ml; TEMED, 20µl; 10% APS, 200µl. The gel was cast horizontally using 0.4mm spacers.

Silver nitrate solution

$AgNO_3$, 2g; 37% formaldehyde, 3.0ml; Milli Q water, 2l.

Sodium carbonate solution

$NaCO_3$, 60g; 37% formaldehyde, 3.0ml, 10µgml^{-1} sodium thiosulphate, 400µl; Milli Q water, 2l.

Nitrate reduction (Williams et al., 1983)

Potassium nitrate 2g, nutrient broth 8g, agar 6g, distilled water 1 litre, pH 7.0; autoclave at 121°C for 20 minutes.

Greiss-Ilovsky Reagents

Reagent I – 0.8ml sulphanilic acid in 100ml 5N acetic acid

Reagent II – 0.6ml dimethyl-α-naphthylamine in 100ml acetic acid

Appendix C Similarity matrix for PyMS data

Strain	Percentage similarity														
12659	100														
12660	96.4	100													
12661	35.4	52.3	100												
12663	15	37.9	95.8	100											
12664	35.7	50.1	99.1	93.8	100										
12665	70.9	86.5	40.5	34.5	31.7	100									
12741	37.1	49.6	97.6	91.5	99.6	26.8	100								
12744	92.1	85.2	43	24.1	47.9	44.9	52.2	100							
12751	39.5	55.8	99.9	95.6	99.1	43.6	97.7	46.9	100						
12754	36.1	52.1	91.9	94.3	93.1	36.7	93.5	51.5	92.8	100					
12882	40.4	45.9	71.2	69.3	77.9	9.6	82.3	67.8	73.2	89.6	100				
12896	48.9	57.2	91.4	84.9	95.3	27.9	97.3	67.5	92.4	94.6	92.6	100			
2223	58.3	78.5	73.1	75.5	67	87.6	63.3	48	75.2	76.5	51.2	63.2	100		
2225	64.4	76.4	95.7	87.7	95	61.7	94.1	67.8	96.7	90.3	75.8	92.7	82.7	100	
43026	73.4	79.7	66.8	63.9	69.2	57	71.5	84.1	70	84.1	88.4	83.2	78.1	81.4	100
43141	70.4	84.4	88.3	84.4	85.9	77.5	84.4	69	90.1	88.7	72.7	85.2	94.1	96.5	88
43814	42.3	50.7	50.3	55.6	54.6	26	58.3	63.2	53.7	80.2	89.9	73.3	61.6	61.5	93.3
44151	67.6	72.6	43.2	43.1	46	50	49.1	78.6	47.4	69.6	79.4	65.7	69.1	62.8	96.6
BDH-76-003	76.4	89	66.1	53.1	59.6	91	55.5	56.5	68	51.5	25.6	52.4	83.9	81.4	59
BDH-76-004	59	74.8	93.9	86.4	90.3	70.3	87.4	54.3	94.6	82.6	58.3	82	86.2	97.4	70.2
BDH-76-006	24.5	43.7	99.5	96.3	97.9	34.9	95.7	31.5	99	89.2	64.5	87.3	69.7	92.5	58.6
BDH-76-007	46.2	63.6	98.7	94.6	96.3	56.7	94	48.2	99	90.7	67.9	88.5	82.8	97.6	71.3
BDH-76-010	35.4	59.5	78	86.5	72.9	68.8	69.5	33.1	79.5	85.9	60	68.2	95.6	80.5	76.2
BDH-76-013	35.7	53.7	80.7	88.1	80.8	44.9	80.8	48.3	82.4	96.4	85.8	84.7	82.9	82.3	88
BDH-76-019	50.8	73.3	50.2	54.5	41.3	92.8	36.2	31.4	52.9	54.5	24.1	36.4	96.2	64.1	63.6
BDH-76-023	73.4	83.9	87.1	73.8	84.4	72.9	82.4	67.1	88.3	73.9	55.1	79.3	80.6	95.8	70.6
BDH-76-028	66.6	78.9	92.6	81.8	90.2	68.3	88.2	63.4	93.5	80.8	61.3	84.4	82	98.2	72.4
BDH-76-035	78.1	78.9	70.2	59.8	74.8	44.4	78.4	92.5	73	81	90.5	89.5	64.6	84.6	95.4
BDH-76-041	72.3	87.5	82.1	77.3	77.6	87.4	74.7	63.3	84	78.9	57.4	74.1	96.4	92.5	80.7
BDH-76-044	95.6	99.5	55.7	43.2	54.2	84.8	54.1	87.6	59.2	58.6	54.3	62.7	80.8	78.7	85.3
NMB-84-001	55.5	70.4	97	89.2	94.6	61.4	92.4	55.1	97.4	86.2	64.6	87.2	81.8	98.6	70.9
NMB-85-001	85.4	94.1	79.7	70	77.9	81.7	77.1	81.4	82	78.5	67.6	80.5	89	93.8	88
NMB-85-006	81.5	83.5	70.4	60.9	74	53.2	76.9	92.7	73.3	81.2	88.2	87.8	70.8	85.7	97.1
NMB-86-003	89.7	93.1	73.8	61.7	74.9	69.7	76.1	92.1	76.5	76.9	76.4	83.7	78.5	90.3	92.5
NMB-86-010	52.9	72.4	91.6	90.9	87.3	74	84.1	49	92.6	88.1	62.8	80.2	94.5	94.5	76.5
NMB-87-004	59.5	79	82.3	79.4	75.9	86.4	71.5	47.1	83.6	75.3	46	67.2	96.3	89.4	69.9
NMB-87-008	98.6	96.6	51	32.4	52.1	69.6	53.6	95.3	54.6	51.9	55.6	64.1	64.7	75.6	81.4
NMB-89-006	76.2	77.2	70.7	60.8	75.5	42.5	79.1	91.5	73.5	82	91.7	90.3	64	84.5	95.4
NMB-89-008	86.1	84.4	58.3	46	62.8	49.6	66.5	97	61.8	70.6	82.7	80.2	62.3	77.9	94.5
NMB-89-009	69	76.6	90.8	82	92.8	52.4	93.9	79	92.3	91.6	87.1	97	75.9	97.5	89.1
NMB-89-010	53.8	71.9	94.6	91.5	90.9	70.3	87.9	50.9	95.4	88.2	63.5	83.3	90.9	96.8	74.6
NMB-90-002	14.4	30.3	70.2	79.5	73.6	11.1	75.7	38.9	71.8	93	91.5	82.7	61	68.9	81.9
NMB-90-004	66.2	72.4	81.9	76.2	85.5	44.1	88	81.7	84	92.3	95.2	95.8	72.1	90.3	95.6
NMB-90-011	87.8	23.7	91.6	98.7	90.8	18.2	89.1	14.6	91.3	94.1	71.3	83.2	66.8	80.8	60

Strain	Percentage similarity														
12659															
12660															
12661															
12663															
12664															
12665															
12741															
12744															
12751															
12754															
12882															
12896															
2223															
2225															
43026															
43141	100														
43814	70.3	100													
44151	74.9	94.5	100												
BDH-76-003	86.1	22.9	42.5	100											
BDH-76-004	95.2	45.3	49.3	88.7	100										
BDH-76-006	84.3	41.9	33.1	62	92.1	100									
BDH-76-007	93.8	52.6	49.9	77.1	97.9	97.6									
BDH-76-010	90.4	68.5	66.4	66.5	81.9	76	100								
BDH-76-013	87.7	88.3	79.6	48.5	74.9	77.1	92.2	100							
BDH-76-019	81.9	44.6	58.3	79.5	71.5	46.4	87.6	66.4	100						
BDH-76-023	92.9	40.6	50.3	93	97.4	83.6	70.7	64.8	66.2	100					
BDH-76-028	94.5	45.8	51.5	89.2	99.1	89.9	75.7	71.6	65.8	99.2	100				
BDH-76-035	84.1	83.4	87.2	57.9	71.2	61.6	60.5	77.6	44.2	75.7	76.6	100			
BDH-76-041	98.2	58	67.5	93.1	94.9	78.3	88.7	79	88.5	93.6	93.6	75.1	100		
BDH-76-044	86.9	59.8	79.2	86.5	75.7	47	64.2	61	74.5	83.6	79.5	83.6	88.6	100	
NMB-84-001	94.1	47.9	49	83.2	99.3	95.3	79.6	76.7	63.8	96.4	98.9	74	91.8	71.9	100
NMB-85-001	97.3	65	76.1	90.7	91.8	73.8	79.1	76.7	77.9	94.7	93.7	87.5	96.9	95.3	89.9
NMB-85-006	87.3	84.3	89.7	63.6	73.9	61.9	65.6	79.5	52.9	78.2	78.6	99.6	79.8	87.9	75.7
NMB-86-003	92.2	72	82.6	80.6	83.4	66.1	68.6	74.2	64.2	89	87.7	95.4	88.9	95.3	83.2
NMB-86-005	92.9	81.9	83.7	68.9	84.2	77	74.3	85.8	57.2	85.5	87.3	97.8	85.5	84.7	86.4
NMB-86-010	97.4	58.8	60.2	83.8	96.8	89.8	93.9	86.3	82.7	90.5	93.6	70.5	96.7	74.8	95.4
NMB-87-004	95.2	46	54.3	92.4	95.2	80.1	90.6	89.8	75.1	89.6	91.3	92	62.6	98.5	79.6
NMB-87-008	79	53.4	73.5	78.8	69.1	41.1	59.4	46.1	49.5	53.7	81.1	76.1	86.9	78.6	96.8
NMB-89-006	83.7	84.3	87.1	56.2	70.8	62.3	72.1	60.8	78.5	43.1	74.9	76.1	100	74.4	82.2
NMB-89-008	80.1	80.3	89.3	58.9	64.6	48.3	62.4	53.9	69.4	45.1	72	71	98.4	72.9	88.4
NMB-89-009	93.7	73.3	73.5	71.8	89.9	85.8	91.5	74.6	84.2	54.8	90.2	92.6	93.9	86.7	80.3
NMB-89-010	97	54.8	56	84.5	98.8	93	98.6	89.8	83.5	76.9	93.5	96.4	71.8	95.7	74
NMB-90-002	71.5	90.7	74.6	17.8	55.4	66	68.1	76.6	95.5	38.3	43.7	52.9	73.2	57.3	40.1
NMB-90-004	89.3	87.1	85.2	58.2	78.6	75.4	82.4	73.3	88.7	50.8	78.3	81.5	97.5	79.7	77.9
NMB-90-011	76.8	57.1	39.7	37.1	77.2	92.4	88.6	82.3	88.1	43.2	62.2	72	55	67	30.5

Strain	Percentage similarity														
NMB-84-001	100														
NMB-85-001	89.9	100													
NMB-85-006	75.7	90.7	100												
NMB-86-003	83.2	97.4	97.1	100											
NMB-86-005	86.4	93.1	98.1	96.5	100										
NMB-86-010	95.4	90.6	74.2	81.1	84	100									
NMB-87-004	91.4	91.2	68	79.5	76.7	97.4	100								
NMB-87-008	67.1	91.1	89.3	95.4	84.6	63.3	66.6	100							
NMB-89-006	73.9	86.7	99.4	94.7	97.8	70.3	61.9	85.4	100						
NMB-89-008	66	87	98.8	95.7	94.1	63.8	58.8	92	98	100					
NMB-89-009	92.5	92.6	93.9	94.1	98.6	87.5	79.9	80.4	94	88.1	100				
NMB-89-010	98.1	90.9	74.8	82.1	85.1	99.4	96.4	64.7	71.6	64.4	89.8	100			
NMB-90-002	60.2	58.4	72.9	60.7	78.6	68.3	50.7	31.5	74.8	62.7	75.9	65.8	100		
NMB-90-004	81.9	87.7	97.2	92.5	99.1	80.3	70.2	77.7	97.9	92.6	97.1	81.1	85.3	100	
NMB-90-011	81.3	59.8	55.4	52.7	70.6	83.7	68.8	19.5	56.5	39.5	76.7	84	83.8	73.4	100

Appendix D TAXON computer program

The TAXON computer program was written in USCD Pascal for Apple II+ computers by Dr. A. C. Ward of the Department of Agricultural and Environmental Science, University of Newcastle. The program was subsequently transferred to IBM PC and compatible personal computers written in standard Pascal the Propas compiler running under MSDOS. The program allows for (i) binary data (+/-) derived from numerical taxonomic studies; (ii) pre-processing of data (iii) analyses of data for clusters of strains defined in numerical analyses, and (iv) identification of unknown strains to clusters defined in the numerical taxonomic analyses.

The taxon program can handle a data matrix containing up to 512 unit characters for 512 organisms. Raw data can be pre-processed to determine the overall percentage distribution of positive characters and the reproducibility of individual tests prior to numerical analysis. Percentage positive results for each character can be determined so that any test that is positive or negative for all of the organisms within the matrix can be identified. In addition, the individual test variances and percentage agreements can be calculated for duplicated strains, and the probability of an erroneous test result, the test error as defined by Sneath and Johnson (1972).

Tests considered to have little if any differential value or which show poor reproducibility can be removed from the data matrix, or marked for selective exclusion in further data analyses.

The data is then transferred to the Clustan program (Wishart, 1978). The clusters of test strains defined during the numerical analysis can be further processed to give the following information (i) determination of the average similarity or dissimilarity of each strain to the other strains in the same cluster; (ii) calculation of mean inter-cluster similarity or dissimilarity; (iii) designation of centrotype strains; (iv) analysis of percentage positive data for clusters using procedures taken from the DIACHAR program (Sneath, 1980).

The inclusion of the DIACHAR routine within the TAXON program allows for the selection of a minimum number of characters to enable each and every cluster to be distinguished from the others. The most differential tests are used as a starting point for the generation of a frequency matrix. Unknown organisms can be identified using the appropriate diagnostic tests generated in the frequency matrix. Identification scores are calculated for each of the unknown strains to every cluster defined in the data matrix using the IDENTIFY procedure, which is based on the MATIDEN program (Sneath, 1980a,b). The identification coefficients calculated include: (i) Willcox probability; (ii) taxonomic distance of the unknown strain to each centroid of every cluster, and (iii) the 95% taxonomic radius of each cluster.

The procedure COMPARE, which is also included in the TAXON program, is used to calculate the identification scores for centrotype strains, hypothetical median organisms, and the outermost member of each cluster. These values can be used to measure the degree of confidence that can be placed in the identification of unknown organisms to defined clusters.

Appendix E Raw data for numerical taxonomic analysis

	D(+) Mannose	D(+) Galactose	Sucrose	Methyl-B-glucopyranoside	Glycogen	Inulin	Xylose	Arabitol	D-Gluconic acid	D-Mannitol	D(+) Cellobiose	L-Sorbose	D-Fructose	D(+) Melizitose	Lyxose	Salicin	Starch	Methyl-A-D-glucopyranoside	N-acetyl-glucosamine	Ferulic acid	L-Proline
12659	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12660	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	1	0	1	0	1
12661	1	1	1	0	1	1	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1
12663	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
12664	1	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0	0	0	1
12665	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
12744	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
12751	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
12754	1	1	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0
2223	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12882	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1
43026	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
43141	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
43814	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1
44151	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
5-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6-001	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0
6-003	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0
6-004	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
7-004	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
7-006	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
7-007	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
7-008	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
9-001	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	1	0	1	0	0
9-003	1	1	1	1	1	1	1	0	1	0	1	0	1	1	0	1	1	0	1	0	1
9-016	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	1	0	1	0	1
9-018	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
9-019	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	1	0	1	0	1
9-026	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
9-030	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0
9-031	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0
9-032	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
9-034	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
9-035	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
10-002	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
10-005	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
10-008	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	0	0	1
10-010	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0
10-011	1	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0

	L-ornithine monohydrochloride	Sebacic acid	m-Hydroxybenzoic acid	p-hydroxybenzoic acid	L-Tyrosine	Sodium propionate	Sodium-n-butyrate	L-Alanine	Sodium acetate	L-Serine	L-Arginine	Mandelic acid	D (-) Mandelic acid	L-Tryptophan	Spermine tetrahydrochloride	Sodium oxalate	L-Phenylalanine	Adenine	Casein	Colloidal chitin	Elastin
12659	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
12660	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1
12661	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0
12663	0	0	0	0	1	1	1	0	0	1	0	1	1	0	0	1	0	0	1	1	1
12664	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1
12665	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1
12744	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1
12751	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1
12754	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1
2223	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2225	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
12882	0	0	0	0	0	1	0	0	0	1	0	1	1	0	1	1	0	1	1	1	1
43026	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
43141	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
43814	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1
44151	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	1	1
5-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
6-001	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1
6-003	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
6-004	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1
7-004	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1
7-006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
7-007	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
7-008	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
9-001	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1
9-003	1	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1
9-016	1	0	0	0	0	1	0	1	1	1	0	1	1	0	1	1	0	0	1	1	1
9-018	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0
9-019	1	0	0	0	0	1	0	1	1	1	0	1	0	0	1	1	0	0	1	0	1
9-026	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
9-030	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1
9-031	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
9-032	1	0	0	0	0	1	1	1	0	0	0	1	0	0	1	1	0	0	1	1	1
9-034	1	0	0	0	0	1	0	1	1	1	0	1	0	0	1	1	0	0	1	1	1
9-035	1	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	1	1	1
10-002	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	1	0	1
10-005	0	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	0	1	0	1
10-008	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
10-010	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1
10-011	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1

	Xylan	Gelatin	Tween 20	Tween 40	Tween 60	Tween 80	Tributyrin	pH 6.0	pH 9.0	3% NaCl	5% NaCl	7% NaCl	Neomycin sulphate 25ug/ml	Neomycin sulphate 5ug/ml	Gentamicin sulphate 2g/ml	Gentamicin sulphate 5ug/ml	Streptomycin sulphate 25ug/	Streptomycin sulphate 5ug/m	Novobiocin 5ug/ml	Penicillin V 25ug/ml	Penicillin V 5ug/ml
12659	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12660	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0
12661	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12663	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
12664	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	1
12665	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	0
12744	1	0	0	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0
12751	1	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	1	0	0
12754	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
2223	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2225	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
12882	1	1	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
43026	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43141	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
43814	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
44151	1	1	0	1	1	1	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0
5-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6-001	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
6-003	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6-004	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7-004	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
7-006	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7-007	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
7-008	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9-003	1	0	0	1	1	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
9-016	1	0	0	1	1	1	0	1	1	0	0	0	1	0	1	1	1	1	1	0	0
9-018	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9-019	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0
9-026	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
9-030	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
9-031	1	0	0	1	1	0	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0
9-032	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0
9-034	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
9-035	1	0	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	0	0	0
10-002	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
10-005	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10-008	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1
10-010	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10-011	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1

	Rifampicin 5ug/ml	Tetracycline 25ug/ml	Tetracycline 5ug/ml	Chloramphenicol 25ug/ml	Nitrate reduction	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterococcus faecalis*	*Candida albicans*	4MUß-D-galactopyranoside	4MU-ß-Dglucopyranoside	4MU-N-ß-D-glucosamide	4MU-ß-D-Glucoronide	4MU-ß-D-Xyloside	4MU-α-D-glucoside	4MU-α-D-galactoside	4MU-ß-D-fucosidase	4MU-α-D-manoside	4MU-phosphate
12659	0	0	0	0	1	1	1	0	1	1	0	0	1	1	1	0	0	0	0	0	1
12660	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
12661	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1
12663	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
12664	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0
12665	1	0	1	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
12744	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
12751	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
12754	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1
2223	0	0	0	0	1	1	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0
2225	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0
12882	0	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0
43026	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0
43141	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	1	0	0	1	0	0
43814	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0
44151	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1
5-001	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	1
6-001	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1
6-003	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
6-004	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0	0
7-004	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0	1
7-006	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1	0	1	1	0	0	1
7-007	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	0
7-008	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	0
9-001	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	0
9-003	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	1	0	1	1	1
9-016	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
9-018	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
9-019	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0
9-026	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	0
9-030	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1	0	1	1	1	0
9-031	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0
9-032	0	0	0	0	1	0	0	0	1	0	0	1	1	0	1	1	0	1	1	0	0
9-034	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0
9-035	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
10-002	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
10-005	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
10-008	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
10-010	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
10-011	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0

	4MU-sulfonide	L-alanayl-amc	Leucyl-amc	Pyrolyl-amc	Pyroglutamyl-amc	4-MU-ß-mannopyranoside	Glutamyl-amc	Lysyl-amc	Asparaginyl-amc	Phenylalanyl-amc	Z-glcyl-proly-amc	Z-arginyl-amc	4-MU-ß-cellobioside	4-MU-butyrate	H-ornithine-amc	L-histidine-amc	4-MU-ß-ribofuranoside	4-MU-α-L-arabinoside	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
12659	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0
12660	1	0	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	1	1
12661	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	1	0
12663	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
12664	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0
12665	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
12744	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
12751	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
12754	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0
2223	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
2225	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0
12882	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
43026	1	0	1	0	0	1	1	0	1	1	0	1	1	0	0	0	0	1	0	0	1
43141	0	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1
43814	0	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1
44151	0	0	1	0	0	1	0	0	1	1	0	1	1	0	1	1	0	1	1	0	0
5-001	0	0	1	0	0	1	1	1	0	1	1	0	1	1	0	0	0	0	1	0	0
6-001	0	1	0	1	0	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1
6-003	0	0	0	1	0	1	0	0	0	1	1	1	1	1	0	1	0	1	0	1	1
6-004	0	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0
7-004	0	1	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	1
7-006	0	1	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0	1	1	0	1
7-007	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0
7-008	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0
9-001	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
9-003	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9-016	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	1	0
9-018	0	1	1	1	0	0	1	0	1	0	1	0	0	1	1	1	0	0	1	0	0
9-019	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0
9-026	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
9-030	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0
9-031	1	0	1	0	0	1	0	0	0	1	1	0	1	1	1	1	0	1	1	1	1
9-032	0	1	1	0	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	0	1
9-034	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
9-035	0	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0	0
10-002	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1
10-005	0	1	1	1	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0
10-008	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
10-010	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
10-011	0	0	1	0	0	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1

	D(+) Mannose	D(+) Galactose	Sucrose	Methyl-B-glucopyranoside	Glycogen	Inulin	Xylose	Arabitol	D-Gluconic acid	D-Mannitol	D(+) Cellobiose	L-Sorbose	D-Fructose	D(+) Melizitose	Lyxose	Salicin	Starch	Methyl-A-D-glucopyranoside	N-acetyl-glucosamine	Ferulic acid	L-Proline
10-014b	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1
10-014b	C	1	1	1	1	0	1	0	1	0	0	0	0	0	1	1	1	1	0	0	0
10-015	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1	0	1	0	0
10-016	1	1	1	1	1	0	1	0	0	1	1	0	1	1	0	1	1	0	0	0	1
10-020	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	1	0	0
11-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11-002	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1
11-003	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	1
12-002	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0
12-004	1	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	1
12-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
12-007	0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	1
12-010	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0
12-011	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	0	1
12-012	1	1	1	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0	1	0	1
12-017	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1
13-027	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13-027	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
13-042	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0
13-042	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
13-047	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
14-003	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
14-026	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0	1	1	0	0	0	1
14-046	NM	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0
14-049	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
14-049	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
14-064	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
14-065	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	0
14-067	1	1	1	1	1	1	1	0	1	0	1	0	1	0	0	1	1	1	0	0	0
14-071	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1
15-005	1	1	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0
15-010	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
15-013	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1
16-002	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0
16-004	1	1	1	0	1	0	1	0	1	1	1	0	1	0	0	1	1	0	1	0	0
16-005	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0
16-007	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	0	0	0	0
16-009	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0
17-001	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0
17-002	1	1	1	1	1	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	0

	L-ornithine monohydrochloride	Sebacic acid	m-Hydroxybenzoic acid	p-hydroxybenzoic acid	L-Tyrosine	Sodium propionate	Sodium-n-butyrate	L-Alanine	Sodium acetate	L-Serine	L-Arginine	Mandelic acid	D (-) Mandelic acid	L-Tryptophan	Spermine tetrahydrochloride	Sodium oxalate	L-Phenylalanine	Adenine	Casein	Colloidal chitin	Elastin
10-014b	0	0	0	0	0	1	0	1	0	1	1	0	1	0	1	0	0	0	1	1	1
10-014b	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1	1
10-015	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1
10-016	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1
10-020	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1
11-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11-002	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	1
11-003	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1
12-002	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1
12-004	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1
12-004	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	0	1
12-007	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0	1	1	1
12-010	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
12-011	0	0	0	0	0	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1
12-012	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1
12-017	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1
13-027	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	1
13-027	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
13-042	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1
13-042	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1
13-047	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
14-003	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	1	0	1	1	1
14-026	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	1	1	1
14-046	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	1	0	1	0	1
14-049	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	0	1	0	1	1	1
14-049	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	0	1	1	1	1	1
14-064	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
14-065	0	0	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1
14-067	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1	1	1
14-071	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
15-005	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	1	1
15-010	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
15-013	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1
16-002	0	0	0	0	0	1	0	1	0	1	0	1	1	0	1	1	0	0	1	1	0
16-004	1	0	0	0	0	1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	1
16-005	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
16-007	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1
16-009	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
17-001	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	1
17-002	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	1	1

	Xylan	Gelatin	Tween 20	Tween 40	Tween 60	Tween 80	Tributyrin	pH 6.0	pH 9.0	3% NaCl	5% NaCl	7% NaCl	Neomycin sulphate 25ug/ml	Neomycin sulphate 5ug/ml	Gentamicin sulphate 25ug/ml	Gentamicin sulphate 5ug/ml	Streptomycin sulphate 25ug/ml	Streptomycin sulphate 5ug/ml	Novobiocin 5ug/ml	Penicillin V 25ug/ml	Penicillin V 5ug/ml
10-014b	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	1
10-014b	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10-015	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1
10-016	1	1	0	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0
10-020	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	1	1	1	0	0
11-001	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
11-002	1	0	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0
11-003	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	0	0	0
12-002	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
12-004	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-004	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-007	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-011	1	0	0	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1
12-012	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
12-017	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0
13-027	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
13-027	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
13-042	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
13-042	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
13-047	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
14-003	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
14-026	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14-046	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14-049	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
14-049	1	0	0	1	0	1	0	1	1	1	0	0	0	1	1	1	0	1	0	0	0
14-064	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0
14-065	1	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1
14-067	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14-071	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15-005	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
15-010	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	0	0
15-013	1	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0
16-002	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1
16-004	1	1	0	1	1	1	0	1	1	0	0	0	0	1	1	1	1	1	0	1	1
16-005	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
16-007	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0
16-009	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0
17-001	1	0	0	1	1	1	0	1	0	0	0	0	1	0	1	1	1	1	0	1	1
17-002	1	0	0	1	1	1	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1

	Rifampicin 5ug/ml	Tetracycline 25ug/ml	Tetracycline 5ug/ml	Chloramphenicol 25ug/ml	Nitrate reduction	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterococcus faecalis*	*Candida albicans*	4MUß-D-galactopyranoside	4MU-ß-Dglucopyranoside	4MU-N-ß-D-glucosamide	4MU-ß-D-Glucoronide	4MU-ß-D-Xyloside	4MU-α-D-glucoside	4MU-α-D-galactoside	4MU-ß-D-fucosidase	4MU-α-D-manoside	4MU-phosphate
10-014b	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0	0
10-014b	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0
10-015	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0
10-016	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
10-020	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1
11-001	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1
11-002	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
11-003	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0
12-002	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
12-004	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
12-004	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0
12-007	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
12-010	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1	1	1	0	1	1	0
12-011	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	0
12-012	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0
12-017	1	1	1	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0	0
13-027	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0
13-027	0	0	1	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0
13-042	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
13-042	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
13-047	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0
14-003	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0
14-026	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	1	1	1	0	0
14-046	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
14-049	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
14-049	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
14-064	0	1	1	0	1	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0
14-065	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0
14-067	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
14-071	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
15-005	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0
15-010	0	0	0	0	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1
15-013	0	0	0	0	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1
16-002	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	0	1	1	0
16-004	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
16-005	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
16-007	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	1	1	1	0
16-009	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1
17-001	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0
17-002	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0	1

	4MU-sulfonide	L-alanayl-amc	Leucyl-amc	Pyrolyl-amc	Pyroglutamyl-amc	4-MU-ß-mannopyranoside	Glutamyl-amc	Lysyl-amc	Asparaginyl-amc	Phenylalanyl-amc	Z-glcyl-proly-amc	Z-arginyl-amc	4-MU-ß-cellobioside	4-MU-butyrate	H-ornithine-amc	L-histidine-amc	4-MU-ß-ribofuranoside	4-MU-α-L-arabinoside	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
10-014b	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
10-014b	0	1	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	1	0	0
10-015	0	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	1	0	1
10-016	0	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	1	1	1
10-020	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	0	1	1	0	0
11-001	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	0	1	1	0	0
11-002	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11-003	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-002	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-004	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
12-004	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
12-007	0	0	1	0	0	1	0	1	1	1	0	0	1	1	1	1	0	1	1	1	1
12-010	0	1	1	1	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	0	0
12-011	0	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	1	0	0
12-012	0	1	1	1	0	0	0	0	1	0	0	0	1	1	0	1	0	1	1	1	0
12-017	0	0	0	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	1
13-027	0	1	0	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	1	0	1
13-027	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	1
13-042	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
13-042	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
13-047	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1
14-003	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
14-026	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1
14-046	0	1	1	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0
14-049	0	1	1	1	0	0	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0
14-049	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1
14-064	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
14-065	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
14-067	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
14-071	0	1	1	1	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	0
15-005	0	1	1	1	0	1	1	0	1	0	0	1	1	0	1	1	0	1	1	0	0
15-010	1	0	1	1	0	0	0	1	1	1	0	1	1	0	1	1	0	1	1	0	0
15-013	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0
16-002	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0
16-004	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16-005	0	1	1	1	0	0	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0
16-007	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0
16-009	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0
17-001	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	1	1	1	1
17-002	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	0	0

	D(+) Mannose	D(+) Galactose	Sucrose	Methyl-B-glucopyranoside	Glycogen	Inulin	Xylose	Arabitol	D-Gluconic acid	D-Mannitol	D(+) Cellobiose	L-Sorbose	D-Fructose	D(+) Melizitose	Lyxose	Salicin	Starch	Methyl-A-D-glucopyranoside	N-acetyl-glucosamine	Ferulic acid	L-Proline
17-003	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	1	1	0	0	0	0
17-004	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
18-019	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
18-030	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
18-032	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
18-047	1	1	1	1	1	0	1	0	0	0	1	0	1	0	1	1	1	1	0	0	0
18-065	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
18-072	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
18-085	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	0
18-251	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
18-251	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
19-003	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
20-001	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	1	0	1
20-002	1	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0
20-006	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0
20-009	1	1	1	0	1	0	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0
21-001	C	1	1	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0
21-003	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21-008	0	0	0	1	1	0	0	0	1	0	0	0	0	0	1	1	0	1	1	0	0
22-001	0	1	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0
23-009	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0
23-012	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
24-002	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
24-006	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0
24-009	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1
24-013	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25-001	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	0
27-001	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
28-001	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
28-001	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
33-003	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
33-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	1
43-022	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	1	0	0	0
43-026	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1
43-038	0	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	0
45-008	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
45-019	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	1
47-004	1	1	1	1	1	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	1
47-018	1	1	1	1	1	0	1	0	0	0	1	0	1	0	1	1	1	1	0	0	0
47-022	1	1	1	1	1	0	1	1	1	0	1	0	1	0	0	1	1	0	0	0	0

	L-ornithine monohydrochloride	Sebacic acid	m-Hydroxybenzoic acid	p-hydroxybenzoic acid	L-Tyrosine	Sodium propionate	Sodium-n-butyrate	L-Alanine	Sodium acetate	L-Serine	L-Arginine	Mandelic acid	D (-) Mandelic acid	L-Tryptophan	Spermine tetrahydrochloride	Sodium oxalate	L-Phenylalanine	Adenine	Casein	Colloidal chitin	Elastin
17-003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
17-004	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
18-019	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
18-030	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
18-032	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	1
18-047	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
18-065	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
18-072	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	1	1	1
18-085	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
18-251	1	0	0	0	1	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1
18-251	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1	1	1
19-003	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	1	1	1
20-001	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
20-002	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0	1	1	1
20-006	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	1	1
20-009	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1
21-001	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	1	1
21-003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
21-008	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1
22-001	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
23-009	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
23-012	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
24-002	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	1	1	1
24-006	0	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	1	0	1	1	1
24-009	0	0	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	1
24-013	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
25-001	1	0	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	1	1
27-001	0	0	0	0	1	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1
28-001	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	1	1
28-001	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	1	1	1
33-003	0	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	1	1
33-004	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1
43-022	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
43-026	1	0	0	0	1	1	0	1	1	1	1	0	0	0	1	0	1	0	1	1	1
43-038	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	1	1	1
45-008	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1
45-019	0	0	0	0	1	0	0	1	1	0	1	0	0	1	1	0	0	0	1	1	1
47-004	1	0	0	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	1	1	1
47-018	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1
47-022	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	1	0	1	0	1

	Xylan	Gelatin	Tween 20	Tween 40	Tween 60	Tween 80	Tributyrin	pH 6.0	pH 9.0	3% NaCl	5% NaCl	7% NaCl	Neomycin sulphate 25ug/ml	Neomycin sulphate 5ug/ml	Gentamicin sulphate 25ug/ml	Gentamicin sulphate 5ug/ml	Streptomycin sulphate 25ug/ml	Streptomycin sulphate 5ug/ml	Novobiocin 5ug/ml	Penicillin V 25ug/ml	Penicillin V 5ug/ml
17-003	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0
17-004	1	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
18-019	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0
18-030	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18-032	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0
18-047	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
18-065	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18-072	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
18-085	1	1	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
18-251	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0
18-251	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
19-003	1	0	0	1	1	1	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0
20-001	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
20-002	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0
20-006	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0
20-009	1	1	0	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1
21-001	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0
21-003	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21-008	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22-001	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23-009	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
23-012	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24-002	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24-006	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
24-009	1	0	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
24-013	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25-001	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1	1	0	0	0
27-001	1	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1	0	0
28-001	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28-001	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33-003	1	0	0	1	0	1	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0
33-004	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43-022	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43-026	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	1	0	0	1	1
43-038	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
45-008	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45-019	1	0	0	0	0	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0
47-004	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47-018	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
47-022	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0

	Rifampicin 5ug/ml	Tetracycline 25ug/ml	Tetracycline 5ug/ml	Chloramphenicol 25ug/ml	Nitrate reduction	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterococcus faecalis*	*Candida albicans*	4MUß-D-galactopyranoside	4MU-ß-Dglucopyranoside	4MU-N-ß-D-glucosamide	4MU-ß-D-Glucoronide	4MU-ß-D-Xyloside	4MU-α-D-glucoside	4MU-α-D-galactoside	4MU-ß-D-fucosidase	4MU-α-D-manoside	4MU-phosphate
17-003	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0
17-004	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
18-019	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
18-030	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
18-032	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
18-047	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	1	1	0
18-065	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
18-072	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	0	0
18-085	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
18-251	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0
18-251	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0
19-003	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0	1	1
20-001	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0
20-002	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1
20-006	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	1	0	1	0
20-009	1	1	1	0	0	1	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0
21-001	0	1	1	0	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1
21-003	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	1	0	0
21-008	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0	1	0	0
22-001	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1
23-009	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1
23-012	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	0	1	0	1	0
24-002	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0
24-006	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0
24-009	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	0	0
24-013	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	1
25-001	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	0
27-001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
28-001	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0
28-001	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
33-003	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	0	0	1	0
33-004	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	1	1	0
43-022	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	0	0
43-026	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
43-038	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	1	0	1
45-008	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	1	0	0
45-019	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0
47-004	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0
47-018	1	0	1	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	0	1
47-022	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	1	1	0	1	0

	4MU-sulfonide	L-alanayl-amc	Leucyl-amc	Pyrolyl-amc	Pyroglutamyl-amc	mannopyranoside	Glutamyl-amc	Lysyl-amc	Asparaginyl-amc	Phenylalanyl-amc	Z-glcyl-proly-amc	Z-arginyl-amc	4-MU-ß-cellobioside	4-MU-butyrate	H-ornithine-amc	L-histidine-amc	4-MU-ß-ribofuranoside	4-MU-α-L-arabinoside	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
17-003	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	1	1	1	1
17-004	1	1	1	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	1	0	0
18-019	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
18-030	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1
18-032	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1
18-047	0	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	1	1	0	0
18-065	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0
18-072	0	1	0	1	0	1	1	0	1	1	0	0	1	0	1	1	0	1	0	0	1
18-085	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
18-251	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
18-251	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
19-003	1	1	1	1	0	0	1	0	1	0	0	1	0	0	1	1	1	1	0	0	0
20-001	0	0	0	0	0	1	0	1	1	1	0	1	1	0	1	1	1	1	1	0	1
20-002	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0	1
20-006	1	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1
20-009	0	1	1	1	0	0	1	0	0	1	1	1	0	0	0	0	1	0	1	0	0
21-001	1	1	0	1	1	1	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0
21-003	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1	0	1	0	0
21-008	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
22-001	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0
23-009	1	1	1	1	1	0	0	0	1	1	0	1	0	0	0	0	1	0	1	1	1
23-012	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1
24-002	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0
24-006	0	0	0	0	0	1	0	0	1	1	0	1	1	0	0	0	0	1	1	0	0
24-009	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0
24-013	0	1	1	1	0	1	1	0	1	0	0	1	1	0	1	1	0	1	0	0	0
25-001	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0
27-001	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0
28-001	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
28-001	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
33-003	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0
33-004	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1
43-022	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	0
43-026	0	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0
43-038	0	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
45-008	1	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	0	0
45-019	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
47-004	0	0	0	0	0	1	1	0	1	1	0	0	1	0	1	1	0	1	0	0	0
47-018	0	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	0
47-022	0	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0

	D(+) Mannose	D(+) Galactose	Sucrose	Methyl-B-glucopyranoside	Glycogen	Inulin	Xylose	Arabitol	D-Gluconic acid	D-Mannitol	D(+) Cellobiose	L-Sorbose	D-Fructose	D(+) Melizitose	Lyxose	Salicin	Starch	Methyl-A-D-glucopyranoside	N-acetyl-glucosamine	Ferulic acid	L-Proline
47-027	1	1	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0
47-042	1	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0	0	0	0
47-047	1	1	1	1	1	0	1	0	0	0	1	0	1	0	1	1	1	1	0	0	0
48-016	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1
48-022	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1	1	1	0	0	0	1
48-022	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	1
48-033	1	0	0	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	1
49-009	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	1	1	0	1
49-011	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
49-016	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
49-025	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0	0	1
50-007	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
53-003	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
55-003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
55-011	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0
56-012	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
57-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
57-020	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	1	0	0	0
63-002	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
63-013	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1
63-034	1	1	1	1	1	1	1	0	1	0	1	0	1	0	0	1	1	1	1	0	1
63-034	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
64-006	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1
64-022	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
73-008	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
73-011	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
76-003	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
76-007	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
76-013	0	0	1	1	1	0	1	0	1	0	1	0	0	0	0	1	1	0	1	0	1
76-019	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
76-028	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
76-035	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0
76-041	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
76-044	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	0
76-044	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
77-005	1	1	0	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
77-012	1	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	0
77-028	1	1	1	1	1	0	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0
77-029	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	1	0	0	0	0
81-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1

	L-ornithine monohydrochloride	Sebacic acid	m-Hydroxybenzoic acid	p-hydroxybenzoic acid	L-Tyrosine	Sodium propionate	Sodium-n-butyrate	L-Alanine	Sodium acetate	L-Serine	L-Arginine	Mandelic acid	D (-) Mandelic acid	L-Tryptophan	Spermine tetrahydrochloride	Sodium oxalate	L-Phenylalanine	Adenine	Casein	Colloidal chitin	Elastin
47-027	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
47-042	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
47-047	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
48-016	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	0	1
48-022	0	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	1	0	1	1	1
48-022	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1	1
48-033	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1
49-009	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
49-011	0	0	0	0	0	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	1
49-016	0	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	1	1
49-025	1	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	1	0	1
50-007	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1
53-003	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	1	1
55-003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
55-011	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1
56-012	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	1
57-004	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0
57-020	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1
63-002	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1
63-013	0	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	0	1	1	1	1
63-034	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1	1	1	1	1
63-034	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1
64-006	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1
64-022	1	0	0	0	0	1	0	1	1	0	1	0	1	1	0	0	1	1	1	1	1
73-008	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	1
73-011	0	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	1	1
76-003	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1
76-007	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1
76-013	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	1	1
76-019	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	1
76-028	1	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	1
76-035	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	1	1	1
76-041	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
76-044	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1
76-044	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1
77-005	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	1	1
77-012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
77-028	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	1	1
77-029	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1
81-004	1	0	0	0	0	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	1

	Xylan	Gelatin	Tween 20	Tween 40	Tween 60	Tween 80	Tributyrin	pH 6.0	pH 9.0	3% NaCl	5% NaCl	7% NaCl	Neomycin sulphate 25ug/ml	Neomycin sulphate 5ug/ml	Gentamicin sulphate 25ug/ml	Gentamicin sulphate 5ug/ml	Streptomycin sulphate 25ug/ml	Streptomycin sulphate 5ug/ml	Novobiocin 5ug/ml	Penicillin V 25ug/ml	Penicillin V 5ug/ml
47-027	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47-042	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0
47-047	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
48-016	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0	0
48-022	1	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
48-022	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
48-033	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49-009	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0
49-011	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
49-016	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49-025	1	1	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	1	1	0	1
50-007	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53-003	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	1
55-003	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55-011	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56-012	1	0	0	1	0	1	0	0	1	0	1	0	1	0	1	1	0	0	0	0	0
57-004	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57-020	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0
63-002	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
63-013	1	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	1	1	0	1	1
63-034	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1
63-034	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1
64-006	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64-022	1	0	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1
73-008	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
73-011	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
76-003	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
76-007	0	0	0	1	1	0	0	1	1	1	0	1	0	0	1	1	0	1	0	0	0
76-013	0	0	0	1	1	0	0	1	1	0	0	0	0	1	1	1	0	1	1	0	0
76-019	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0
76-028	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
76-035	1	0	0	1	1	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0
76-041	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0
76-044	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0
76-044	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0
77-005	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
77-012	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
77-028	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
77-029	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	1
81-004	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	0	0

	Rifampicin 5ug/ml	Tetracycline 25ug/ml	Tetracycline 5ug/ml	Chloramphenicol 25ug/ml	Nitrate reduction	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterococcus faecalis*	*Candida albicans*	4MUß-D-galactopyranoside	4MU-ß-Dglucopyranoside	4MU-N-ß-D-glucosamide	4MU-ß-D-Glucoronide	4MU-ß-D-Xyloside	4MU-α-D-glucoside	4MU-α-D-galactoside	4MU-ß-D-fucosidase	4MU-α-D-manoside	4MU-phosphate
47-027	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0
47-042	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0
47-047	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0
48-016	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1
48-022	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	1
48-022	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1
48-033	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	1	1	1
49-009	0	0	0	1	1	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0
49-011	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0
49-016	0	0	0	0	1	1	1	0	0	1	0	1	0	0	1	0	1	1	0	0	0
49-025	1	1	1	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	0	0	1
50-007	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0
53-003	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1
55-003	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	1	1	0	0	0	0
55-011	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	1
56-012	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	1
57-004	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1
57-020	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
63-002	0	1	0	0	1	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0
63-013	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1
63-034	1	1	1	1	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1
63-034	1	1	1	1	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1
64-006	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0
64-022	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	1	1	1
73-008	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
73-011	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0
76-003	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
76-007	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	1	1	0	0	0
76-013	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	1	1	0	1	1
76-019	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	1	1
76-028	0	0	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	1	0	1	1
76-035	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	1	1	0	1	1
76-041	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	1	1	0	1	0
76-044	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0	1	0	0
76-044	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1
77-005	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0	1
77-012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0
77-028	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1	1	0	1	1	0	0
77-029	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1
81-004	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0

	4MU-sulfonide	L-alanayl-amc	Leucyl-amc	Pyrolyl-amc	Pyroglutamyl-amc	4-MU-ß-mannopyranoside	Glutamyl-amc	Lysyl-amc	Asparaginyl-amc	Phenylalanyl-amc	Z-glcyl-proly-amc	Z-arginyl-amc	4-MU-ß-cellobioside	4-MU-butyrate	H-ornithine-amc	L-histidine-amc	4-MU-ß-ribofuranoside	4-MU-α-L-arabinoside	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
47-027	0	1	1	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	1
47-042	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
47-047	1	1	0	0	1	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0	1
48-016	1	1	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	0	1
48-022	0	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0
48-022	0	1	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
48-033	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1
49-009	0	1	1	1	0	0	1	0	1	0	0	0	1	1	1	1	0	0	1	1	1
49-011	1	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	1
49-016	0	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	1	1	1
49-025	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0
50-007	0	1	1	1	0	0	0	0	0	1	1	0	0	1	1	1	0	0	1	0	0
53-003	0	1	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0
55-003	1	1	0	1	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1
55-011	0	1	1	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0
56-012	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0
57-004	0	0	0	0	0	0	1	0	0	1	1	0	1	1	0	0	0	0	1	0	0
57-020	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
63-002	0	1	1	1	0	0	1	0	1	0	0	1	0	1	1	1	0	0	1	0	1
63-013	0	1	1	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0
63-034	0	1	1	1	0	1	1	0	0	1	0	0	1	1	1	1	0	1	1	0	0
63-034	0	1	1	1	0	1	1	0	0	1	0	0	1	1	1	1	0	1	1	0	0
64-006	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	0	0	1	0	0
64-022	0	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	0
73-008	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
73-011	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1
76-003	0	0	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0
76-007	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0
76-013	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0
76-019	1	1	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0
76-028	1	0	1	1	0	1	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
76-035	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0
76-041	0	1	1	1	0	1	1	0	1	0	0	0	0	1	0	1	0	0	1	0	0
76-044	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
76-044	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	1	0	1	0	1
77-005	0	1	1	0	0	1	1	0	1	1	0	1	1	0	0	1	0	0	0	0	0
77-012	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
77-028	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
77-029	0	1	1	1	0	0	1	0	1	1	0	0	0	0	0	1	0	1	0	0	1
81-004	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0

	D(+) Mannose	D(+) Galactose	Sucrose	Methyl-B-glucopyranoside	Glycogen	Inulin	Xylose	Arabitol	D-Gluconic acid	D-Mannitol	D(+) Cellobiose	L-Sorbose	D-Fructose	D(+) Melizitose	Lyxose	Salicin	Starch	Methyl-A-D-glucopyranoside	N-acetyl-glucosamine	Ferulic acid	L-Proline
81-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
81-015	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0
81-015	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
84-001	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	0
86-003	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
86-005	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
86-010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
87-004	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
87-008	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0
89-006	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	0	1
89-008	1	1	1	0	1	1	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1
89-009	1	1	1	1	1	1	1	0	1	0	1	0	1	1	0	1	1	0	1	0	1
90-002	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1
90-011	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	0
91-001	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	0	0	1
91-002	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0
91-004	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	1	0	1	0	1
91-005	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1
93-008	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0
93-010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
93-011	0	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	0	1
100-002	1	1	0	1	1	1	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0

	L-ornithine monohydrochloride	Sebacic acid	m-Hydroxybenzoic acid	p-hydroxybenzoic acid	L-Tyrosine	Sodium propionate	Sodium-n-butyrate	L-Alanine	Sodium acetate	L-Serine	L-Arginine	Mandelic acid	D (-) Mandelic acid	L-Tryptophan	Spermine tetrahydrochloride	Sodium oxalate	L-Phenylalanine	Adenine	Casein	Colloidal chitin	Elastin
81-004	0	0	0	0	0	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1
81-015	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1
81-015	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	1	0	1	1
84-001	0	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	1	1	1
86-003	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1
86-005	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1
86-010	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1
87-004	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
87-008	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1
89-006	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	1
89-008	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	1	1	1
89-009	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	1	1
90-002	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	0	0	1	1	1
90-011	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	1	0
91-001	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	1	1
91-002	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
91-004	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	1	1	1	1	1
91-005	1	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
93-008	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1
93-010	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1
93-011	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
100-002	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1

	Xylan	Gelatin	Tween 20	Tween 40	Tween 60	Tween 80	Tributyrin	pH 6.0	pH 9.0	3% NaCl	5% NaCl	7% NaCl	Neomycin sulphate 25ug/ml	Neomycin sulphate 5ug/ml	Gentamicin sulphate 25ug/ml	Gentamicin sulphate 5ug/ml	Streptomycin sulphate 25ug/ml	Streptomycin sulphate 5ug/ml	Novobiocin 5ug/ml	Penicillin V 25ug/ml	Penicillin V 5ug/ml
81-004	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
81-015	1	1	0	1	0	1	0	1	1	0	0	0	0	1	0	0	1	1	0	0	0
81-015	0	1	0	1	0	0	0	1	1	1	0	1	0	1	1	1	1	1	0	0	0
84-001	1	0	0	1	1	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0
86-003	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	0	0
86-005	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86-010	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
87-004	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
87-008	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
89-006	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
89-008	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
89-009	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0
90-002	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	1	0	1	0	0	0
90-011	1	0	0	1	0	0	0	1	1	0	0	0	0	1	0	1	1	1	0	0	0
91-001	1	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1
91-002	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
91-004	1	1	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0
91-005	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
93-008	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
93-010	1	1	0	1	0	0	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0
93-011	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
100-002	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Rifampicin 5ug/ml	Tetracycline 25ug/ml	Tetracycline 5ug/ml	Chloramphenicol 25ug/ml	Nitrate reduction	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterococcus faecalis*	*Candida albicans*	4MUß-D-galactopyranoside	4MU-ß-Dglucopyranoside	4MU-N-ß-D-glucosamide	4MU-ß-D-Glucoronide	4MU-ß-D-Xyloside	4MU-α-D-glucoside	4MU-α-D-galactoside	4MU-ß-D-fucosidase	4MU-α-D-manoside	4MU-phosphate
81-004	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
81-015	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1
81-015	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1
84-001	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1
86-003	0	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	1	1	1	0	0
86-005	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1	0	1	0	0	1	0
86-010	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1	0
87-004	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1	0	0	1	1	1	1
87-008	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	0	1	1	1	0	0
89-006	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	1	0
89-008	0	0	0	0	1	1	0	0	1	0	0	0	0	1	1	0	0	1	1	1	0
89-009	0	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	1	1	1	1	1
90-002	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	1	1	0
90-011	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	1	1
91-001	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
91-002	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	0	0
91-004	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	1	0	0	0
91-005	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0	0
93-008	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
93-010	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
93-011	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0
100-002	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0

	4MU-sulfonide	L-alanayl-amc	Leucyl-amc	Pyrolyl-amc	Pyroglutamyl-amc	4-MU-ß-mannopyranoside	Glutamyl-amc	Lysyl-amc	Asparaginyl-amc	Phenylalanyl-amc	Z-glcyl-proly-amc	Z-arginyl-amc	4-MU-ß-cellobioside	4-MU-butyrate	H-ornithine-amc	L-histidine-amc	4-MU-ß-ribofuranoside	4-MU-α-L-arabinoside	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
81-004	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
81-015	0	1	1	1	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0
81-015	0	1	1	1	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0
84-001	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
86-003	0	1	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1	1	0	1
86-005	0	1	1	0	0	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	1
86-010	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
87-004	1	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	1	1	0	0
87-008	0	0	1	0	0	1	0	0	0	1	0	1	0	1	1	1	0	1	1	0	1
89-006	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
89-008	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
89-009	1	1	1	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0	1	0	1
90-002	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
90-011	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0
91-001	0	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	1
91-002	0	1	1	1	0	0	1	0	1	1	0	1	0	0	1	0	0	1	0	1	1
91-004	0	1	1	0	0	0	1	0	1	1	0	1	0	0	1	0	0	1	0	0	1
91-005	0	1	1	1	0	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	0
93-008	0	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0
93-010	0	0	0	1	0	0	0	1	0	1	1	0	0	1	1	1	0	0	1	0	1
93-011	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1
100-002	0	1	0	1	0	1	0	0	1	1	1	0	1	1	0	1	1	1	1	0	1

Appendix F Raw data for rapid enzyme profiling

Strain code	4mu-β-galactopyranoside	4mu-β-glucopyranoside	4mu-β-D-N-β-D-glucosamide	4mu-β-D-Glucuronide	4mu-7-β-xyloside	4mu-α-D-glucoside	4mu-α-D-galactosi	4mu-β-D-fucoside	4mu-α-D-mannoside	4mu-phosphate
1 AUS-6-003	43958	24727	52989	15	19136	57432	27636	43379	656	1398
2 AUS-6-004	27944	33416	5189	6	18135	57475	4215	31237	266	396
3 AUS-6-001	23818	30099	11667	-21	26971	58266	3476	23126	241	1044
4 AUS-5-001	5195	12596	16481	-9	2435	58022	0	24242	186	864
5 JPN-100-002	26852	35964	2594	-34	11856	56276	1071	18874	135	348
6 JPN-90-011	26980	8219	34957	-94	1129	55873	10426	30155	873	1914
7 NMB-89-009	28796	16822	12668	28	2848	59560	13084	33630	531	2262
8 NMB-89-008	17849	16090	46973	21	183	52025	17870	29229	687	369
9 NMB-89-006	22229	11561	28441	12	12598	56905	6858	26241	824	3
10 NMB-87-004	39115	38834	24284	30	6363	50642	18373	49064	443	793
11 NMB-86-010	2796	9070	56096	12	61	58953	48	15380	425	-85
12 NMB-86-003	29431	20115	31954	6	3702	58129	14558	39820	233	174
13 NMB-84-001	19003	11283	11894	9	7223	58864	6995	27499	366	754
14 BDH-76-044	27593	15733	1464	6	30227	59188	8732	30261	198	61
15 BDH-76-041	20376	12254	4383	-21	13938	58919	12846	27960	385	9
16 BDH-76-018	28326	10472	22109	-49	6509	56975	9702	20843	571	83
17 BDH-76-019	27993	16173	22710	-33	27340	55772	17504	24382	428	729
18 CZR-16-004	13955	10484	11869	-134	1031	55125	5710	27154	482	336
19 IND-64-022	27725	21803	21254	27	11923	57543	16609	41282	1483	1795
20 IND-64-006	14436	15601	20332	3	15415	54646	3223	8354	416	88
21 IND-63-034	35788	25740	16807	12	11436	52751	24724	42692	562	1843
22 IND-63-013	44208	25274	31338	30	8695	50962	28753	42573	1157	2228
23 IND-63-002	2543	3775	15064	18	5781	60094	1596	8467	238	-49
24 IND-57-020	16991	8927	16688	9	8811	57909	4587	25329	242	-22
25 IND-57-004	18792	32238	16380	-12	29732	56193	27	40293	150	1242
26 IND-56-002	6892	10557	8503	39	31344	54719	55	20779	245	262
27 IND-55-011	20470	28618	19048	-3	6846	58217	9263	46012	452	684
28 IND-55-003	26974	35400	4319	-6	13654	56679	2225	21249	171	302
29 IND-53-003	32672	9970	11897	-21	15363	58455	8030	26998	342	461
30 IND-50-007	13695	5146	15135	-122	22872	56508	4328	8063	455	217
31 USA-49-025	5414	11320	4459	40	27956	56990	67	17370	217	1224
32 USA-49-016	25491	9299	5994	9	4309	58577	21593	19512	345	64
33 USA-49-011	37567	9397	4962	21	6549	58842	20668	22937	300	0
34 USA-49-009	28797	16886	10911	12	21831	57729	9250	29602	330	138
35 USA-48-033	25176	35049	12889	-3	13849	59588	18453	43958	470	403
36 USA-48-022	39933	10581	19286	34	19695	44505	6638	23815	211	1343
37 USA-48-016	47999	44153	23641	6	6732	58708	23766	56506	702	968
38 UK-10-015	30130	15485	24385	18	8380	58858	19435	34060	296	65
39 UK-10-014B	44682	30501	35781	-24	23238	56477	22750	46571	269	284
40 UK-10-011	23125	8155	26738	7	16157	57524	4947	25036	311	-9
41 UK-10-010	24819	5624	29687	-18	18675	53050	6150	18898	245	-138

Strain code	4mu-ß-glucopyranoside	4mu-ß-D-N-ß-D-glucosamide	4mu-ß-D-Glucuronide	4mu-7-ß-xyloside	4mu-α-D-glucoside	4mu-α-D-galactosi	4mu-ß-D-fucoside	4mu-α-D-mannoside	4mu-phosphate	4mu-ß-glucopyranoside
42 UK-10-005	23092	22209	314	-128	4232	48710	8976	39755	153	-12
43 UK-10-002	25869	11625	10758	9	3937	58849	14899	21984	312	-15
44 AUS-9-035	26813	8991	5463	-6	4980	57897	25365	22804	235	-21
45 AUS-9-034	27285	13184	7889	3	5740	59157	17708	20522	318	117
46 AUS-9-031	11396	17661	900	3	9000	48292	6028	30136	171	-150
47 AUS-9-026	27398	35498	2236	21	14560	59334	5051	47691	425	-79
48 AUS-9-019	43397	11508	8753	12	13657	58739	22756	33481	361	146
49 AUS-9-018	20980	5896	18123	-3	9901	56102	4504	8906	437	238
50 JPN-93-010	6589	7810	14747	12	2392	58379	1855	15667	190	-58
51 AUS-9-016	21706	6928	3559	-46	4043	56694	18529	18617	226	-138
52 AUS9-003	30489	18944	18321	-15	4620	57521	7328	42457	638	858
53 AUS-7-008	39167	18022	46137	-42	13895	56542	25328	35916	503	735
54 CZR-17-002	39100	24349	36810	-159	26049	55788	15855	39277	281	568
55 CZR-17-001	39121	6937	12748	24	8881	58611	32412	18435	388	-94
56 CZR-16-009	27502	27724	45804	-3	17762	55180	14228	44450	428	8607
57 CZR-16-007	46501	42829	44040	-12	1395	56969	25923	57305	2976	7313
58 CZR-16-005	22961	6941	25701	12	5584	58455	9049	18242	287	296
59 CZR-16-002	29065	10319	33498	30	6165	54613	10547	23281	485	437
60 UK-12-017	33414	42068	4095	21	18610	59816	5457	32217	284	33
61 UK-12-011	34727	19929	35223	-6	11814	58220	19694	40271	354	140
62 UK-12-010	30386	44611	7083	28	15696	58147	4401	44175	486	-97
63 UK-12-008	34888	10127	35272	-3	26482	58373	4825	30054	358	446
64 UK-12-007	25128	4346	22182	-21	17219	53453	7450	15709	241	-103
65 UK-12-004	32595	31759	1682	-18	11106	52119	5890	47968	226	-149
66 UK-11-002	30062	7142	2225	-158	9534	55854	17946	19674	193	-122
67 UK-10-020	29291	14232	26867	28	17018	53359	7352	29064	269	903
68 UK-10-016	14967	6791	3174	36	2173	47230	7673	15962	122	-89
69 ARG-14-064	23538	7746	5341	9	11338	58922	12873	22766	300	-39
70 ARG-14-049	41975	11759	11707	-3	9314	57613	19096	23620	333	193
71 ARG-14-046	16258	8887	11487	0	5725	57088	5069	19427	342	-18
72 ARG-14-054	37180	19835	30086	-21	12397	55931	10859	37223	452	-61
73 ARG-14-071	23153	5853	18077	27	22057	57033	6833	6971	339	21
74 ARG-14-026	37198	17143	18892	30	18531	55242	28555	35245	361	110
75 ARG-14-065	37034	10673	6500	-54	14085	54869	20384	30498	333	-24
76 ARG-13-047	8479	16490	18699	-8	2609	54259	91	20998	2170	-143
77 AUS-7-001	26724	9354	7380	-9	9955	55635	23680	37278	327	-21
78 ARG-13-037	17696	12501	29668	-134	3051	56130	8158	22621	354	357
79 ARG-13-042	24456	13819	26608	18	9239	57167	4279	24624	208	-138
80 ARG-13-001	24847	12886	22368	-9	17042	57592	12900	20943	178	-161
81 ARG-13-027	28442	13712	29559	-128	18184	58302	11354	26642	178	-155
82 JPN-73-011	28903	15196	14249	12	4586	56480	30392	19899	263	-64
83 USA-47-022	21856	21364	6549	70	4435	50840	15077	27895	686	-104

Strain code	4mu-ß-glucopyranoside	4mu-ß-D-N-ß-D-glucosamide	4mu-ß-D-Glucuronide	4mu-7-ß-xyloside	4mu-α-D-glucoside	4mu-α-D-galactosi	4mu-ß-D-fucoside	4mu-α-D-mannoside	4mu-phosphate	4mu-ß-glucopyranoside
84 NMB-91-003	31549	14714	3766	-3	20332	56816	11427	34506	293	433
85 NMB-91-004	34629	13933	15639	-128	17704	51536	19969	31906	357	330
86 NMB-91-005	24883	20122	4172	-134	31600	58336	12281	27991	235	510
87 IND-18-047	27157	24603	40985	-180	23238	57964	9544	37580	418	397
88 POR-20-006	24236	17179	30846	-155	10841	56926	16755	25314	538	461
89 JPN-18-050	42680	28817	31374	-143	14198	44996	25738	37234	400	305
90 NMB-87-008	27023	18471	30953	-289	5404	57896	14292	34481	236	455
91 SPN-77-006	21829	27541	38851	-116	317	45511	6263	39182	680	134
92 IND-63-034	34934	26491	20915	-158	12885	52305	24764	40861	550	3202
93 NZD-33-001	32809	18907	46246	-146	17342	54012	14845	35974	288	-128
94 JPN-18-080	19304	8106	6162	-143	9852	58534	5320	22811	290	177
95 POR-24-002	37748	18711	8704	-167	21208	56154	12001	27077	162	168
97 POR-23-009	32950	16941	15840	39	16847	54265	14421	32470	238	610
98 POR-23-012	24459	7297	4248	68	5935	56923	27172	18437	241	183
99 BDH-76-044	25192	15900	1797	24	27929	57273	9406	29162	141	681
100 POR-21-003	29812	30996	34020	7	21031	50559	16157	44334	330	155
101 POR-21-001	35626	18180	26061	52	22478	56047	27895	41135	528	1239
102 POR-22-001	25094	36533	45471	-15	50215	158	2042	53300	534	1129
103 POR-20-006	21011	10878	23949	64	6934	57335	14970	24755	290	476
104 POR-20-009	7951	13871	5060	34	2411	48051	1105	31204	690	271
105 POR-20-001	26038	12147	37790	34	12345	57262	23363	31592	216	482
106 POR-20-002	29098	19066	32577	52	24340	56456	19685	34128	342	1206
107 JPN-18-085	11024	3391	1129	40	2597	33730	9895	15126	74	10
108 POR-19-003	38034	14918	19930	12	7288	55616	11991	28927	422	1459
109 JPN-18-050	40284	23647	26027	24	21022	46681	24261	29743	323	364
110 JPN-18-019	13603	7541	4138	43	5539	46787	5496	24264	144	73
111 JPN-18-032	13341	7077	7233	15	6112	58140	6812	21154	202	147
112 CZR-17-003	44871	6998	17222	0	14918	56905	46546	32461	364	280
113 CZR-17-004	9504	6940	1559	12	4968	55516	8820	22469	113	37
114 BDH-76-003	24695	15709	20500	-149	5030	82	12025	27929	318	345
115 BDH-76-007	32391	8454	7468	55	6516	58077	23318	21212	239	156
116 43814	9477	16746	21016	43	183	55659	3986	42896	528	833
117 44151	27090	39578	18977	37	15052	54616	17702	48908	913	2255
118 43026	28146	13844	14497	43	18718	57362	9983	30169	135	391
119 12882	14351	5766	3906	55	6049	56212	8686	19487	229	94
120 12751	13448	4230	3299	31	5094	55071	11872	15706	184	275
121 12754	19908	8604	13682	43	6723	55549	21703	18361	312	678
122 12744	15059	4060	3430	55	4890	54735	9162	23687	205	131
123 12664	32138	28313	17271	49	11680	57231	6662	24810	247	290
124 12665	25152	4538	2920	37	6393	54616	9876	28438	293	85
125 12659	8006	25384	27226	40	708	43705	39	22789	174	696
126 12660	25418	14278	10306	-61	19273	62	12001	29327	330	360
127 USA-47-027	22652	15379	26031	79	11051	56758	9733	31808	360	92

Strain code	4mu-ß-glucopyranoside	4mu-ß-D-N-ß-D-glucosamide	4mu-ß-D-Glucuronide	4mu-7-ß-xyloside	4mu-α-D-glucoside	4mu-α-D-galactosi	4mu-ß-D-fucoside	4mu-α-D-mannoside	4mu-phosphate	4mu-ß-glucopyranoside
128 USA-47042	20907	13820	29345	37	8197	55445	8240	138	348	83
129 USA-45-019	26479	8066	7779	64	26009	52790	8558	18678	251	248
130 UK-12-004	22152	43631	1584	43	10822	49873	5695	50942	229	61
132 USA-45-008	33261	25017	47624	65	38937	52183	7908	41486	287	103
133 USA-43-022	15902	18467	52973	31	25575	50328	3250	30136	287	501
134 USA-43-026	10814	18553	10257	80	22463	30492	3845	21105	135	82
135 NZD-33-001	39166	20445	44669	33	18678	56493	14650	37998	315	77
136 ANT-28-001	17711	5918	4489	43	14866	58259	11341	17257	214	180
137 POR-24-013	42930	48471	48063	40	19489	53483	22102	55974	309	9669
138 ANT-25-001	20812	17982	4538	-64	5859	42	12433	38074	620	278
139 POR-24-006	20217	13053	41879	64	9647	55186	17699	28250	159	230
140 POR-24-009	19554	10666	22069	55	6485	57561	15522	23250	239	486
141 JPN-18-251	24001	9131	17512	22	8774	54899	12129	18889	391	486
142 JPN-18-080	20208	11194	10642	68	11612	58494	6998	23565	226	650
143 JPN-93-008	23217	13480	5866	19	7502	55269	14030	23168	184	156
144 JPN-18-072	32443	18852	11597	43	20021	58531	12842	36444	254	269
145 JPN-91-002	50602	20821	5826	19	19523	56550	12831	31873	232	281
146 JPN-91-001	19542	11539	4706	55	15016	58333	6491	26458	199	250
147 JPN-73-008	7814	2918	885	3	2454	23815	8396	15403	58	15
148 IND-81-015	28769	21199	198	43	290	58424	20805	31695	198	1251
149 IND-81-004	17177	6866	2328	-6	2695	59032	18361	19509	165	146
150 SPN-77-029	25439	16048	5569	-88	20054	36	10016	30752	254	598
151 SPN-77-028	30930	20616	44425	40	23833	58	15287	43226	232	186
152 SPN-77-006	14916	30004	28158	13	329	53428	4639	41483	690	632
153 SPN-77-005	32486	18346	8203	40	23317	56588	12049	32528	287	665
154 SPN-77-012	5986	15559	21364	28	201	53578	2430	23366	431	486
155 BRA-15-005	20677	26677	41510	55	36008	51909	4926	32983	275	449
156 BRA-15-013	29843	25218	22926	-6	7327	56486	12901	39298	937	2170
157 BRA-15-010	33918	30019	31701	65	12833	55168	12816	45420	1160	2540
158 IND-66-007	38230	19114	836	15	10481	57033	473	39435	419	168
159 USA-47-004	28092	26446	61876	6	22625	57606	11140	45375	522	357
160 USA-47-018	32984	35913	56617	49	37890	58077	20381	46138	364	1413
161 BDH-76-044	26186	16334	1953	22	28850	59185	9519	30438	141	262
162 NZD-33-001	41090	25762	52345	-106	18379	36	19457	42451	296	116
163 JPN-91-005	34525	22826	7151	31	26369	52	19454	40434	278	415
164 JPN-91-003	31469	19203	5649	25	22695	57506	15279	37714	275	318
165 USA-48-002	43620	15397	27676	67	20747	45627	8997	26919	193	2179
166 IND-53-003	27545	13899	18511	28	15864	58104	8591	35956	272	623
167 USA-47-027	24209	17146	24035	52	11121	56148	9852	30688	354	82
168 IND-63-013	38169	22526	23055	9	8228	54518	25292	37461	794	1730
169 NMB-89-006	21505	13172	25643	46	14451	54142	8350	27557	708	153
170 JPN-18-019	14122	7852	4239	128	5255	47437	5463	23201	131	31

Strain code	4mu-α-D-mmannoside	4mu-phosphate	4mu-sulfate	L-alanyl-7amc	Leucyl-7amc	Prolyl-7amc	Pyroglutamyl-7amc	4-muB-mannopyranoside	Glutamyl-7amc	Lysyl-7amc
1 AUS-6-003	656	1398	22	9082	13747	45227	-1095	4032	-1136	-2805
2 AUS-6-004	266	396	6	39679	44083	28560	-1303	5833	-2317	-5377
3 AUS-6-001	241	1044	25	44223	33423	42551	-1086	2130	229	-3601
4 AUS-5-001	186	864	6	22862	49916	15201	-809	7108	28450	2054
5 JPN-100-002	135	348	15	44489	20925	44565	-1193	2491	1205	-5108
6 JPN-90-011	873	1914	21	2936	5836	9296	-1120	95	-2177	-27831
7 NMB-89-009	531	2262	43	34942	44605	39187	-1290	326	-1341	1960
8 NMB-89-008	687	369	12	24312	29529	25743	-1300	-983	-2671	-24449
9 NMB-89-006	824	3	27	9894	36197	24461	-1229	-1065	-1960	-6040
10 NMB-87-004	443	793	61	2356	5836	13105	-1360	858	378	-21986
11 NMB-86-010	425	-85	25	6967	10966	39611	-1169	3943	-2384	-25087
12 NMB-86-003	233	174	12	30422	51909	22755	-1244	-152	482	-10163
13 NMB-84-001	366	754	18	18986	48378	35699	-576	1209	-1514	-1367
14 BDH-76-044	198	61	9	45075	44584	49124	-619	-1434	-2454	-49579
15 BDH-76-041	385	9	3	38147	51036	43841	-992	5866	8164	-7773
16 BDH-76-018	571	83	34	16782	45502	24181	-735	1764	-1975	-7397
17 BDH-76-019	428	729	49	34875	51793	39902	-1113	4124	-1120	-933
18 CZR-16-004	482	336	30	18419	49400	28892	-1568	-1388	-2384	-49461
19 IND-64-022	1483	1795	18	26186	50536	25847	-1211	790	-1719	-6495
20 IND-64-006	416	88	-15	37484	47358	37475	-1437	-1126	534	-6244
21 IND-63-034	562	1843	12	39868	38575	41931	-839	7059	9882	-2612
22 IND-63-013	1157	2228	18	35748	48799	38870	-1156	2720	9790	-10285
23 IND-63-002	238	-49	12	44678	44712	37295	-1019	-1388	11475	-20405
24 IND-57-020	242	-22	-3	22386	50703	29376	-1202	-602	-1618	-2429
25 IND-57-004	150	1242	15	12397	21279	22471	-1181	1929	41677	-9418
26 IND-56-002	245	262	21	8054	14553	21046	-1233	-1395	-2409	-49557
27 IND-55-011	452	684	15	25393	50645	34899	-1012	122	452	373
28 IND-55-003	171	302	27	44882	467	44669	-900	195	2942	-11792
29 IND-53-003	342	461	31	32723	27419	42929	-903	2637	2960	-890
30 IND-50-007	455	217	15	33028	48313	34771	-1061	-1019	-510	-2008
31 USA-49-025	217	1224	6	7770	17370	14283	-943	668	38635	-10098
32 USA-49-016	345	64	19	25667	51640	28615	-723	738	-1045	-2072
33 USA-49-011	300	0	43	22622	49962	25835	-677	2311	-1783	-2059
34 USA-49-009	330	138	31	32680	53413	37612	-662	358	10141	1670
35 USA-48-033	470	403	9	41065	42427	49070	-962	15486	3537	-6385
36 USA-48-022	211	1343	12	47352	46442	48432	-656	2124	4553	-433
37 USA-48-016	702	968	259	34235	48878	46966	-344	110	9827	-14899
38 UK-10-015	296	65	18	30663	44431	34722	-1169	2106	6738	-3555
39 UK-10-014B	269	284	15	40848	52788	41549	-906	-1407	-2454	-49460
40 UK-10-011	311	-9	18	11729	35116	11677	-824	5924	18046	2249
41 UK-10-010	245	-138	0	14387	43394	13529	-1113	4938	1464	-18147
42 UK-10-005	153	-12	21	39407	51985	47284	-747	-714	2884	-2963

Strain code	4mu-α-D-mmannoside	4mu-phosphate	4mu-sulfate	L-alanyl-7amc	Leucyl-7amc	Prolyl-7amc	Pyroglutamyl-7amc	4-muB-mannopyranoside	Glutamyl-7amc	Lysyl-7amc
43 UK-10-002	312	-15	15	8772	30203	17613	-573	1831	-1151	-2463
44 AUS-9-035	235	-21	6	26268	51533	29378	-202	132	-1759	-3826
45 AUS-9-034	318	117	27	18218	44889	19627	-549	-507	-2305	-19520
46 AUS-9-031	171	-150	36	16407	50883	23243	-842	5023	-492	-488
47 AUS-9-026	425	-79	12	43274	51485	38406	-481	-1413	-2420	-49481
48 AUS-9-019	361	146	9	20845	15871	12199	-790	-269	-2494	-28697
49 AUS-9-018	437	238	12	34893	49760	36117	-1028	-974	2716	-14912
50 JPN-93-010	190	-58	24	3519	11293	34490	-1218	-1364	-1606	1261
51 AUS-9-016	226	-138	6	18279	43843	22874	-945	232	-1722	-4431
52 AUS9-003	638	858	12	28651	50789	35235	-882	-1373	-2494	-49466
53 AUS-7-008	503	735	9	5249	8866	40698	-811	19282	-2182	-11286
54 CZR-17-002	281	568	9	38125	53066	34555	-927	-418	7678	-4004
55 CZR-17-001	388	-94	3	9623	20162	13960	-674	2341	-2134	-11848
56 CZR-16-009	428	8607	3	4700	25939	49033	-900	-668	-2104	-25181
57 CZR-16-007	2976	7313	12	4312	20010	18583	-692	-357	-2573	-23836
58 CZR-16-005	287	296	24	29323	49959	34157	-922	522	-251	-5686
59 CZR-16-002	485	437	27	4947	23092	27279	-616	1325	-2223	-8292
60 UK-12-017	284	33	9	8216	34916	40924	144	2485	-2033	-2871
61 UK-12-011	354	140	18	29491	51500	33388	-707	2213	2532	-1190
62 UK-12-010	486	-97	6	32653	46726	40231	-1035	3339	-1503	2033
63 UK-12-008	241	-103	6	23070	47477	21299	-1367	9257	20451	568
64 UK-12-007	226	-149	-12	16786	51383	29824	-1258	-1373	-2488	-49478
65 UK-12-004	193	-122	6	38586	51543	42947	-1041	-1389	-2494	-49515
66 UK-11-002	269	903	18	45438	45942	46634	-784	8119	23421	-1147
67 UK-10-020	122	-89	0	46985	46506	48298	-882	-775	6339	-5771
68 UK-10-016	300	-39	18	5155	6913	4937	-1102	-528	-2613	-35085
69 ARG-14-064	333	193	36	7902	10228	11756	-851	1862	-2305	-10072
70 ARG-14-049	342	-18	12	24608	50407	30513	-799	2402	-233	-1535
71 ARG-14-046	452	-61	19	30666	44871	35089	-891	6376	7923	1612
72 ARG-14-054	339	21	16	41742	48063	34808	-1175	-1154	7575	-18644
73 ARG-14-071	361	110	21	17778	20244	14564	-870	-98	-2271	-14576
74 ARG-14-026	333	-24	37	16575	11567	12189	-875	-888	-2750	-39392
75 ARG-14-065	2170	-143	3	7334	15563	37951	-820	-1007	-2503	-36493
76 ARG-13-047	327	-21	-6	21065	45286	20171	-927	-632	-2512	-25178
77 AUS-7-001	354	357	-9	38278	50590	40823	-1248	7029	3961	598
78 ARG-13-037	208	-138	0	6165	8506	19077	-1061	-870	-2393	-30016
79 ARG-13-042	178	-161	6	6027	10093	19102	-1309	-839	-2494	-37185
80 ARG-13-001	178	-155	9	12131	10386	19755	-1275	-980	-2488	-30871
81 ARG-13-027	263	-64	12	19203	37744	24464	-1007	119	-1511	-2377
82 JPN-73-011	686	-104	-9	34652	46449	35973	-760	262	8026	-20384
83 USA-47-022	293	433	-3	44504	51225	48093	-1000	-76	3555	1716
84 NMB-91-003	357	330	49	38684	51079	44199	-992	-592	1324	596

Strain code	4mu-α-D-mmannoside	4mu-phosphate	4mu-sulfate	L-alanyl-7amc	Leucyl-7amc	Prolyl-7amc	Pyroglutamyl-7amc	4-muB-mannopyranoside	Glutamyl-7amc	Lysyl-7amc
85 NMB-91-004	235	510	37	43353	49950	48599	-924	-333	2420	1044
86 NMB-91-005	418	397	6	38950	52138	37155	-1052	4139	4767	192
87 IND-18-047	538	461	82	27773	50044	38879	-1006	-46	-1679	-2402
88 POR-20-006	400	305	15	25062	41959	32876	-198	745	-837	-1263
89 JPN-18-050	236	455	3	5775	37119	11680	-1006	1673	-1380	-3186
90 NMB-87-008	680	134	6	29882	29886	28164	-1495	-1139	-2100	-12980
91 SPN-77-006	550	3202	-15	37435	39591	39892	-928	8570	16981	-3052
92 IND-63-034	288	-128	16	43577	40815	45181	-1440	4230	10373	-9351
93 NZD-33-001	290	177	37	37390	48616	42420	-1138	238	10904	-878
94 JPN-18-080	238	610	28	28533	52467	30492	360	528	10126	-9473
95 POR-24-002	241	183	12	11533	24364	13999	-25	64	-1657	-10068
97 POR-23-009	141	681	25	43293	29668	49232	1	-625	3418	-1315
98 POR-23-012	330	155	-15	32260	48197	34292	455	2536	3628	-1223
99 BDH-76-044	528	1239	37	35623	18032	42203	65	1276	235	897
100 POR21-003	534	1129	33	12565	39487	30776	-186	55	-1207	-23451
101 POR-21-001	290	476	58	31109	47416	34545	-378	-68	-614	199
102 POR-22-001	690	271	-6	27132	49088	33386	-760	617	17378	-3729
103 POR-20-006	216	482	21	8667	34271	14430	-2102	11182	515	1654
104 POR-20-009	342	1206	70	37979	54179	43176	-2118	150	-1371	-3613
105 POR-20-001	74	10	15	7684	20195	13981	-2115	-452	-1322	-2750
106 POR-20-002	422	1459	55	41162	48927	45196	-2109	-141	3137	-3185
107 JPN-18-085	323	364	21	17655	45310	30367	55	1178	-379	-244
108 POR-19-003	144	73	18	28966	40820	38217	-247	-329	-690	-3842
109 JPN-18-050	202	147	24	2820	827	11832	-473	178	5163	-29553
110 JPN-18-019	364	280	34	10038	18410	14131	-167	1676	-1789	-7380
111 JPN-18-032	113	37	39	35561	51387	45746	-235	708	8267	2198
112 CZR-17-003	318	345	3	22786	45231	22035	-320	2277	-864	-1141
113 CZR-17-004	239	156	24	13208	36170	17835	-283	-9	-1578	-7776
114 BDH-76-003	528	833	21	12855	25274	21333	-1016	-1389	-1963	-49512
115 BDH-76-007	913	2255	24	21559	39731	29739	-2115	5240	-389	-1617
116 43814	135	391	31	15147	43006	22081	-2115	2518	12559	-775
117 44151	229	94	229	11155	24599	13209	-2127	-67	-1621	-9388
118 43026	184	275	205	14335	34396	21364	-2109	-396	-1576	-10880
119 12882	312	678	24	6897	9782	6415	702	1300	-1886	-17649
120 12751	205	131	275	12937	30520	14069	-6	-382	-1557	-9082
121 12754	247	290	18	3884	12355	13852	-488	2787	-1771	-3311
122 12744	293	85	339	11329	39246	21086	-103	-265	-1251	-5756
123 12664	174	696	-9	2902	-23589	33825	-161	-1330	-1575	-28771
124 12665	330	360	76	14796	39347	28807	-354	736	12875	-5765
125 12659	360	92	15	31424	53096	34783	-512	2207	-205	1578
126 12660	251	248	18	15492	48051	21169	-2136	-721	-1847	-18641
127 USA-47-027	229	61	21	12785	49409	36108	-2109	-376	-1670	-6695

Strain code	4mu-α-D-mmannoside	4mu-phosphate	4mu-sulfate	L-alanyl-7amc	Leucyl-7amc	Prolyl-7amc	Pyroglutamyl-7amc	4-muB-mannopyranoside	Glutamyl-7amc	Lysyl-7amc
128 USA-47042	290	974	3	27483	35959	31857	-2108	3332	-1411	-1724
129 USA-45-019	287	103	37	18397	33582	24513	-2099	696	1611	1935
130 UK-12-004	287	501	-9	26598	46626	30877	654	4484	4645	-1077
132 USA-45-008	135	82	15	41703	51113	43820	15	120	19969	989
133 USA-43-022	315	77	24	33233	47614	35421	-18	873	9384	-3845
134 USA-43-026	214	180	214	9323	29232	9967	-210	-332	-1450	-9858
135 NZD-33-001	309	9669	24	40176	49907	41833	-366	8741	6802	-2118
136 ANT-28-001	620	278	3	37680	46107	40609	-503	-622	-745	1072
137 POR-24-013	159	230	12	4348	21013	8536	-433	1803	-1371	-2990
138 ANT-25-001	239	486	70	28039	51426	39392	-2145	-311	-938	-628
139 POR-24-006	391	486	34	26342	52535	34282	-2099	18	-1453	-4843
140 POR-24-009	226	650	27	36910	51087	44058	-2130	-342	5808	-1907
141 JPN-18-251	184	156	3	35418	45914	41574	-2115	851	-275	-131
142 JPN-18-080	254	269	-3	27513	53810	38418	-2118	5073	35372	-1572
143 JPN-93-008	232	281	-9	29711	41871	36672	-104	986	12885	-1468
144 JPN-18-072	199	250	15	32300	52864	38067	156	-360	6018	998
145 JPN-91-002	58	15	42	7520	20986	8874	-122	-790	-1603	-10647
146 JPN-91-001	198	1251	15	28194	50874	37487	-186	28	5106	-4306
147 JPN-73-008	165	146	24	10691	36857	16852	-73	-577	-1456	-12317
148 IND-81-015	254	598	12	37473	49369	36400	-531	440	9839	-644
149 IND-81-004	232	186	18	43399	53425	44775	-497	-1141	-1963	-49323
150 SPN-77-029	690	632	-21	31472	34726	28591	-2115	-1380	-2076	-15199
151 SPN-77-028	287	665	12	34237	53792	19126	-2109	1593	5899	-1174
152 SPN-77-006	431	486	15	23924	44416	15647	-2124	-1382	-1923	-49512
153 SPN-77-005	275	449	12	45230	53185	49182	-2130	2722	19493	-4999
154 SPN-77-012	937	2170	10	15583	47700	27956	-2112	-159	-1716	-2853
155 BRA-15-005	1160	2540	36	18815	47426	37198	-241	-61	-1130	1804
156 BRA-15-013	419	168	-3	10575	26879	24153	-225	-1385	-1935	-49460
157 BRA-15-010	522	357	12	7414	11217	11435	-256	3101	4297	-104
158 IND-66-007	364	1413	24	42380	54830	43631	-216	2454	4984	-570
159 USA-47-004	141	262	6	42658	52452	48086	-195	-461	4403	-1205
160 USA-47-018	296	116	9	39343	47065	42718	556	1095	11076	-8295
161 BDH-76-044	278	415	15	43812	47514	49458	-323	-360	5966	818
162 NZD-33-001	275	318	3	40090	40424	48182	-2130	-180	5545	-958
163 JPN-91-005	193	2179	6	39154	53163	43350	-2139	16	8307	1719
164 JPN-91-003	272	623	9	28280	53316	44455	-2124	1260	9086	-2469
165 USA-48-002	354	82	3	32705	52214	36334	-2106	1477	5417	-1321
166 IND-53-003	794	1730	9	27398	49955	35693	-2133	3898	2810	-345
167 USA-47-027	708	153	27	9745	31399	25276	101	4907	24827	-943
168 IND-63-013	131	31	12	26973	49730	35989	-88	-1053	-1383	-7593
169 NMB-89-006	364	1413	24	42380	54830	43631	-216	2454	4984	-570
170 JPN-18-019	141	262	6	42658	52452	48086	-195	-461	4403	-1205

Strain code	Asparaginyl-7amc	Pheylanalyl-7amc	Z-glycyl-prolyl-amc	Z-arginyl-7amc	4-mu-B-celobioside	4-mu-butyrate	H-ornithine-7amc	L-hisidine-7amc	4mu-B-D-4MU-A-L-ribofuranoside	4-mu-α-L-arabinoside
1 AUS-6-003	-1013	4582	6384	244	7352	4946	10746	18422	-40	3970
2 AUS-6-004	-4865	-16373	101	-305	2918	6113	-16911	-6333	-43	-995
3 AUS-6-001	-809	11663	8961	89	10474	5686	15993	25697	-52	2719
4 AUS-5-001	-3946	4988	4614	-571	4709	6845	-803	-2252	-58	-1163
5 JPN-100-002	-46	9034	11848	-125	10536	6174	17149	30031	324	7905
6 JPN-90-011	-4886	-22810	46	-604	2255	5975	-15534	-5442	-25	1464
7 NMB-89-009	-547	8940	-83	-280	1043	5707	7642	18084	-58	717
8 NMB-89-008	-5484	2314	-100	-229	1626	4627	-12675	-3022	-79	1246
9 NMB-89-006	-5008	9910	253	-211	7023	5984	-6900	-1791	-74	-348
10 NMB-87-004	-2692	3126	61	39	16700	6125	-13801	-4392	-43	1883
11 NMB-86-010	-5106	-7916	1340	-495	-393	5633	-10709	-3937	-86	-2099
12 NMB-86-003	927	-4306	-58	250	1182	5237	17540	32153	-52	1657
13 NMB-84-001	-2924	9648	174	-385	-189	5820	-9125	-2566	-33	-726
14 BDH-76-044	-5332	-36730	-101	-433	-570	2731	-21627	-8302	-89	-2323
15 BDH-76-041	461	-14823	58	-375	934	5856	11930	15233	-76	897
16 BDH-76-018	-3952	10918	49	-442	201	5167	-9165	1298	-43	579
17 BDH-76-019	-2991	1316	12941	-220	2878	5609	-1349	1798	-49	1321
18 CZR-16-004	-5439	-36699	-190	-393	-583	3442	-21608	-8354	-61	-2311
19 IND-64-022	-2213	10621	-76	-436	2979	5594	95	8683	1920	2649
20 IND-64-006	-2997	2522	-46	-269	1068	6180	17134	24852	-27	-638
21 IND-63-034	-1081	1896	-9	-333	4371	4571	7102	23354	-55	4446
22 IND-63-013	-1526	13496	-16	-345	1978	6921	-7795	4206	-40	201
23 IND-63-002	3937	-27079	-107	116	-436	5761	14219	36425	-49	-2005
24 IND-57-020	-5110	8412	953	-128	2262	5310	-2417	-137	-10	-113
25 IND-57-004	-2240	3782	9186	-497	8015	6064	-3762	-876	-34	595
26 IND-56-002	-5354	-36754	-125	-387	-592	3085	-21727	-8317	-48	-2320
27 IND-55-011	-858	5046	-51	-396	3641	6506	-5313	629	-98	-274
28 IND-55-003	2863	5757	17769	-347	9537	5844	17882	34161	437	6919
29 IND-53-003	-3262	-1339	12543	-485	406	5234	-1526	1059	-37	470
30 IND-50-007	-3979	7581	7584	-336	-272	6613	14696	18486	-64	-724
31 USA-49-025	-4276	-11631	61	-644	5314	4697	-8756	-2661	-37	-888
32 USA-49-016	-3461	1725	10670	-333	433	4821	632	2951	-46	1957
33 USA-49-011	-2542	2289	14876	-235	1608	5783	-2487	2744	-73	1819
34 USA-49-009	1630	-10831	-42	-424	3131	6506	9403	17702	-37	753
35 USA-48-033	2679	5436	3266	2155	15699	6970	18548	30288	-15	12495
36 USA-48-022	2390	-1996	10	-244	2201	5676	13349	11222	-55	18278
37 USA-48-016	-5207	12035	80	-315	5652	7110	-13843	-4453	-40	2762
38 UK-10-015	4166	6416	720	513	4786	6269	19453	28338	-33	452
39 UK-10-014B	-5417	-36773	-168	-357	-620	4010	-21645	-8375	-85	-2332
40 UK-10-011	-497	11815	189	-214	6724	5172	8628	6922	-36	1691
41 UK-10-010	-3547	1648	12	-476	4361	6555	-14051	-5356	-34	650
42 UK-10-005	3784	-20807	-33	-336	27545	5231	14082	17256	-34	7477

Strain code	Asparaginyl-7amc	Pheylanalyl-7amc	Z-glycyl-prolyl-amc	Z-arginyl-7amc	4-mu-B-celobioside	4-mu-butyrate	H-ornithine-7amc	L-hisidine-7amc	4mu-B-D-4MU-A-L-ribofuranoside	4-mu-α-L-arabinoside
43 UK-10-002	-1862	13341	70	480	1871	6406	-5204	3555	-64	451
44 AUS-9-035	-4266	2842	5924	-296	-89	5606	-5155	-131	-58	89
45 AUS-9-034	-4044	11986	1981	-397	49	7767	-13966	-4141	-55	-1520
46 AUS-9-031	-3928	12230	27386	272	13310	6341	6049	11640	-46	2853
47 AUS-9-026	-5655	-36700	-177	-387	-601	5209	-21788	-8316	-77	-2332
48 AUS-9-019	-6806	-11563	-30	-336	567	7065	-17173	-2490	-61	2038
49 AUS-9-018	1404	3263	5930	-40	128	7391	18422	24098	-68	375
50 JPN-93-010	-2612	11442	17772	-436	-113	7568	11024	8463	-92	-842
51 AUS-9-016	-4289	7878	4892	-296	-112	6347	-6251	-405	-85	61
52 AUS9-003	-5374	-36718	-156	-333	-573	3867	-21941	-8341	-80	-2329
53 AUS-7-008	-4121	-2529	217	245	8875	7209	-9650	-1068	-46	4178
54 CZR-17-002	-3287	13316	-88	-443	6320	7309	-4422	-1709	-43	4981
55 CZR-17-001	-5860	10548	199	-241	702	6638	-8524	6736	-52	7596
56 CZR-16-009	-5536	9632	296	24	317	6912	-12397	1453	-73	-1007
57 CZR-16-007	-2115	10765	18361	-409	2643	7773	-18108	-3424	-34	689
58 CZR-16-005	1599	-2947	64	-290	4911	7593	10279	20620	-30	5570
59 CZR-16-002	-2466	7109	85	-125	2545	6747	-10505	1978	-37	4251
60 UK-12-017	-5264	12563	2484	-289	9218	6113	-9299	207	-43	2710
61 UK-12-011	3906	10536	131	739	7557	5155	18676	23207	-37	827
62 UK-12-010	-3598	12367	4768	-222	24111	6122	-244	7032	-43	5186
63 UK-12-008	-5603	-36757	-207	-363	-583	4025	-21810	-8341	-67	-2332
64 UK-12-007	33	8803	171	-363	8408	5365	14141	10679	-45	2576
65 UK-12-004	-5616	-36706	-220	-412	-592	3622	-21870	-8329	-73	-2332
66 UK-11-002	-5531	-36748	-223	-384	-577	3747	-21916	-8353	-67	-2302
67 UK-10-020	4306	-2526	177	79	7240	6012	17275	19853	-12	8549
68 UK-10-016	5667	-25902	3	-412	5014	4540	16936	28615	-37	1486
69 ARG-14-064	-6684	-18513	-21	-510	104	5396	-19355	-4797	-64	-266
70 ARG-14-049	-3778	1417	101	-88	919	7596	-12363	-1642	-27	2759
71 ARG-14-046	-3	-8103	15229	-116	2090	6427	7230	9650	-25	1618
72 ARG-14-054	3372	-5508	15	4431	5908	6756	18200	14780	-15	1324
73 ARG-14-071	4068	-5469	9748	-204	141	5309	17779	31246	-55	211
74 ARG-14-026	-5594	10698	-39	-33	-73	6329	-14542	-4156	-34	275
75 ARG-14-065	-6803	-23995	-48	-504	-97	4804	-20277	-5814	-80	-949
76 ARG-13-047	-3931	-16856	6363	-287	28	6421	-17488	-5219	-82	-1996
77 AUS-7-001	-5793	10033	2243	-427	-333	5938	-17867	-5597	-73	-1285
78 ARG-13-037	949	8537	-18	-326	1361	6268	10782	13615	-55	-46
79 ARG-13-042	-2308	-3934	30523	-534	827	6649	-14628	-3733	-64	-555
80 ARG-13-001	-4434	-15717	27141	-531	-168	5963	-18446	-4627	-61	-1053
81 ARG-13-027	-3421	-12134	26162	-558	28	6461	-15394	-3806	-58	-729
82 JPN-73-011	-3494	5522	-25	-372	174	5899	-735	113	-88	46
83 USA-47-022	-5591	10048	403	-330	552	5182	-16587	-4682	162	-702
84 NMB-91-003	-1767	-6500	-107	-461	192	5368	4856	4315	-52	327

Strain code	Asparaginyl-7amc	Pheylanalyl-7amc	Z-glycyl-prolyl-amc	Z-arginyl-7amc	4-mu-B-celobioside	4-mu-butyrate	H-ornithine-7amc	L-hisidine-7amc	4mu-B-D-4MU-A-L-ribofuranoside	4-mu-α-L-arabinoside
85 NMB-91-004	-2545	-1550	9	-402	-369	6201	1453	1685	-77	-845
86 NMB-91-005	-2591	-4547	-30	-467	-286	5420	2869	3184	-76	-558
87 IND-18-047	2362	8173	40	-329	3891	5868	15403	14656	-58	4703
88 POR-20-006	-4163	3633	-3	-351	-144	5268	-6760	-1716	-46	-254
89 JPN-18-050	-540	-2868	186	156	5286	6354	2970	8979	-55	3372
90 NMB-87-008	-3302	5864	101	217	790	6134	11867	13749	-67	2411
91 SPN-77-006	-5729	7350	-55	-327	6186	4721	-8021	-308	-110	-339
92 IND-63-034	439	-79	77	-367	8066	4871	10277	25903	-55	4007
93 NZD-33-001	4642	6162	101	339	1389	6784	14247	36560	-34	3665
94 JPN-18-080	-1587	-4962	-71	-384	-141	5915	7218	4581	-52	-498
95 POR-24-002	-2551	2409	-150	-415	1435	6469	-11380	-3040	-54	1221
97 POR-23-009	305	10096	95	48	1819	1007	-9098	-1999	18	1230
98 POR-23-012	-949	11022	15	86	21	1959	-12732	-2958	30	-376
99 BDH-76-044	4187	-8755	-30	28	-241	2508	9977	7948	16	-839
100 POR21-003	1975	6474	24	147	9861	1397	9962	10578	15	1370
101 POR-21-001	2304	-8670	4	854	583	1691	2643	3910	27	1413
102 POR-22-001	391	7033	-37	-106	1273	1340	-15333	21455	3	8662
103 POR-20-006	760	2165	0	116	110	1306	-3330	223	6	-9
104 POR-20-009	-7	5378	2301	607	1053	1287	-9583	809	488	-919
105 POR-20-001	1963	12754	150	1077	4136	1858	11119	10556	12	10938
106 POR-20-002	387	9608	125	195	1331	1696	-6266	-1380	3	16
107 JPN-18-085	-1090	3089	1566	55	-199	1739	-6848	-1068	-6	-458
108 POR-19-003	8326	-1312	155	305	2018	1599	11103	24404	92	5100
109 JPN-18-050	3128	5458	168	409	4724	2538	818	5531	18	2558
110 JPN-18-019	372	6898	-28	-55	33	1788	-3522	-2777	22	-659
111 JPN-18-032	-675	-12570	-27	428	-223	1703	-17881	-5524	9	-882
112 CZR-17-003	-1752	11265	122	1948	1438	985	-11170	7734	43	2011
113 CZR-17-004	4156	-6729	-9	293	9	1818	7716	8018	19	-504
114 BDH-76-003	-739	7082	49	287	1285	1372	998	2009	-3	1837
115 BDH-76-007	-229	11406	-45	135	202	1690	-11338	-3076	18	1986
116 43814	-1755	-36651	-116	61	-552	-1026	-21592	-7874	-6	-2299
117 44151	2747	5455	-27	366	8240	204	10328	25405	-3	6254
118 43026	2777	4612	-67	116	4578	2166	-2262	2311	16	3296
119 12882	-1023	8016	138	67	-91	2013	-11952	-2457	19	-754
120 12751	-1130	10744	77	128	-235	1223	-12629	-3275	24	-1105
121 12754	-241	358	97	43	858	1950	-12550	-2198	49	1807
122 12744	-970	10102	31	-16	-150	1635	-12848	-2588	15	-864
123 12664	-1471	-1330	7185	146	2072	2902	-9382	-1520	886	3400
124 12665	-848	8730	-6	12	-21	1544	-11188	-2158	31	-751
125 12659	-1746	-24809	-18	852	867	1520	-17796	-6210	2930	-1102
126 12660	2243	10817	699	-143	6495	2508	-7065	-1715	22	213

Strain code	Asparaginyl-7amc	Pheylanalyl-7amc	Z-glycyl-prolyl-amc	Z-arginyl-7amc	4-mu-B-celobioside	4-mu-butyrate	H-ornithine-7amc	L-hisidine-7amc	4mu-B-D-4MU-A-L-ribofuranoside	4-mu-α-L-arabinoside
127 USA-47-027	2560	12992	34	168	2134	2435	8735	8527	24	271
128 USA-47042	-1022	-20500	-116	67	-299	1882	-16935	-6196	15	-1874
129 USA-45-019	-1242	10863	34	-58	-73	1840	-14591	-2743	15	-409
130 UK-12-004	-553	1575	736	98	-64	1382	-11726	-3512	-6	366
132 USA-45-008	-15	4387	6227	77	5216	937	-3537	3434	31	1114
133 USA-43-022	2842	4969	64	-36	8747	2667	9989	-183	43	1154
134 USA-43-026	6611	3944	171	501	5268	2139	14415	21303	24	1743
135 NZD-33-001	7242	8043	24697	143	7771	2075	15147	23790	171	4532
136 ANT-28-001	8442	6102	37	277	836	1229	18871	32290	25	3107
137 POR-24-013	-1050	10033	12	-6	-119	915	-11979	-2308	3	-656
138 ANT-25-001	8244	-2267	608	101	18794	1611	18581	29076	34	3070
139 POR-24-006	2764	5611	27	-167	613	2136	-2848	9663	15	1511
140 POR-24-009	311	10185	64	193	3925	876	-3045	-311	15	2018
141 JPN-18-251	488	5308	-6	21	-76	887	-4496	-94	10	-259
142 JPN-18-080	434	7408	9	64	110	500	-7923	342	10	986
143 JPN-93-008	2572	-5026	-58	77	-58	680	8125	9244	9	-211
144 JPN-18-072	1548	8229	144	205	501	2426	-1138	-428	-19	2432
145 JPN-91-002	7215	867	138	-76	9201	1690	12855	18199	40	4434
146 JPN-91-001	1883	8442	52	58	2448	3100	2015	2973	24	4340
147 JPN-73-008	2405	-5227	-12	-37	128	1599	3934	4972	0	192
148 IND-81-015	-1697	8018	-61	-40	-251	503	-14305	-3461	-12	-840
149 IND-81-004	9162	-23386	40	-89	2353	1800	16588	30770	5805	4681
150 SPN-77-029	-1127	8644	-34	125	-247	1605	-11338	-2292	21	-824
151 SPN-77-028	1764	5006	9	299	2469	1922	-1114	13802	15	4980
152 SPN-77-006	-1654	-36675	-94	85	-525	-498	-21959	-7853	-10	-2302
153 SPN-77-005	-2500	3529	-6	162	5298	512	-9956	1142	6	18
154 SPN-77-012	1099	6606	77	15	6314	1657	-2912	20683	9	1254
155 BRA-15-005	-1666	-36639	-97	83	-519	-993	-21876	-7722	3	-2308
156 BRA-15-013	13285	-14277	268	2793	5201	1462	18038	25667	40	4462
157 BRA-15-010	2317	8872	116	-67	2186	924	-3015	6900	6	4822
158 IND-66-007	2756	5955	113	464	5665	2660	7816	14219	-12	2582
159 USA-47-004	-1877	-36630	-116	61	-519	-1163	-21611	-7828	-15	-2311
160 USA-47-018	3946	4597	-6	12	6470	1010	15761	6590	22	8329
161 BDH-76-044	6876	4859	40	256	3889	1482	15135	15797	42	5833
162 NZD-33-001	2994	-11072	-113	-18	33	1363	7972	7893	9	-354
163 JPN-91-005	10160	4325	61	232	4608	1714	16191	38125	12	3772
164 JPN-91-003	1877	-5193	-85	21	149	1129	3104	8149	25	9
165 USA-48-002	1306	-5166	-33	88	88	1495	3712	4935	33	33
166 IND-53-003	2374	-1779	-61	39	259	1962	9824	19087	15	2927
167 USA-47-027	8774	-4678	89	311	1737	1992	15801	16707	22	11567
168 IND-63-013	839	4069	8897	122	253	604	-2884	1935	22	900
169 NMB-89-006	4493	6422	192	-42	3381	1348	14275	18801	51	1807
170 JPN-18-019	2817	2531	129	235	4105	1492	3702	19051	36	1532

Strain code	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
1 AUS-6-003	-1596	1062	146
2 AUS-6-004	5104	-7538	-189
3 AUS-6-001	2952	1423	6
4 AUS-5-001	3349	-2313	-156
5 JPN-100-002	3339	-323	24
6 JPN-90-011	4670	-3104	-250
7 NMB-89-009	2289	-843	85
8 NMB-89-008	1145	-3528	-216
9 NMB-89-006	1019	-3180	-198
10 NMB-87-004	1535	-3381	-210
11 NMB-86-010	1807	-3552	-88
12 NMB-86-003	2546	-1996	800
13 NMB-84-001	1704	-5524	-141
14 BDH-76-044	-20225	-14140	-324
15 BDH-76-041	3369	-805	-86
16 BDH-76-018	4923	2192	213
17 BDH-76-019	2586	-1407	-86
18 CZR-16-004	-20225	-14033	-299
19 IND-64-022	1154	-2634	-189
20 IND-64-006	1651	-2021	-195
21 IND-63-034	50	-6025	-247
22 IND-63-013	2018	-3631	-189
23 IND-63-002	2564	-4452	323
24 IND-57-020	574	-2814	-131
25 IND-57-004	3092	-3491	-192
26 IND-56-002	-20222	-14094	-284
27 IND-55-011	3962	-3379	-271
28 IND-55-003	3379	2057	196
29 IND-53-003	4151	-2029	3
30 IND-50-007	1447	-2677	-180
31 USA-49-025	2350	5011	-94
32 USA-49-016	791	3699	476
33 USA-49-011	-162	4154	3
34 USA-49-009	3672	6824	393
35 USA-48-033	2445	3431	192
36 USA-48-022	2024	-4129	-15
37 USA-48-016	34	-4984	-226
38 UK-10-015	3721	-510	25
39 UK-10-014B	-20216	-13917	-305
40 UK-10-011	1841	2991	869
41 UK-10-010	3181	-2429	-207
42 UK-10-005	1145	3004	-101
43 UK-10-002	2223	2750	241

Strain code	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
44 AUS-9-035	1999	-18	-21
45 AUS-9-034	1914	-3528	-55
46 AUS-9-031	1090	5192	454
47 AUS-9-026	-20109	-14060	-320
48 AUS-9-019	-405	-1236	-183
49 AUS-9-018	648	-3559	-214
50 JPN-93-010	4389	-4334	171
51 AUS-9-016	2347	611	-3
52 AUS9-003	-20198	-13914	-290
53 AUS-7-008	3373	-2103	-107
54 CZR-17-002	2295	-5185	-244
55 CZR-17-001	1462	4231	18
56 CZR-16-009	3702	-3525	-39
57 CZR-16-007	193	-2088	-131
58 CZR-16-005	1028	-1022	-171
59 CZR-16-002	626	-2606	-168
60 UK-12-017	1520	2201	366
61 UK-12-011	1810	-2270	-147
62 UK-12-010	1939	-192	-12
63 UK-12-008	-20183	-13948	-293
64 UK-12-007	1767	3012	167
65 UK-12-004	-20194	-14036	-320
66 UK-11-002	-20225	-13938	-293
67 UK-10-020	1490	-394	-49
68 UK-10-016	2366	940	183
69 ARG-14-064	983	-2762	-6
70 ARG-14-049	1618	3784	61
71 ARG-14-046	-98	-1016	-113
72 ARG-14-054	2768	-2442	83
73 ARG-14-071	-143	-4120	-260
74 ARG-14-026	2668	-3183	28
75 ARG-14-065	1865	-5460	-198
76 ARG-13-047	4004	-555	208
77 AUS-7-001	3168	-5527	-31
78 ARG-13-037	2732	-2908	-186
79 ARG-13-042	3736	-809	558
80 ARG-13-001	1847	4600	1423
81 ARG-13-027	2854	4951	1819
82 JPN-73-011	2699	2524	137
83 USA-47-022	132	-7410	-168
84 NMB-91-003	1563	-1028	158
85 NMB-91-004	1227	-4743	-3
86 NMB-91-005	2872	-1813	375

Strain code	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
87 IND-18-047	3721	-4059	-265
88 POR-20-006	3553	-1474	67
89 JPN-18-050	-250	-921	-9
90 NMB-87-008	3694	-2112	821
91 SPN-77-006	1114	-6619	-250
92 IND-63-034	-21	-3049	-251
93 NZD-33-001	2106	-3616	-192
94 JPN-18-080	2433	-1190	369
95 POR-24-002	846	2591	546
97 POR-23-009	168	3049	116
98 POR-23-012	837	-1114	-88
99 BDH-76-044	1478	-3708	177
100 POR21-003	2057	-4178	-256
101 POR-21-001	1795	-1632	-107
102 POR-22-001	223	-3571	-131
103 POR-20-006	977	-1044	-12
104 POR-20-009	492	-5005	-214
105 POR-20-001	1347	-2676	229
106 POR-20-002	726	-4596	21
107 JPN-18-085	-277	-3461	-15
108 POR-19-003	-817	-2762	-52
109 JPN-18-050	-946	-2829	-95
110 JPN-18-019	1630	-3720	-76
111 JPN-18-032	1749	-1910	104
112 CZR-17-003	415	1807	183
113 CZR-17-004	361	-3531	-15
114 BDH-76-003	-702	-1171	-79
115 BDH-76-007	107	-332	-9
116 43814	-20344	-14064	-314
117 44151	-1366	-5024	-204
118 43026	-512	1557	1185
119 12882	1713	-1920	-46
120 12751	2445	-4395	-97
121 12754	-1242	-2451	-174
122 12744	1490	-2549	-155
123 12664	895	-1617	-104
124 12665	1096	-3739	-89
125 12659	657	-2374	-195
126 12660	-1885	2787	192
127 USA-47-027	516	-5295	18
128 USA-47042	-509	-11530	-332
129 USA-45-019	461	-4013	-171
130 UK-12-004	22	-1425	-30

Strain code	4-MU-nonanoate	4-MU-laurate	4-MU-palmitate
132 USA-45-008	-1089	-3436	-415
133 USA-43-022	34	-4297	-226
134 USA-43-026	1341	-5249	-162
135 NZD-33-001	785	-5047	-272
136 ANT-28-001	1572	-4422	-223
137 POR-24-013	1129	-867	-141
138 ANT-25-001	-632	-2588	-253
139 POR-24-006	141	-3528	-223
140 POR-24-009	601	-3400	-91
141 JPN-18-251	175	46	-122
142 JPN-18-080	-515	-2673	-79
143 JPN-93-008	-1096	-2319	-42
144 JPN-18-072	-1559	-3778	-135
145 JPN-91-002	-2625	-1148	113
146 JPN-91-001	-1034	120	235
147 JPN-73-008	583	-1316	92
148 IND-81-015	125	-4838	-125
149 IND-81-004	-79	-2909	-189
150 SPN-77-029	224	-3583	-49
151 SPN-77-028	-4251	-1913	67
152 SPN-77-006	-20326	-14030	-308
153 SPN-77-005	-2020	-8989	-266
154 SPN-77-012	-601	-2841	-18
155 BRA-15-005	-20384	-13926	-336
156 BRA-15-013	5106	-3019	-21
157 BRA-15-010	205	-4172	-251
158 IND-66-007	171	-3299	-204
159 USA-47-004	-20461	-14155	-299
160 USA-47-018	-310	-4394	-223
161 BDH-76-044	971	-4181	-187
162 NZD-33-001	-741	-3623	110
163 JPN-91-005	1111	-4270	-256
164 JPN-91-003	-1385	-2536	-21
165 USA-48-002	-927	-2591	-34
166 IND-53-003	-1886	-2555	46
167 USA-47-027	647	-4880	-158
168 IND-63-013	-378	-2500	0
169 NMB-89-006	-885	-2963	101
170 JPN-18-019	-235	-4260	-259