A STUDY OF THE ECOLOGY AND TAXONOMY OF *MICROMONOSPORA* IN THE NATURAL ENVIRONMENT

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Finally I feel it appropriate to dedicate this thesis to my husband, Paul and to my father, William Hodges for putting up with me for the past 6 and 30 years, respectively!

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself and all sources of information have been specifically acknowledged by source of reference.

Jane Mullins

ABBREVIATIONS

AGS	Arginine glycerol salts medium
DNA	Deoxyribonucleic acid
HSD	Honestly significant difference
MSD	Minimum significant difference
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
SD	Standard deviation
S_{Dice}	Dice coefficient
SDW	Sterile distilled water
S_J	Jaccard coefficient
SL	Single linkage
S_{sm}	Simple matching coefficient
UPGMA	Unweighted pair-group arithmetic average clustering
WPGMA	Weighted pair-group arithmetic average clustering

SUMMARY

Selective isolation procedures were developed for the isolation of *Micromonospora* from natural environments. Methods developed involved the use of heat treatment and the use of antibiotic incorporated media using lincomycin and novobiocin. It was found that heat treating air dried soil samples in a dry oven at 120°C for 60 minutes drastically reduced the number of both bacteria and streptomycetes occurring on the isolation plates thereby allowing for the selective isolation of *Micromonospora*. Additionally the most effective concentration of antibiotics for the selective isolation of *Micromonospora* was found to be 10μl/ml novobiocin and 10μl/ml lincomycin.

A spore specific extraction procedure was also developed, again exploiting the ability of *Micromonospora* spores to withstand heat treatment, to follow the fate of *Micromonospora* spores and mycelia in artificial soil microcosms. Comparison of the survival of both the spore and mycelial component of *M. chalcea* and *M. fulvopurpurea* populations indicated that heat treating 10g microcosms for 20 minutes at 70°C allowed the maximum recovery of spores present whilst deselecting completely the mycelial population. The ability of *Micromonospora chalcea* and *M. fulvopurpurea* to survive in sterile soil microcosms was studied over 30 days. Both species showed marked germination and sporulation cycles mirroring streptomycetes. Both species consistantly showed significant germination at Day 1 with spore numbers starting to increase by Day 2 (ca. 10^5 spores/g soil). Following rapid sporulation at Day 5. a plateau at ca. 10^7 to 10^8 c.f.u./g soil was attained.

Using phenotypic data, including antibiotic resistance profiles, *Micromonospora* strains, comprising both type strains and wild isolates, were clustered using numerical taxonomic methods. Clustering of the largest set of data (121 strains/179 characteristics) using the NTSYS clustering package, gave 14 distinct species-groups. The character state data obtained for clusters defined at the 77.5% S_{sm} similarity level were then used to develop a probabilistic identification matrix for the rapid identification of *Micromonospora*.

CHAPTER 1

General Introduction

Aims of research

Despite their abundance and industrial importance relatively little work has been published concerning the classification and ecology of *Micromonospora*. The primary aim of this research was therefore to initiate a study of the phenotypic diversity of *Micromonospora* by developing a probabilistic identification matrix enabling rapid characterisation of putative *Micromonospora* isolates. On the basis of phenotypic data, we aimed to classify a range of *Micromonospora* species including both type strains and wild isolates with the objective of producing a number of probabilistic identification matrices. As a direct result of these phenotypic studies we hoped to select a number of representative *Micromonospora* for 16S rRNA sequencing providing the basis for future genotypic studies of the genus.

Initial studies, however, were aimed mainly at studying a range of methods for the selective isolation of *Micromonospora* from a variety of terrestrial environments. This provided a varied source of *Micromonospora* isolates for further studies.

An additional important objective of this research was to gain a greater understanding of the ecology of *Micromonospora* by studying the survival of *M. chalcea* and *M. fulvopurpurea* in soil microcosms.

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1.1 The actinomycetes

The actinomycetes are a widely distributed group of Gram-positive bacteria which are predominantly saprophytic but also include species forming parasitic or mutualistic associations with plants, animals and humans. They occur in a wide range of natural and man-made environments including soil, plant litter, manure compost, biodeteriorated materials, fresh and salt water, and the live tissue of plants, animals and humans. The most widely distributed genera are *Corynebacterium*, *Nocardia*, *Streptomyces*, *Rhodococcus* and *Micromonospora*, but other genera such as *Frankia* occupy important, clearly defined ecological niches. Detailed reviews have been provided by Cross and Goodfellow (1973), Goodfellow and Williams (1983), Williams and Wellington (1982) and Williams *et al.* (1984).

Actinomycetes are characterised by the formation of branching hyphae (usually 0.5-1.0μm diameter) that may develop into a mycelium. Reproduction is by almost total fragmentation of the hyphae or by production of spores in specialized areas of the mycelium. Most species are chemoorganotrophic, aerobic and mesophilic and grow optimally at a pH near neutrality.

1.2 The monosporic actinomycetes

Actinomycetes with single spores are very common in soil (Cross, 1981a). They primarily include members of the genus *Micromonospora*, which have the ability to actively degrade complex polymers such as cellulose and chitin in soil; a number are also able to produce valuable antibiotics (Wagman and Weinstein, 1980, Table 1). In contrast, other monosporic actinomycetes include the thermophilic genera

Table 1 **Examples of antibiotics produced by *Micromonospora***

Species	Antibiotic	Reference
M.purpurea	Gentamicin[1]	Weinstein *et al.* (1964)
M.echinospora	Gentamicin[1]	Weinstein *et al.* (1964)
M.sagamiensis	Sagamicin[1]	Okachi *et al.* (1974)
M.inyoensis	Sisomicin[1]	Weinstein *et al.* (1970)
M.olivasterospora	Fortimicin[1]	Nara *et al.* (1977); Kawamoto *et al.* (1983b)
M.grisea	Verdamicin[1]	Weinstein *et al.* (1975)
M.rosaria	Rosaramicin[2]	Reimann & Jaret (1972)
M.fastidiosus	Analog of rosaramicin-6108A[2]	Nakijima *et al.* (1990)
M.narashinoensis	Rustmicin[2]	Takatsu *et al.* (1985)
M.chalcea	Neorustmicin[2]	Abe *et al.* (1985)
M.capillata	M4365[2]	Furumai *et al.* (1977)
M.chalceaizumensis subsp.	Juvenimicin[2]	Hatono *et al.* (1976)
M.megalomicea	Megalomicin$_2$	Weinstein *et al.* (1969)
M.sp 1225	Erythromicin B[2]	Marquez *et al.* (1976)
M.halophytica	Halomicin[3] (ansamysin)	Weinstein *et al.* (1968)
M.carbonacea	Everninomicin	Weinstein *et al.*, (1964)
M.globosa	Deoxydynemicin[4]	Shiomi *et al.* (1990)
M.chersina	Dynemicin[4]	Tomita *et al.* (1992)
M.citrea	Citreamicin[5]	Carter *et al.* (1990)

[1]Antibiotic belonging to aminoglycoside group.

[2]Antibiotic belonging to macrolide group

[3]Antibiotic belonging to ansamycins.

[4]Antitumor antibiotic related to calicheamicins.

[5]Related to family of antibiotics including cervinomycin and simaomicin.

Thermomonospora and *Saccharomonospora*. Both probably survive as dormant spores in temperate soils, but are more active in high temperature composts or stores of fodder or grains.

1.2.1 The genus *Micromonospora*

The genus *Micromonospora* was first proposed by Orskov in 1923 as a sub group of the order *Actinomycetales*. Originally the genus *Micromonospora* contained only one species, *Streptothrix chalcea*, which was isolated by Foulerton (1905) from the air. This strain formed characteristic deep cinnabar red colonies on nutrient peptone agar and assumed a brownish colour on other media (Orskov, 1923). Aerial mycelium was absent and the spores were borne singly at the tip of short side branches on the substrate mycelium. This species was characterised by the formation of single spores and the lack of aerial mycelium and therefore considered distinct within the collection of actinomycetes belonging to Orskov.

Morphologically, *Micromonospora* are characterised by well-developed, branched, septate mycelia averaging 0.5μm in diameter. Nonmotile spores are usually borne singly, sessile, or on short or long sporophores that often occur in branched clusters. Characteristically, aerial mycelia is absent, however in some cultures it may occasionally appear as an irregular, white bloom (Kawamoto, 1989). On agar media most species of *Micromonospora* produce colonies which are initially light orange, maturing to a progressively dark hue, often accompanied by the production of dark spores. Diffusible pigments are sometimes produced. Further descriptive information is given in Table 2.

In recent years members of the genus *Micromonospora* have attracted attention as producers of many anti-bacterial compounds.

Table 2 **Differential characteristics of examples of species of the genus *Micromonospora***

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Mycelial pigment					B-G			Pu	Pu	Br	Y
Diffusible pigment	Y	R	Y			Br	G			R	Y
Growth on Czapek-sucrose agar	-	+	-	-	-	+	+	+	+	+	+
Growth on potato slice	+	-	+	+	+	+	-	-	-	-	+
Utilisation of melibiose	+	+	+	+	+	+	-	-	-	+	+
Utilisation of D-mannitol	-	-	-	+	+	-	-	-	-	+	-
Utilisation of glycerol	-	-	-	-	-	+	-	-	-	+	-
ß-Xylosidase	+	+	+	+	-	-	-	+	+		-
α-Galactosidase activity	+	+	+	+	+	+	-	-	-		+
α-Mannosidase activity	-	-	-	+	+	-	-	-	-		-
Tolerance of $\geq$ 4% (w/v) NaCl	-	+	+	-	-	-	-	-	-	-	-
Main menaquinone (MK)	9	9	10	10	10	10	10	10	10	10	9

Y, yellow; R, red; B, blue; G, green; Pu, purple; Br, brown

1 *M.carbonacea* (Luedemann & Brodsky, 1965).

2 *M.halophytica* (Weinstein *et al.* 1968).

3 *M.chalcea* (Foulerton, 1905).

4 *M.inositola* (Kawamoto *et al.*, 1974).

5 *M. coerulea* (Jensen *et al.*, 1932).

6 *M.purpureochromogenes* (Waksman & Curtis, 1916).

7 *M.olivasterospora* (Kawamoto *et al.*, 1983).

8 *M.echinospora* (Luedemann & Brodsky, 1964).

9 *M.purpurea* (Luedemann & Brodsky, 1964).

10 *M.rosaria* (Wagman *et al.*, 1972).

11 *M.chersina* (Tomita *et al.*, 1992).

Although *Streptomyces* produce a great range of antibiotics, *Micromonospora* are now recognized as the second most important group of antibiotic-producing actinomycetes. Many aminoglycoside (gentamicins, sisomicin, sagamicin) and macrolide type compounds (megalomicins, rosamicin), as well as many others (halomycins, everninomicin) have been found to be present in strains of *Micromonospora* (Table 1).

1.2.2 The taxonomy of *Micromonospora*

Micromonospora undoubtedly play a significant role in the soil ecosystem as well as being commercially important antibiotic producers despite this, however, there is a suprising lack of published information concerning the taxonomy of this important genus.

Chemotaxonomic data have shown *Micromonospora* to be related to *Actinoplanes*, *Amorphosporangium*, *Ampullariella* and *Dactylosporangium* (Lechevalier and Lechevalier, 1970; Lechevalier *et al.*, 1977). These relationships have been further supported by results from DNA-DNA pairing and 16S rRNA cataloguing studies (Stackebrandt and Woese, 1981; Stackebrandt *et al.*, 1981, 1983), consequently *Micromonospora* are now classified, along with the above, under the suprageneric group, the actinoplanetes (Goodfellow, 1989). Complementary work by Goodfellow *et al.* (1990) has proposed that the genera *Actinoplanes*, *Ampullariella*, *Amorphosporangium*, *Dactylosporangium*, *Micromonospora* and *Pilimelia* should be classified in the family Micromonosporaceae Krassilnikov 1938. All members of the actinoplanetes share a common cell wall chemotype containing meso- and/or 3-hydroxy diaminopimelic acid and glycine in combination with xylose and arabinose (Goodfellow and Cross, 1984), all also share the

Table 3 **Chemotaxonomical properties of the actinoplanetes[a]**

GENUS	Cell wall chemotype[b]	Whole cell sugar pattern[c]	Phospho lipid pattern[d]	Major menaquinones[e]			Fatty acids[f]		DNA G + C (mol. %)
				MK9	MK10	MK12	S	U	
Micromonospora	II	D	PII	+	+	+	+	+	71-73
Actinoplanes	II	D	PII	+	+		+	+	72-73
Ampullariella	II	D	PII	+	+		+	+	71-73
Dactylosporangium	II	D	PII	+			+	+	ND
Glycomyces	II	D	PI		+		ND	ND	71-73
Catellatospora	II	D	PII	+	+		ND	ND	71-73

[a]Data taken from Vobis (1991) & Goodfellow *et al.* (1988)

[b]cell wall chemotype II, glycine & meso- and/or 3-hydroxy diaminopimelic acid

[c]whole cell sugar pattern D, xylose and arabinose

[d]phospholipid pattern: PI, nitrogenous phospholipid absent
PII, phosphatidylethanolamine present

[e]number, e.g MK9, refers to isoprene units

[f]saturated and unsaturated fatty acids

same peptidoglycan structure, sugar composition and polar lipid pattern as is found in *Micromonospora* (Kawamoto *et al.*, 1981; Lechevalier *et al.*, 1977). The GC content of DNA belonging to the actinoplanetes is, in general, between 71-73 mol% (Vobis, 1989), recent studies, however, have shown the range to be as great as 67-76 mol% (Kothe, 1987). Table 3 summarises the most useful chemotaxonomical properties of the actinoplanetes.

The classification of species groups within the genus *Micromonospora* remains, as it has been for the past thirty years, in a fairly uncertain state (Cross, 1981a). Many species, named over 50 years ago, have only ever been briefly described with no type or neotype cultures existing; such examples include *Micromonospora parva* (Jensen, 1932), *M. gallica* (Erikson, 1935), *M. globosa* (Kriss, 1939), *M. elongata* and *M. bicolor* (Krassilnikov, 1941).

According to Cross (1981a), one reason for the state of confusion existing in *Micromonospora* classification is that many species were originally isolated in one of two laboratories; one in the USA (Luedemann, 1971a,b; Luedemann and Brodsky, 1964, 1965; Weinstein *et al.*, 1968) and one in the USSR (Sveshnikova *et al.*, 1969, 1970), with little exchange of information between the two. In addition, many strains, isolated and found to produce novel bioactive compounds, have been given new specific names (Sveshnikova *et al.*, 1970) resulting in an over classification of the genus.

The description of many species of the genus *Micromonospora*, including *M. echinospora*, *M. purpurea* and *M. chalcea*, began with taxonomical studies of the gentamicin producing strains (Luedemann and Brodsky, 1964). More recent publications (Kawamoto, 1989) include descriptions of over 12 species and subspecies of *Micromonospora*, some examples of which are illustrated in Table 2. Such descriptions were

based largely on chemotaxonomical markers and morphological and physiological characteristics.

On the basis of cell wall composition, Kawamoto *et al.* (1981) were able to divide the species of *Micromonospora* into three broad groups; the first group contained only diaminopimelic acid (DAP) and included *M. chalcea* and *M. globosa*; the second one contained a significant amount of both DAP and 3-hydroxy DAP (included *M. parva* and *M. inositola*) and the third group contained mainly 3-hydroxy DAP and a very small amount of DAP (included *M. olivasterospora* and *M. sagamiensis*).

Menaquinone composition has also been used in an attempt to broadly classify the species of the genus *Micromonospora*; the predominant menaquinones of *M. carbonacea* and *M. halophytica* having 9 isoprene units, those of *M. echinospora* subsp. *pallida* having 12 units, and those of most remaining species having 10 units (Collins *et al.*, 1984; Kawamoto, 1989).

Mycelial pigments and colony coloration appear also to be of diagnostic value (Kawamoto, 1989; Table 2). On the basis of these features Bibikova *et al.* (1990) were able to divide the genus *Micromonospora* into 9 groups; representatives of each of the groups were additionally found to be characterised by both their sensitivities to, and their capacity to produce, a number of antibiotics.

A number of species have been characterised, in part, by the production of soluble pigments of various colours, examples include *M. rosaria* (wine red pigment; Horan and Brodsky, 1986), *M. olivasterospora* (olive green pigment; Kawamoto *et al.*, 1983b), *M. chersina* (yellow pigment; Tomita *et al.*, 1992), *M. chalcea* (yellow pigment; Luedemann, 1971a) and *M. purpureochromogenes* (olive green pigment; Luedemann, 1971b). Further physiological parameters such as

growth on certain types of media, carbon and nitrogen utilisation patterns (Kawamoto *et al.*, 1983a) and the ability to tolerate growth inhibiting chemicals, have also played an important role in the identification and classification of species and subspecies groups of *Micromonospora* (Kawamoto, 1989).

1.3 Actinomycetes in the natural environment

The soil and aquatic environments are natural reservoirs for a multitude of diverse prokaryotic and eukaryotic organisms. Many actinomycete species have been isolated from soil and aquatic environments and identified, and their roles in ecosystem processes have been determined. This has led to an increased understanding of microbial ecology (Goodfellow and Williams, 1983).

1.3.1 *Micromonospora* in the natural environment

Micromonospora constitute a significant part of the actinomycete population in many environments. They can be isolated, with relative ease, from many soils, indeed, according to Shearer (1987), two or three strains may be expected from most soil samples. Their predominant incidence, however, is in aquatic systems where there has been evidence of proliferation in both freshwater and marine habitats (Cross, 1981a; Goodfellow and Haynes, 1984).

1.3.2 *Micromonospora* in the terrestrial environment

Since the discovery of antibiotic-producing *Micromonospora* species, various attempts have been made to isolate a greater variety of

strains (Hamdi *et al.*, 1980). Although saprophytic *Micromonospora* exist with greater frequency in aquatic ecosystems (Goodfellow and Haynes, 1984), it is, perhaps, the soil from which most *Micromonospora* have been isolated. Indeed, *Micromonospora*, as the second most commonly isolated sporogenous actinomycete, constitute a significant component of the microbial population in most soils and counts of up to 10^6 per gram are commonly obtained (Lechevalier, 1964; Nonomura and Hayakawa, 1988). Obviously such figures need to be treated with caution as such high numbers may be a reflection of spore resistance or longevity. Because the soil is a prolific source of *Micromonospora* isolates it is not suprising that it is the most intensively studied habitat (Goodfellow and Williams, 1983). Despite this, however, there are still many gaps in our understanding of the roles played by *Micromonospora*, indeed by all actinomycetes, in many soil processes. Several reviews covering the isolation and abundance of actinomycetes, including *Micromonospora*, in the soil are available (Williams and Wellington, 1982; Williams *et al.*, 1984). The occurrence of *Micromonospora* in neutral and alkaline soils was initially reported by Jensen (1930, 1932). Kriss in 1939 then isolated eight strains of this genus from Russian soils (Kriss, 1939) using an artificial medium. More recently work has shown that representative *Micromonospora* strains, although distributed widely in soils of various geographical regions, occur preferably in moist soils (Ishizawa *et al.*, 1969; Solovieva and Singal, 1972). Singal *et al.* (1978) showed that *Micromonospora* strains predominated in soils with a high silt content; their content with respect to all isolated *Streptomycetaceae* amounted to 67 to 83% in moist soils and silts, but only 6 to 11% in ordinary soil samples. Interest in the selective isolation of this group of bacteria from soil was initiated by Nonomura and Ohara (1957) who

isolated *Micromonospora* strains by the direct spreading of soil particles over soil-extract agar plates (Section 1.5.1).

1.3.3 *Micromonospora* in the aquatic environment

A considerable amount of attention has been given to the study of actinomycetes in terrestrial systems, so much so that those species encountered in aquatic habitats have been relatively neglected. In recent years, however, it has become apparent that many actinomycetes, in particular members of the genus *Micromonospora*, form a significant proportion of the heterotrophic bacterial population in many aquatic environments (Al-Diwany and Cross, 1978).

1.3.3(a) Freshwater habitats

It is now widely accepted that members of the genus *Micromonospora* are common inhabitants of many freshwater sites (Colmer and McCoy, 1943; Cross and Collins, 1966). Erikson (1941) was the first to suggest that the micromonosporae may be indigenous to such habitats and that they may play an important role in the turnover of cellulose, chitin and lignin. Since then the occurrence of *Micromonospora* in lake systems has been confirmed by workers in many countries (Corberi and Solani, 1960; Johnston and Cross, 1976; Potter and Baker, 1956; Willoughby, 1969). Indeed early work on Wisconsin lake samples showed that micromonosporae accounted for as much as 10 to 50% of the total microbial spore population in the water mass (Colmer and McCoy, 1943; Umbreit and McCoy, 1940). It has also been shown that micromonosporae are frequently present in samples from streams, rivers, and river sediments (Al-Diwany and Cross, 1978; Rowbotham

and Cross, 1977). For example counts of *Micromonospora* have been obtained as high as 1500 c.f.u./ml from stored water from the River Thames (Burman, 1973).

1.3.3(b) Marine habitats

One of the first reports of actinomycetes inhabiting marine environments can be dated back to when Rubentschik (1928) isolated a cellulose decomposing streptomycete from saline mud of an inshore locality of the Black Sea. Actinomycetes have since been frequently found in shallow and inshore areas as well as in the deep sea (Cross, 1981b; Goodfellow and Haynes, 1984; Goodfellow and Williams, 1983; Williams *et al.*, 1984). The most extensive investigations concerned with the isolation of actinomycetes, in particular *Micromonospora*, from marine sites have been carried out by Weyland (1969, 1970, 1981) who found that numbers of actinomycetes as a whole, in marine environments were substantially lower than in terrestrial environments. In addition to uncertainties about the origin of actinomycetes in marine habitats, it is still not known to what extent these bacteria represent a physiologically active component of the marine microbial community (Jensen *et al*, 1991). It has been shown that many actinomycetes, including *Micromonospora*, can grow in a sea-water based medium (Weyland, 1981) as well as at increased hydrostatic pressures (Helmke, 1981). It has also been reported that increased numbers of *Micromonospora* occur with increasing depth in deep-sea sediments (Weyland, 1981; Weyland and Helmke, 1988).

1.3.4 *Micromonospora* in other environments (including the anaerobic *Micromonospora*)

In 1942 Hungate observed, in the rumen of sheep, bacteria morphologically resembling the *Micromonospora*. Later he described *Micromonospora propionici* as a new species of anaerobic cellulolytic bacterium isolated from the gut of a termite (Hungate, 1946).

Pasti and Belli (1985) have, more recently, shown that *Micromonospora* and other mesophilic actinomycetes with vigorous cellulolytic activity can be isolated from the guts of higher termites of the subfamilies Macrotermitinae and Termitinae, using aerobic enrichment media containing natural or microcrystalline cellulose. *Micromonospora* have also been isolated from the gut, parent soil and mound materials of the termites *Procubitermes aburiensis* and *Cubitermes severus* (Bignall *et al.*, 1991).

Many of the above *Micromonospora* are obligately anaerobic and can be separated from other members of the genus by their ability to utilize cellulose and by the nature of their fermentation products. The three species recognised are *M. acetoformici* (Sebald and Prevot, 1962) which is unable to utilize cellulose but can ferment glucose and starch, *M. ruminantium* (Maluszynska and Janota-Bassalik, 1974), a cellulose fermenter and *M.propionici* (Hungate, 1946) which has the ability to ferment glucose and cellulose to form propionic and acetic acids.

1.4 The physiology, biochemistry and genetics of *Micromonospora*

1.4.1 The growth of *Micromonospora*

The genus *Micromonospora* has a complex developmental cycle in which a monopodially branched mycelium, divided at intervals by cross-walls, develops from one asexual spore. On this mycelium, sessile spores or spores on short sporophores are produced either singly or in pairs. Under appropriate conditions, these germinate and repeat the cycle (Suarez *et al.*, 1980).

One of the more common species of *Micromonospora*, *M. carbonacea* is often characterised by it's sympodial sporophore structure - a morphological characteristic peculiar to this species (Luedmann and Brodsky, 1965). This can occasionally be seen to a lesser degree in other *Micromonospora* species (Luedmann, 1971a,b,c). Although in most species single spores form on substrate mycelium, infrequently spores also occur in longitudinal pairs and more infrequently as multiple longitudinal spores (Luedmann and Casmer, 1973). Often bizarre-shaped and multiple septate cells that resemble chlamydospores are found among certain enlarged vegetative hyphae.

Stevens (1975) studied sporogenesis in two species of *Micromonospora*, *M. globosa* and *M. fusca*. He observed that spore formation began as a blowing-out of a hyphal tip with the subsequent centripetal invagination of the plasma membrane. This sporulation process closely resembles sporangial formation, as the individual cells which are formed do not exhibit the wall thickening typical of singly produced spores (Stevens, 1975).

Spore surface ornamentation of species of *Micromonospora* has been studied in depth by Luedemann and Casmer (1973) who found that

spore shape and surface ornamentation varies with the age of the spore. The spores of almost all species were highly refractile with blunt and spiny surfaces. Protruberances of varying sizes have often been observed on the outer layer of the spore wall, thus differing in origin from the surface ornamentation found on the sheath of some *Streptomyces* spores (Luedemann and Casmer, 1973). Further complementary work has been done by Dietz and Ulrich (1990) who used a non-critical point drying method, to examine the surface texture of *Micromonospora* spores electron microscopically.

Germinating spores of *Micromonospora* species pass through three morphological stages: darkening, swelling and germ tube emergence, as has been reported for *Streptomyces* (Attwell and Cross, 1973; Ensign, 1978; Hardisson *et al.*, 1978; Kalakoutskii and Agre, 1976). The process of germination has a pH and temperature optima of 8.0 and 40°C, respectively, and is unaffected by activation treatments (Suarez *et al.*, 1980).

1.4.2 Spore resistance of *Micromonospora*

It is well known that many bacterial spores are resistant to mechanical disruptions such as ultrasonication. Johnston and Cross (1976) investigated *Micromonospora* in lake mud and found that the spores of isolates were unaffected by ultrasonication treatment, but that the mycelia were quickly killed. Similar results were obtained by Kawamoto *et al.* (1982).

In previous studies *Micromonospora* spores have been characterized by their relatively high resistance to polar solvents and to heat treatments. More than 10% spore survival is observed after treatment for 30 minutes at 30°C with a solution containing 60% (v/v) of

tert-butyl alcohol, formamide, dimethylformamide and acetone (Kawamoto *et al.*, 1982). Additionally spores of many *Micromonospora* species are absolutely resistant to temperatures below 75°C. Indeed the decimal reduction time at 80°C for *M. chalcea* is 12.5 minutes.

The investigation of microelectrophoresis of some actinomycetes spores by Douglas *et al.* (1970) indicated that spores of *Micromonospora* may have different properties to those of *Streptomyces*. Slight differences in mobility have been observed between the respective spores at both pH 5 and 8. However, studies by Kawamoto *et al.* (1982) contrastingly, revealed no significant differences between spores of both genera tested through a wide range of pH. The physiological relationships underlying resistance mechanisms of spores still remain to be studied. For example, whilst the phenomenon of heat resistance in many bacterial spores has been established for a long time, its physiological mechanism has still not been fully elucidated. Additionally, even less attention has been paid to the action of polar solvents on spores.

Interestingly, electron-microscopic observations (Hardisson and Suarez, 1979; Luedemann and Casmer, 1973; Stevens, 1975) have revealed that although the spore wall of *Micromonospora* is devoid of the multilaminar coat present in *Thermoactinomyces*, it does possess a significant stratified deposition; a property lacking in *Streptomyces*. It may therefore be possible to explain the heat resistance of *Micromonospora* spores by such differences in spore wall structure as described above.

Unlike *Micromonospora* spores, those of *Streptomyces* are enclosed by a hydrophobic sheath, of which some superficial components seem to be displaced easily with acetone (Bradshaw and Williams, 1976). In addition, again unlike *Micromonospora*, *Streptomyces* spores were

affected by a number of organic solvents (Erikson, 1946). The resistance of *Micromonospora* spores to polar solvents compared to *Streptomyces* might therefore be explained by the fact that *Micromonospora* spores both lack a spore sheath and have a significant layer of stratified deposition.

Kawamoto *et al.* (1982) carried out an extensive study on the resistance of *Micromonospora* spores to a variety of conditions. Of the thirteen species tested all had similarly resistant spores, however, they all exhibited small differences in detail with each other. For example, *Micromonospora purpureochromogenes* and *Micromonospora fusca* which are thought to be synonymous, were not identical in the resistance of their respective spores. From this it can be concluded that the resistance of spores might depend mostly on the strains or at least be altered to some extent by the manipulations of the cultural conditions.

It is quite obvious from the above studies, therefore, that members of the genus *Micromonospora* are far superior with respect to spore resistance and, consequently, survivability in soil, to their more common counterparts, the *Streptomyces*.

1.4.3 Chemotaxonomic properties of *Micromonospora*

Relatively little is known about the physiological and biochemical properties of *Micromonospora* strains, even though many produce industrially important antibiotics (Wagman and Weinstein, 1980; Table 1).

Members of the genus *Micromonospora* can be characterized broadly by a number of chemotaxonomic properties including cell wall analysis (Table 3). They contain glycine and *meso* (or *meso*-3-hydroxy)-diaminopimelic acid in the cell wall and have been classified as cell wall type II by Lechevalier and Lechevalier (1970). In addition to

Micromonospora, *Streptomyces* also contain glycine and diaminopimelic acid in their cell wall, however in *Streptomyces* the configuration of the diaminopimelic acid is in the LL-form. Although the primary structure of some *Streptomyces* cell walls were studied in detail (Arima *et al.*, 1968; Leyh-Bouille *et al.*, 1970) until relatively recently (Kawamoto *et al.*, 1981) little was known about the structure of peptidoglycan in *Micromonospora*.

Kawamoto *et al.* (1981) undertook a study to examine the amino acid composition of the cell walls of recently discovered aminoglycoside producing *Micromonospora*. The cell walls of the 19 strains studied contained glycine, glutamic acid, *meso*-diaminopimelic acid (including its 3-hydroxy derivative), and alanine in a molar ratio of 1:1:1:0.6-0.6. The cell wall of *Micromonospora* contains D-alanine but not L-alanine. Based on this evidence, a primary structure has been proposed that is characterised by the presence of glycine in the first position of the peptide subunit, a direct interbridge between D-alanine and DAP, and the presence of N-glycolylmuramic acid. Further chemotaxonomic information concerning the *Micromonospora* is given in Table 3.

1.4.4 Genetic systems in *Micromonospora*

In spite of their commercial importance and their ability to produce and modify a large variety of antibiotics (Berdy and Jari, 1986a; Wagman and Weinstein, 1980), reports on the genetics of *Micromonospora* are few (Berdy and Jari, 1986b). In order to address this lack of knowledge, techniques are constantly being developed enabling insights to be gained into genetic systems governing these important organisms.

Genetic recombination occurs in a number of actinomycete genera including *Streptomyces* (Sermonti and Spada-Sermonti, 1955) and *Nocardia* (Adams and Bradley, 1963). In addition, a number of investigations have studied genetic recombination in *Micromonospora* (Beretta *et al.*, 1971; Kim *et al.*, 1983; Ryu *et al.*, 1983). Beretta *et al.* (1971) obtained biochemical mutants from *Micromonospora chalcea*, *M. purpurea* and *M. echinospora*. Crosses carried out between complementary nutritional mutants of the same species showed positive genetic interaction, this was later found to be due to genetic recombination. No evidence, however, for interspecific genetic recombination has been found.

Much work has been done to develop a gene cloning system for *Micromonospora*, providing a potentially useful tool for the study of the organisation and regulation of antibiotic biosynthesis and resistance genes (Keleman *et al.*, 1989; 1991, respectively).

Efficient host-vector systems and transformation methods have been developed for many *Streptomyces* species (Garcia-Dominguez *et al.*, 1987; Yamamoto *et al.*, 1986), but in experiments using *Micromonospora* strains with plasmid DNA, until recently, relatively low transformation frequencies have been observed (Lotvin *et al.*, 1986; Matsushima and Baltz, 1988). However, efficient transformation of *M. purpurea*, a prolific gentamicin producer, using pIJ702 plasmid was successfully achieved by Kelemen *et al.* in 1989. In addition, since then, much work has been done in the development and improvement of methods for protoplasting, regenerating and transforming *Micromonospora* species (Love *et al.*, 1992).

Recent work has also been concerned with the cloning and characterisation of antibiotic resistance genes into *Micromonospora*. Aminoglycoside resistance genes (grm) were cloned from gentamicin

producer, *M. purpurea* and sisomicin producer, *M. rosea* by Kelemen *et al.* (1991) as a primary step in the study of the genetic organisation of aminoglycoside production genes. A similar study was undertaken by Goldberg *et al.* (1990) using the sisomicin resistance gene from *M. inyoensis*.

In addition to the above techniques, a broad host range temperate phage could also provide the basis for the development of a gene cloning system. Such studies have been done with the streptomycete specific phi C31 derivatives (Chater *et al.*, 1981; Rodicio *et al.*, 1985) and the phage R4 derived cosmid (Morino *et al.*, 1985). Many *Micromonospora* specific actinophage have been described including lytic phages, phiUW21 and phiUW51 (Kikuchi and Perlman, 1977, 1978). Several other lytic *Micromonospora* phages have been used to screen for the presence of restriction enzymes (Meyertons *et al.*, 1987). Additionally, a number of temperate phages specific for *Micromonospora* species have been partially characterised (Caso *et al.*, 1986) and used in the development of a transfection system, allowing the propagation of deletion mutants generated *in vitro*, through partial restriction and religation of the phage DNAs. This was seen as a necessary step in the construction of a phage cloning vector (Caso *et al.*, 1987).

More recent work of this nature has been done by Tilley *et al.* (1990a) who have reported on a temperate phage (MPphi WR-1) capable of transfecting *M. purpurea* and suitable for development as a cloning vector. In addition a cryptic phage (MPphi WR-2) has also been described in association with *M. purpurea* (Tilley *et al.*, 1990b).

1.5 The selective isolation of actinomycetes

The isolation of actinomycetes from terrestrial environments has traditionally been made using conventional dilution plating techniques. However, such methods often lead to an underestimation of the actinomycete population present, due either to their inhibition or out-competition by other more abundant or proliferate microorganisms. Many attempts have therefore made to overcome these problems including the use of pretreatment techniques, such as heat treatment and the use of selective agents such as antibiotics (Williams and Davies, 1965).

Prior to the mid 1960's published information concerning the isolation of actinomycetes dealt almost exclusively with methods suitable for *Streptomyces* (Shearer, 1987) reflecting their growing importance to the pharmaceutical industry as producers of important antibacterial compounds (Goodfellow, 1988; Okami and Hotta, 1988). However, as the search for novel bioactive compounds increased (Woodruff *et al.*, 1979), bringing with it the discovery of commercially successful antibiotics from other rarer genera such as *Micromonospora* (Luedemann and Brodsky, 1964) and *Actinoplanes* (Palleroni, 1980), more emphasis was placed on the development of selective methods for the isolation of non-streptomycete actinomycetes (Nonomura and Ohara, 1969, 1971 a-d; Shearer, 1987). More recent examples include the use of polyvalent phage to reduce the streptomycete population on soil dilution plates (Kurtboke *et al.*, 1992) and the use of dry heat treatment in conjunction with phenol and chlorhexidine gluconate treatments to isolate *Microbispora* from soil (Hayakawa *et al.*, 1991).

1.5.1 The selective isolation of *Micromonospora* from the natural environment

Although members of the genus *Micromonospora* are widely distributed in a variety of terrestrial and aquatic environments, their isolation from soil by conventional dilution plate techniques is generally hindered by, not only bacterial and fungal colonies, but also by the faster growing, more abundant *Streptomyces* colonies (Goodfellow and Williams, 1983; Williams and Vickers, 1988). Efficient and effective recovery of *Micromonospora* therefore requires the use of specific growth media and pretreatment techniques allowing the depression of the growth of other competing microorganisms on the isolation plates. As most actinomycetes survive in the natural environment mainly as spores (Mayfield *et al.*, 1972; Skinner, 1951), many techniques used tend to exploit their differential resistance properties in order to selectively isolate a chosen actinomycete genus. This has indeed been the case with *Micromonospora*.

Many pretreatments have been used, exploring the heat resistant properties of *Micromonospora* (Rowbotham and Cross, 1977; Sandrak, 1977), as well their tolerance to treatments with chlorine (Burman *et al.*, 1969; Willoughby, 1969) and sodium hydroxide (Wakisaka *et al.*, 1982). In addition to pretreatment techniques, novobiocin (Orchard, 1980) and tunicamycin (Wakisaka *et al.*, 1982) have been used to improve the selectivity of isolation media for micromonosporae.

Table 4 **Selective isolation procedures developed for *Micromonospora***

Source	Isolation Method	Isolation/Treatment Medium	References
Water/soil	dilution plating	Treated 70°C in a water bath for 10 minutes	Rowbotham & Cross (1977) Sandrak (1977)
Water	dilution plating	Treated with 4 ppm ammonia, then 2 ppm sodium hypochlorite	Burman *et al.*, (1969) Willoughby (1969)
Water/soil/ sediment	dilution plating	colloidal chitin agar	Hsu & Lockwood (1975)
Water/soil/ sediment	dilution plating	M3 agar + cycloheximide (50 mg/l) + thiamine (4 mg/l)	Rowbotham & Cross (1977)
Water/soil/ sediment	dilution plating	Kadota's cellulose benzoate agar + sodium benzoate (20 g/l)	Sandrak (1977)
Marine sediment	dilution plating	Cellulose asparagine agar plus artificial seawater with cycloheximide (50 mg/l) and novobiocin (50 mg/l)	Goodfellow & Haynes (1984)
Soil & mud of salt marsh	stamping	Arginine glycerol salts agar with cycloheximide (75 mg/ml) & nystatin (75 mg/l) plus artificial seawater	Hunter *et al.* (1984) Hunter-Cevera *et al.* (1986)
Plant material	washing	Colloidal chitin agar with cycloheximide	Willoughby (1968, 1969)
Soil	dilution	Starch-casein agar	Küster & Williams (1964) Williams & Davies (1965)
Soil	dilution plating	Humic acid/vitamin agar with either tunicamycin (20 mg/l) & nalidixic acid (30 mg/l) or cycloheximide (50 mg/l)	Nonomura & Hayakawa (1988) Hayakawa & Nonomura (1984)
Soil	dilution plating	Modified Bennett's agar with tunicamycin (25 mg/l) and cycloheximide (30 mg/ml)	Wakisaka *et al.* (1982)
		also incorporation with novobiocin (25-50 μg/ml)	Sveshnikova *et al.* (1976) Orchard (1980)
		gentamicin 1-10 mg/ml	Ivanitskaya *et al.* (1978)
		lincomycin	Ivanitskaya *et al.* (1978)

Micromonospora spores have been found to germinate and grow well on a number of defined agar media including starch-casein (Kuster and Williams, 1974; Williams and Davies, 1965) and colloidal chitin (Hsu and Lockwood, 1975; Lingappa and Lockwood, 1962). More recent studies have seen the use of HV agar as a basal isolation medium for *Micromonospora* (Hayakawa and Nonomura, 1987) used in conjunction with both chemical and physical pretreatments (Hayakawa *et al.*, 1991). A summary of isolation procedures developed for the selection of *Micromonospora* is given in Table 4.

1.6 The growth and survival of microorganisms in the terrestrial environment

Soils and sediments are comprised of five major components: mineral and organic matter, water, air and living organisms (reviewed by Alexander, 1977; Gray and Williams, 1971; Lynch, 1979; Nedwell and Gray, 1987; Stotzky, 1989) which exist together in varying proportions. Generally soils and sediments represent environments in which microorganisms, despite the possible presence of large amounts of organic matter, are energy or nutrient limited (Nedwell and Gray, 1987). Both environments are subject to significant variations in pH, temperature, ionic concentration and nutrient availability, leading to the development of environmental heterogeneity resulting in diversity of the microbial population.

1.6.1 Microorganisms in the soil

The soil represents a source of a diverse range of microorganisms. Indeed, many species and genera exist in this environment, some

obviously being more common than others. More than 40 genera of aerobic actinomycetes have been isolated from soil (Goodfellow and Cross, 1984) and representatives of new genera are isolated and described every year.

The presence and activity of a given genus, species or subspecies in any terrestrial environment is dependant on many features, some of which have been outlined above. These ecological factors naturally differ quite markedly from soil to soil operating very much together as a system rather than individually. One important factor influencing microbial metabolic activity in soil is temperature, which is affected primarily by sunlight. Temperature varies significantly with latitude, climatic conditions and altitude, the diurnal fluctuation at the soil surface, sometimes being as great as 35°C. The pH of soil is also important when considering the diversity of microorganisms in the terrestrial environment. Due to the leaching of base metals, CO_2 production by microbes and the formation of organic acids by dead plant material, soil has a tendency to be acidic rather than alkaline which has obvious implications for the microbial community.

The critical requirement, however, for the growth of most heterotrophic microorganisms in the soil is usually the supply of nutrients, the most growth limiting substrate generally being nitrogen. There are many possible sources of nutrients including dead plant and animal material, leaf litter and root exudates, all of which can provide microorganisms with a variety of energy sources (Chapman and Gray, 1986; Zeph and Casida, 1986).

1.6.2 *Micromonospora* in soil

The abundance of *Micromonospora* in the soil has been widely reported (Section 1.3.2), and it is in this environment where they form, along with their counterparts the *Streptomyces*, a significant part of the microbial population.

It is known that active growth in most actinomycetes is limited to sites of nutrient availability (Goodfellow and Simpson, 1985; Kutzner, 1981) and, that where organic substrates are colonised by hyphal growth, they are rapidly replaced by spores following nutrient depletion (Lloyd, 1969). The successful survival and subsequent growth of actinomycetes in soil, therefore, is due to the resistance of their spore population and its replenishment by germination followed by growth and eventual sporulation at sites of high nutrient concentration (Lloyd, 1969).

It is thought, although little work has been done to confirm it, that *Micromonospora*, like *Streptomyces* (Lloyd, 1969; Mayfield *et al.*, 1972; Skinner, 1951) and other spore-forming actinomycetes, exist in the soil mainly as spores. If this is the case, the survival and subsequent growth of *Micromonospora* in soil is likely to be largely influenced by the properties of their spores, details of which are given in Section 1.4.2.

Although work has been done on the biochemical and physiological properties of *Micromonospora* spores, little however, has been done to clarify the nature of their growth and survival in soil systems. One aim of the present study, therefore, was to study the fate of two species of *Micromonospora* in soil (Chapter 3).

1.6.3 Bacteriophage in soil

The abundance of any species in the soil is determined, in part, by the resources available to it. For bacteriophage, this resource is primarily the presence of susceptible host species (Williams *et al.*, 1986). For a phage to survive, therefore, a balance must exist between itself and the host species; the bacteriophage must complement the life style of it's host so as not to eliminate it (Alexander, 1981). For example bacteriophage with copiotrophic hosts undergo rapid replication during short periods of bacterial growth, thereby mimicing their hosts to ensure continued survival (Williams *et al.*, 1986). Phage may be involved in either virulent or lysogenic associations with their host. However, as current detection procedures rely on the virulent reaction of the virus with it's host, no indications are available of the proportions that these two associations exist in the natural environment (Reanney, 1968).

The survival of free phage in soil systems may be affected by a number of factors. Williams and Lanning (1984) found that sterilisation of soil along with increased temperature and moisture content seemed to decrease the survival of free phage, whilst Cheo (1980), found that desiccated soil quickly destroyed virus particles. Reanney and Marsh (1973) have suggested that if phages occurred in soil at 0.1% of the titre obtained under optimum laboratory conditions, they must be one of the most abundant genetic objects in that habitat. Phages are, therefore, potential agents of prime ecological importance. Not suprisingly, therefore, phages now play an increasingly important role in biochemical, genetic and taxonomic research.

Many methods have been developed for the direct isolation of phage from soil (Lanning and Williams, 1982) including the enrichment of soil with either the potential host (Willoughby *et al.*, 1972; Zachary,

1974), with nutrients (Germida and Casida, 1981) or with both (Retinskaya and Rautenstein, 1960). However, due to problems such as either host inactivity (Gray and Williams, 1971) or phage inactivity, the direct isolation of phages can often yield low titres (Tan and Reanney, 1976). Many methods, however, have been developed by Lanning and Williams (1982) to overcome some of these problems. In addition, the successful isolation of phage is dependant on soil pH. Sykes *et al.* (1981) were unable to detect phage in soils below pH 6.1, phage survival was also found to be negligible in acidic soils. Phage do, however, exert some degree of resistance to acidity when adsorbed to clay particles (Bystricky *et al.*, 1985).

Although bacteriophage capable of infecting actinomycetes have been studied for over 50 years (Wellington and Williams, 1981; Anne *et al.*, 1990), the focus has been primarily on phages attacking streptomycetes. Until recently little published information on phage infecting *Micromonospora* has been available (Bradley *et al.*, 1961) consequently few have been described and knowledge concerning them is poor. Phage that have been described in more detail include a number of temperate actinophage capable of infecting *Micromonospora purpurea* (Kikuchi and Perlman, 1977, 1978; Tilley *et al.*, 1990a,b) as well as a number of other species of *Micromonospora* (Caso *et al.*, 1986).

1.6.4 Problems in studying microbial ecology in soil

One of the greatest problems still faced by microbial ecologists is the ability to isolate the entire population present in any natural habitat despite the increasing number of methods available for this purpose.

Most techniques used in microbial ecology studies involve the isolation and culturing of microorganisms. Although these have the

advantage of being rapid and relatively reproducible to use, such methods have the disadvantage of relying on the ability of the target organism to grow following extraction. As only viable cells can be cultured, those microbes that are viable but non-culturable are undetectable (Rozack and Colwell, 1987). Viable counts of bacteria can be as low as 2% of the values obtained by direct counting (Bakken, 1985) indicating that only a small fraction of the microbial community can be isolated using viability based methods. Consequently, an increasing number of techniques are now available for the direct counting of microorganisms which allow enumeration without cultivation of a sample; examples include the staining of cells with fluorescent dyes (Porter and Feig, 1980) and the use of specific antibodies (Brookner and Stokes, 1990). Such methods are discussed in more detail in Section 3.1.2.

In addition, recent years have seen the development of molecular biological techniques to study recalcitrant bacterial populations in natural systems, again without their isolation or cultivation (Holben *et al.*, 1988; Sommerville *et al.*, 1989). Methods have been developed which allow the rapid recovery of DNA and RNA from highly heterogeneous environmental samples (Sayler *et al.*, 1989), thereby representing the microbial community genome (Ogram and Sayler, 1988; Sayler and Stacey, 1986). This genetic material can subsequently be used to measure the quantitative abundance of genes associated with community structure and function and additionally DNA based reassociation kinetics can be used to estimate microbial diversity and species difference among communities (Ogram and Sayler, 1988; Torsvik *et al.*, 1990a,b).

An alternative to DNA as the target for identification of microbial communities is the use of rRNA in hybridization experiments (Giovannoni *et al.*, 1988; Stahl *et al.*, 1988). Much work has been done on the development of synthetic oligonucleotides for use as specific

probes in hybridisation experiments against 16S rRNA sequences in order to discriminate between different strains or species (Hahn *et al.*, 1989; Hahn *et al.*, 1990).

1.7 Bacterial systematics

Classifications are constructed to serve a variety of purposes. Phylogenetic, genealogical or cladistic classifications, often based on sequence data, attempt mainly to trace evolutionary pathways, whilst taxonomic classifications aim to make generalisations, with respect to a wide range of characters, about their members (Sneath, 1962); the final result often being identification.

Methods available to characterise, classify and subsequently identify microorganisms have evolved significantly over recent years (Cain, 1962; Cowan, 1962; Sneath, 1962), accompanying developments in biology and other scientific disciplines such as chemistry and physics. The application of mathematical, biological and chemical methods have provided invaluable data for the assignment of microorganisms to species, generic and suprageneric groups (Alderson, 1985; Austin and Priest, 1986; Sneath and Sokal, 1973).

1.7.1 Numerical taxonomy

Numerical taxonomy may be defined as the computer assisted numerical evaluation of similarities between groups of organisms and the ordering or arranging of these groups into clusters or taxa based on these similarities (Jones and Sackin, 1980). By quantitatively defining these clusters and assessing their relationships to each other a classification can eventually be constructed.

Although numerical taxonomic methods, which are based primarily on assessing phenetic similarities between organisms, have been used to classify many major groups of organisms, they have been found to be of most use in the classification of bacteria. This is perhaps due to the fact that over many years data, based on numerous physiological, biochemical, chemical, serological and genetic tests, has become available for the classification of bacteria making its evaluation, other than by computer, difficult if not impossible.

Numerical taxonomic methods were first applied to bacteria over 30 years ago by Sneath (1957). Since that time numerous publications have appeared on numerical taxonomic studies of a wide range of bacteria (Athalye *et al.*, 1985; Goodfellow *et al.*, 1990; McCarthy and Cross, 1984; Schofield and Schaal, 1981; Table 5). Many techniques have been used by numerical taxonomists for establishing a bacterial classification or identification ranging from classification by pyrolysis mass spectrometry (Gutteridge *et al.*, 1985) to classification by protein electrophoresis (Kersters, 1985). Traditionally however, numerical classifications have been mainly based on results from morphological and physiological tests assessing microscopic, nutritional and metabolic characters.

All numerical taxonomic studies, for whatever purposes they are designed, should be planned very carefully, the strength of any technique being dependant on the ability of computers to handle large amounts of data on large numbers of strains. The steps taken in a numerical taxonomic study are outlined in Section 4.1.1.

1.7.2 Phenotypic methods used in bacterial systematics

In 1971 Stanier pointed out that the question of relatedness can be probed at two fundamentally different levels; firstly at the strictly genetic level where general organisation and base sequence homologies of the DNAs or RNAs of different organisms are compared, and the epigenetic, or phenotypic level, where properties that are expressed at the level of translation are compared.

Since the seventies the genotypic study of microorganisms has attracted an increasing amount of attention, leading many to believe that future work on the taxonomy and phylogeny of prokaryotes will eventually solely reflect their genotypic makeup. Genotypic relationships have been assessed at many levels and are discussed in greater detail in Section 1.8.3.

However, at the present moment in time, it is completely impracticable to define genera solely on the basis of genotypic or phylogenetic data. Bacterial genera need to be characterised and subsequently classified by the use of phenotypic properties, even if the choice of phenotypic markers might change as a result of development of improved methods. A degree of flexibility is always necessary in any classification study, and in cases where there is a disparity between phylogenetic and phenotypic data, although priority is generally given to the latter, further detailed phenotypic studies are usually encouraged so that, if possible, the classification reflects phylogenetic relationships (Murray *et al.*, 1990).

Our increasing knowledge and development of techniques to assess morphological and physiological characters, as well as the chemical composition of cell constituents, in recent years has

considerably improved the phenotypic classification and identification of prokaryotes, in particular the actinomycetes.

1.7.2(a) Chemotaxonomic methods

Our increasing knowledge of the chemical composition of cell constituents has provided invaluable data for the improved classification and identification of prokaryotes. Chemotaxonomy may be described as the study of chemical variation in living organisms which may subsequently be used to classify them. However, when using chemical properties to distinguish between microorganisms it is important that any variations that exist are shown to be an expression of innate differences and not just due to inconsistences in cultivation conditions, as it is well known that bacterial cell wall contents are often affected by growth conditions such as media and pH (Goodfellow *et al.*, 1988).

The application and development of chemotaxonomic methods have profoundly influenced developments in prokaryotic systematics at both sub- and supra- generic levels. Indeed, in recent years chemotaxonomic methods have provided invaluable data for the classification of a wide range of actinomycete genera (summarised in Table 6), examples include analyses based on cell wall chemotype, whole sugar type, peptidoglycan and phospholipid type and fatty acid analysis (Goodfellow *et al.*, 1988).

More recent chemotaxonomic techniques have included the classification of prokaryotes on the basis of electrophoretic whole cell protein patterns (reviewed by Jackman (1987) and Kersters (1985)) and electrophoretic enzyme patterns (Selander *et al.*, 1986). More distant taxonomic relationships can be measured by analysis of ribosomal proteins (Bock, 1985; Ochi, 1989).

Table 5 **Methods used in numerical classification of bacteria**

Method	Microorganism	Reference
Morphological and physiological characterisation	*Streptomyces*	Williams *et al.* (1983)
	Nocardia	Orchard & Goodfellow (1980)
	Actinomyces	Schofield & Schaal (1981)
	Actinomadura & *Nocardiopsis*	Athalye *et al.* (1985)
	Thermomonospora	McCarthy & Cross (1984)
	Micromonospora	Bibikova *et al.* (1989)
Substrate utilisation	*Pseudomonas*	Sneath *et al.* (1981)
Morphological, physiological and chemotaxonomic characterisation	*Actinoplanes*	Goodfellow *et al.* (1990)
Electrophoretic protein patterns	*Campylobacter*	Owen *et al.* (1989)
	Zymomonas & *Agrobacterium*	Swings *et al.* (1976)
Fatty acid analysis	*Streptococcus*	Drucker (1974)
	Rhizobium	MacKenzie *et al.* (1978)
	Nocardia	Bousfield *et al.* (1983)
Cell wall analysis	*Nocardia*	Alderson *et al.* (1981)
Pyrolysis mass spectrometry	*Serratia* & *Acinetobacter*	MacFie & Gutteridge (1982)

Table 6 **Chemotaxonomic criteria used in the classification of actinomycetes**

Chemotaxonomic criteria	Genus	Reference
Whole cell sugar pattern	*Micromonospora* *Actinoplanes* and other actinomycetes	Lechevalier & Lechevalier (1970)
	Catellatospora	Asano *et al.* (1989)
Fatty acid analysis	*Micromonospora* and other actinomycetes	Kroppenstedt & Kutzner (1978) Kroppenstedt (1979)
	Nocardia & *Rhodococcus*	Minnikin & Goodfellow (1980)
	Saccharopolyspora	Embley *et al.* (1987)
Cell wall chemotype	*Micromonospora*	Kawamoto *et al.* (1981)
	Actinoplanes	Horan & Brodsky (1986) Tille *et al.* (1982)
	Streptomyces, *Micromonospora* & other actinomycetes	Lechevalier & Lechevalier (1970)
	Coryneforms	Keddie & Bousfield (1980)
Phospholipids	*Micromonospora*, *Streptomyces* and other actinomycetes	Lechevalier *et al.* (1977, 1981)
	Glycomyces	Labeda *et al.* (1985)
	Actinoplanes	Hasegawa *et al.* (1979)
	Ampullariella	Stackebrandt & Kroppenstedt (1987)
Mycolic acids	*Mycobacterium*, *Nocardia*, *Rhodococcus*, *Micromonospora*, *Actinoplanes* and others	Minnikin & O'Donnell (1984)
Menaquinones	*Micromonospora*, *Streptosporangium*, *Actinoplanes* and others	Collins *et al.* (1984)
Electrophoretic protein patterns	*Nocardia*	Mauff *et al.* (1981)
	Bifidobacterium	Biavati *et al.* (1982)
	Corynebacterium	Carlson & Vidaver (1982)

Table 6 continued

Chemotaxonomic criteria	Genus	Reference
DNA homology	*Nocardia*	Mordarski *et al.* (1978)
	Streptomyces and other actinomycetes	Stackebrandt *et al.* (1981)
Ribosomal protein heterogeneity	*Streptomyces*	Ochi (1989)
Cytochrome analysis	*Streptomyces* and other actinomycetes	Meyer & Jones (1973)

1.7.2(b) Other methods

Bacteriophages have been used for some years to investigate relationships between prokaryotes. The susceptibility of bacteria to bacteriophages depends on the presence of specific receptor structures in the surface of the cell to which phage can adsorb. On the basis of their interaction with known phages, strains of bacteria can be classified into groups.

Actinophages have been used widely to investigate relationships between different genera of the Actinomycetales (Bradley and Anderson, 1958; Bradley *et al.*, 1961; Wellington and Williams, 1981) as well as other Gram-positive bacteria (Loessner, 1991). Indeed, phages which exhibit polyvalency to a number of species are a useful tool in the identification of a wide range of prokaryote genera, particularly the actinomycetes (Prauser, 1976b; Williams *et al.*, 1980). Some of the earliest, most extensive studies of phage susceptibility among actinomycete genera were those of Prauser and Falta (1968), who tested 19 phages against members of 20 actinomycete genera and found that phages were active only on hosts with identical cell wall chemotypes. Polyvalent phages isolated to *Streptomyces* species therefore lysed only members of wall chemotype I genera (Prauser, 1976a). Further studies on the taxonomic implications of actinophage host ranges have been completed by Wellington and Williams (1981) and Williams *et al.* (1980).

The resistance or susceptibility of microorganisms to inhibitors such as bismuth sulfite and bile salts are also generally stable characters for use in characterisation studies. Additionally, recent work has focused on patterns of susceptibility to antibiotics. Many actinomycetes have

distinctive antibiosis and resistance patterns which have been used in classification studies (Phillips, 1992; Phillips *et al.*, 1992; Wellington *et al.*, 1987). Characterisation by resistance to a wide variety of macrolide and aminoglycoside antibiotics has shown that many actinomycetes, in particular streptomycetes, have individual patterns of multiple resistance (Fujizawa and Weisblum, 1981). The use of antibiotic resistance as a tool in classification has become more widespread following studies reporting the genetic linkage of biosynthesis and resistance genes (Chater and Bruton, 1985; Murakami *et al.*, 1986; Skeggs *et al.*, 1987); indeed an increasing number of studies are now becoming concerned with the distribution of antibiotic production and resistance pheno- and genotypes in natural populations.

1.7.3 Genotypic methods used in bacterial systematics

Properties of genetic material have long been used as systematic criteria, providing insights into the phylogenetic relationships of prokaryotes. Initially, organisms were characterised by guanine-plus-cytosine contents of their DNA (Lee *et al.*, 1956; Mandel *et al.*, 1968; Marmur and Doty, 1962), this however, tends to permit only a very general grouping of strains, species or genera. Other methods frequently used are DNA-DNA hybridisation (Doty *et al.*, 1960; Schildkraut *et al.*, 1961; Stanzak *et al.*, 1990) and rRNA-DNA hybridisation (Gillespie and Spiegelman, 1965; Stackebrandt *et al.*, 1981), both of which allow differentiation of closely related organisms. In addition, the specific fragmentation of DNA by restriction endonucleases and the separation of the fragments by agarose gel electrophoresis have provided useful tools for DNA characterisation (Crameri *et al.*, 1983).

Many studies on the genetic relationships of the actinomycetes have been performed by using DNA-DNA reassociation techniques (Bradley *et al.*, 1973; Farina and Bradley, 1970). These methods, however, can detect only the closest genealogical relationships. Most studies have included *Streptomyces* (Okanishi *et al.*, 1972; Tewfik and Bradley, 1967) although a number have been published including members of other actinomycete genera (Farina and Bradley, 1970).

After the conserved character of the nucleotide sequences of ribosomal RNAs was realised (Doi and Igarashi, 1965; Moore and McCarthy, 1967), reassociation experiments in which rRNA's and rRNA cistrons were used were introduced in taxonomic studies (Moore and McCarthy, 1967; Pace and Campbell, 1971a, 1971b). The conserved primary structures of these molecules, with their regions of high variability along with their genetic stability and ubiquity have therefore made them a superior choice for phylogenetic studies.

It is the opinion of many workers (Fox and Stackebrandt, 1987) that the best methods for exploring phylogenetic relations among microorganisms are the use of 16S rRNA oligonucleotide cataloguing and 5S rRNA complete sequencing (Stahl *et al.*, 1985). Indeed, as a result of the application of these methods, amendments have been made to the classification of several groups of bacteria (Wellington *et al.*, 1992) and detailed hierarchical structures of many bacterial groups are now available.

1.7.4 Methods used in actinomycete systematics

Since the mid 1940's, when interest in the bioactive nature of their products began, the actinomycetes have attracted much attention . This subsequently lead to a quest for better methods to characterise these

microorganisms, perhaps the most significant outcome of this being the development of the International Streptomyces Project which began in 1964 (Shirling and Gottlieb, 1976) and led to publications redescribing over 400 cultures.

Numerous criteria have been used in the classification of the actinomycetes, some of which are summarised in Table 6. Many numerical taxonomic studies have used traditional morphological, physiological and biochemical tests as tools for classification (Williams *et al.*, 1983, Table 5). More recently developed methods, which have been useful in the classification of actinomycete genera, include the use of chromogenic and fluorogenic substrates for detecting enzyme activity (Goodfellow *et al.*, 1987) and the use of cytochrome patterns using spectrophotometry (Jones, 1980). Protein profiling has also proved to be an important tool in taxonomic studies allowing comparisons to be made of banding patterns produced after electrophoresis of soluable cellular proteins (Kersters, 1985). Ochi (1989) used a similar technique by producing ribosomal protein patterns using two-dimensional gels inorder to illustrate specific profiles within *Streptomyces* species.

DNA hybridisation studies on actinomycetes have been carried out by various workers. Mordarski *et al.* (1985) found partial congruence between his study of *Streptomyces albidoflavus*, *S. halstedii* and *S. griseus*, using DNA hybridisation studies and the study of Williams *et al.* (1983) using phenotypic methods of characterisation. Similar studies, however, with contrasting results, have been carried out by Labeda and Lyons (1991) who found that DNA hybridisation values spanned a wide range (40%-60%) and were inconsistant with other strains belonging to the same species. DNA hybridisation studies have also been carried out with *Micromonospora* (Stackebrandt *et al.*, 1981; 1983) which have

proved useful in elucidating generic relationships, supporting previously stated chemotaxonomic relationships.

Ribosomal RNA sequencing and cataloguing studies using many actinomycetes, including *Micromonospora*, have provided workers with information concerning both 16S and 5S rRNA sequences which have proved invaluable in recent classification studies (Fox and Stackebrandt, 1987; Stackebrandt *et al.*, 1981, 1983, Section 1.3). Such studies have led to the production of a number of species specific oligonucleotide probes for representatives of many actinomycete genera including *Streptomyces* (Kemmerling *et al.*, 1989; Stackebrandt and Charferitag, 1990; Witt *et al.*, 1990) and *Frankia* (Hahn *et al.*, 1990), providing new methods of *in situ* identification. Studies to date have concentrated on 16S rRNA sequences in three regions of hypervariability, however Stackebrandt *et al.* (1991a,b) reported that only one third of strains tested could be distinguished using this method.

1.8 Diversity studies

The soil and aquatic environments are natural reservoirs for a diversity of prokaryotic organisms, many of which play major roles in the nutrient cycles of the biosphere. Knowledge of such microbial processes has increased significantly in recent years, in part, due to the development of new molecular biological techniques. One area, however, that has lagged behind in this field is a knowledge of the diversity of the microorganisms that make up these natural communities. Such information is necessary in order to develop an understanding of natural variability; a subject at the heart of many basic and applied questions.

In order to study microbial communities either on a macro or micro level, it is often firstly necessary to produce a comprehensive "species list" for each sample. Perhaps the simplest, most widely used methods to accomplish such tasks, remain by classical identification techniques. Assessing the diversity of microbial groups, such as the actinomycetes, in the natural environment by traditional methods present a number of difficulties. One major problem, discussed in more depth in Section 1.6.4, is that most techniques require the direct culturing of organisms, of which only a small number are actually culturable (Ferguson *et al.*, 1984). Although actinomycetes can be cultured on normal bacteriological media, many selective methods have been developed and are used regularly (Williams and Cross, 1971). The results of such methods when assessing microbial diversity, must however be interpreted in the knowledge of their effects.

The classical approach to the study of species variation in the natural environment, following isolation, requires the identification of bacteria. Comprehensive identification typically requires completing a number of physiological, morphological and biochemical tests for each organism. Many such probabilistic identification matrices have been developed for a wide range of actinomycete genera including *Streptomyces* (Williams *et al.*, 1983), *Nocardia* (Orchard and Goodfellow, 1980) and *Actinomadura* (Athalye *et al.*, 1985). Little work, however, has been done to develop a similar rapid identification system for *Micromonospora*.

Complementing the above classical approach, much work has been done to study natural microbial species variations at the DNA level (Fuhrman and Lee, 1989; Torsvik *et al.*, 1990a,b). The study of naturally occurring microorganisms at the genotypic level has provided an invaluable complement to phenetic studies, particularly for elucidating

community composition. Sequences from rRNA have been particularly helpful for this purpose. The introduction of such molecular biological techniques to characterise unidentified microorganisms from natural habitats was pioneered by Pace *et al.* (1986), using 5S rRNA extracted and sequenced directly from the organisms. The small size, however, of these molecules has allowed only limited sequence comparisons to be made. The larger 16S rRNA molecules, consequently, have proved more useful for these purposes as they contain "universally conserved" regions that allow for selection and amplification. The strategy of polymerase chain reaction (PCR)-mediated amplification of 16S rRNA genes, using either rRNA or rDNA isolated from the natural habitat, in recent years has provided an invaluable approach to the study of microbial ecology (Olsen *et al.*, 1986; Steffan and Atlas, 1991). PCR is generally followed by gene cloning, sequencing and comparative data analysis allowing insights to be gained into the compositions of microbial communities. Although such strategies have been used successfully on samples from aquatic environments (DeLong *et al.*, 1989; Fuhrman and Lee, 1989), until recently, little had been done to test them on samples from terrestrial environments (Liesack and Stackebrandt, 1992).

The above techniques are primarily aimed at obtaining sequence data from otherwise uncharacterised organisms, complementary to this, however, is the search, by the use of taxon-specific nucleic acid probes, for organisms with known sequences of interest (Pace *et al.*, 1986). Such methods require either bulk extracted nucleic acid or single cells as samples for probing.

Many methods have been developed for the extraction of good quality, representative DNA from aquatic (Fuhrman *et al.*, 1988; Pace *et al.*, 1986; Somerville *et al.*, 1989) and terrestrial environments (Holben *et al.*, 1988; Torsvik, 1980). Indeed, it is thought that DNA can be

isolated from bacterial fractions from terrestrial environments, containing as much as 80% of the soil bacteria (Holben *et al.*, 1988; Steffan and Atlas, 1988; Torsvik, 1980) providing a potential source of genetic information concerning nonculturable bacteria in soil. Not suprisingly, therefore, such samples are being increasingly used to assess genotypic diversity in natural samples (Torsvik *et al.*, 1990a).

Molecular ecological studies, using species specific probes or sequence analysis on naturally isolated nucleic acids, in marine (Britschgi and Giovannoni, 1991; Giovannoni *et al.*, 1990), thermophilic (Ward *et al.*, 1990; Weller *et al.*, 1991) and terrestrial (Liesack and Stackebrandt, 1992) environments have supported the widely held idea that culturable prokaryotes constitute only a small percentage of the microbial population present in any environment. A recent study by Liesack and Stackebrandt (1992) using 16S rDNA sequencing data along with taxon-specific nucleic acid probes pioneered the study of the diversity of prokaryotes in the terrestrial environment. Using isolated DNA from an Australian soil sample they were able to amplify a large fragment of the 16S rRNA gene which was subsequently used to generate a gene library. Comparative sequence analysis and dot blot hybridisation with defined oligonucleotide probes enabled in depth analysis of the genotypic diversity of a terrestrial environment; a habitat previously uninvestigated by these techniques.

Another approach to assessing genetic diversity has been to study the heterogeneity of DNA isolated from natural samples by thermal denaturation and reassociation techniques. Torsvik *et al.* (1990a and 1990b) have successfully used this approach to gain some measure of the genotypic diversity of bacterial communities in soil. Interestingly, they were also able to show that a good correlation existed between phenotypic and genotypic diversity measurements (Torsvik *et al.*, 1990b). Additionally Lee and Fuhrman (1989) have used DNA hybridisation

techniques to measure the relatedness of species compositions between paired samples from aquatic environments. They were able to use these methods to compare rapidly the species compositions of samples taken from Long Island Sound and the Caribbean and Sargasso Seas - such studies are significant as many important questions can be answered by simply knowing if species composition is different between two samples rather than knowing the composition in detail.

An alternative method frequently used to study the genetic diversity of natural microbial populations is multilocus enzyme electrophoresis. Natural populations of a variety of bacterial species have been studied using such methods including *Escherichia coli* (Selander *et al.*, 1986), *Rhizobium* (Harrison *et al.*, 1989; Young, 1985) and *Pseudomonas* (Vaun McArthur *et al.*, 1988). Findings indicate considerable circumstantial evidence for the existance of a positive relationship between genetic diversity and environmental variability (Vaun McArthur *et al.*, 1988).

CHAPTER 2

The Selective Isolation of *Micromonospora* from the Natural Environment

2.1 Introduction

Micromonospora strains occur in a wide range of environments in which they have the ability to grow on most naturally occurring substrates (Luedmann, 1970). Some form mutualistic associations in the rumen of sheep and in the gut of termites (Pasti and Belli, 1985), but the vast majority of *Micromonospora* are saprophytes in soil, water, composts and other habitats (Hamdi *et al.*, 1980; Williams and Wellington, 1982). These isolates have often been exploited commercially to produce important antibacterial compounds such as gentamicin and sisomicin (Luedmann and Brodsky, 1964; Wagman and Weinstein, 1980).

Micromonospora, next to *Streptomyces*, are the second most frequently isolated of the sporogenous actinomycetes but despite their abundance there is a suprising lack of published infomation concerning the isolation of this important genus. The following study was therefore designed to investigate methods used to selectively isolate *Micromonospora* and to determine if improvements could be made to allow isolation of a wider range of species from the natural environment.

Members of the genus *Micromonospora* occur frequently in soils and in high numbers in aquatic habitats such as lake and river sediments. Their occurrence was first reported in Australian soils (Jensen, 1932) and then in Russian soils (Kriss, 1939). Early investigations using Wisconsin Lake samples indicated that *Micromonospora* comprised 10-50% of the microbial population in the water mass (Colmer and McCoy 1943; Umbreit and McCoy, 1940). Indeed the occurrence of *Micromonospora* in lake systems has been confirmed by workers in many countries (Coberi and Solaini, 1960; Potter and Baker, 1956; Johnston and Cross, 1976; Willoughby, 1969). It has also been shown that *Micromonospora* are

frequently present in water samples from streams and rivers (Burman, 1973), marine environments such as beach sand (Watson and Williams, 1974), deep oceanic marine sediments (Weyland, 1969) and sediments from the White sea and Black sea (Solovieva, 1972; Solovieva and Singal, 1972). In marine environments, recent work indicates that numbers of *Micromonospora* increase in proportion to the depth of the sediment (Jensen *et al.*, 1991; Weyland, 1981; Weyland and Helmke, 1988).

Anaerobic representatives of the genus have been isolated less frequently, however three strains were recovered from the intestinal tract of termites (Hungate, 1946; Sebald and Prevot, 1962) and the rumen of sheep (Maluszynska and Janoto-Bassalik, 1974). These form acid fermentation products but their role in the host has not yet been established.

2.1.1 Enrichment and isolation procedures

Many pretreatments have been applied to enhance recovery of *Micromonospora*. These include the heat treatment of samples at 70°C in a water bath for 10 minutes (Rowbotham and Cross, 1977; Sandrak, 1977) and chlorine treatment with 4μg/l ammonia followed by 2μg/l chlorine for 30 minutes (Burman *et al.*, 1969; Willoughby, 1969). Whilst *Micromonospora* spores are not as resistant to heat as *Bacillus* endospores, they germinate and grow well on defined agar media such as starch-casein (Kuster and Williams, 1974; Williams and Davies, 1965), collodial chitin (Hsu and Lockwood, 1975; Lingappa and Lockwood, 1962) and Kodota's cellulose benzoate medium (Sandrak, 1977).

In addition many workers have used antibacterial antibiotics in the media to select for a wide range of actinomycetes (Athalye *et al.*, 1981;

McCarthy and Cross, 1981; Williams and Davies, 1965). The incorporation of selected antibiotics into the isolation medium has been very effective for the selection of *Micromonospora* (Table 7).

The main aim of the following study was to improve the above published methods, developing effective selective isolation procedures capable of isolating a wide range of *Micromonospora*. The procedures were then used to investigate *Micromonospora* populations in a variety of terrestrial environments.

Table 7. The selective isolation of *Micromonospora* using antibiotics

ANTIBIOTICS	CONC^{n}	REFERENCES
Novobiocin	25-50μg/ml	Sveshnikova *et al.* 1976 Orchard, 1980
Gentamicin	1-10μg/ml	Ivanitskaya *et al.* 1978 Bibikova *et al.* 1981
Tunicamycin	25-50μg/ml	Wakiskaya *et al.* 1982 Ivanitskaya, 1986
Lincomycin	10-20μg/ml	Ivanitskaya *et al.* 1978

2.2 General methods

2.2.1 Bacterial strains

The bacterial strains used in the following study, both *Micromonospora* and *Streptomyces* are summarised in Tables 8 and 9 respectively.

2.2.2 Growth and maintenance of cultures

Micromonospora and *Streptomyces* strains were grown on oatmeal agar. A well-sporulating plate was flooded with 10 ml sterile distilled water (SDW) and the spores scraped off the mycelia with a sterile inoculating loop. The resulting suspension was transferred to a sterile 20ml Universal bottle and vortexed for 1 minute before being filtered through non-absorbant cotton wool. The suspension was then centrifuged for 10 minutes at 3000g in a MSE benchtop centrifuge. The pellet was then vortexed briefly before being mixed with 20% (v/v) glycerol and stored at -20°C. This was then used as an inoculum for the following experiments. Cultures were also stored at 4°C as slope cultures on oatmeal agar.

2.2.3 Media

A list of media, including antibiotics, and their constituents is given in the Appendices 1, 2, 3 and 4, along with the source and type of chemicals used in this study.

All media were made up with double distilled water and sterilised by autoclaving at 121°C for 15 minutes unless otherwise stated.

Table 8. A selection of named and identified isolates and type strains used to investigate heat susceptibility and antibiotic resistance within the genus *Micromonospora*.

1	*M. carbonacea* var. *aurantiaca*	ATCC 27115
2	*M. chalcea* var. *chalcea*	ATCC 12452
3	*M. chalcea*	ATCC 27344
4	*M. fulvopurpurea*	JCM 5696
5	*M. echinoaurantiaca*	JCM 3257
6	*M. purpureochromogenes*	ATCC 27007
7	*M. nigra*	JCM 3328
8	*M. narashinoensis*	IFM 110-76
9	*M. floridensis*	NRRL 8020
10	*M. halophytica*	NRRL 3097
11	*M. globosa*	JCM 3358
12	*M. chalcea* var. *flavida*	ATCC 27084
13	*M. globosa*	NRRL B2673
14	*M. melanosporea*	IFM 12515
15	*M. parva*	NRRL B2676
16	*M. echinospora* var. *pallida*	ATCC 15838
17	*M. megalomicea* var. *nigra*	ATCC 27598
18	*M. chalcea* var. *izumensis*	ATCC 21561
19	*M. sagamiensis*	ATCC 21826
20	*M. echinospora* var. *ferruginea*	NRRL 2995

ATTC: American Type Culture Collection
JCM : Japan Collection of Microorganisms
IFM : International Fermentation Organisation
NRRL: Northern Regional Research Laboratories

Table 9. A selection of isolates used as a control to study antibiotic resistance within the genus *Micromonospora*.

1	*Streptomyces 1*	Warwick soil
2	*Streptomyces 2*	Warwick soil
3	*Streptomyces 3*	Bicutan soil
4	*Streptomyces 4*	Pond sediment
5	*Streptomyces 5*	Warwick soil
6	*Streptomyces 6*	Italian soil
7	*Streptomyces 7*	Warwick soil
8	*Streptomyces 8*	Warwick soil
9	*Streptomyces 9*	Bicutan soil
10	*Streptomyces 10*	Bicutan soil

2.2.4 Isolation of soil bacteria

One gram of soil (wet weight) was transferred to 9 ml of Ringer's solution and shaken at maximum speed for 10 minutes on a Griffin wrist action shaker. The supernatant was serially diluted and 100μl aliquots were aseptically dispensed into Petri dishes (Sterilin) prior to the addition of the appropriate media.

The antifungal agents, nystatin and cycloheximide (Sigma; 50μg/ml; Appendix 4) were added to all solid media, prior to pouring. All plates were incubated at 30°C for 2 to 6 weeks prior to enumeration.

2.3 The isolation of *Micromonospora* from soil

The effects of different isolation methods, and pretreatments on the recovery of *Micromonospora* were studied. In order that a valid conclusion could be reached 15 methods were investigated using a variety of media and pretreatment techniques. Two contrasting soils were chosen for the study enabling a comparison to be made between isolations methods. The soils differed markedly in composition (Section 2.3.1(b)).

2.3.1 Methods

2.3.1(a) Identification of *Micromonospora* isolates

Micromonospora isolates were readily recognised on pour plates. The colonies were a characteristic orange/yellow colour after incubation for 2 weeks at 30°C becoming a darker orange/brown on sporulation.

The surface of the colonies often appeared to be smooth and glistening, with the absence of aerial mycelium.

Putative *Micromonospora* colonies were examined at x200 with a long working distance objective lens for the presence of fine, branching hyphae, averaging 0.5μm in diameter around the colony margin with the characteristic single spores. After 3-4 weeks, colonies growing on isolation plates were enumerated and for the purposes of this study actinomycete colonies on plates were assigned to three major groups on the basis of their morphology.

(i) *Micromonospora* colonies

As described above.

(ii) *Streptomycete*-like colonies

Identified as hard, firm colonies producing unfragmented substrate mycelium, with a moderate to abundant spore mass.

(iii) Others

This group contained *Nocardia* (substrate mycelium soft, orange/pink. Poor to moderate aerial spore mass which was normally white), *Rhodococcus* (soft substrate mycelium, moist bacterial-like appearance, pink/orange colour), bacterial-like actinomycetes (soft substrate mycelium, moist, bacterial-like appearance) and unidentifiable actinomycete colonies. For each isolation method 5 plates were counted, at an appropriate dilution (up to 100 colonies per plate). The mean number of isolates per plate was calculated.

2.3.1(b) Soils

Two contrasting soils were chosen for the study.

(i) Soil A - Soil was taken from a local field site (Cryfield Hall, University of Warwick). Analysis of particle size gave a composition of (percent, dry weight) 63% sand, 18% silt, 14% clay and 5% loss on ignition (Allen *et al.*, 1974). The pH of the fresh soil was 6.5-6.8. For all isolation studies soil was air dried for two weeks at room temperature.

(ii) Soil B - A calcareous soil was taken from a permanent pasture field near Salisbury in Wiltshire. Analysis of particle size gave a composition of (percent, dry weight) 60% sand, 30% silt, 7% clay and 3% loss on ignition (Allen *et al.*, 1974). The pH of the fresh soil was 7.0.

2.3.1(c) Isolation methods

For each of the 2 soil types the following isolation methods were used:

(i) Sedimentation method using arginine glycerol salts medium (AGS)

Arginine glycerol salts has been used widely as a general isolation media for actinomycetes (El-Nakeeb and Lechevalier, 1963). It was used in this study for comparison with other selective media.

The sedimentation method (Makkar and Cross, 1982) involved the shaking of 1 gram of soil suspended in Ringer's solution for 30 minutes on a Griffin wrist action shaker followed by a settling period of 30 minutes. 100μl of the suspension was then removed from the surface layer and used as an inoculum for pour plates (Section 2.2.4).

(ii) Pour plate method using AGS.

For standard pour plate method see Section 2.2.4.

(iii) Tap water agar (pour plate method)

Tap water agar, as with the above, was used for comparative purposes.

(iv) Kadota's cellulose benzoate medium (pour plate method).

Kadota's cellulose benzoate medium has been used as a selective isolation procedure for *Micromonospora* (Sandrak, 1977).

(v) M3 agar medium (pour plate method).

M3 agar has been used extensively for the isolation of actinomycetes (Rowbotham and Cross, 1977).

The following 4 types of media were used for comparative purposes to isolate *Micromonospora* (the pour plate method was used for all media - see Section 2.2.4.).

(vi) Complex carbon/inorganic nitrogen medium (Medium A)

(vii) Complex carbon/organic nitrogen medium (Medium B)

(viii) Simple carbon/inorganic nitrogen medium (Medium C)

(ix) Simple carbon/organic nitrogen medium (Medium D)

(x) Heat treatment 120°C for 30 minutes

(xi) Heat treatment 120°C for 60 minutes

The heat treatment of soil has been frequently used to decrease the numbers of bacteria on actinomycete isolation plates, thereby selecting for rarer genera (Nonomura and Ohara, 1969, 1971). Heat treating soil at 70°C in a water bath for 10 minutes has been shown to be a good

selective method for isolating *Micromonospora* (Rowbotham and Cross, 1977; Sandrak, 1977).

In this study air dried soils were heat treated at 120°C in a dry oven in glass Petri dishes for 30 and 60 minutes. AGS agar was then used as an isolation medium using the standard pour plate method (Section 2.2.4).

(xi) Chitin enrichment of soil (AGS medium)

(xii) Chitin enrichment of soil (Colloidal chitin agar)

The use of chitin to enrich soil prior to isolation was a method developed by Williams and Mayfield (1971) for the selection of *Streptomyces*. This method was tested as a selective isolation procedure for *Micromonospora*.

Crude chitin (from crab shells, Sigma Chemical Company) was added to air dried soil at a concentration 1% (w/w). Soil was then incubated at 55°C for 7 days. The standard pour plate method (Section 2.2.4) was then used for isolation. Two media were used for this study, AGS (El-Nakeeb and Lechevalier, 1963) and colloidal chitin agar (Burman *et al.*, 1969; Hsu and Lockwood, 1975; Lingappa and Lockwood, 1962; Makkar and Cross, 1982).

Colloidal chitin agar

Crude chitin (from crab shells, Sigma Chemical Company) was washed alternately in 1M NaOH and 1M HCl for 24 hour periods on five separate occasions. It was then washed four times with ethanol (95% v/v). 15 g of the purified chitin was treated with 100 ml concentrated HCl and stirred for 20 minutes in an ice bath. The mixture was then filtered through glass wool and the filtrate poured into cold distilled water to precipitate the chitin. The deposit on the filter was further treated with concentrated HCl and the process repeated until no more precipitate was

obtained when the filtrate was added to cold water. The colloidal chitin was allowed to settle overnight and the supernatant decanted. The remaining suspension was neutralised with NaOH to pH. 7.0 and the precipitated chitin centrifuged, washed and stored as a paste at 4°C (Burman *et al.*, 1969).

Hsu and Lockwood (1975) found that colloidal chitin agar showed significant selectivity superior to that of several other media for isolating *Micromonospora* from water and soil, by favouring these organisms and suppressing development of most bacteria and fungi. Consequently it was selected for this study as a comparative medium to AGS.

(xiii) Chelex method (untreated slurry)

(xiv) Chelex method (dried slurry)

The Chelex extraction method (Herron and Wellington, 1990) is based on the concentration of soil microflora. The procedure makes use of an iminodiacetic ion exchange resin to disperse soil particles and allow greater access for an eluent to remove any actinomycetes present. Subsequently, the organisms are concentrated by differential centrifugation.

Soil (100 g) was transferred to a Beckman 500 ml centrifuge pot, to which were added 20 g Chelex-100 and 100 ml 0.1% (w/v) sodium deoxycholate/ 2.5% (w/v) PEG. This was shaken for 2 hours on a Griffin wrist action shaker (4°C, setting 7), before being centrifuged to remove the larger soil particles (2000g, 30 seconds). The supernatant was filtered through a 5 cm diameter metal filter holder (Millipore) and stored at 4°C, whilst the pellet was re-extracted with fresh sodium deoxycholate/ PEG (4°C, 1 hour). After filtration the two supernatants were pooled and spores pelleted by centrifugation (3500g, 15 minutes).

The pellet was resuspended in 9 ml Ringer's solution which was subsequently divided into two. One sample was taken immediately and used for isolation, the other was dried for 12 hours at 65°C.

Selective isolations were made using the pour plate method (Section 2.2.4) with the Complex carbon/inorganic nitrogen medium (Medium A).

2.3.2 Results and discussion

The identification data for isolates obtained from the 15 treatments showed marked differences in the microorganisms recovered from the 2 soils, A and B.

The 2 soils selected for the study differed on the basis of geographical location; one soil was taken from a field site in close proximity to the University of Warwick (soil A), the other soil was taken from a permanent pasture field near Salisbury in Wiltshire (soil B). Analysis of particle size (Allen *et al.*, 1974) indicated that the two soils were quite different. most noticably with respect to clay content (soil A: 14%; soil B: 7%). The 2 soils varied on the basis of numbers of actinomycetes isolated, the variance was not significant enough to hinder any comparisons likely to be made between isolation methods. As only 2 soils were used, it was inappropriate to hypothesize as to why such variance occurred.

Most of the 15 treatments varied significantly in the numbers of *Micromonospora* isolated (Figure 6). A total of 9 media were used altogether in the study. Tap water agar (Figure 1C) was the least successful for the isolation of *Micromonospora*; Soil B yielding none; Soil A yielding only 1% *Micromonospora*, compared to 96% streptomycete-like colonies.

Arginine glycerol salts medium (El-Nakeeb and Lechavalier, 1963) as expected, proved to be a good general medium for the isolation of a wide range of actinomycetes. Using this agar, the pour plate method was significantly more successful for isolating *Micromonospora* than the sedimentation method of Makkar and Cross (1982), (Figures 1A and 1B).

However, of the 9 media used, the combination of a complex carbon source (yeast extract) and an inorganic nitrogen source (sodium nitrate)(Medium A) gave the highest percentages of *Micromonospora* from both soils A (51%) and B (47%)(Figure 3). Consequently it was decided that this medium was superior to Kadota's cellulose benzoate medium and M3 agar (Figures 1D and 2) for the isolation of *Micromonospora*. It was therefore chosen to be used as a selective medium in future studies.

The work of Hsu and Lockwood (1975) indicated that colliodal chitin agar was a highly selective medium for the isolation of actinomycetes. This was based on the widespread ability of actinomycetes to hydrolyze chitin (Jeuniaux, 1955; Reynolds, 1954) coupled with the inability of majority of bacteria and fungi among the soil population to utilize it. However, our results, using this medium (Figures 4A and 4B) in conjunction with the enrichment of soil with chitin (Williams and Mayfield, 1971), indicated that whilst it supported good growth of a wide range of actinomycetes, especially in comparison to AGS (Figure 6B), it was by no means an ideal medium or treatment for the selective isolation of *Micromonospora*.

The use of the 'Chelex extraction' method of Herron and Wellington (1990) provided some interesting results. The method makes use of an iminodiacetic ion-exchange resin (Edwards and Brenner, 1965; Macdonald, 1986) to disperse soil particles and allow greater access for an eluent to remove the actinomycetes present which are then

concentrated by differential centrifugation. The wet slurry obtained at the end of the treatment was divided in 2. The sample which was used immediately for isolation work did not yield *significantly* high numbers of *Micromonospora* (Soil A: 40%; Soil B: 46%) in comparison to other actinomycetes (Figure 5B). Significant numbers of bacteria seemed to be present in slurries from soils A and B (approximately 6×10^6 c.f.u./g soil). However, after drying the slurry for 12 hours at 65°C, the percentage of *Micromonospora* in the sample increased to 80% and 92% for soils A and B respectively (Figure 5A). In addition the numbers of bacteria in the samples decreased dramatically to approximately 1.5×10^5 c.f.u./g soil. The effect of heating the sample for a prolonged period of time seemed therefore, to be greatly beneficial for the selection of *Micromonospora*, working to reduce significantly the relative numbers of other actinomycetes and bacteria in the slurry.

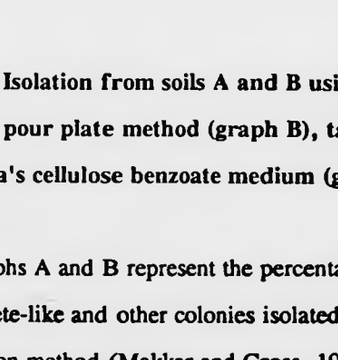
The pretreatment of soil by heating to 120°C for 30 and 60 minutes provided the most conclusive results for the selective isolation of *Micromonospora* (Figures 4C and 4D). Heating dry soil for both amounts of time greatly reduced the survival of other actinomycetes in the soil, thus giving high percentage values for the number of *Micromonospora*. Heating both soils, A and B, at 120°C for 60 minutes yielded 100% values for *Micromonospora*. This indicated that the heat treatment of soil was an ideal method for the selective isolation of *Micromonospora*.

On the basis of the above results the selection of *Micromonospora* using heat treatment was explored further in Section 2.4.

Figure 1. Isolation from soils A and B using a sedimentation method (graph A), pour plate method (graph B), tapwater agar (graph C) and Kadota's cellulose benzoate medium (graph D)

Graphs A and B represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using a sedimentation method (Makkar and Cross, 1982) and a pour plate method with AGS medium, respectively.

Graphs C and D represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using tap water agar and Kadota's cellulose benzoate medium, respectively.



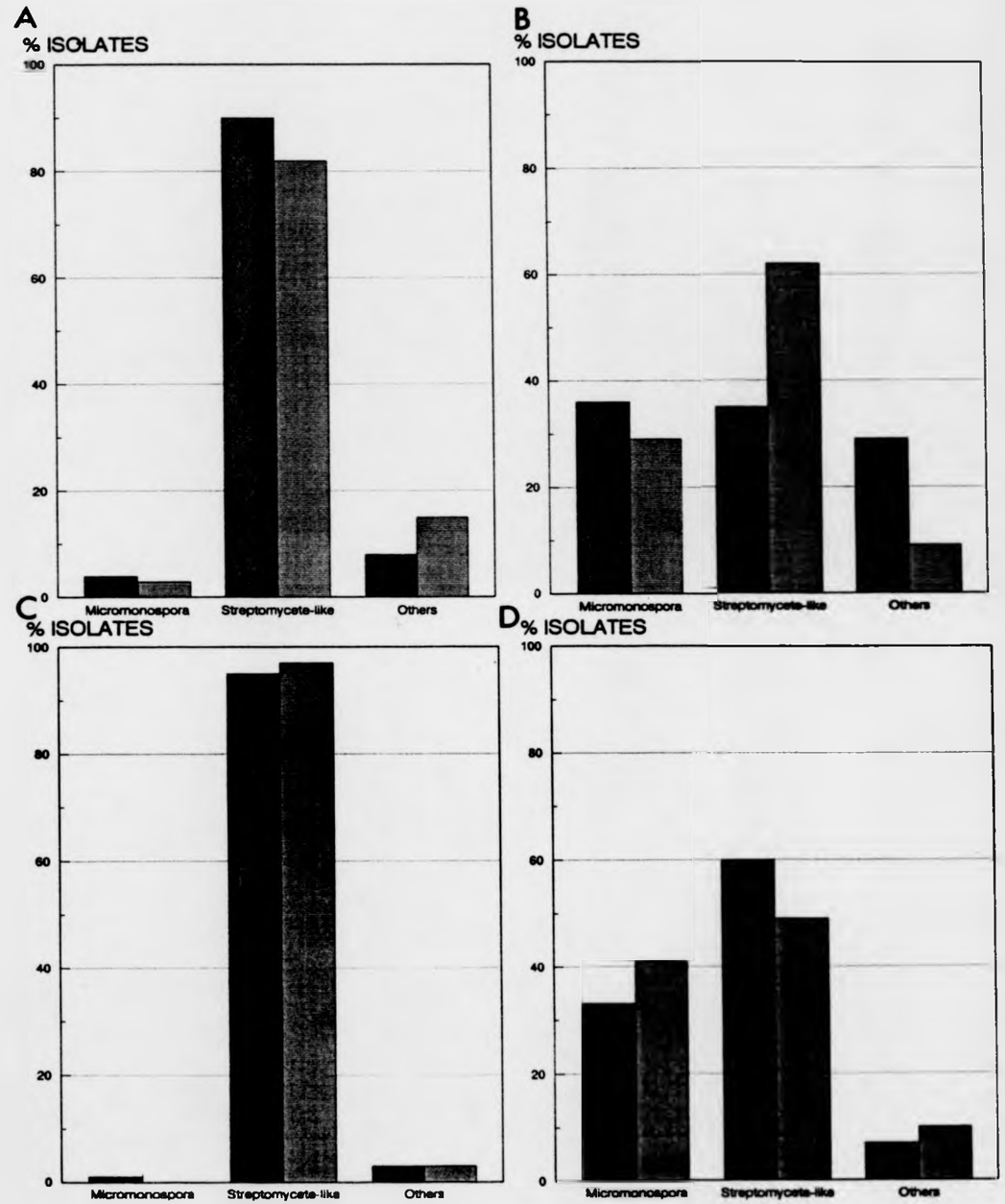
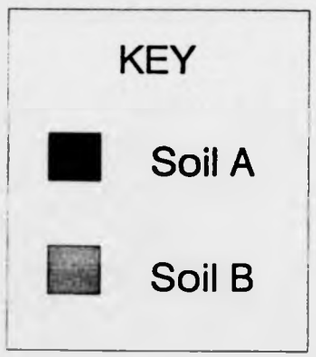


Figure 2. Isolation from soils A and B using M3 agar

Figure 2 represents the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using M3 agar.



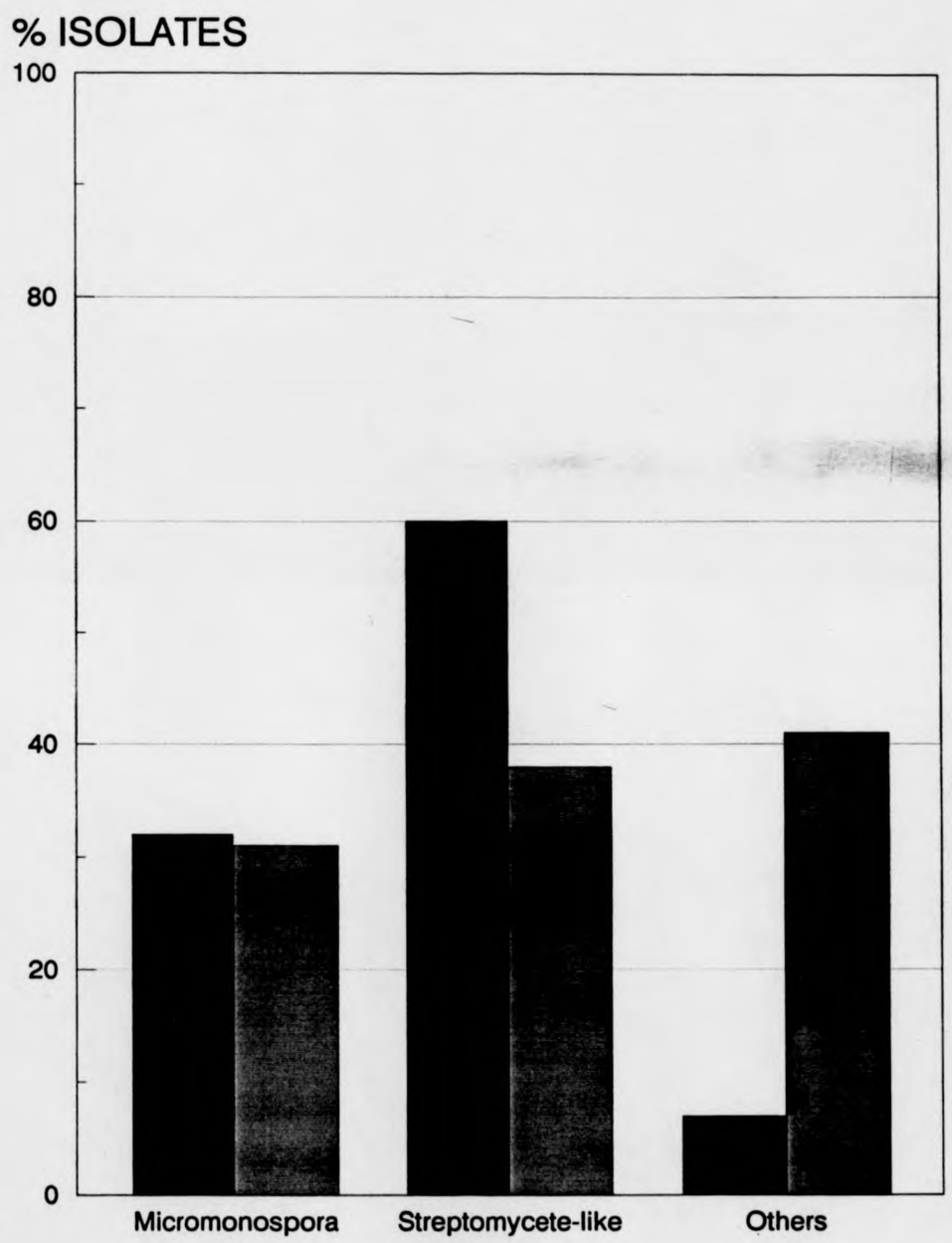
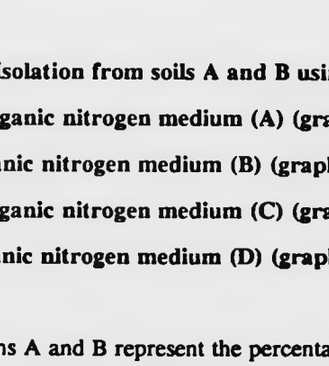


Figure 3. Isolation from soils A and B using a complex carbon/inorganic nitrogen medium (A) (graph A), a complex carbon/organic nitrogen medium (B) (graph B), a simple carbon/inorganic nitrogen medium (C) (graph C) and a simple carbon/organic nitrogen medium (D) (graph D).

Graphs A and B represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using a complex carbon/inorganic nitrogen medium (A) and a complex carbon/organic nitrogen medium (B), respectively.

Graphs B and C represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using a simple carbon/inorganic nitrogen medium (C) and a simple carbon/organic nitrogen medium (D), respectively.



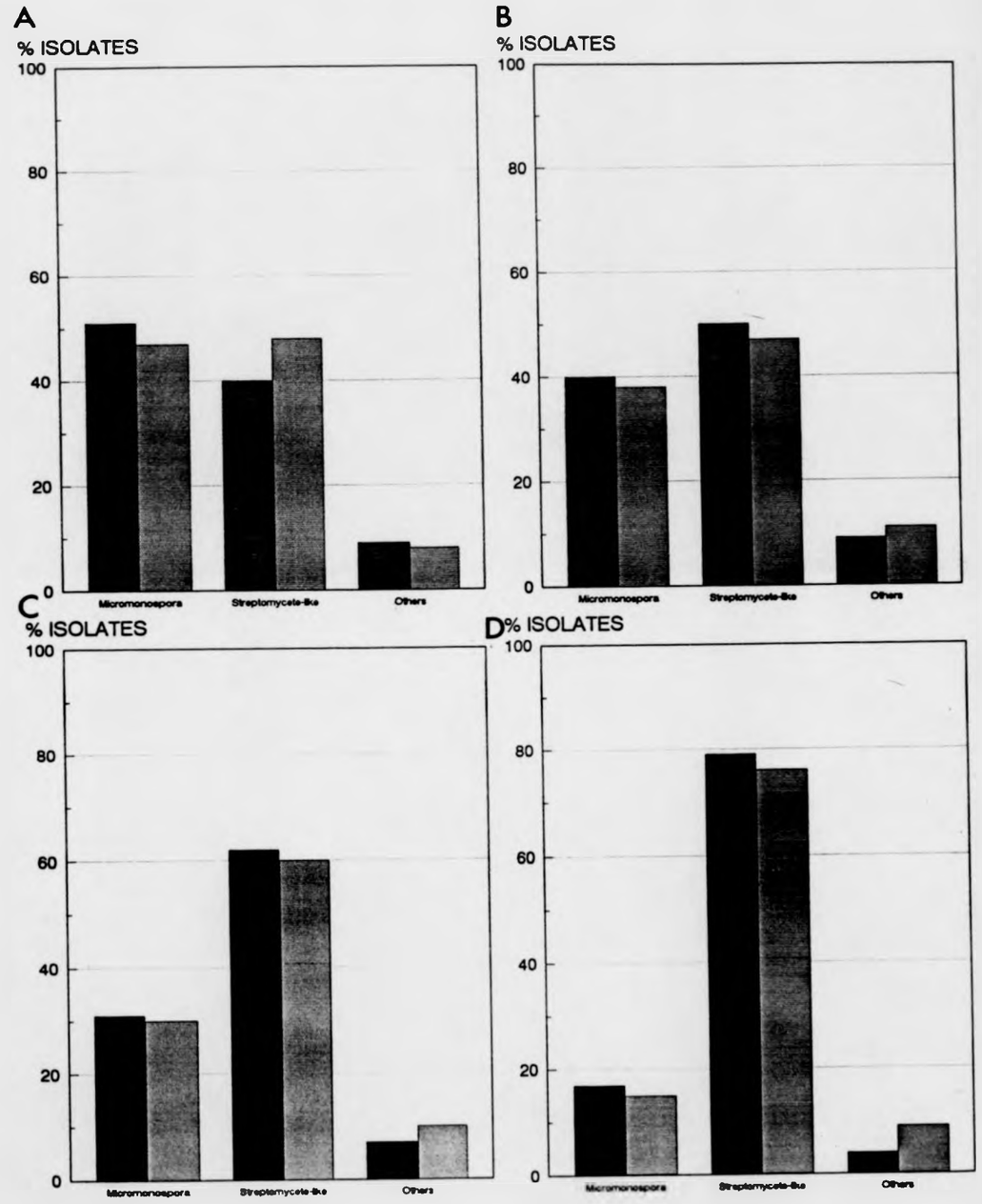
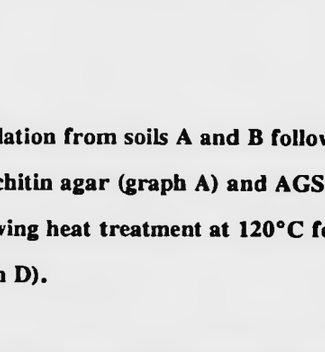


Figure 4. Isolation from soils A and B following chitin enrichment, using collidal chitin agar (graph A) and AGS medium (graph B) and isolation following heat treatment at 120°C for 30 (graph C) and 60 minutes (graph D).

Graphs A and B represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B after chitin enrichment, using collidal chitin agar and AGS medium, respectively.

Graphs C and D represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B after heat treatment at 120°C for 30 and 60 minutes, respectively. AGS medium was used in this study.



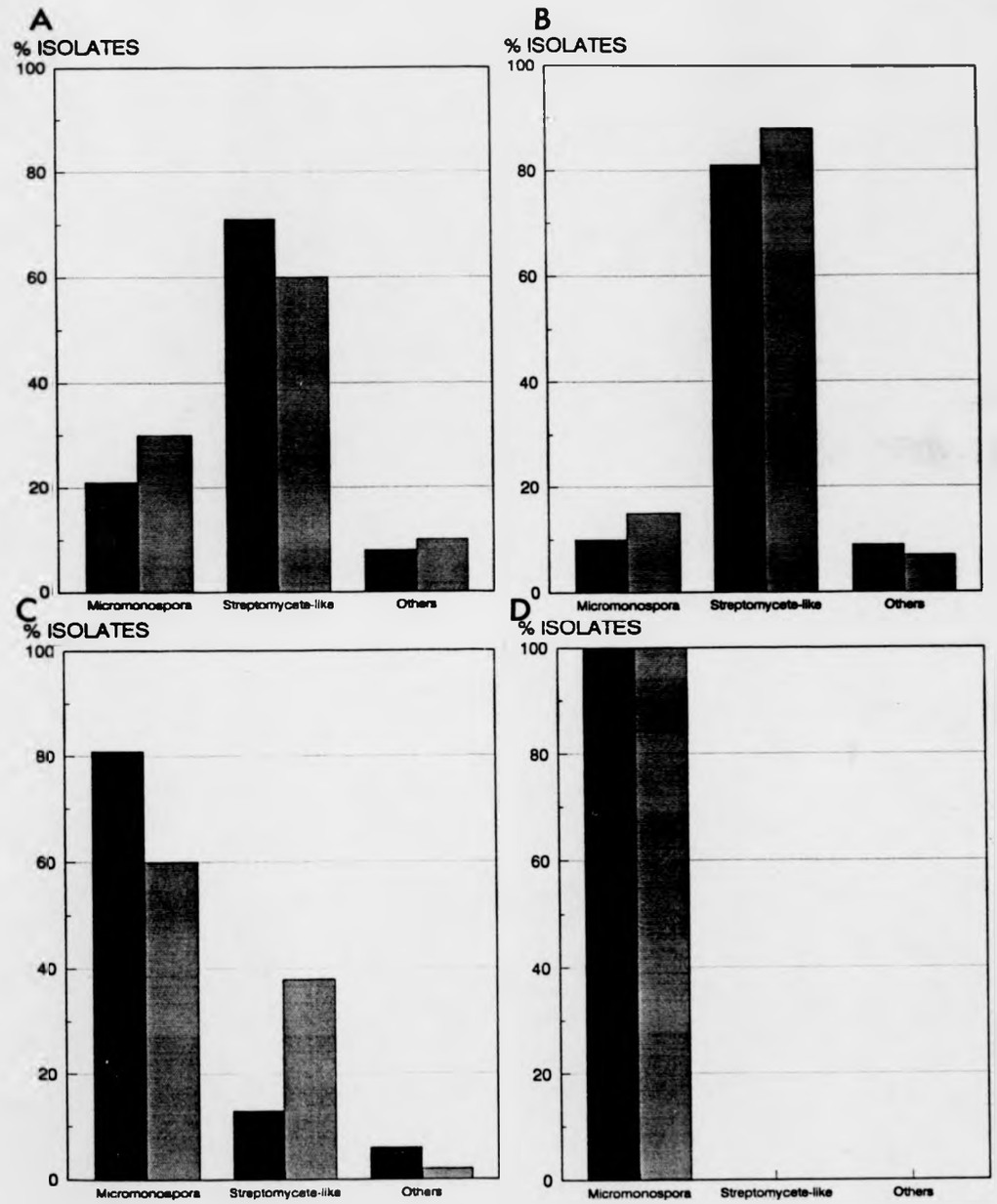
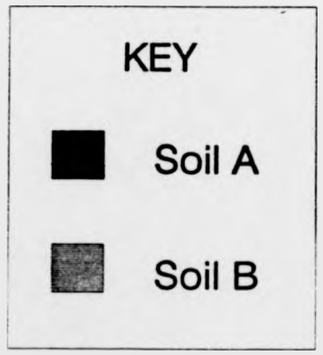


Figure 5. Isolation from soils A and B using the Chelex extraction method

Graphs A and B represent the percentage of *Micromonospora*, streptomycete-like and other colonies isolated from soils A and B, using the Chelex extraction method (Herron and Wellington, 1990). Graph A represents isolation from a slurry dried for 12 hours at 65°C, Graph B represents isolation from a wet slurry.



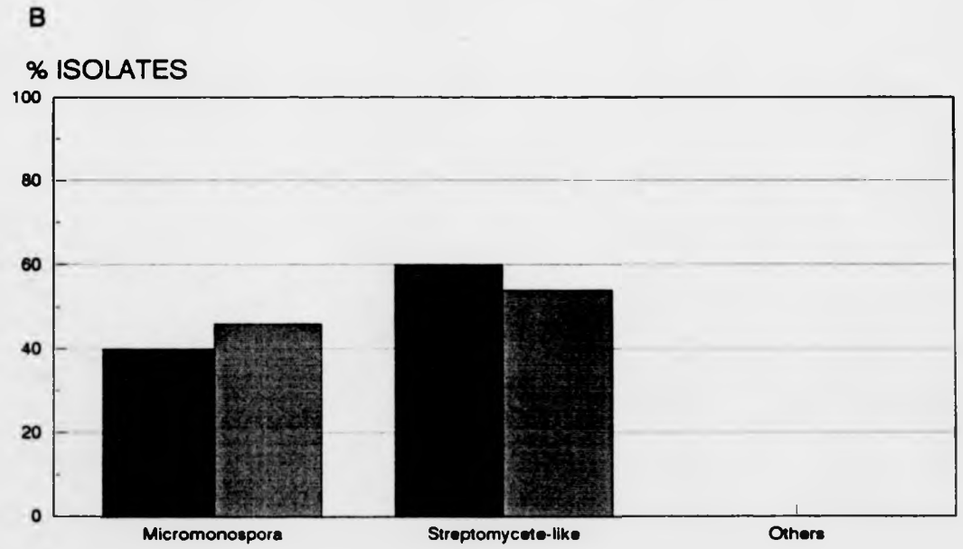


Figure 6. The Isolation of *Micromonospora* from soils A and B using 15 methods

Figure 6 represents the percentage of actinomycete-like colonies that are *Micromonospora* isolated from soils A and B using the 15 methods described.

1 AGS medium/sedimentation method

2 AGS medium/pour plate method

3 Tap water agar

4 Kadota's cellulose benzoate medium

5 M3 medium

6 Complex carbon/inorganic nitrogen medium

7 Complex carbon/organic nitrogen

8 Simple carbon/inorganic nitrogen

9 Simple carbon/organic nitrogen

10 Chitin enrichment/colloidal chitin agar

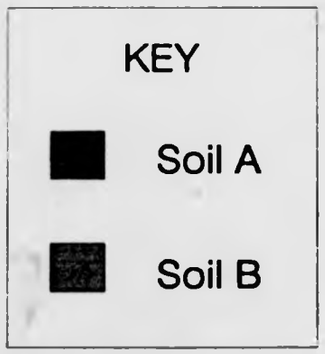
11 Chitin enrichment/AGS medium

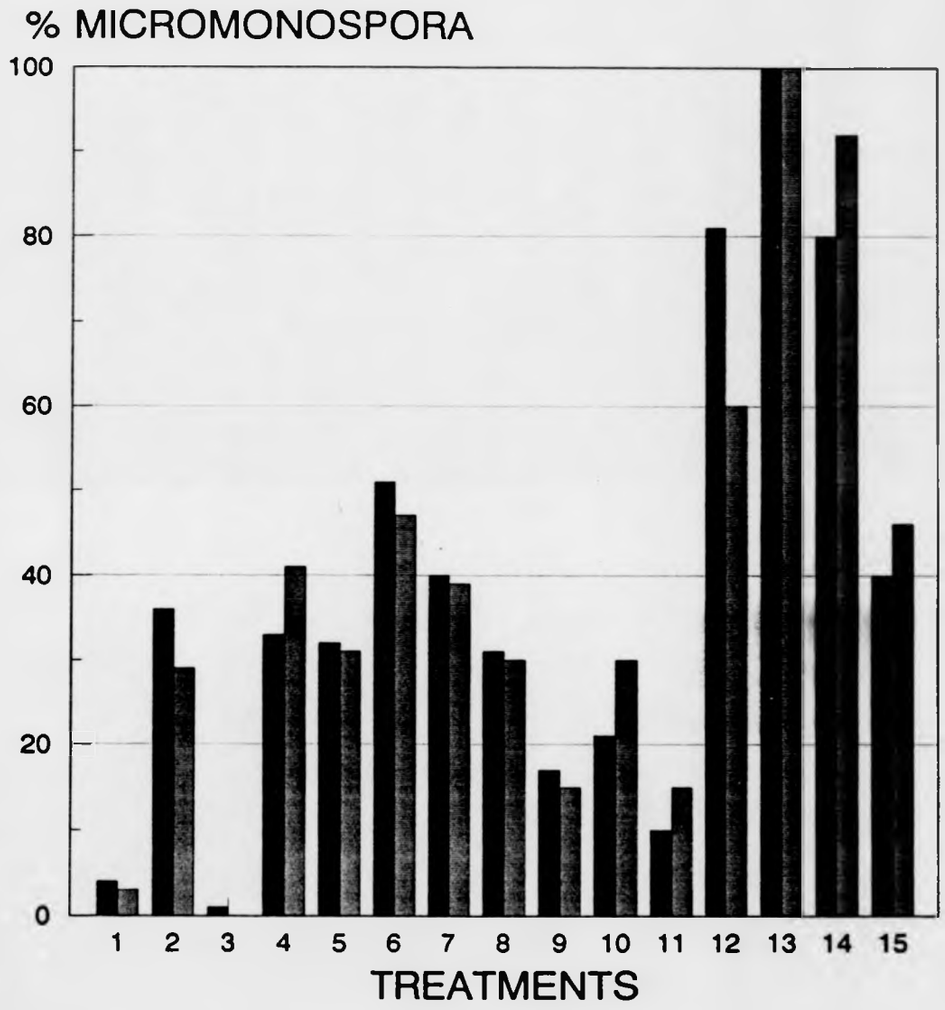
12 Heat treatment 120°C for 30 minutes

13 Heat treatment 120°C for 60 minutes

14 Chelex extraction/dry slurry

15 Chelex extraction/wet slurry





2.4 The selective isolation of *Micromonospora* using heat treatment

The following study explored in more detail the use of heat treatment as a selective isolation method for *Micromonospora*. In addition to the heat treatment isolation methods used in the previous Section (2.3.1(c)), the effect of treating soil at the same temperature (120°C) for more variable lengths of time was investigated. Section 2.3.1(c) showed that heat treatment was effective for the selective isolation of *Micromonospora*, however, this may have been the result of the presence of a small number of highly resistant species in the soil. To exclude this possibility 20 *Micromonospora* type strains, representing the main species groups of the genus (Kawamoto, 1991) were selected. The ability of these organisms to withstand severe dry heat treatment was examined.

2.4.1 Methods

2.4.1(a) The effect of heat treatment and time on the survival of indigenous *Micromonospora* spores

One gram samples of air dried Warwick soil were pre-heated at 120°C in a dry oven for varying lengths of time from 10 minutes to 100 minutes at 10 minute intervals. The soils were then added to sterile Ringer's solution (1g/100mls). The soils were shaken for 30 minutes on a Griffin hand shaker (4°C, setting 7) and used to prepare a dilution series from 10^{-2} to 10^{-5}, again using Ringer's solution. Isolation was then achieved using the pour plate method (Section 2.2.4), using AGS agar.

After 5 weeks the number of *Micromonospora* colonies were enumerated and compared to the total number of actinomycete-like colonies growing on each plate. Triplicate samples were taken for each treatment.

2.4.1(b) Heat susceptibility studies

Twenty *Micromonospora* type strains were used for this study representing all of the main species groups of the genus (Kawamoto, 1991)(Table 8). The ability of the spores to withstand severe heat treatment was tested to examine the efficacy of using heat treatment methods to selectively isolate most *Micromonospora* species from the natural environment.

Sterile Warwick soil was taken and divided into three 1g samples. Each sample was inoculated with 10^7/spores/ml and dried overnight in a 60°C drying oven. One sample was used as a control, one sample oven-heated at 120°C for 50 minutes and one sample at 120°C for 70 minutes. Each sample was then suspended in 10mls Ringer's solution, shaken for 30 minutes on a Griffin wrist action shaker (4°C, setting 7) and serially diluted. The pour plate method was then used for isolation with AGS agar. Plates were examined after 5 weeks. Triplicate samples were taken for each treatment and *Micromonospora* colonies were enumerated and the percentage survival of each strain was calculated for each treatment.

After 5 weeks the number of *Micromonospora* colonies were enumerated and compared to the total number of actinomycete-like colonies growing on each plate. Triplicate samples were taken for each treatment.

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2.4.2 Results and discussion

The heat treatment of soil for varying lengths of time, ranging from 10 to 100 minutes at 120°C has proved to be an effective method for the selective isolation of *Micromonospora* (Table 10 and Figure 7).

After heating for 70 minutes at 120°C, Warwick soil gave an average number of 60 *Micromonospora* colonies per plate (Table 10), which represented 80% of the total number of colonies on each plate. Heat treating at this temperature for longer than 80 minutes greatly decreased the viability of *Micromonospora* spores in the soil. The results therefore provided conclusive evidence that the heat treatment of soil at 120°C for between 50 and 70 minutes worked well for the deselection of streptomycetes and other common actinomycetes in the soil, thereby allowing the indigenous *Micromonospora* population to grow without competition. The possibility that the relatively large number of *Micromonospora* spores seen on isolation plates after the heat treatment of soil may have been the result of the presence of a small number of highly resistant species in the soil was investigated (Section 2.3.1(ii)).

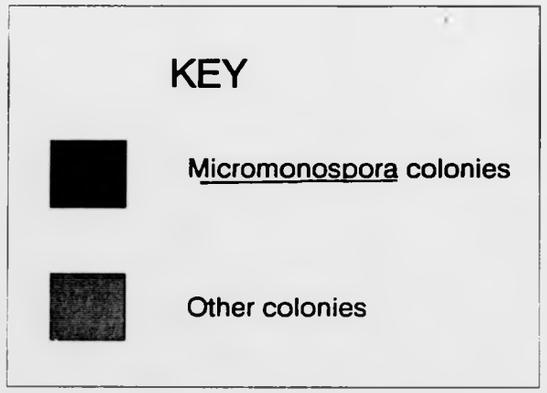
It was shown that all 20 *Micromonospora* type strains tested (Table 8) were capable of surviving both heat treatment methods (120°C for 50 and 70 minutes) to significant extents (Figure 8). However some variability in the levels of survival at the higher temperatures were observed, indicating that if this method was used solely as a means of selective isolation, the range of species obtained would not give a true picture of diversity.

Table 10 A Table to show the percentage of Micromonospora colonies isolated from soil after heat treatment at 120°C

Time(mins) Heat 120°c	Micromonospora cols (average/plate)	Total no. of cols. (average/plate)	Percentage Micromonospora
0	120	824	4.5
10	40	600	7.0
20	36	241	13.0
30	60	165	37.0
40	100	216	48.0
50	37	60	60.0
60	65	71	70.0
70	60	82	80.0
80	45	69	64.0
90	9	15	60.0
100	0	0	0.0

Figure 7. The isolation of *Micromonospora* from soil using heat treatment

Figure 7 represents the number of *Micromonospora* per plate, expressed as a percentage of the total number of colonies, after heat treatment at 120°C for 0 to 100 minutes, at 10 minute intervals.



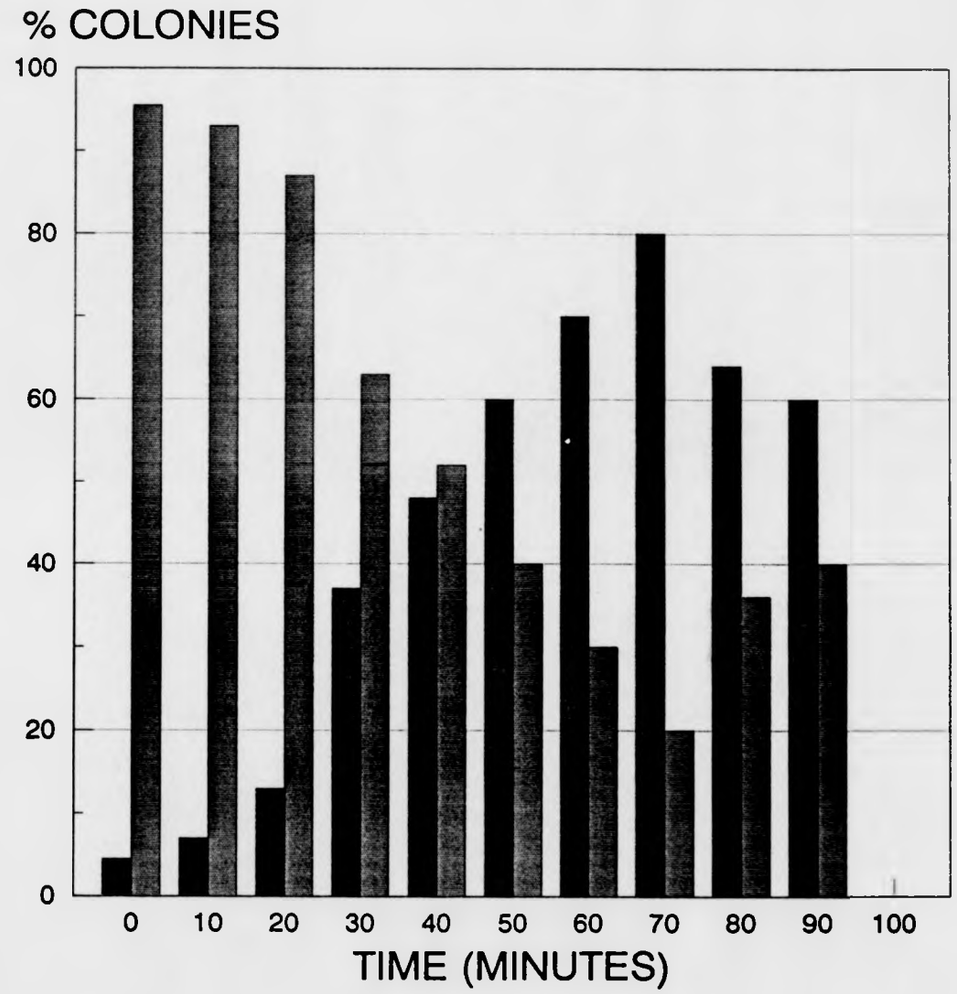
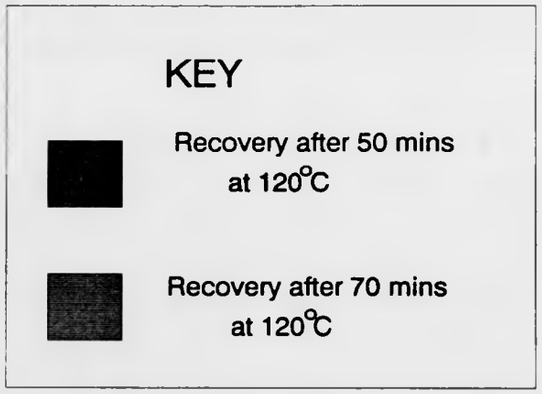
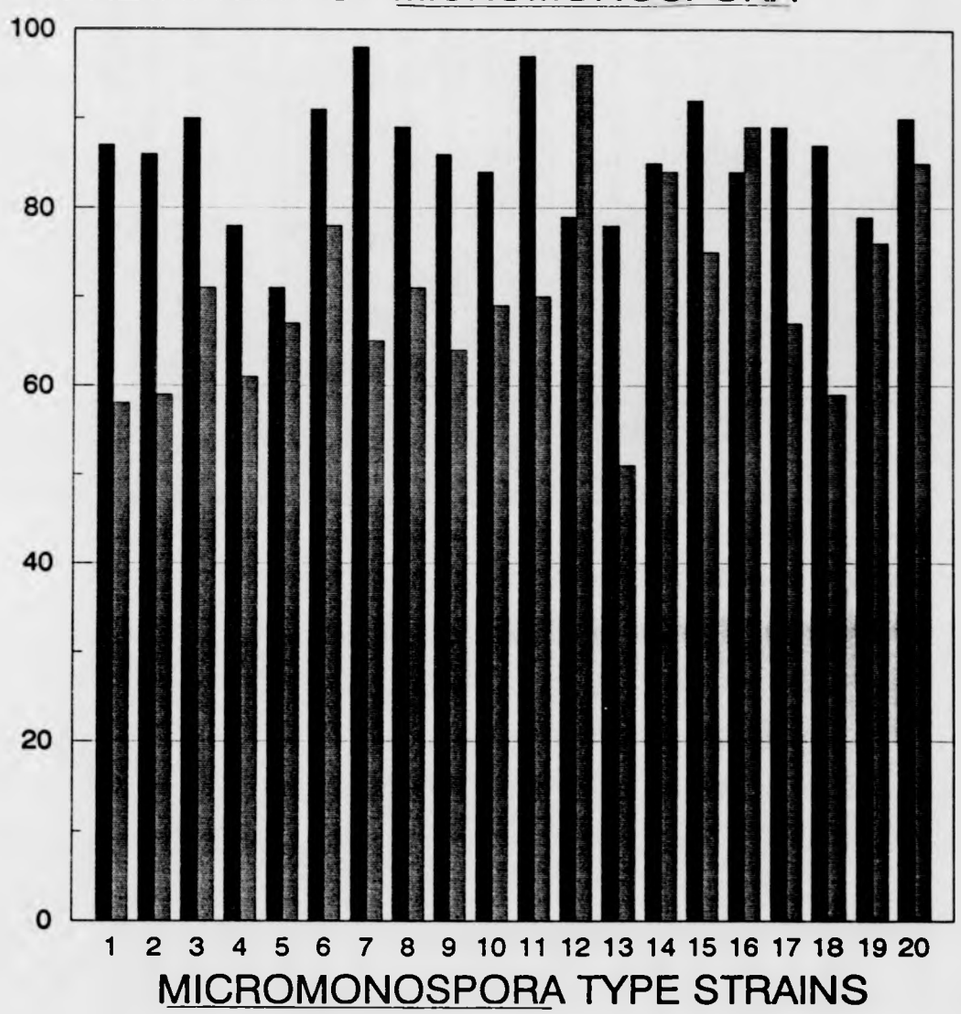


Figure 8. The recovery of *Micromonospora* after heat treatment

Figure 8 represents the percentage recovery of 20 species of *Micromonospora* after heat treatment of sterile, seeded soil at 120°C for 50 and 70 minutes in a dry oven.





2.5 The selection of *Micromonospora* using antibiotics

This study was initiated to devise a more efficient isolation method for *Micromonospora* by the incorporation of a selection of antibiotics into a suitable medium. Previous work has shown that a medium consisting of a complex carbon source (yeast extract) and an inorganic nitrogen source (sodium nitrate)(Medium A) was effective for the isolation of *Micromonospora* (Section 2.3.1(c)). It was therefore decided to use this medium for the following study.

A preliminarily examination was undertaken of 20 *Micromonopora*, representing each of the main species groups of the genera (Kawamoto, 1989) for their sensitivities to 4 antibiotics (Table 8). Ten representative streptomycetes were tested as a control (Table 9). The use of the above medium with the addition of antibiotics was then tested as a selective isolation procedure for *Micromonospora* using Warwick soil.

2.5.1 Methods

2.5.1(a) Development of a selective medium using antibiotics

A medium consisting of a complex carbon source and an inorganic nitrogen source (Section 2.3.1(c) and Appendix 2) was supplemented with concentrations ranging from 10 - 40μg/ml of novobiocin, lincomycin, gentamicin and penicillin.

Twenty type strains (Table 8) were inoculated onto the above media to test their antibiotic sensitivities. Ten *Streptomyces* wild isolates

(Table 9) were used as a control, to test the selectivity of each antibiotic mixture for the growth of *Micromonospora*.

2.5.1(b) Isolation of *Micromonospora* using antibiotic incorporated media

The above medium found to be most selective for *Micromonospora* was used to selectively isolate this genus from Warwick soil. The standard pour plate method was used (Section 2.2.4), using the complex carbon source and inorganic nitrogen source medium (Appendix 2).

2.5.2 Results and discussion

Novobiocin, gentamicin, penicillin and lincomycin were individually incorporated into a complex carbon and inorganic nitrogen source medium, and sensitivities of 20 *Micromonospora* tested (Table 11). The most effective concentration of antibiotic for growth of all 20 type strains of *Micromonospora* was a mixture of 10μg/ml lincomycin and 10μg/ml novobiocin. None of the 10 *Streptomyces* isolates tested were able to grow on this medium. These results indicated that a medium containing these antibiotics would be effective for the selective isolation of *Micromonospora* from the natural environment.

This method was then tested using Warwick soil (Table 12 and Figure 9). The above medium incorporated with lincomycin (10μg/ml) and novobiocin (10μg/ml) yielded the best results, 87% of the isolates being *Micromonospora*, thus proving the efficacy of using antibiotics to selectively isolate *Micromonospora* from soil.

Table 11 The ability of 20 Micromonospora identified strains and 10 Streptomyces isolates to grow on antibiotic incorporated media

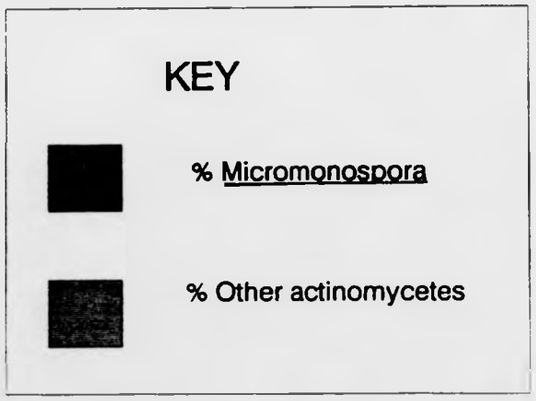
ANTIBIOTIC	CONCENTRATION (UG/ML)	GROWTH (No. +ve) Micromonospora	GROWTH (No. +ve) Streptomycetes
Novobiocin	10	20	7
	20	20	4
	30	19	5
	40	0	3
	50	0	0
	60	0	0
Gentamicin	1	15	7
	10	1	0
	15	0	0
	30	0	0
Penicillin	1	0	5
	5	0	5
	10	0	1
Lincomycin	5	20	5
	10	20	0
	20	5	0
	30	0	0
Novobiocin/ Lincomycin	10 / 10	20	0

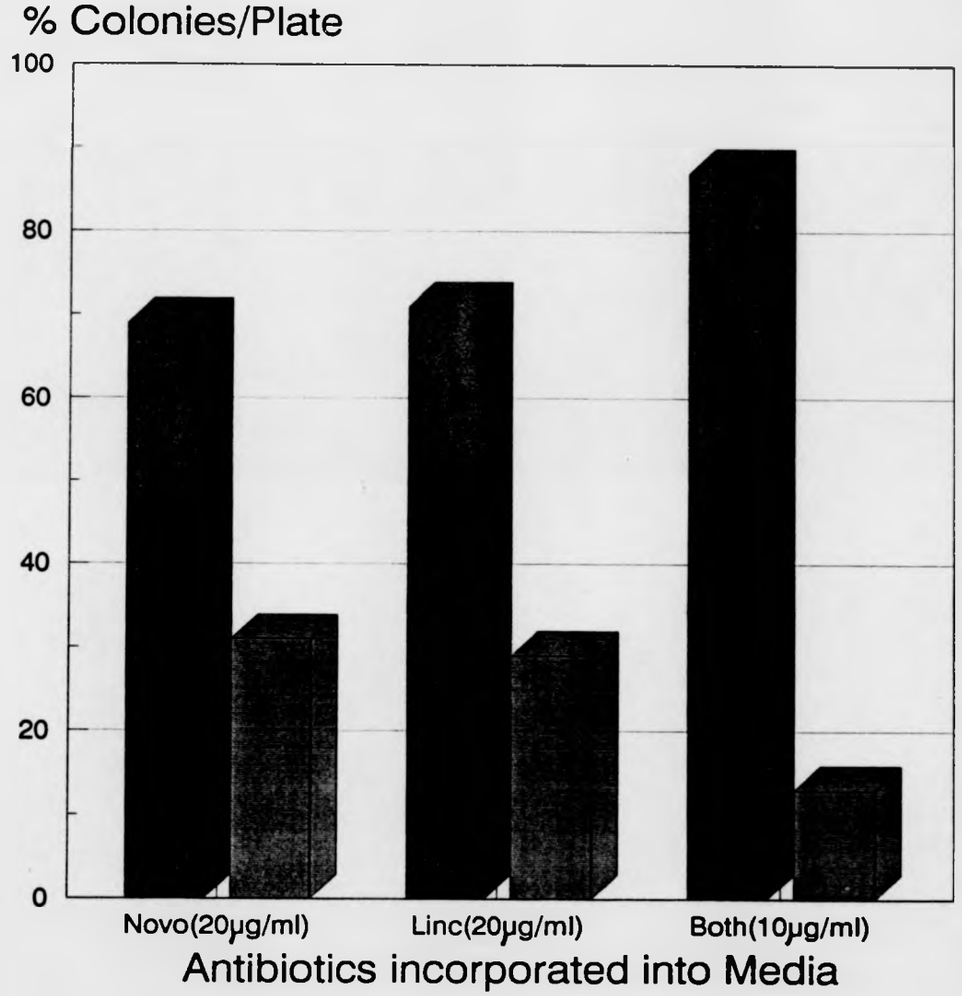
Table 12. The percentage of Micromonospora colonies isolated from Warwick soil using antibiotic incorporated media

ANTIBIOTIC CONC. IN MEDIUM	MICROMONOSPORA AVE.NO. COLS/PLATE	OTHER COLONIES AVE.NO./PLATE	% MICROMONOSPORA
Novobiocin (20µg/ml)	37	17	69
Lincomycin (20µg/ml)	43	17	71
Nov+Lin(10µgeach/ml)	44	7	87

Figure 9. The selective isolation of *Micromonospora* from Warwick soil using novobiocin and lincomycin

Figure 9 represents the percentage of *Micromonospora* isolated compared to other actinomycetes, from Warwick soil using the complex carbon/inorganic nitrogen medium (A) incorporated with novobiocin (20μg/ml), lincomycin (20μg/ml) and both (10μg/ml each).





2.6 The isolation of *Micromonospora* from different soil types

It has been reported that representative *Micromonospora* strains are distributed widely in soils of various geographical regions (Kawamoto, 1989), and that they often occur in moist soils (Solovieva and Singal, 1972). Singal *et al.* (1978) showed that *Micromonospora* strains predominated in moist soils and silts and that their content with respect to all isolated *Streptomyces* amounted to 67 to 87% in moist soils and silts but only 6 to 11% in ordinary soil samples.

The following investigation aimed to use the isolation methods developed in Sections 2.3 and 2.4, to study the distribution of *Micromonospora* in different soil types. Eight soils were sampled from a variety of locations, and using the media and pretreatments previously described, *Micromonospora* were isolated. The main aim of this study was to test, in full, the isolation procedures developed. Whilst definite conclusions could not be reached on the distribution of *Micromonospora* by using such a limited range of soil types, it was possible to hypothesize on the types of soil that *Micromonospora* may be prevalent in.

2.6.1 Methods

2.6.1(a) Soils and sediments

Eight soils were selected for this study, chosen to represent different soil types.

An analysis of particle size was performed for each soil (Allen *et al.*, 1974), in addition soil moisture content and pH were measured (Table 13). These measurements were taken immediately after sampling. Moisture content was determined to give a rough estimate of the nature of

the soil. It was realized that solid conclusions could not be made from such measurements as the moisture content of many soils is dependant on weather conditions and seasonal variations. However, from the results, hypotheses were made as to the type of environment that may favour the survival *Micromonospora* populations.

Soils selected for study

A. Pond sediment, Warwick University.

B. Soil from Tocil Wood, Warwick University.

C. Sandy soil from heathland, New Forest, Hampshire.

D. Soil from river bank, River Bourne, Salisbury, Wiltshire.

E. Field soil recently cultivated with potatoes, Warwickshire.

F. Compost soil, Kenilworth, Warwickshire.

G. Warwick soil, Cryfield site, Warwickshire.

H. Cotswold soil (Broadway Hill) from south facing hilltop.

2.6.1(b) Isolation procedures

The complex carbon/inorganic nitrogen (yeast extract/sodium nitrate) medium (Medium A) was used to isolate *Micromonospora* from the above soils (Appendix 2). All soils were air dried at room

temperature for 2 weeks and then pretreated by heating at 120°C for 50 minutes. The pour plate method was then used for isolation (Section 2.2.4). Plates were incubated at 30°C for 2 to 4 weeks, any colonies present on plates were then enumerated and the *Micromonospora* population in each soil was determined.

The total number of detectable actinomycetes present in each soil was then determined by using the using the above medium, with the elimination of the pretreatment step. This enabled an estimation to be made of the number of *Micromonospora* in the soil in relation to other actinomycetes present.

2.6.2 Results and discussion

The results satisfactorily proved the efficacy of the heat treatment of soil as a selective isolation procedure for *Micromonospora*. Using this method *Micromonospora* populations were enumerated from 8 contrasting soil types (Figure 10) enabling comparisons to be made as to the types of environments in which *Micromonospora* may be most prevalent.

From Table 13, it can be seen that the 8 soils vary significantly in moisture content, with values ranging from 10% (H. Cotswold hill soil) to 50% (A. Warwick pond sediment). As expected from the results of Solovieva and Singal (1972) *Micromonospora* populations seemed to be higher in wet soils than in dry ones; this was reflected in results shown in both Figures 10 and 11. Samples A (pond sediment) and D (river bank soil) yielded 9.8×10^4 and 10×10^4 *Micromonospora* c.f.u./g soil respectively, compared to a dry soil (sample H) which yielded 2×10^4 c.f.u./g soil. The 2 samples, A and D (moisture content, A - 50%, D - 23%) with no selective isolation steps yielded 60% *Micromonospora* (A) compared to other actinomycetes, and 64% *Micromonospora* (D).

Table 13 Particle size analysis of 8 different soils used in isolation studies

SOIL	TYPE	pH	MOISTURE CONTENT	CLAY (%)	SILT (%)	SAND (%)	ORGANIC MATTER (%)
A	WET	6.1	50	6	15	40	39.0
B	WOODLAND	6.7	31	7	27	46	20.0
C	SANDY	7.0	15	4	17	72	7.0
D	WET	7.9	23	5	34	55	6.0
E	CULTIVATED	7.1	16	10	22	61	7.0
F	COMPOST	7.2	19	5	12	20	63.0
G	CLAY	6.9	15	14	18	53	6.5
H	DRY	7.1	10	7	21	62	10.0

Warwick soil, which had the highest clay content (14%), using the heat pretreatment method, yielded relatively low numbers of *Micromonospora* (3×10^4 c.f.u./g soil). These results supported the findings of Singal *et al.* (1978), that *Micromonospora* survive best in soils with a high silt content. Indeed samples B and D (Silt, B-27%, D-34%), yielded significantly higher numbers of *Micromonospora* in comparison to the other samples (Figure 10).

2.7 Conclusions

The above study was primarily concerned with developing the use of heat treatment and antibiotics in the selection of *Micromonospora* from the natural environment.

Methods developed will be used throughout the whole of this study, both in the isolation of *Micromonospora* and in the studies of the survival of this genus in soil microcosms.

Figure 10. ***Micromonospora*** **isolated from 8 soils after heat treatment at 120°C for 50 minutes**

Figure 10 represents the number of *Micromonospora* isolated from 8 different environments. Soils and sediments after air drying, were heat treated in a dry oven for 50 minutes at 120°C.

A Pond sediment, Warwick University

B Top soil, Tocil Wood, Warwick University

C Sandy, heathland soil, New Forest, Hampshire

D Riverbank soil, River Bourne, Salisbury

E Field soil recently cultivated with potatoes, Warwickshire

F Compost soil, Kenilworth, Warwickshire

G Warwick soil, Cryfield site, Warwick

H Cotswold soil, Broadway Hill (south facing hilltop)

KEY

Number of Micromonospora

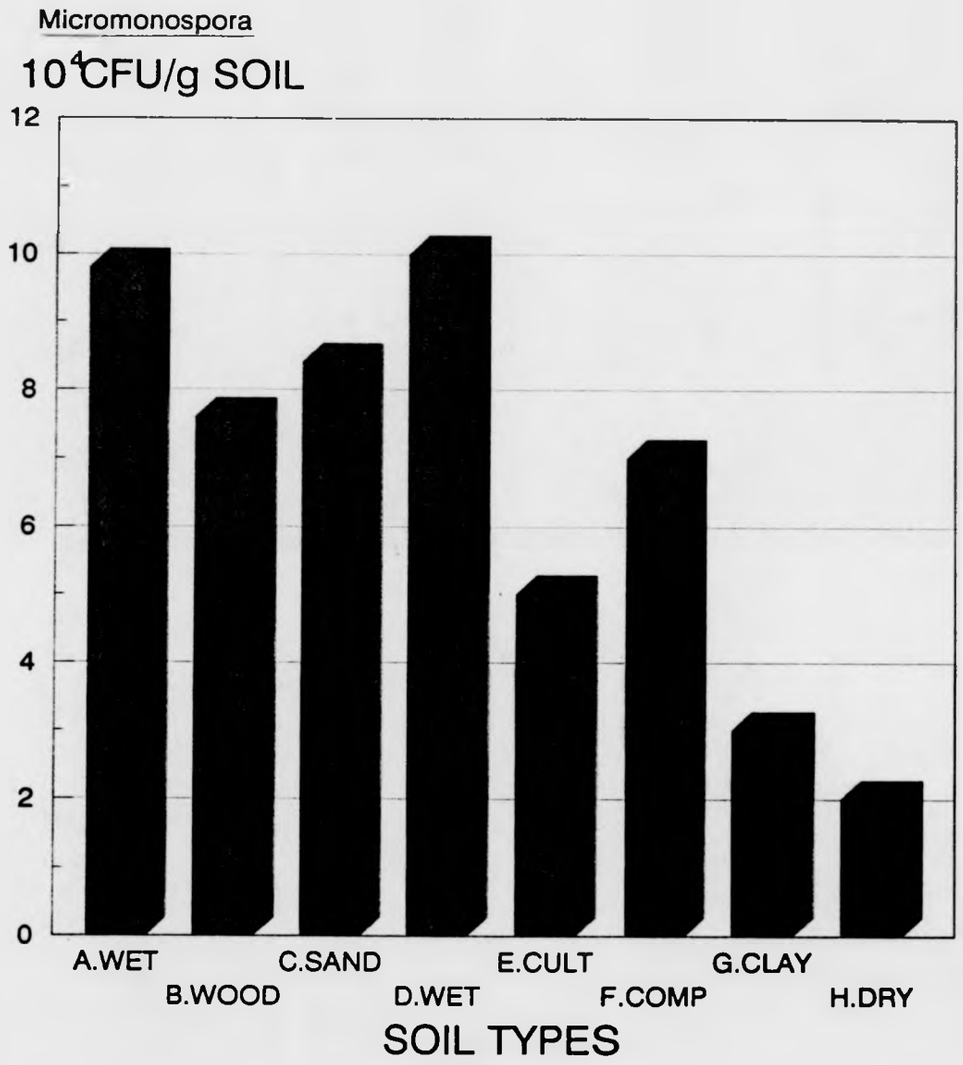


Figure 11. The isolation of actinomycetes from 8 soils

Figure 11 represents the percentage of *Micromonospora* and other actinomycetes isolated from 8 soils.

A Pond sediment, Warwick University

B Top soil, Tocil Wood, Warwick University

C Sandy, heathland soil, New Forest, Hampshire

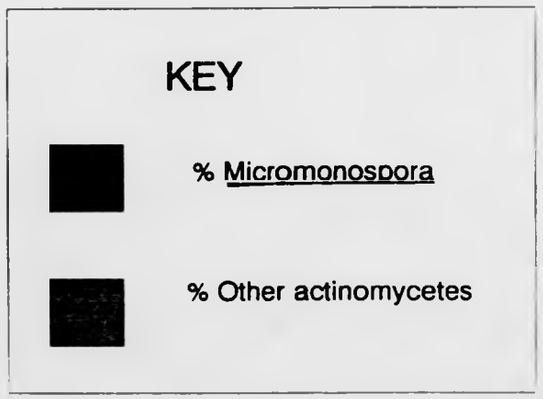
D Riverbank soil, River Bourne, Salisbury

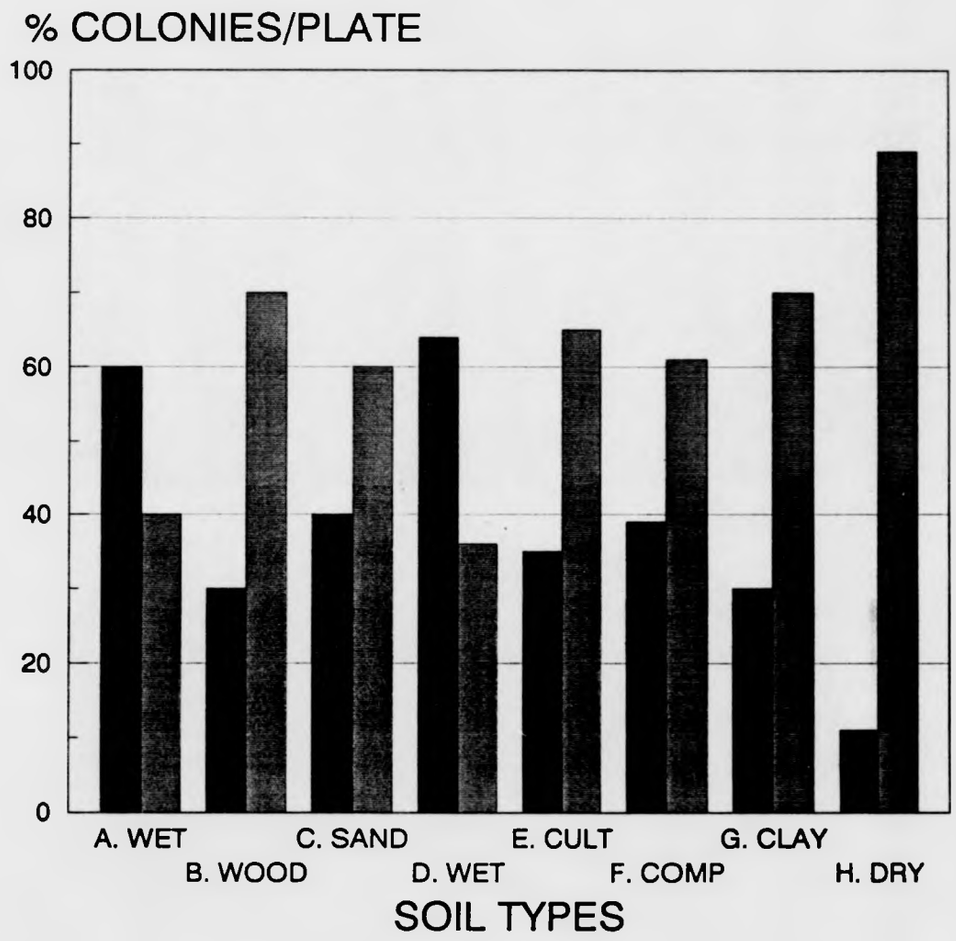
E Field soil recently cultivated with potatoes, Warwickshire

F Compost soil, Kenilworth, Warwickshire

G Warwick soil, Cryfield site, Warwick

H Cotswold soil, Broadway Hill (south facing hilltop)





CHAPTER 3

The Survival of *Micromonospora* in Soil

3.1 Introduction

Growth of most heterotrophic microorganisms in the soil is usually limited by the supply of nutrients, the most limiting substrate generally being nitrogen. Dead plant material, leaf litter, root exudates and dead organisms all provide rich sources of nutrients (Chapman and Gray, 1986; Zeph and Casida, 1986), providing microbes with a variety of energy sources, some of which are readily available and others that require extensive degradation (Williams, 1985).

3.1.1 *Micromonospora* in soil: Aims

Organisms of the genus *Micromonospora* can be readily isolated from soil, sediment and aquatic environments (Section 1.3). Their abundance in soils has been widely reported (Ishizawa *et al.*, 1969; Lechevalier, 1964) as has their occurrence in marine and freshwater environments (Johnston and Cross, 1976; Solovieva and Singal, 1972) (Section 1.3.3). The soil environment is the natural habitat for growth and proliferation and is suitable for the survival of many *Micromonospora* populations which have adapted survival strategies for variations in nutrient supply. It contains many resources that allow *Micromonospora* and other actinomycetes to compete successfully with other members of the soil microflora (Kutzner, 1981).

Micromonospora, although to a lesser extent than *Streptomyces*, are able to survive on inorganic nitrogen and are able to utilise efficiently soil infrastructures in order to support their mycelial growth. Although it is known that the spore-forming actinomycetes, particularly *Streptomyces*, exist in soil mainly as spores (Lloyd, 1969; Mayfield *et*

al., 1972; Skinner, 1951), little is known about the survival of *Micromonospora* in soil systems.

It is known that active growth in many actinomycetes is limited to sites of nutrient availability (Goodfellow and Simpson, 1985; Kutzner, 1981) and that organic substrates, such as root fragments, are rapidly colonised by hyphal growth which is subsequently replaced by spores after nutrient depletion (Lloyd, 1969). Thus, it appears that the main reason for the successful survival of actinomycetes in soil is the resistance of their spore population and its inevitable replenishment by mass germination at sites of high nutrient concentration (Lloyd, 1969).

The survival of *Micromonospora*, indeed all actinomycetes, is therefore largely influenced by the properties of their spores; The spores of *Micromonospora* are borne singly on substrate mycelia; their physiological properties and structures being significantly different from those of *Streptomyces* spores which are formed on long chains on aerial mycelia. Indeed electron microscopic examination has revealed that the *Micromonospora* spore is a specialized, broad-based type of aleuriospore which differs in spore wall structure from the *Streptomyces* spore (Hardisson and Suarez, 1979; Luedmann and Casmer, 1973; Stevens, 1975). Unlike *Thermoactinomyces*, the spore wall of *Micromonospora* has no inner multilaminar coat; it does, however, have a much more stratified deposition than that of *Streptomyces* spores. This probably accounts for their high refractility and their high resistance to physical and chemical treatments (Kawamoto *et al.*, 1982) (Section 1.4.2). Actinomycetes generally are able to withstand considerable desiccation (Williams *et al.*, 1972); some being able to grow in a limited form at a water potential of -100 bars.

Although work has been done on the biochemical and physiological properties of *Micromonospora* spores, few studies have

investigated the nature of the survival of *Micromonospora* in soil. Previous work with closed microcosm systems has shown that streptomycete growth in soil follows a cyclical pattern of germination and sporulation after inoculation into sterile nutrient-amended soil and, to a lesser extent, in nonsterile soil (Herron and Wellington, 1990; Wellington *et al.*, 1990). The following study aimed to use a similar set of conditions to follow the fate of two *Micromonospora* species in the soil, *M. chalcea* and *M. fulvopurpurea* to investigate similarities in life style and also survival of mycelium.

A spore specific extraction procedure was firstly developed enabling enumeration of the spore and mycelial populations present in the soil over a 30 day period. The study was aimed at proving that *Micromonospora* occur in soil mainly in the form of spore structures thus complementing the study outlined in Chapter 2 which involved the development of a spore specific method of isolation.

Two *Micromonospora* were chosen as representatives of the genus, *M. chalcea* subsp. *chalcea* ATCC 12452, and *M. fulvopurpurea* JCM 5696. Previous work has shown that members of the genus *Streptomyces* generally have a similar pattern of behaviour (Cresswell *et al.*, 1992; Wellington *et al.*, 1990). *M. chalcea* and *M. fulvopurpurea* both represent typical members of the genus; both have been frequently isolated from air, soil and aquatic environments. Phenogram 5 (Section 4.3.5) shows the dissimilarity between the two strains, *M. chalcea* falling in cluster group 2 at the 74 % S_{sm} S-level, *M. fulvopurpurea* falling in cluster group 7.

3.1.2 Detection and monitoring of microorganisms in the environment

The isolation and enumeration of microorganisms from soil has traditionally relied upon methods which use the mechanical desorption of bacteria from soil particles by shaking the soil with an eluent, often $^1/_4$ strength Ringer's solution (Wellington *et al.*, 1990). However, other molecular biological detection and monitoring methods have recently been developed (Trevors and Van Elsas, 1989) which rely on the subsequent growth of the organism after isolation, rendering undetectable those organisms that are viable but non-culturable (Rozack and Colwell, 1987). Indeed, from soil, plate counts can be between 2 and 3 orders of magnitude below direct counts (Bakken, 1985). Torsvik *et al.* (1990a) found that isolating microorganisms from soil by a traditional method yielded 200 strains which on phenotypic analysis represented *ca.* 20 clones. However extraction of DNA from the soil followed by DNA reassociation analysis suggested that *ca.* 4000 different clones were present in the soil sample (Torsvik *et al.*, 1990b).

Many techniques are available for the direct counting of microorganisms, allowing enumeration without cultivation of a sample (Pickup, 1991). Examples include staining cells with fluorescent dyes such acridine orange (Porter and Feig, 1980) and the use of antibodies to determine direct counts for a specific subpopulation (Howgrave-Graham and Steyn, 1988; Brookner and Stokes, 1990). Detection and enumeration may also be achieved by ELISA and flow cytometry respectively (Bashan et al., 1991; Saunders et al., 1990, respectively). A problem faced by all methods for direct counting however, apart from the chance of enumerating 'dead' cells, is that of non-specific binding of the

stain to nontarget organisms requiring therefore, a high degree of specificity for the target organism.

Recent years have seen the development of 16S rRNA gene probes (Amann *et al.*, 1990a; Lane *et al.*, 1985) that can be used along with microautoradiography for the detection of microorganisms *in situ*. Such methods involve the production of species or genus specific probes (Woese, 1987) which can be used for detection purposes. Developed probes can also be used to differentiate between species by the use of flow cytometry (Amann et al., 1990b).

Such techniques however, are considerably time consumming and detection limits are relatively low (10^4 cells/g soil) (Hahn *et al.*, 1990), for the purposes, therefore, of monitoring *Micromonospora* in soil microcosms the traditional method of shaking soil with an eluent was used.

3.2 General methods

3.2.1 Bacterial strains

Micromonospora chalcea subsp. *chalcea* ATCC 12452 and *Micromonospora fulvopurpurea*, JCM 5696 were used for the following soil microcosm experiments. Strains were grown on *Micromonospora* media (see Appendix) or oatmeal agar plates. Cultures were stored at 4°C as slope cultures. Spore suspensions, prepared as described below, were suspended in 20% glycerol and stored at -20°C.

(a) Preparation of Spore Suspension

A sporulating plate culture was flooded with 10ml sterile distilled water (SDW), the spores were then scraped off with an inoculating loop. The spore suspension was transferred to a sterile 20ml Universal bottle

and vortexed for approximately 1 minute after which it was then filtered through non-absorbant cotton wool. The suspension was examined at X400 with a phase-contrast microscope (Kyowa) for the presence of spores only, before use as an inoculum.

(b) Preparation of Mycelial Suspension

Liquid cultures of *M. chalcea* and *M. fulvopurpurea* were grown in 25 ml TSB in 250 ml baffled flasks using a Gallenkamp orbital shaker (180-200 r.p.m.) at 30°C. Growth was checked after 2-3 days for the absence of sporing structures. The suspension was centrifuged at 3,000 r.p.m. for 15 minutes and subsequently washed 3 times with phosphate buffered saline (PBS). The resulting suspension was then passed up and down a sterile syringe needle prior to use as an inoculum in the microcosm experiments.

3.2.2 Sterile soil microcosm conditions

Soil was taken from a local field site (Cryfield Hall, University of Warwick). Analysis of particle size gave a composition of (percent, dry weight): 63.6% sand, 18.4% silt, 11.7% clay and 6.2% loss on ignition. The pH of fresh soil was 6.5-6.8 (Wellington *et al.*, 1990). Before use in microcosm experiments, soil was air dried for two weeks and sieved through a 2 mm. sieve, it was then sterilised by autoclaving in containers at 121^{o}C for 15 minutes on two separate days. The soil was wetted to 15% moisture (40% moisture holding capacity, -3 bar [-300 kPa]) with SDW which carried the inoculum if appropriate. Soil was amended, when appropriate, with sterilised 1% (w/w) chitin and 1% (w/w) soluble starch.

All microcosm experiments were performed in sterile 50 ml Falcon tubes (Sarstedt, West Germany) containing 10 g soil. The

mycelial or spore inoculum was thoroughly mixed into the soil with a sterile spatula to ensure adequate dispersal of inoculum.

3.2.3 Isolation of total *Micromonospora* from microcosm

One gram of soil (wet weight) was transferred asceptically to 9 ml of $^1/_4$ strength Ringer's solution. This was shaken at maximum speed for 10 minutes on a Griffin wrist action shaker. The supernatant was serially diluted and 100μl aliquots spread onto oatmeal plates.

3.2.4 Statistical analysis

Statistical analysis was by the MINITAB software package (Minitab statistical software, State College, Pa., USA).

All points on graphs are means of three replicate samples counted in triplicate. The method of Petersen (1985) was used to calculate minimum significant differences from the analysis of variance.

Two calculations can be used (see below). The most commonly used one is the M.S.D. calculation, for when the number of replicates for each sample point differs between sample days (e.g. contamination, unreadable plates). If however, the number of replicates remains the same for each day of sampling Tukey's H.S.D. was used.

Minimum Significant Difference

$$M.S.D. = Q_a \sqrt{[(m.s.e.[1/n_i + 1/n_j])/2]}$$

Tukey's Honestly Significant Difference

$$H.S.D. = Q_a \sqrt{(m.s.e./r)}$$

Where,

Q_a	value from Studentised range (Q) table (95%) confidence limits. Depending on u (error degrees of freedom from ANOVA) and n (number of sample points in days)
m.s.e.	mean square error (from ANOVA)
r	sample size (number of replicates)
n_i	lowest number of replicates
n_j	highest number of replicates

3.3 The development of a spore specific extraction procedure for *Micromonospora*

Hopkins and O'Donnell (1991) have outlined that the extraction of microorganisms from soil can be divided into four areas : dispersion of soil aggregates, dissociation of microorganisms from soil particles, separation of microorganisms from soil material and the purification of bacteria from the extract. Many methods are available that involve some or all of these steps for the specific isolation of actinomycetes from soil (Herron and Wellington, 1990; Hopkins *et al.*, 1991; MacDonald 1986). However, the simplest and least time consumming method still remains that of shaking a given mass of soil with an eluent, a common choice being Ringer's solution. This method gives reliable and good recovery of organisms from seeded soil microcosms with detection limits as low as 10^2c.f.u./g soil.

In order to study the fate of *Micromonospora* in soil microcosms it was firstly necessary to develop a method which allowed the determination of the spore population only in soil. This was based on the heat resistance of *Micromonospora* spores and enabled both spore and mycelial numbers to be calculated.

3.3.1 Optimisation of heat treatment method to allow detection of *Micromonospora* spores only in soil

To be able to assess both mycelial and sporing populations in a soil sample at any one time, a method was developed which involved an assessment of the optimum length of time for which soil microcosms

could be heat treated, allowing for the survival of all *Micromonospora* spores, whilst completely deselecting for the mycelial population.

3.3.2 Survival of *Micromonospora* spores in soil microcosms after heat treatment

Ten grams of sterile, unamended soil microcosms were seeded with 10^8 c.f.u./g soil of *Micromonospora chalcea* subsp. *chalcea*, ATCC 12452 or *Micromonospora fulvopurpurea*, JCM 5696. Spore suspensions were checked microscopically at x400 for the absence of mycelial fragments prior to inoculation.

Triplicate soil samples were heated at five different temperatures, 65°C, 70°C, 75°C, 80°C and 85°C, in a water bath for 10, 20 and 30 minutes, for each set of microcosms a non-heat treated control was included . Three 1 gram of soil samples from each microcosm were then suspended and shaken in 9 ml sterile Ringer's solution on a Griffin wrist action shaker at maximum speed for 30 minutes at 4°C (Section 3.2.3). The suspension was serially diluted and 100μl of diluted samples were then spread on to oatmeal agar plates (Appendix 2).

Colony counts for each temperature and time were compared to counts obtained from a control (an inoculated, un-heat treated, soil sample) which had been left at room temperature for 20 minutes. It was therefore possible to calculate the optimal temperature and longevity of heat treatment which allowed for the survival of all of the spore populations in the soil.

3.3.3 Survival of *Micromonospora* mycelial fragments in soil microcosms after heat treatment

Ten gram sterile, unamended soil microcosms were seeded with 10^5 c.f.u./g soil of *Micromonospora chalcea* subsp. *chalcea*, ATCC 12452 and *Micromonospora fulvopurpurea*, JCM 5696. All samples used as inoculua were checked microscopically at x400 for the absence of sporing structures, confirming that the sample comprised of mycelial fragments only.

Triplicate soil samples were heated at four temperatures, 65°C, 70°C, 75°C and 80°C in a water bath for 10, 20 and 30 minutes, for each set of microcosms a non-heat treated control was included . Three 1 gram samples of soil from each microcosm were then suspended and shaken in 9 ml sterile Ringer's solution on a Griffin wrist action shaker at maximum speed for 30 minutes 4°C. The suspension was serially diluted and 100μl of diluted samples were then spread on to oatmeal agar plates.

Colony counts for each heat treatment for each length of time were then compared to counts obtained from a control (an inoculated, un-heat treated, soil sample) which had been left at room temperature for 20 minutes. It was then, therefore, possible to work out the optimal temperature and longevity of heat treatment which allowed for the survival of no mycelial fragments, allowing therefore, for the enumeration of the spore population only.

3.3.4 The development of a spore specific extraction procedure for *Micromonospora*: Results and discussion

Figures 12 and 13 show the recovery and survival of *Micromonospora chalcea* subsp. *chalcea*, ATCC 12452 (Figure 12) and

Micromonospora fulvopurpurea, JCM 5696 (Figure 13) from sterile soil microcosms after heat treatment in a water bath. Each figure shows the survival of populations of both spores and mycelia. Graphs are displayed as logged counts at each day.

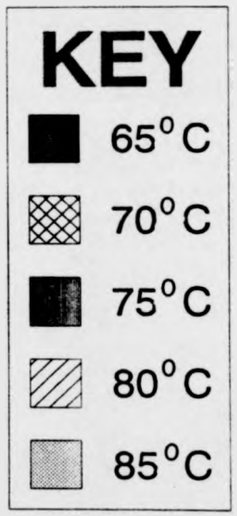
Figures 12A and 13A show the survival of the spore populations only of *M. chalcea* and *M. fulvopurpurea* after heat treatment in a water bath. Both organisms were seeded into the microcosms at an initial concentration of 10^6 c.f.u./gram soil. Immediate recovery of *M. chalcea* and *M. fulvopurpurea* gave counts of 3.3 and 4.0 x 10^5 c.f.u./g soil respectively. This apparent decline in numbers following addition of spores to soil has been reported by many workers (Ramsay, 1984; Hopkins and O'Donnell, 1991) and may to due to adhesion of spores to soil particles or damage to spore structures following vigorous shaking.

Spore numbers in both *M. chalcea* and *M. fulvopurpurea* remain constant after heat treatment at 65°C for up to 30 minutes, falling only slightly to 2.5 and 3.9 x 10^5 c.f.u./g soil respectively after 30 minutes at 70^oC. Heat treating in the water bath at 75°C, 80°C and 85°C, however, gave increasingly lower numbers of both organisms after 10, 20 and 30 minutes. Indeed heat treatment for 30 minutes at 85°C gave numbers of 3.2 x10^2 and 3.6 x 10^2 c.f.u./g soil for *M. chalcea* and *M. fulvopurpurea* respectively.

Figures 12B and 13B show the survival of the mycelial populations only of *M. chalcea* and *M. fulvopurpurea* after heat treatment in a water bath. Both organisms were seeded into the microcosms at an initial concentration of 10^5 c.f.u./g soil. Immediate recovery of *M. chalcea* and *M. fulvopurpurea* gave counts of 9.0 and 9.5 x 10^5 c.f.u./g soil respectively. This decline in numbers following addition of mycelia to soil was significantly less than that was observed above for the recovery of spores. This was not surprising, as due to the nature of their

Figure 12. The survival of *Micromonospora chalcea* subsp. *chalcea* ATCC 12452, after heat treatment in sterile, unamended soil.

Graphs A and B show the logged counts of *M. chalcea* spores and mycelia respectively, after heat treatment of seeded soil microcosms in a water bath.



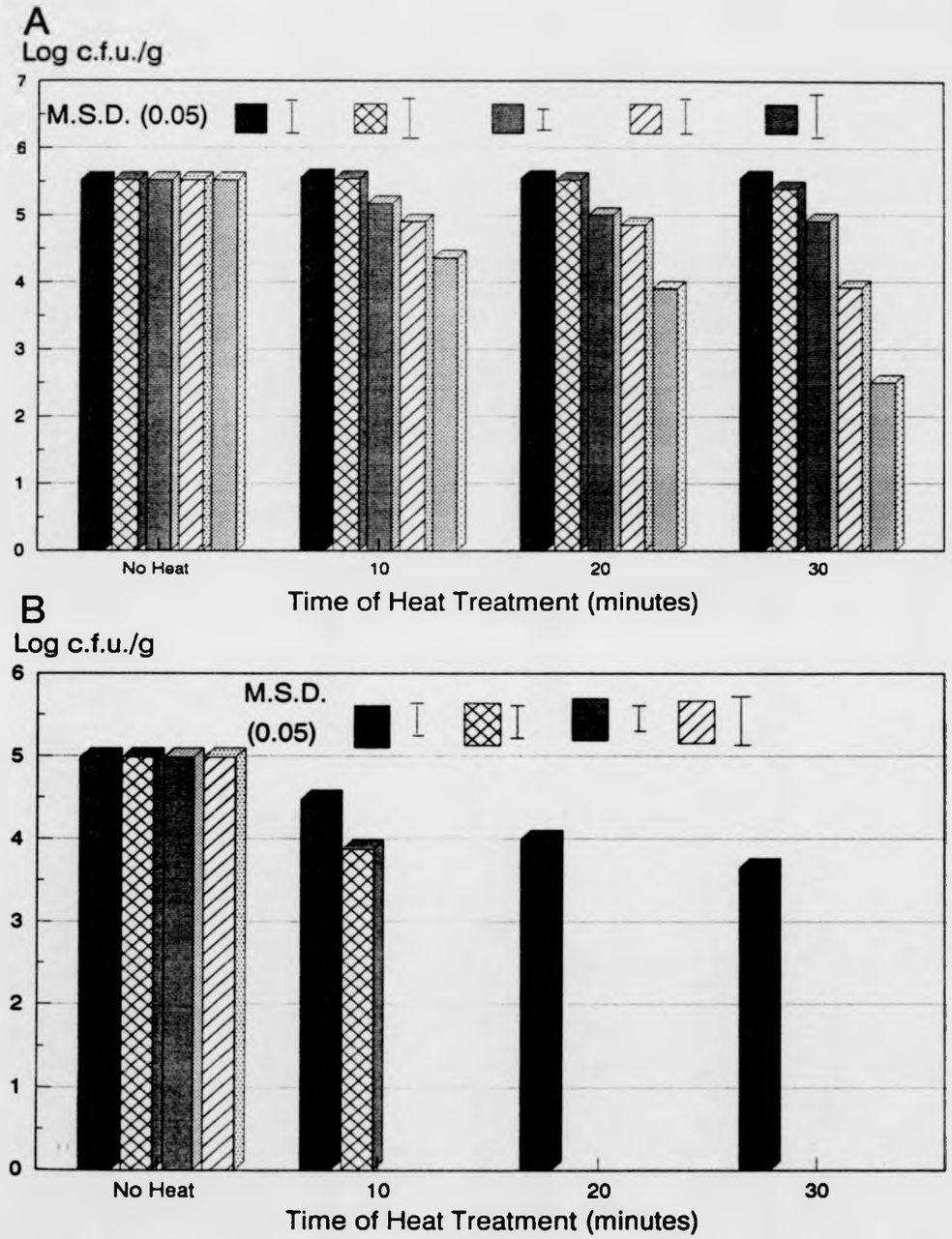
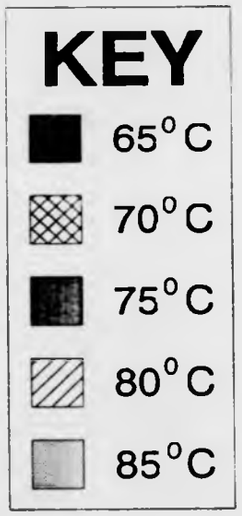
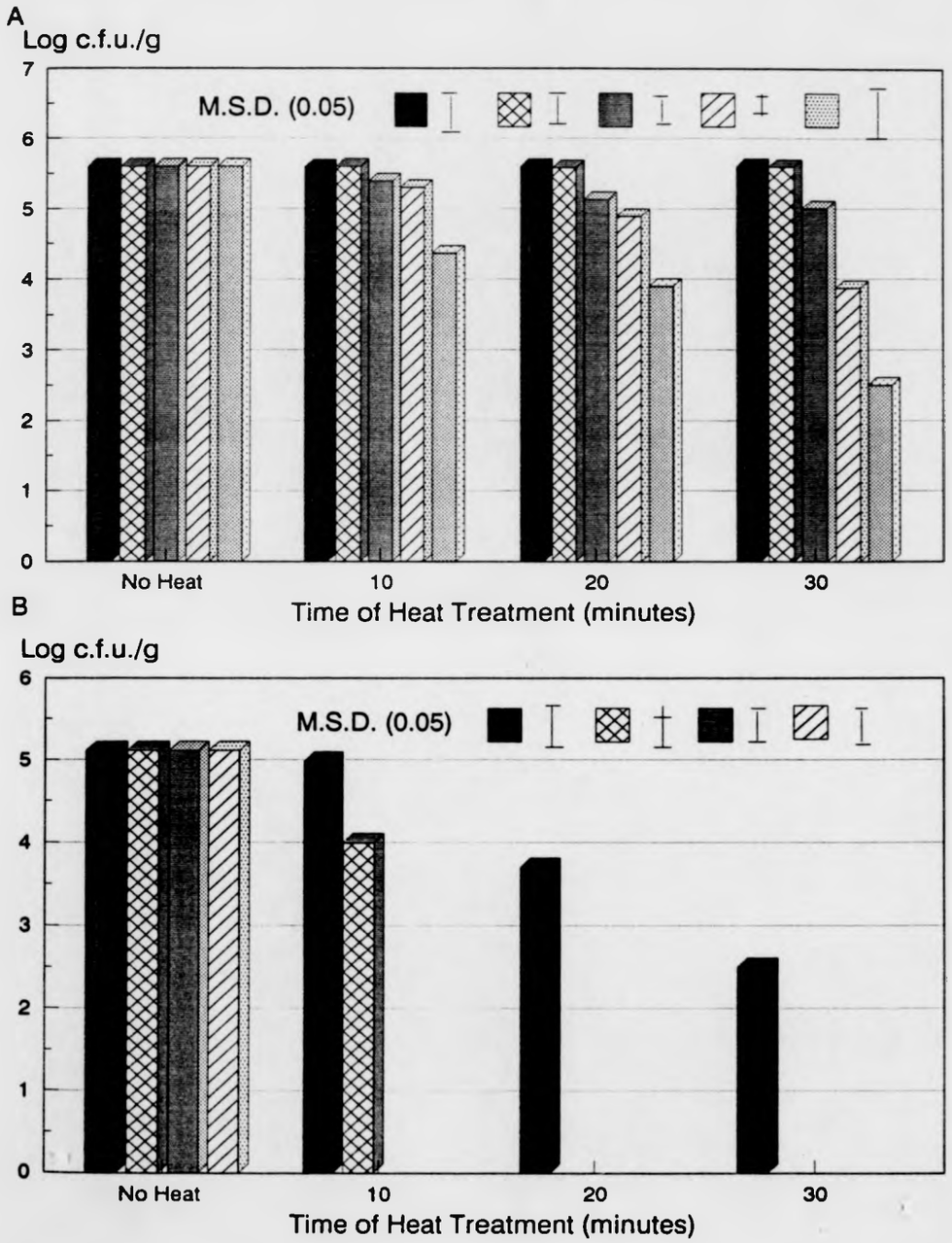


Figure 13. The survival of *Micromonospora fulvopurpurea* JCM 5696, after heat treatment in sterile, unamended soil.

Graphs A and B show the logged counts of *M. fulvopurpurea* spores and mycelia respectively, after heat treatment of seeded soil microcosms in a water bath.





structure, mycelia will often fragment on agitation so that initial counts may often be higher than the original inoculum.

Heat treatment at 65°C for 30 minutes gave a significant drop in mycelial numbers to 4.5×10^3 and 3.1×10^3 c.f.u./g soil for *M. chalcea* and *M. fulvopurpurea* respectively. This was followed by a complete death of all mycelial fragments after heat treatment for 20 minutes at 70°C.

Comparison of the survival of both the spore and mycelial component of *M. chalcea* and *M. fulvopurpurea* populations (Figures 12 and 13), indicated that heat treating 10g soil microcosms for 20 minutes at 70°C would allow the maximum recovery of spores present whilst deselecting completely, the mycelial population. This method was therefore suitable to use when requiring the enumeration of spores and mycelia in soil populations.

3.4 The survival of *M. chalcea* and *M. fulvopurpurea* in sterile amended and unamended soil

Using the above developed method which allowed the detection of sporing structures only, the fate of the spores and mycelia of *Micromonospora chalcea* subsp. *chalcea*, ATCC 12452 and *Micromonospora fulvopurpurea*, JCM 5696 were followed in soil microcosms using both sterile amended and unamended soil.

3.4.1 The survival of *M. chalcea* and *M. fulvopurpurea* in sterile amended and unamended soil: Methods

Amended and unamended soil was weighed out into 10 gram microcosms as described in Section 3.2.2. Seven sets of three

microcosms were prepared for each organism for both amended and unamended soil. *M. chalcea* ATCC 12452 and *M. fulvopurpurea* JCM 5696 were added, separately, to aliquots of S.D.W. that when added to soil gave a final moisture content of 15%. Inocula of both organisms consisted of spore suspensions, prepared as outlined in Section 3.2.1 (a). 10^6 c.f.u./g soil of both organisms were added to the soil. At days 0, 1, 2, 5, 10, 15 and 30 three microcosms were destructively sampled, and at each sample day, three 1 gram aliquots were removed from each microcosm and extracted with Ringer's solution as outlined in Section 3.2.3. Recovered *Micromonospora* were enumerated after two weeks incubation at 30°C.

3.4.2 The survival of *M. chalcea* and *M. fulvopurpurea* in sterile amended and unamended soil: Results and discussion

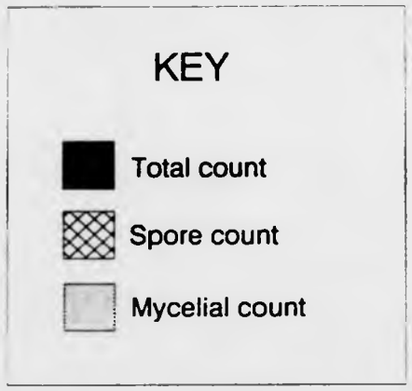
The growth and survival of *M. chalcea* and *M. fulvopurpurea* in amended and unamended sterile soil are shown in Figures 14 and 15. Graphs are displayed as logged counts at each day.

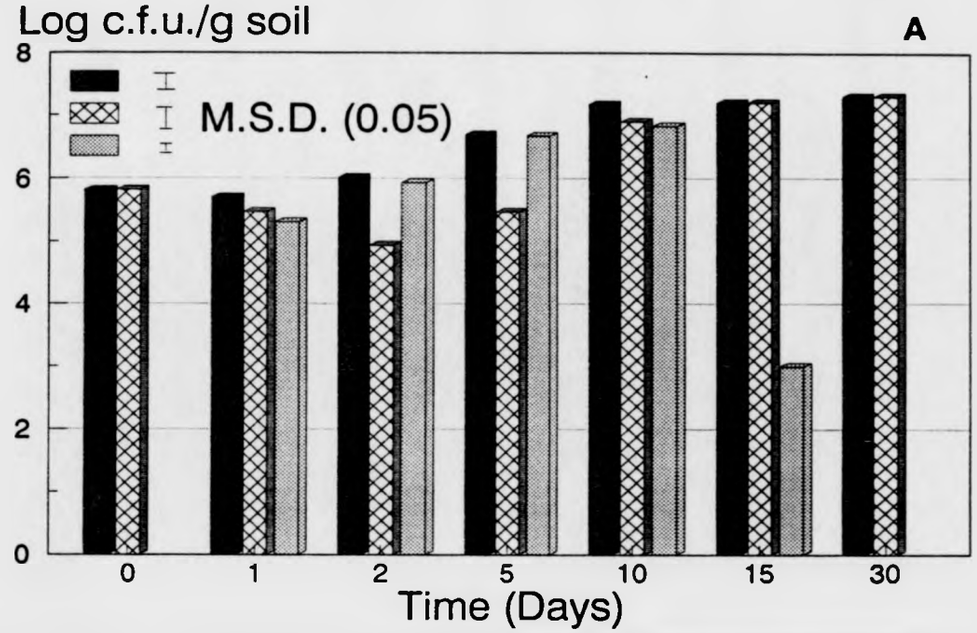
Initial extraction of both organisms from both sets of soil yielded good recovery rates. For *M. chalcea* recovery rates at Day 0 were 66% and 55% for unamended and amended soil respectively. For *M. fulvopurpurea* recovery was slightly less successful at 11% and 30%.

Little difference was observed in the survival of *M. chalcea* and *M. fulvopurpurea* in amended and unamended soil, trends discussed therefore refer to both experiments. Small differences occurring in survival between sterile amended and unamended soil are discussed in the Section 3.4.2 (c).

Figure 14. The survival of *Micromonospora chalcea* subsp. *chalcea* ATCC 12452, in sterile amended and unamended soil.

Graphs A and B show the logged total, spore and mycelial counts at each day of *M. chalcea*. Graph A shows survival in unamended soil, graph B in amended soil.





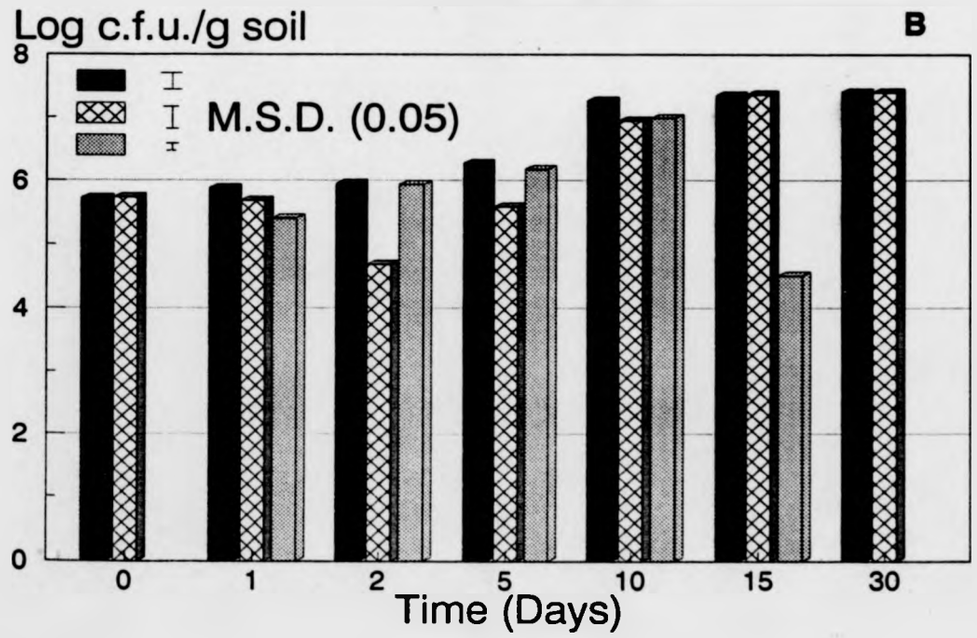
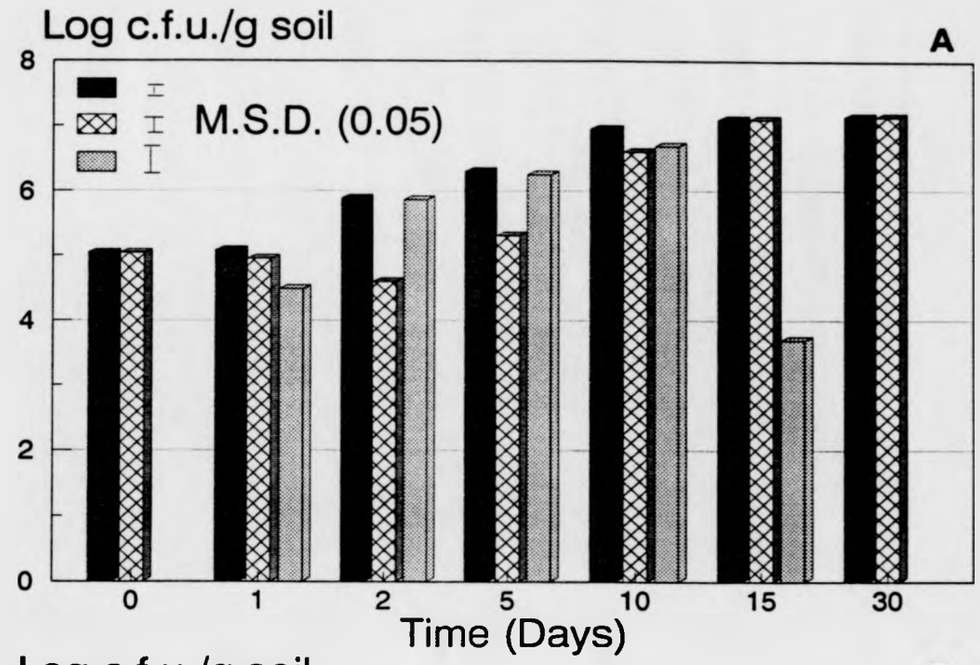


Figure 15. The survival of *Micromonospora fulvopurpurea* JCM 5696, in sterile amended and unamended Soil.

Graphs A and B show the logged total, spore and mycelial counts at each day of *M. fulvopurpurea*. Graph A shows survival in unamended soil, graph B in amended soil.

KEY

- Total count
- Spore count
- Mycelial count



3.4.2(a) The survival of *M. chalcea*

Over the course of the experiment total *M. chalcea* ATCC 12452 counts increased from *ca.* 6 x 10^5 c.f.u./g soil to *ca.* 10^7 c.f.u./g soil in both amended and unamended soil (Figure 14). Spore counts tended to drop progressively to *ca.* 10^4 c.f.u./g soil, rising at day 5 to *ca.* 10^5 c.f.u./g soil. This fall in spore numbers correlated, therefore, with an increase in mycelial numbers to *ca.* 10^6 c.f.u./g soil indicating germination of the spore population. After this initial drop, however, spore numbers continued to increase at day 30 to *ca.* 10^7 c.f.u./g soil. Mycelial numbers peaked at day 10 (*ca.* 10^7 c.f.u./g soil) rapidly declining to an undetectable number by day 30, indicating the onset of sporulation between days 10 and 15. The fact that mycelia could not be detected at day 30 indicates the inability of these structures to survive for prolonged periods of time in soil.

3.4.2(b) The survival of *M. fulvopurpurea*

Over the course of the experiment total *M. fulvopurpurea*, JCM 5696 counts increased from *ca.* 10^5 c.f.u./g soil to *ca.* 10^7 c.f.u./g soil in both amended and unamended soil (Figure 15). Spore counts tended to drop progressively to *ca.* 10^4 c.f.u./g soil, rising at day 5 to *ca.* 10^5. This fall in spore numbers correlated, therefore, with an increase in mycelial numbers to *ca.* 10^6 c.f.u./g soil indicating germination of the spore population. After this initial drop, however, spore numbers continued to increase at day 30 to *ca.* 10^7 c.f.u./g soil. Mycelial numbers peaked at day 10 (*ca.* 10^6 c.f.u./g soil) rapidly declining to an undetectable number by day 30, indicating the onset of sporulation

between days 10 and 15. The fact that mycelia could not be detected at day 30, again indicates the inability of these structures to survive for prolonged periods of time in soil. Trends observed in the survival of *M. fulvopurpurea* mirrored those observed with *M. chalcea*.

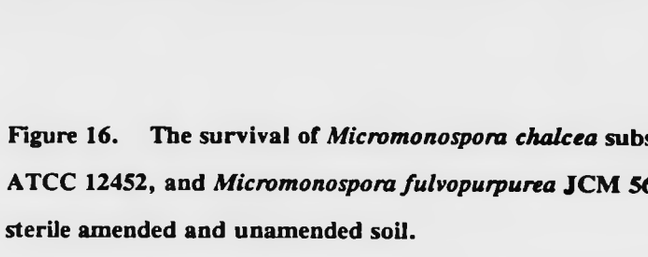
3.4.2(c) Comparison of the survival of *M. chalcea* and *M. fulvopurpurea* in amended and unamended soil

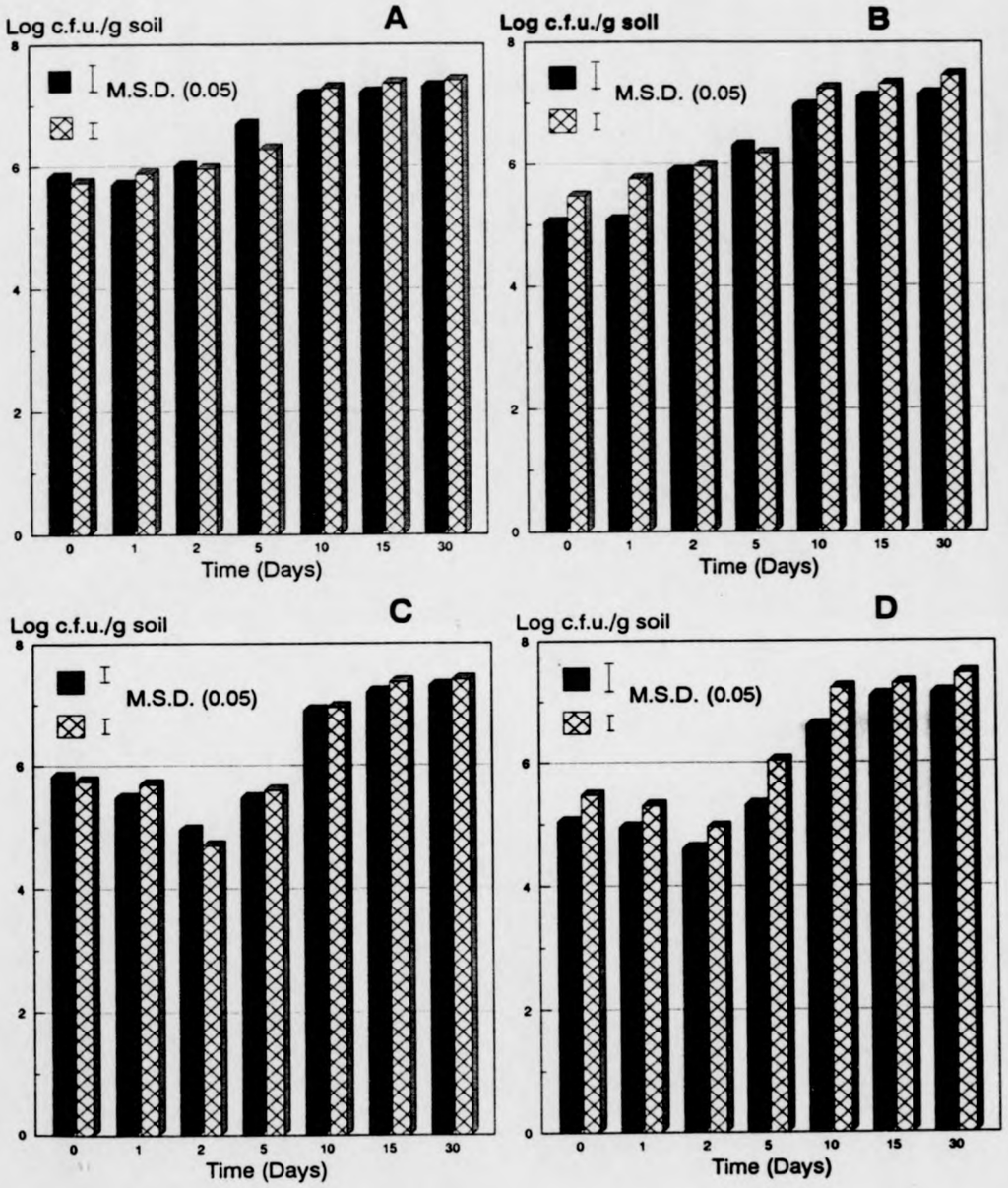
Figure 16 shows the comparison between the survival of *M. chalcea* and *M. fulvopurpurea* in amended and unamended sterile soil.

Graphs A and C show the logged total and spore counts respectively of *M. chalcea*, ATCC 12452, graphs B and D of *M. fulvopurpurea*, JCM 5696. Although total and spore numbers at day 30 were slightly higher in amended soil, little change was observed between the survival of both organisms in amended and unamended soil, the effects of amending soil with starch and chitin having therefore little effect on the growth and proliferation of *Micromonospora* in soil.

Figure 16. The survival of *Micromonospora chalcea* subsp. *chalcea* ATCC 12452, and *Micromonospora fulvopurpurea* JCM 5696, in sterile amended and unamended soil.

Graphs A and C show the logged total and spore counts respectively of *M. chalcea* in unamended and amended sterile soil. Graphs B and D show the logged total and spore counts respectively for *M. fulvopurpurea* in unamended and amended sterile soil.





3.5 Discussion

The method used for the extraction and subsequent enumeration of *Micromonospora* was that based on shaking a given mass of soil with $^1/_4$ strength Ringer's solution (Wellington *et al.*, 1990). The percentage recovery of *Micromonospora* using this method was generally between 50 and 80%. Although the method was limited by only being capable of detecting numbers greater than *ca.* 10^2 c.f.u./g., it had the advantage of being rapid and simple to use to monitor *Micromonospora* populations in soil microcosms.

The development of the above technique to select for *Micromonospora* spores only, was developed from the selection isolation procedures used in Chapter 2, exploiting the resistance of *Micromonospora* to heat treatment. Using these methods we were able to enumerate both the sporing and the mycelial population in soil enabling us to follow the survival of two species of *Micromonospora* in soil. In addition, an obvious conclusion to be made from the development of this method was that it enabled us to definitively prove that heat treatment of soil, as used in Chapter 2, was selective for the spore population of *Micromonospora* only.

The growth and survival of *Micromonospora chalcea* subsp. *chalcea* ATCC 12452 and *Micromonospora fulvopurpurea* JCM 5696 in sterile amended and unamended soil showed a marked germination and sporulation cycle when inoculated as a spore suspension as has been found for streptomycetes (Cresswell *et al.*, 1992; Herron and Wellington, 1990; Wellington *et al.*, 1990). In all cases significant germination was seen at Day 1 increasing by Day 2 (*ca.* 10^5 spores/g soil recovered for both treatments for both organisms). Following rapid sporulation at Day 5, a plateau at *ca.* 10^7-10^8 c.f.u./g soil was generally reached. The

plateau continued, in all cases, up until the end of the experiment, showing no evidence of a decrease in numbers at Day 30. Survival experiments with *Streptomyces* show that although trends in growth were similar to those of *Micromonospora*, plateaus were reached at generally higher concentrations. Bleakey and Crawford (1989) found that *Streptomyces lividans* reached a maximum of *ca.* 10^9 c.f.u./g soil whilst *Streptomyces parvulus* reached counts of *ca.* 10^{10} in sterile amended soil. The counts of *M. chalcea* and *M. fulvopurpurea* reached after Day 10 were generally independant of the host inoculum size indicating that both organisms grew until the soil could support no more growth propagules. This effect could be due either to nutrient depletion or to the inability of the soil to hold any more microbes.

It is known that both *M. chalcea* and *M. fulvopurpurea* can utilise both starch and chitin, amendment of the soil with these compounds, however, appeared to have only a slight effect on the survival of both species of *Micromonospora*. Both organisms appeared to grow a little more extensively in amended than unamended soil. This may be expected although the reason why amendment did not cause higher levels of growth is not understood, as Mayfield *et al.* (1972) found that amendment of soil with glucose caused a significant increase in the percentage of *Streptomyces* spores germinating in soil. It is widely understood, however, that the act of autoclaving soil does provide a means for amending soil in itself, acting to release dissolved nutrients as well as those trapped inside dead organisms allowing the germination of spores (Salonius *et al.*, 1967; Lloyd, 1969).

Work with *Streptomyces* (Herron, 1991; Lloyd, 1969; Mayfield *et al.*, 1972; Skinner, 1951) supports the widely held idea that in the natural soil environment actinomycetes exist largely as spores that are able to survive adverse environmental conditions such as severe nutrient

depletion and drought. Any subsequent drop in spore numbers being replaced by germination at sites of nutrient availability.
However, as similar studies had never been done to follow the fate of *Micromonospora* in the natural soil environment it was impossible to speculate as to their likely survival pattern. The results of the present study therefore have revealed that *Micromonospora* have a similar pattern of behaviour to *Streptomyces*, and that they exist in the soil primarily as sporing structures.

In addition, an obvious conclusion to be made from the development of the heat treatment method was that it enabled us to definitively prove that heat treatment of soil, as used in Chapter 2, is selective for the spore population of *Micromonospora* only. As it appears, from the results of this study that *Micromonospora* occur in the soil primarily as spores the implications are that the use of this method is unlikely to deselect for certain species of the genus.

CHAPTER 4

Cluster Analysis

4.1 Introduction

Recent years have seen the reexamination of traditional classification schemes in biology, due mainly to the development of computer assisted data analysis techniques. Microbiological systematics is dominated by numerical analysis, which provides a basis for the creation of phenetic classifications. A large number of properties and traits characterising a microorganism can be incorporated into a matrix, each character being treated with equal importance. Many actinomycete genera have been examined using such methods, including *Actinomyces* (Schofield and Schaal, 1981), *Actinomadura* (Athalye *et al.*, 1985), and *Nocardia* (Orchard and Goodfellow, 1980). However taxonomically, the most widely studied actinomycete group is the *Streptomyces*. Indeed, arguably, the most comprehensive taxonomic study published was that of Williams *et al.* (1983) for the classification of this genus.

Numerical analysis of a genus makes it possible to determine its degree of homogeneity and, perhaps more importantly, to identify groups or clusters of species occurring within the genus. In some cases numerical analysis may serve to distinguish different clusters necessitating revision of classification. For example, numerical analysis of data on the biological and chemical characteristics of actinomycetes containing meso-DAP in their cell walls, justified the transfer of the species *Micropolyspora brevicatena* to the genus *Nocardia* as *Nocardia brevicatena* comb. nov. (Goodfellow and Pirouz, 1982).

Among actinomycetes containing meso-DAP in their cell walls, surprisingly little research has been done on *Micromonospora*, although this genus accounts for the third largest number of strains isolated from various soils, after streptomycetes and nocardioforms (Lechevalier and Lechevalier, 1967).

The chemotaxonomic characteristics that distinguish *Micromonospora* from genera, such as *Actinoplanes* and *Dactylosporangium*, that are morphologically and chemically related, are summarised in Table 3. It is interesting to note that results from DNA-DNA pairing and 16S rRNA cataloguing studies have shown that the genus *Micromonospora* is closely related to *Actinoplanes*, *Amorphosporangium*, *Ampullariella* and *Dactylosporangium* (Stackebrandt *et al.*, 1981, 1983), thus supporting the chemotaxonomic relationships elucidated in the Table 3 (Section 1.2.2).

In the 4th Volume of Bergey's Manual of Systematic Bacteriology a key is given for the determination of twelve species of *Micromonospora* (Kawamoto, 1989). However, its practical use is limited (Shomura *et al.*, 1983) and somewhat misleading as the main diagnostic test is based on the capacity of the *Micromonospora* to utilise carbohydrates. Work by Bibikova *et al.* (1990) indicated that insufficient attention has been devoted to cultural features such as coloration of aerial mycelium and spores *en masse*. A number of workers, seeking to correct this situation, have focused on the cultural-morphological features as well as the antibiotic producing abilities of the *Micromonospora* (Bibikova and Ivanitskaya, 1986; Bibikova *et al.*, 1990; Ivanitskaya *et al.*, 1985).

The objective of the present study, described in this, and following Chapter, was to make a comparative analysis of the strains belonging to the genus *Micromonospora*. The analysis made was with respect to cultural, biochemical and morphological features, as well as to the capacity of the cultures to grow in the presence of antibiotics. The overall aim of the study was to establish the degree of similarity among members of the genus and to further assist the classification and identification of the *Micromonospora* by the development of a probabilistic identification matrix.

4.1.1 Cluster analysis

Classification may be simply defined as the grouping of a collection of organisms, or Operational Taxonomic Units (OTU's). The history and development of taxonomic classification has been thoroughly reviewed by Cain (1962) and concise overviews of techniques used have been given by Alderson (1985), Austin and Priest (1986) and Sneath and Sokal (1973).

The aim of the work presented in this chapter was to classify *Micromonospora* by using a number of hierarchical clustering methods, the results of which were then used as the basis for the production of a probabilistic identification matrix (Chapter 5). The cluster phenograms obtained gave a clearer understanding of the intra- and suprageneric relationships of the *Micromonospora*.

Phenetic classifications, such as the ones used in this study, arrange OTU's into groups based on high overall similarities, using both phenotypic and genotypic characters (Sneath, 1962). Comprehensive reviews of methods used in classification studies have been given by Everitt (1980) (hierarchical and non-hierarchical clustering), Gordon (1981) (measuring similarity, hierarchical clustering, ordination), Dunn and Everitt (1982) (measuring similarity, hierarchical classification), Alderson (1985) (ordination), and Gordon (1987) (hierarchical clustering).

Sneath and Sokal (1973) have suggested that in order to obtain a reliable classification, taking account of strain variation and sampling error, the number of OTU's used in any study should be greater than 60. Similarly, the optimal number of characterisation tests for a taxonomic study should be between 100 and 150 (Austin and Priest, 1984). Lower

numbers of either OTU's or characters could result in poor and unreliable classifications.

The primary basis of any phenotypic classification is the calculation of similarity between all combinations of OTU's comprising the matrix. Many coefficients of similarity have been devised. Possibly the most comprehensive studies published concerning such measurements, have been by Sneath and Sokal (1973) and Legendre and Legendre (1983). In addition, a detailed critical survey of coefficients on binary data alone has been provided by Hubalek (1982).

The choice of coefficients used in a taxonomic study are determined by the nature of the original data matrix. For qualitative binary data, the simple matching coefficient (S_{sm}) (Sokal and Michener, 1958) is most commonly used, measuring simply the proportion of characters that, for a pair of OTU's, have the same state. Other common examples include the Jaccard coefficient (S_J) which is the same as the S_{sm} except that matching negative states are ignored, and the Dice coefficient (S_D) (Dice, 1945). All 3 coefficients have been used and therefore compared in the following study which deals just with binary data.

Taxonomic structure is subsequently obtained by ordering OTU's into groups of high overall similarity (Alderson, 1985; Goodfellow and Dickinson, 1985). Hierarchical clustering uses an algorithm to search the similarity data matrix for the pair of OTU's which have the highest degree of similarity. The similarities between this pair and each of the remaining OTUs are computed and the process cycles back, treating the newly formed groups as an OTU, to find the new highest similarity. The final output is a hierarchical taxonomic tree. Algorithms used in cluster analysis, differ over the definition of similarity between an OTU and a group. A common algorithm, according to Goodfellow and Dickinson

(1985) is single linkage or nearest neighbour clustering (Sneath, 1957). This joins an OTU to an established group at the highest similarity level of any member of that group. In contrast, complete linkage or furthest neighbour clustering (Sorenson, 1948) joins an OTU at the lowest similarity. However, perhaps the most commonly used algorithm and the one predominantly chosen for use in this study, is that of average linkage. This takes the average of all the similarities across the groups and is thought to give high within-group similarity (Sokal, 1985). The most widely used variant of average linkage is unweighted average linkage (UPGMA) the simple arithmetic average of the similarities across the two groups is taken, each similarity thus having equal weight. In weighted average linkage (WPGMA) each of the two subgroups forming the group has equal weight. Other examples of average linkage are centroid sorting (WPGMC) and median sorting (WPGMS) (Sneath, 1973).

The hierarchical taxonomic trees which can result from the above, diagrammatically summarise the salient points of cluster analysis, illustrating clearly the taxonomic ranks and relationships of given OTUs. It has been suggested by Alderson (1985), however, that such trees can often distort the taxonomic distances between major groups.

In order for a classification to be valid and reliable, it should remain, to all intents and purposes, stable under variations in character coding, coefficient and algorithm (Sackin, 1985). It has been recommended that duplicate strains, amounting to 5% of the total, should be included as an internal check (Sackin, 1985).

The taxonomic map produced should adequately represent the original data matrix. Such representations can be assesed by calculating cophenetic values (Rohlf and Sokal, 1962, 1981).

4.2 Methods

4.2.1 Strains

Two sets of test strains were used giving two sets of phenograms.

Set 1

135 test strains were obtained from Schering-Plough Corporation (Table 14); 10 duplicates were also included. 121 strains represented the *Micromonospora* genus, 13 strains represented the *Actinomadura* genus. All strains were stored on oatmeal slopes at 5°C. Suspensions of spores and mycelial fragments were stored in glycerol (20% v/v) at -20°C (Wellington and Williams, 1978). Phenograms 2, 3 and 4 comprised the *Actinomadura* strains which were included as marker strains in the study. Phenogram 1, however was produced using the 121 *Micromonospora* strains only. This phenogram was used in Chapter 5 to produce the probabilistic identification matrix. *Actinomadura* were not included in this study as it was thought a possibility that they may distort the clustering pattern.

Set 2

55 test strains in total (Table 15): 29 test strains (type cultures) were obtained from both public and private collections. 26 natural isolates were obtained by a variety of isolation methods (Table 15).

4.2.2 Characterisation tests

The 2 sets of strains described in Section 4.2.1 were examined using 2 sets of unit characters, respectively (Tables 16 and 17). Thawed glycerol suspensions served as inocula for all tests. All tests were carried out at least once on each strain, being repeated where ambiguous or

clearly unexpected results were obtained. Inoculated media were usually incubated at 30-32°C. Strains were grown on media at a pH close to 7.0. Morphological, degradation and antibiotic sensitivity tests were carried out in Petri dishes. The 2 sets of characterisation tests are summarised in Tables 16 and 17. All chemicals used in the following tests were obtained from Sigma unless otherwise stated (see also Appendix 1).

Some results obtained from testing Test Set 1 using Characterisation Set 1 were donated by Dr. Ann Horan of Schering Plough Corporation, Bloomfield, New Jersey in most of these cases, however, tests were repeated.

4.2.2(a) Growth on carbohydrates

Appropriate weights of each carbohydrate were sterilised in 50ml flasks with loose fitting bungs by covering the solid with diethyl ether which was allowed to evaporate at room temperature in a fume cupboard for 18 hours. Final traces of ether were removed by placing the flasks in a laminar flow cabinet for 10 minutes. Alternatively carbon sources were sterilised by filtration. Each of the 8 sterilised carbon sources was added, aseptically, to give a concentration of 1% (w/v), to a basal medium containing (l^{-1}): 5g Yeast extract, 1g $CaCO_3$, agar, 15g; adjusted to pH 7.0 (modified from Pridham and Gottlieb, 1948). Repli dishes were inoculated with 0.1 to 0.2 ml of cells. Cultures were incubated at 30-32°C and read at 14, 21 and 28 days. The ability of a strain to use a carbon source was a determined by comparison of its growth with that on a negative control (no carbon source).

4.2.2(b) Growth on nitrogen sources

Strains were tested for their ability to use various nitrogen sources (0.1%, w/v) in a basal medium consisting of D-glucose, 1.0% (w/v); $MgSO_4.7H_2O$, 0.05%; NaCl, 0.05%; $FeSO_4.7H_2O$, 0.001%; K_2HPO_4, 0.1%; Bacto-agar (Difco), 1.2%; pH 7.4. Growth was scored after 15 days by comparing test plates with both negative and positive controls, the latter consisting of the basal media supplemented with either L-asparagine or L-proline.

4.2.2(c) Utilisation of organic acids

The ability of strains to use organic acids as sole carbon sources was examined on Koser's Citrate Agar (Modified)(Appendix 2). A solution of 0.04% phenol red (20ml/litre) was added to the media. Carbon sources were then added at 0.2% (w/v), except for lactate which was added at the concentration 3.3 ml/litre. The final colour of the agar was a very light orange neutral. Each slant of medium was inoculated with 0.1 to 0.2 ml of washed inoculum and incubated at 30-32°C. Growth was scored at 7, 14, 21 and 28 days. A positive result was indicated by the agar turning a rose pink.

4.2.2(d) Degradative tests

The degradation of hypoxanthine and xylan (0.5 and 0.4%, w/v respectively) was detected in plates containing (per litre) Yeast extract 10g, Dextrose 10g, Agar 15g. Plates were inoculated with cells and incubated at 30-32°C. After 3 weeks plates were examined for growth;

clearing of the insoluble compounds from under and around areas of growth was scored as positive.

Arbutin degradation was studied in tubes containing (l^{-1}): 10g yeast extract (Oxoid), 1g arbutin, 0.5g ferric ammonium citrate, and 15g agar, pH 7.2 (Kutzner, 1976). Controls without arbutin were also inoculated. After 3 weeks, a positive result was indicated by a brown-black pigment. Comparison with a control was necessary to avoid confusion with melanin production.

The degradation of adenine, tyrosine (0.5%, w/v), xanthine, keratin (0.3%, w/v), casein (1%, w/v, skimmed milk) was detected in modified Bennett's agar (MBA)(Appendix 2) after 21 days; clearing of the insoluble compounds from under and around areas of growth was scored as positive. Gelatin (0.4%, w/v) and starch (1.0%, w/v) degradation were detected in the same basal medium after 7 days by flooding plates with acidified $MgCl_2$ solution (Frazier, 1929) and iodine solution (Cowan, 1974), respectively, and scoring zones of clearing as positive. The degradation of DNA (0.2%, w/v) was observed using Bacto DNase Test agar (Difco). 7 day plates were flooded with 1M HCl and clearance zones were recorded as positive. Chitinolytic activity was observed after 21 days by the appearence of zones of clearing in colloidal chitin agar (Hsu and Lockwood, 1975); only zones of >4.5mm were scored as positive, as weak positive reactions were not reproducible. The degradation of allantoin and urea was recorded after 21 days using the media and methods of Gordon (1966, 1968). The breakdown of esculin (0.1%, w/v) was determined by the method of Kutzner (1976) using a modified medium containing yeast extract (Oxoid), 0.3% (w/v); ferric ammonium citrate, 0.05% and Lab M agar, 0.75%. Tubes were examined after 21 days; any slight production of melanin was detected in

the negative control. Blackening of the test media resulted in a positive result.

4.2.2(e) Growth inhibition

Growth inhibition tests were carried out in Repli dishes containing (l^{-1}): 10g yeast extract (Oxoid), 10g Dextrose, 1g $CaCO_3$, and 15g agar, pH 7.3. Each potential inhibitor was added to this medium before autoclaving to give the appropriate final dilution. After 2 weeks growth was compared with that on the medium without the inhibitor; absence of growth, or very weak growth was recorded as negative.

Growth at 10, 27, 35, 40, 42 and 45°C was determined in Repli dishes using the same basal medium, as was growth at a range of pHs (5, 6, 7, 8, 9 and 10). Plates were incubated also for 14 days, enclosed in a polythene bag to prevent desiccation, and presence or absence of growth was recorded.

4.2.2(f) Antibiotic resistances

Resistances to a range of antibiotics including gentamicin 50mg/ml, kanamicin 10mg/ml, novobiocin 50mg/ml and cycloserine 50mg/ml were determined on the same basal medium as above. Each antibiotic was filter sterilised and added to the medium aseptically after autoclaving to the appropriate concentration. Plates were examined for growth after 2 weeks.

4.2.2(g) Biochemical tests

Catalase production was detected by adding a few drops of 20 volume H_2O_2 on to 7 day old colonies grown on MBA; evolution of oxygen was detected under a binocular microscope. Nitrate reduction was determined after 7 and 14 days by addition of 0.2ml each of Griess-Ilosvay reagents I and II to stab cultures in a sloppy nitrate medium composed of nutrient broth (Oxoid CM1) supplemented with KNO_3 (0.2%, w/v) and Bacto-agar (0.6%; Difco).

Hydrolysis of hippurate was determined by using a Hippurate medium (Thirst, 1957). The medium was dispensed into 25mm tubes stoppered with Morton closures. Tubes were inoculated with the test culture, incubated at 30°C on a rotary shaker at 250rpm. After 14 days the culture was tested for benzoic acid by mixing 1ml of culture broth with 1.5ml of 50% sulphuric acid in a 16mm test tube. The appearence of crystals in the acid mixture after 4 hours at room temperature indicated hydrolysis of the hippurate.

4.2.2(h) Ability to use 4-methyl-umbelliferone-linked substrates

This test is based on the generation of fluorescent 4-methylumbelliferone from the appropriate substrate indicating activity of enzymes. Identification of 4-MU is achieved by adjustment of pH (Goodfellow *et al.*, 1987).

Strains were tested for their ability to use a range of 4-methylumbelliferone linked substrates. *Micromonospora* were grown on Bennett's agar at 25°C for 6 days. The 4-methylumbelliferone conjugated substrates were used to detect the following enzymes: BL-fucosidase, BD-fucosidase, BD-glucosidase, BD-mannosidase,

phosphatase, BD-glucuronidase, AD-mannosidase, BD-galactosidase, BD-cellobiosidase and BD-xylosidase. The 4-MU substrates (5mg) were dissolved in 200ul dimethyl sulphoxide to give approximately 2×10^{-3} molar solutions which were diluted to 10ml in 0.05M acetate buffer, pH 5.2.

Micromonospora inocula from 6-day old Bennett's agar plates were transferred to ringed areas on Whatman filter paper No. 3 (ca. 90mm) held in a petri dish. 8 inocula were rubbed into the paper and a drop of substrate added. The reactants were incubated for 20 minutes at 37°C. A drop of saturated sodium bicarbonate was then added to each inoculated substrate to enhance fluorescence which was visualised at 366nm under UV light. Controls consisted of substrate only and *Micromonospora* growth plus the solvent and buffer used in preparing the substrate. Positive reactions were recorded when a light blue fluorescence was observed.

The following tables (14 to 17) show sets of strains and sets of characterisation tests used for cluster analysis.

Strain histories

ATTC: American Type Culture Collection

JCM : Japan Collection of Microorganisms

IFM : International Fermentation Organisation

NRRL: Northern Regional Research Laboratories

All other strains were obtained from Schering Plough Corporation, Bloomfield, New Jersey.

Table 14 Strain Set 1

1. *Micromonospora grisea*	G55
2. *M. zionensis*	G52
3. *M. olivasterospora*	ATCC 21819
4. *M. olivasterospora*	ATCC 31010
5. *M. olivasterospora*	ATCC 31009
6. *M. rosaria*	ATCC 29337
7. *rosaria*-like	S9683
8. *rosaria*-like	Q319
9. *rosaria*-like	Q1578
10. *rosaria*-like	4215
11. *rosaria*-like	9603
12. *rosaria*-like	S2742
13. *rosaria*-like	T816
14. *rosaria*-like	X70
15. *rosaria*-like	T5771
16. *rosaria*-like	T5804
17. *M. purpureochromogenes*	ATCC 27007
18.	SCC 969 (M1021)
19.	SCC 1000 (68-I)
20. *M. fusca*	CBS SCC 821
21. *M. fusca*	SCC 900
22. *M. narashinoensis*	SCC 939 IFO
23. *M. purpureochromogenes*	ATCC 10026
24. *M. echinospora* var. *echinospora*	817
25.	TS 8
26. *M. echinospora* var. *ferruginea*	782
27. *M. echinospora* var. *pallida*	TS 5

28.		795
29.		774
30.		799
31.		805
32.		10-408-C
33.		10-408-35
34.		11 89
35.		10 245
36.		11 224
37.		11 365
38.		12 46
39.		12 123
40.		12 125
41.		12 44
42.	*M. echinospora* var. *challensensis*	14 261
43.	*M. polytrota*	AR 5
44.	*M. polytrota*	15 368
45.	*M. capillata Ferm. P*	2598
46.	*M. carbonacea* var.*carbonacea*	ATCC 27114
47.	*M. carbonacea* var.*aurantiaca*	ATCC 27115
48.	*M. carbonacea* var.*africana*	ATCC 39149
49.	*M. chalcea*	ATCC 12452
50.	*M. chalcea*	ATCC 27333
51.	*M. chalcea*	ATCC 21994
52.		91 372
53.		91 1306
54.	*M. chalcea*	ATCC 27083
55.	*M. chalcea*	ATCC 27084
56.		R 460
57.		R 461
58.		69 683
59.		1020
60.		1062
61.		1063
62.	*M. halophytica* var. *halophtica*	SCC 807 SP-30
63.	*M. halophytica* var. *nigra*	SCC 760
64.		SCC 763
65.		785 (R476)
66.		818 (R476)
67.		737 (SP14)
68.		815 (SP14)
69.	*M. sagamiensis*	ATCC 21803
70.	*M. sagamiensis*	ATCC 21826
71.	*M. sagamiensis*	ATCC 21827
72.	*M. sagamiensis*	ATCC 21949
73.	*M. purpurea*	NTS 9
74.	*M. purpurea*	ATCC 31119
75.	*M. purpurea*	SCC 1038
76.		SCC 1039
77.		71-1225
78.		H334-0
79.		334-5
80.		334-13
81.		11-90
82.		12-33
83.		12-34
84.		12-35
85.		12-36

86.	12-37
87. *Micromonospora sp.*	NRRL 3543
88. *M. inyoensis*	ATCC 27600
89.	NRRL 5742
90.	6640
91.	1081
92.	72-469
93.	10-8
94.	10-9
95.	984
96.	990
97.	71-64
98. Nocardicin (22JC) producer	SCC 895
99. Nocardicin (22JC) producer	13 859
100. Gilvocarcin producer	13 304
101. Gilvocarcin producer	13 335
102. Gilvocarcin producer	12 402
103. Phenazine-type producer	124
104. Phenazine-type producer	12 414
105. Toxic compound producer	68-742
106. Toxic compound producer	68-742-12
107. Toxic compound producer	68-742-1284
108. Thiostrepton producer	68-1147
109. Thiostrepton producer	68-1147-134
110. Thiostrepton producer	10-259
111. Myxin producer	13-16
112. Myxin producer	13-17
113. Myxin producer	13-20
114. Myxin producer	13-22
115. Myxin producer	13-23
116. Myxin producer	13-26
117. Myxin producer	13-34
118. Myxin producer	13-36
119. Myxin producer	13-40
120. Myxin producer	13-83
121. Myxin producer	13-96
122. *Actinomadura*	SCC 906
123. *Actinomadura*	SCC 918
124. *Actinomadura*	A 27295
125. *Actinomadura*	A 27296
126. *Actinomadura*	A 27297
127. *Actinomadura*	A 27298
128. *Actinomadura*	A 27299
129. *Actinomadura*	A 27887
130. *Actinomadura*	A 27888
131. *Actinomadura*	A 29553
132. *Actinomadura*	13-363
133. *Actinomadura*	AA-6
134. *Actinomadura*	BL-6
135. *Actinomadura*	A 23218

Table 14A. Strain Set 1 (Subset A)

Consisting of type strains only.

1. *Micromonospora grisea*	G55
2. *M. zionensis*	G52

86.	12-37
87. *Micromonospora sp.*	NRRL 3543
88. *M. inyoensis*	ATCC 27600
89.	NRRL 5742
90.	6640
91.	1081
92.	72-469
93.	10-8
94.	10-9
95.	984
96.	990
97.	71-64
98. Nocardicin (22JC) producer	SCC 895
99. Nocardicin (22JC) producer	13 859
100. Gilvocarcin producer	13 304
101. Gilvocarcin producer	13 335
102. Gilvocarcin producer	12 402
103. Phenazine-type producer	124
104. Phenazine-type producer	12 414
105. Toxic compound producer	68-742
106. Toxic compound producer	68-742-12
107. Toxic compound producer	68-742-1284
108. Thiostrepton producer	68-1147
109. Thiostrepton producer	68-1147-134
110. Thiostrepton producer	10-259
111. Myxin producer	13-16
112. Myxin producer	13-17
113. Myxin producer	13-20
114. Myxin producer	13-22
115. Myxin producer	13-23
116. Myxin producer	13-26
117. Myxin producer	13-34
118. Myxin producer	13-36
119. Myxin producer	13-40
120. Myxin producer	13-83
121. Myxin producer	13-96
122. *Actinomadura*	SCC 906
123. *Actinomadura*	SCC 918
124. *Actinomadura*	A 27295
125. *Actinomadura*	A 27296
126. *Actinomadura*	A 27297
127. *Actinomadura*	A 27298
128. *Actinomadura*	A 27299
129. *Actinomadura*	A 27887
130. *Actinomadura*	A 27888
131. *Actinomadura*	A 29553
132. *Actinomadura*	13-363
133. *Actinomadura*	AA-6
134. *Actinomadura*	BL-6
135. *Actinomadura*	A 23218

Table 14A. Strain Set 1 (Subset A)

Consisting of type strains only.

1. *Micromonospora grisea*	G55
2. *M. zionensis*	G52

3. *M. olivasterospora*	ATCC 21819
4. *M. olivasterospora*	ATCC 31010
5. *M. olivasterospora*	ATCC 31009
6. *M. rosaria*	ATCC 29337
7. *M. purpureochromogenes*	ATCC 27007
8. *M. fusca*	SCC 821
9. *M. fusca*	SCC 900
10. *M. narashinoensis*	SCC 939 IFO
11. *M. purpureochromogenes*	ATCC 10026
12. *M. echinospora* var. *echinospora*	817
13. *M. echinospora* var. *ferruginea*	782
14. *M. echinospora* var. *pallida*	TS 5
15. *M. echinospora* var. *challensensis*	14 261
16. *M. polytrota*	AR 5
17. *M. polytrota*	15 368
18. *M. carbonacea* var. *carbonacea*	ATCC 27114
19. *M. carbonacea* var. *aurantiaca*	ATCC 27115
20. *M. carbonacea* var. *africana*	ATCC 39149
21. *M. chalcea*	ATCC 12452
22. *M. chalcea*	ATCC 27333
23. *M. chalcea*	ATCC 21994
24. *M. chalcea*	ATCC 27083
25. *M. chalcea*	ATCC 27084
26. *M. halophytica* var. *halophytica*	SCC 807 SP-30
27. *M. halophytica* var. *nigra*	SCC 760
28. *M. sagamiensis*	ATCC 21803
29. *M. sagamiensis*	ATCC 21826
30. *M. sagamiensis*	ATCC 21827
31. *M. sagamiensis*	ATCC 21949
32. *M. purpurea*	TS 9
33. *M. purpurea*	ATCC 31119
34. *Micromonospora sp.*	NRRL 3543
35. *M. inyoensis*	ATCC 27600
36. *Micromonospora sp.*	NRRL 5742
37. *Actinomadura*	SCC 906
38. *Actinomadura*	SCC 918
39. *Actinomadura*	ATCC 27295
40. *Actinomadura*	ATCC 27296
41. *Actinomadura*	ATCC 27297
42. *Actinomadura*	ATCC 27298
43. *Actinomadura*	ATCC 27299
44. *Actinomadura*	ATCC 27887
45. *Actinomadura*	ATCC 27888
46. *Actinomadura*	ATCC 29553
47. *Actinomadura*	13-363
48. *Actinomadura*	AA-6
49. *Actinomadura*	BC-6
50. *Actinomadura*	ATCC 23218

Table 14B. Strain Set 1 (subset B)

Consisting of type strains and 8 unclassified antibiotic producing *Micromonospora*.

1. *Micromonospora grisea*	G55
2. *M. zionensis*	G52
3. *M. olivasterospora*	ATCC 21819

4. *M. olivasterospora*	ATCC 31010
5. *M. olivasterospora*	ATCC 31009
6. *M. rosaria*	ATCC 29337
7. *M. purpureochromogenes*	ATCC 27007
8. *M. fusca*	SCC 821
9. *M. fusca*	SCC 900
10. *M. narashinoensis*	SCC 939 IFO
11. *M. purpureochromogenes*	ATCC 10026
12. *M. echinospora* var. *echinospora*	817
13. *M. echinospora* var. *ferruginea*	782
14. *M. echinospora* var. *pallida*	TS 5
15. *M. echinospora* var. *challensensis*	14 261
16. *M. polytrota*	AR 5
17. *M. polytrota*	15 368
18. *M. carbonacea* var. *carbonacea*	ATCC 27114
19. *M. carbonacea* var. *aurantiaca*	ATCC 27115
20. *M. carbonacea* var. *africana*	ATCC 39149
21. *M. chalcea*	ATCC 12452
22. *M. chalcea*	ATCC 27333
23. *M. chalcea*	ATCC 21994
24. *M. chalcea*	ATCC 27083
25. *M. chalcea*	ATCC 27084
26. *M. halophytica* var. *halophytica*	SCC 807 SP-30
27. *M. halophytica* var. *nigra*	SCC 760
28. *M. sagamiensis*	ATCC 21803
29. *M. sagamiensis*	ATCC 21826
30. *M. sagamiensis*	ATCC 21827
31. *M. sagamiensis*	ATCC 21949
32. *M. purpurea*	TS 9
33. *M. purpurea*	ATCC 31119
34. *Micromonospora sp.*	NRRL 3543
35. *M. inyoensis*	ATCC 27600
36. *Micromonospora sp.*	NRRL 5742
37. *Actinomadura*	SCC 906
38. *Actinomadura*	SCC 918
39. *Actinomadura*	ATCC 27295
40. *Actinomadura*	ATCC 27296
41. *Actinomadura*	ATCC 27297
42. *Actinomadura*	ATCC 27298
43. *Actinomadura*	ATCC 27299
44. *Actinomadura*	ATCC 27887
45. *Actinomadura*	ATCC 27888
46. *Actinomadura*	ATCC 29553
47. *Actinomadura*	13-363
48. *Actinomadura*	AA-6
49. *Actinomadura*	BC-6
50. *Actinomadura*	ATCC 23218
51. Nocardicin producer	SCC 895
52. Gilvocarcin	13 304
53. Phenazine-type producer	12 414
54. Toxic compound producer	68-742
55. Thiostrepton producer	68-1147
56. Thiostrepton producer	10-259
57. Myxin producer	13-16
58. Myxin producer	13-17

Table 15. Strain Set 2

All strains belong to the *Micromonospora* genus

1. *M. carbonacea* var. *aurantiaca*	ATCC 27115
2. *M. chalcea* var. *chalcea*	ATCC 12452
3. *M. chalcea*	ATCC 27344
4. *M. fulvopurpurea*	JCM 5696
5. *M. echinoaurantiaca*	JCM 3257
6. *M. purpureochromogenes*	ATCC 27007
7. *M. nigra*	JCM 3328
8. *M. narashinoensis*	IFM 110-76
9. *M. floridensis*	NRRL 8020
10. *M. yulogensis*	ATCC 43540
11. *M. globosa*	JCM 3358
12. *M. rubra*	ATCC 27031
13. *M. chalcea* var. *flavida*	ATCC 27084
14. *M. globosa*	NRRL B2673
15. *M. melanosporea*	IFO 12515
16. *M. parva*	NRRL B2676
17. *M. echinospora* var. *pallida*	ATCC 15838
18. *M. megalomicea* var. *nigra*	ATCC 27598
19. *M. chalcea* var. *izumensis*	ATCC 21561
20. *M. sagamiensis*	ATCC 21826
21. *M. echinospora* var. *ferruginea*	NRRL 2995
22. *M. zionensis*	NRRL 5466
23. *M. halophytica*	NRRL 3097
24. *M. viridifaciens*	ATCC 31146
25. *M. inyoensis*	ATCC 27600
26. *M. lacustris*	ATCC 21974
27. *M. fulvoviridis*	JCM 3259
28. *M. purpurea*	ATCC 15835
29. *M. echinobrunnea*	JCM 3327
30. Wild Isolate A	Warwick soil/Heat treatment
31. Wild Isolate B	Permanent Pasture soil/Heat
32. Wild Isolate C	Permanent Pasture soil/Heat
33. Wild Isolate D	Warwick soil/Antibiotic treatment
34. Wild Isolate E	Regina soil/Heat treatment
35. Wild Isolate F	Warwick soil/Heat treatment
36. Wild Isolate G	Regina soil/Heat treatment
37. Wild Isolate H	Pond Sediment
38. Wild Isolate I	Bicutan soil/Heat treatment
39. Wild Isolate J	Italian soil/Antibiotic treatment
40. Wild Isolate K	Warwick soil/Heat treatment
41. Wild Isolate L	Bicutan soil/Heat treatment
42. Wild Isolate M	Permanent Pasture soil/Heat
43. Wild Isolate N	Permanent Pasture soil/Heat
44. Wild Isolate O	Permanent Pasture soil/Heat
45. Wild Isolate P	Italian soil/Antibiotics
46. Wild Isolate Q	Warwick soil/Heat treatment
47. Wild Isolate R	Warwick soil/Heat treatment
48. Wild Isolate S	Warwick soil/Heat treatment
49. Wild Isolate T	Warwick soil/Heat treatment
50. Wild Isolate U	Warwick soil/Heat treatment
51. Wild Isolate V	Warwick soil/Heat treatment
52. Wild Isolate W	Warwick soil/Heat treatment

53. Wild Isolate X	Warwick soil/Heat treatment
54. Wild Isolate Y	Warwick soil/Heat treatment
55. Wild Isolate Z	Warwick soil/Heat treatment

NB. For details of isolation methods used see **Chapter 2.**

Table 15A. Strain Set 2 (subset A)

***Micromonospora* type cultures only.**

1. *M. carbonacea* var. *aurantiaca*	ATCC 27115
2. *M. chalcea* var. *chalcea*	ATCC 12452
3. *M. chalcea*	ATCC 27344
4. *M. fulvopurpurea*	JCM 5696
5. *M. echinoaurantiaca*	JCM 3257
6. *M. purpureochromogenes*	ATCC 27007
7. *M. nigra*	JCM 3328
8. *M. narashinoensis*	IFM 110-76
9. *M. floridensis*	NRRL 8020
10. *M. yulogensis*	ATCC 43540
11. *M. globosa*	JCM 3358
12. *M. rubra*	ATCC 27031
13. *M. chalcea* var. *flavida*	ATCC 27084
14. *M. globosa*	NRRL B2673
15. *M. melanosporea*	IFO 12515
16. *M. parva*	NRRL B2676
17. *M. echinospora* var. *pallida*	ATCC 15838
18. *M. megalomicea* var. *nigra*	ATCC 27598
19. *M. chalcea* var. *izumensis*	ATCC 21561
20. *M. sagamiensis*	ATCC 21826
21. *M. echinospora* var. *ferruginea*	NRRL 2995
22. *M. zionensis*	NRRL 5466
23. *M. halophytica*	NRRL 3097
24. *M. viridifaciens*	ATCC 31146
25. *M. inyoensis*	ATCC 27600
26. *M. lacustris*	ATCC 21974
27. *M. fulvoviridis*	JCM 3259
28. *M. purpurea*	ATCC 15835
29. *M. echinobrunnea*	JCM 3327

Table 16. Characterisation Test Set 1

Growth on Carbohydrates

1. Adonitol
2. Amygdalin
3. D-Arabinose
4. L-Arabinose
5. Arbutin
6. Cellobiose
7. Dextrin
8. Dulcitol
9. Erythritol
10. Fructose
11. L-Fucose
12. Galactose
13. Glucose
14. D-2 Glucose
15. a B Glucopyranose
16. Glycerol
17. Glycogen
18. Inositol
19. Inulin
20. Lactose
21. Maltose
22. Mannitol
23. Mannose
24. Melibose
25. Melezitose
26. Raffinose
27. Rhamnose
28. Ribose
29. Salicin
30. Sorbitol
31. Sucrose
32. Trehalose
33. D-Xylose
34. L-Xylose

Utilisation of Organic Acids

35. Acetate
36. Benzoate
37. Butyrate
38. Caprylate
39. Citrate
40. Formate
41. Gluconate
42. Glucuronate
43. Glutamate
44. Lactate
45. Malate
46. Oleate
47. Oxalate
48. Propionate
49. Pyruvate
50. Succinate
51. Tartrate
52. Calcium glycerate
53. Fumarate

54. a-Ketoglutarate
Hydrolysis of:
55. Adenine
56. Hypoxanthine
57. Tyrosine
58. Xanthine
59. Xylan
60. Chitin
61. Casein
62. Starch
63. DNA
64. Gelatin
65. Arbutin
66. Esculin
67. Urea, 7 days
68. Urea, 28 days
69. Allantoin
70. Hippurate
71. Catalase
72. Oxidase
73. Nitrate.
Growth at:
74. pH 5.0
75. pH 6.0
76. pH 7.0
77. pH 8.0
78. pH 9.0
79. pH 10.0
80. 10°C
81. 27°C
82. 35°C
83. 40°C
84. 42°C
85. 45°C
86. 50°C/8 hours
Resistance to:
87. Lysozyme
88. Sodium salicyclate
89. Sodium Chloride 1%
90. 1.5%
91. 2%
92. 3%
93. 4%
94. 5%
95. $Na_2S_2O_3$ 1%
96. 2%
97. 3%
98. 4%
99. $(NH_4)SO_4$ 1%
101. 3%
102. 4%
103. Sodium azide 0.001%
104. 0.005%
106. 0.02%
107. 0.03%
108. Phenyl EtOH 0.05%
109. 0.1%
110. 0.2%

111. 0.3%
112. 0.4%
113. $CoCl_2$ 1×10^{-4}
114. 5×10^{-4}
115. 1×10^{-3}
116. 5×10^{-3}
117. 1×10^{-2}
118. Phenol 0.025%
119. 0.05%
120. 0.075%
121. 0.1%
122. Thallium acetate 0.0001%
123. 0.001%
124. 0.01%
125. Tetrazolium 0.001%
126. 0.005%
127. 0.01%
128. 0.1%
129. Crystal violet 0.000001%
130. 0.0005%
131. 0.0001%
132. 0.005%
133. 0.001%

Antibiotic Resistances:

134. Gentamicin 10mg/ml
135. 50mg/ml
136. Sisomicin 10
137. 50
138. Neomycin 10
139. 50
140. Kanamycin 10
141. 50
142. Erythromicin 10
143. 50
144. Halomicin 10
145. 50
146. Evernomicin 10
147. 50
148. Rosamicin 10
149. 50
150. Novobiocin 10
151. 50
152. Chloroamphenicol 5
153. 10
154. Bacitracin 2
155. 10
156. Nalidixic acid 10
157. 50
158. Cycloserine 10
159. 50
160. Streptomicin 10
161. 50
162. Gantisin 100
163. 150
164. Tetracycline 10
165. 50
166. Penicillin G 10
167. 50 168. Lincomycin 10 169. 50

170. Clindamycin 10
171. 50
172. Genta A 10
173. 50
174. Paromycin 10
175. 50
176. Cephalothin 10
177. 50
178. Sulphadiazin 100
179. 150

Table 16A. Characterisation Test Set 1 (subset A)
Antibiotic Resistances only:
1. Gentamicin 10mg/ml
2. 50mg/ml
3. Sisomicin 10
4. 50
5. Neomicin 10
6. 50
7. Kanamicin 10
8. 50
9. Erythromicin 10
10. 50
11. Halomicin 10
12. 50
13. Evernomicin 10
14. 50
15. Rosamicin 10
16. 50
17. Novobiocin 10
18. 50
19. Chloroamphenicol 5
20. 10
21. Bacitracin 2
22. 10
23. Nalidixic Acid 10
24. 50
25. Cycloserine 10
26. 50
27. Streptomicin 10
28. 50
29. Gantisin 100
30. 150
31. Tetracycline 10
32. 50
33. Penicillin G 10
34. 50
35. Lincomycin 10
36. 50
37. Clindamycin 10
38. 50
39. Genta A 10
40. 50
41. Paromycin 10
42. 50
43. Cephalothin 10 44. 50
45. Sulphadiazin 100 46. 150

Table 17 Characterisation Test Set 2

Resistance to Antibiotics:
1. Chloroamphenicol
2. Erythromicin
3. Fusidic Acid
4. Methicillin
5. Novobiocin
6. Penicillin G
7. Streptomycin
8. Tetracycline
9. Clindamycin
10. Gentamicin
11. Trimethoprim
12. Sulphamethoxazole
13. Ampicillin
14. Cephaloridine
15. Cloxacillin
16. Lincomycin

Ability to utilise enzymes (Using 4 Methyl Umbelliferyl Conjugated Substrates):
17. B L Fucosidase
18. B D Fucosidase
19. B D Glucosidase
20. B D Mannosidase
21. Phosphatase
22. B D Glucuronidase
23. a D Mannosidase
24. B D Galactosidase
25. B D Cellobiosidase
26. B D Xylosidase

Growth at:
27. 10°C
28. 45°C

Ability to Degrade:
29. Xylan

Resistance to:
30. Sodium chloride, 4%
31. Sodium azide, 0.01%
32. Sodium azide, 0.02%

Utilization of Carbohydrates:
33. Glucose
34. Ribose
35. Rhamnose
36. Adonitol
37. Mannitol
38. Lactose
39. Xylose
40. Initol
41. Raffinose
42. Cellobiose
43. Fructose
44. Asparagine

Utilisation of Nitrogen Sources:
45. a Aminobutyric Acid
46. Histidine
47. Hydroxyproline

Growth at:
48. pH 4.5
49. pH 10.0

Table 17A. Characterisation Test Set 2 (subset A)

Resistance to Antibiotics:
1. Chloroamphenicol
2. Erythromicin
3. Fusidic Acid
4. Methicillin
5. Novobiocin
6. Penicillin G
7. Streptomycin
8. Tetracycline
9. Clindamycin
10. Gentamicin
11. Trimethoprim
12. Sulphamethoxazole
13. Ampicillin
14. Cephaloridine
15. Cloxacillin
16. Lincomycin

4.2.3 Data handling

4.2.3(a) Data coding

Nearly all of the characters existed in one of two mutually exclusive states and were scored plus (1) or minus (0). Quantitative multistate characters, such as tolerance to chemical inhibitors, were coded using the additive method of Sneath and Sokal (1973).

4.2.3(b) Test reproducibility

For analysis of Strain Set 1 using Test Set 1 (the largest data set) 20 duplicate cultures were included in the study. The duplicate cultures and their partners (Table 18) were compared for all characters tested and an estimate of test variance (s_i^2) calculated (formula 15; Sneath & Johnson, 1972). The average test variance was determined to obtain a pooled variance (s^2), which was used to calculate the average probability (p) of an erroneous test result (formula 4; Sneath and Johnson, 1972).

4.2.3(c) Cluster analysis

The binary data was stored in Lotus 123 (Lotus Development Corporation, Cambridge, MA 02142). Data matrices, taken from the database, provided input for NTSYS-pc (Exeter Publishing, Ltd., 100 North Country Road, Building B, Setauket, New York 11733), where the SIMQUAL programs were used to give similarity and dissimilarity matrices. Three coefficients were used and compared in SIMQUAL; the simple matching coefficient (S_{sm}; Sokal and Michener, 1958), which

includes both positive and negative matches, the Jaccard coefficient (S_J; Sneath, 1957) including positive matches only, and the Dice coefficient (S_D; Dice, 1945).

Hierarchical clustering was performed in SAHN, using algorithms for single linkage (Dunn and Everitt, 1982) and average linkage (Sneath and Sokal, 1973). Two algorithms were used in average linkage; the Unweighted Pair Group method (UPGMA; Sneath and Sokal, 1973) and the Weighted Average pair group method (WPGMA; Sneath and Sokal, 1973). Graphic hardcopies of phenograms were provided by the TREEG program. Cophenetic matrices were obtained using COPH and cophenetic correlations using MXCOMP and MXCOMPG for phenograms produced using S_{sm} and UPGMA. By calculation of the Mantle statistic this enabled assessment of the phenographic representation of the similarity data.

4.3 Hierarchical clustering using physiological and biochemical tests and resistance profiles

Using the two sets of strains and the two respective sets of characterisation tests, the following phenograms were produced.

4.3.1 Description of Phenogram 1 to Phenogram 7

4.3.1(a) Phenogram 1

Using **Strain Set 1** (*Micromonospora* strains-135) and **Test Set 1**, five phenograms were produced (Figures 17 and 18). The simple matching coefficient, the Jaccard and Dice coefficients were used to produce similarity matrices which were then clustered using the UPGMA

algorithm. Using the similarity matrix produced from the simple matching coefficient, two further phenograms were produced using the algorithms, single linkage and WPGMA. A comparison of different clustering algorithms and coefficients was made to minimise the chances of accepting misleading results. The dendrograms were compared for stability of clustering.

Fourteen groups were defined on the phenogram produced using the S_{SM} coefficient and the UPGMA algorithm at the 77.5% S_{sm} similarity level.

4.3.1(b) Phenogram 2

Using **Strain Set 1 (Subset A)** and all characterisation tests, the same phenograms as above were produced (Figures 19 and 20). **Subset A** consisted of just ATCC strains, representing each of the main *Micromonospora* species plus representative *Actinomadura* species for comparative purposes (50 strains in total). This enabled relationships between the species to be clearly observed and allowed comparisons to be made between this and the above set of phenograms.

Nine groups were defined at the 75.5% S_{sm} similarity level (S_{sm}/UPGMA phenogram).

4.3.1(c) Phenogram 3

This set of phenograms were produced by using **Strain Set 1** strains (135 strains). **Test Set 1 (Subset A)** were used, consisting of just antibiotic resistance tests (Figures 21 and 22). Antibiotic resistance profiles of the *Micromonospora* were then compared to the conventional taxonomic profile of the organisms.

The phenograms enabled the relationship between phenetic resistance and antibiotic production to be explored.

As above (Section 4.3.1(a)), five combinations of algorithms and coefficients were used, enabling an assessment to be made of the reproducibility of the clusters produced. Eighteen groups were defined at the 78% S_{sm} similarity level (S_{sm}/UPGMA phenogram).

4.3.1(d) Phenogram 4

Phenogram 4 was produced by using, again, just the antibiotic resistance tests (**Test Set 1 (Subset A)**).
However, just **Strain Set 1 (Subset B)**, which were all ATCC strains (*Actinomadura* reference strains included), plus 8 prolific antibiotic-producing *Micromonospora* isolated by Scherings but, as yet, uncharacterised were used (58 strains). The simple matching coefficient and the UPGMA algorithm were used for clustering (Figure 23). Eleven distinct groups were formed at the 72.7% S_{sm} similarity level.

Comparisons were then made between this and phenogram 3 (Section 4.3.1(c)).

4.3.1(e) Phenogram 5

Strain Set 2, consisting of 29 type strains and 26 natural isolates were clustered using **Test Set 2**. Five phenograms were produced using these data (see 4.3.1(a)-Phenogram 1)(Figures 24 and 25).

Ten groups were formed at the 74% S_{sm} similarity level (S_{sm}/UPGMA phenogram).

4.3.1(f) Phenogram 6

Strain Set 2 (Subset A) consisting of 29 type cultures only, were clustered using **Test Set 2.**

The simple matching coefficient and the UPGMA algorithm were used to produce the phenogram (Figure 26). Five groups were formed at the 72.5% S_{sm} similarity level. These clusters were compared to those produced using all strains.

4.3.1(g) Phenogram 7

Once again, using the simple matching coefficient and the UPGMA algorithm, a phenogram was produced from using all strains **(Strain Set 2)** and **Test Set 2 (Subset A)**, consisting of antibiotic resistance tests only (Figure 27). Eleven groups were formed at the 77% S_{sm} similarity level.

4.4 Test error and comparison of clustering coefficients and algorithms (Phenograms 1, 2, 3 and 5)

Twenty duplicate strains were included in the analysis of the largest strain set, Strain set 1/Test set 1. This enabled experimental test error to be calculated (Table 18). The average probability (*p*) of an erroneous test result was 3.59%, which was well within the 10% limit suggested by Sneath and Johnson (1972).

Phenogram 1a-1e **(Strain Set 1/Test Set 1)**

Phenogram 2a-2e **(Strain Set 1(Subset A)/Test Set 1)**

Phenogram 3a-3e **(Strain Set 1/Test Set 1(Subset A))**

Phenogram 5a-5e **(Strain Set 2/Test Set 2)**

Table 18. **Reproducibility of groups of tests calculated from comparison of data on 20 duplicate cultures**

Character states	Test	Agreement between duplicates (%)	Test variance (Si^2)
1-34	**Growth on carbohydrates** (Mean of 34 carbohydrates)	95.5	0.023
35-54	**Utilisation of organic acid** (Mean of 20 organic acids)	96.7	0.017
55-73	**Hydrolysis tests** (Mean of 19 Substrates)	93.2	0.031
74-133	**Growth at various temperatures and in the presence of chemical inhibitors**	92.6	0.037
134-179	**Resistance of antibiotics** (Mean of 45 tests)	97.8	0.010

A selection of available coefficients and algorithms were examined for use with the above sets of data. Five phenograms were created using a combination of three similarity coefficients (Dice, Jaccard and simple
matching) and three clustering algorithms (single linkage, UPGMA and WPGMA) in order to avoid the possibility of accepting misleading results.

The nature of the original data was carefully considered prior to clustering. It was thought that using a coefficient which takes into consideration positive matches only, as the Dice and Jaccard coefficients do, might not necessarily give a true measure of similarity between strains. This is due to the possibility that some species may have been measured as falsely positive for a number of characters. The Dice coefficient is marginally preferable to that of Jaccard because it weights against mismatches relative to matches and would prevent the underestimation of similarity values.

The error calculated in the above section (Table 18) relates to clustering using the simple matching coefficient. It is thought that the error might exert a greater effect on the clustering when using a coefficient which ignores positive matches. Consequently, the simple matching coefficient , which considers both positive *and* negative matches, along with the clustering algorithm, UPGMA, were chosen for the production of the first phenograms (1a, 2a, 3a and 5a).

Using the similarity matrix produced from the simple matching coefficient, three phenograms for each set of data were produced using algorithms based on average linkage (UPGMA and WPGMA) and single linkage (Phenograms a, d and e, respectively, for each set).

The initial group of phenograms (1a-8a), were tested for cluster stability by comparison with counter-part phenograms, produced from the same data set using alternative coefficients and algorithms. Group formation was achieved by the recognition of clearly well separated, tight clusters of strains. This occurred at a variety of similarity levels ranging from 70 to 80%, depending on the data set. This will be discussed in greater detail in the following section.

Comparisons of the UPGMA, WPGMA and single linkage algorithms revealed good reproduction of clusters, the basic topology of the three phenograms (a, d and e) being broadly similar. Single linkage and WPGMA showed marginally poorer group integrity, when compared to UPGMA. For example, in several cases (Phenograms 3d, 3e, 5d and 5e), clusters were split into several distinct groups dispersed throughout the phenogram.

However, on close examination, all five possible phenograms representing each data set appeared to show adequate agreement and, in most cases, excellent reproduction of the clustered groups.

Cophenetic correlations of the phenograms 1, 2, 3 and 5, produced using the S_{sm} and UPGMA gave Mantle statistics of 0.76, 0.74, 0.69, 0.75 and 0.69, which show reasonable agreement with the similarity matrices.

The 11 groups elucidated for phenogram 1a, based on the largest set of data, were distinctly and reliably reproduced in the remaining four phenograms (1b-1e). This was particularly pertinent as this phenogram provided the basis of the development of the probabilistic identification matrix discussed in the following chapter.

4.5 Hierarchical clustering patterns using physiological and biochemical tests

4.5.1 Phenograms 1 and 2

122 *Micromonospora* strains were investigated representing 16 different species groups. The strains were characterised on the basis of 179 traits, including cultural, morphological and physiological characters including sensitivity to antibiotics.

On the basis of the above, 2 phenograms were produced one using all 122 *Micromonospora* strains (**Strain Set 1** - excluding *Actinomadura* strains), the other using just ATCC strains including *Actinomadura* species (**Strain Set 1(Subset A)**).

The taxonomic analysis of phenogram 1 (**Strain Set 1/Test Set 1**) defined clusters at the 77.5% S_{sm} similarity level. At this level of similarity, 14 clusters were formed, providing the basis for the development of a probabilistic identification matrix (Chapter 5). Clusters were assigned to 8 major groups (consisting of 4 or more strains), and 6 minor ones (fewer than four strains).

Phenogram 2 (**Strain Set 1(Subset A)/Test Set 1**) also showed good cluster formation at the 77% S_{sm} similarity level, at which 5 major clusters (six strains or more) and 4 minor clusters (fewer than six strains) were distinguished.

4.5.1(a) Composition and characteristics of major clusters: Phenogram 1

Cluster 1 was the smallest of the major clusters containing only four strains. *Micromonospora grisea* G55 had a similarity of over 85%

with three *M. olivasterospora* strains. Both species utilise similar carbon sources, for example, D-fructose and D-galactose and both are unable to sustain growth on D-arabinose, lactose or salicin. *M. olivasterospora* and *M. grisea* produce the fortimicin and verdamicin antibiotic complexes respectively; both antibiotics are members of the aminoglycoside family.

Cluster 2 was the largest cluster group, containing 30 strains. The strains consisted largely of *Micromonospora echinospora* and *Micromonospora purpurea* type and subspecies. All strains are prolific producers of the aminoglycoside, gentamicin (Wagman and Weinstein, 1980). Microscopic observations of both subsets of species revealed long, branched, nonseptate mycelia, of approximately 0.5μm in diameter. In contrast to *M. echinospora*, spores or sporophores were rarely found in cultures of *M. purpurea*, sporulation generally being atypical or abortive. However, the taxonomic position of *M. purpurea* is uncertain due to lack of descriptive infomation on single spore formation (Kawamoto, 1989), but it is generally accepted that if a sporulating variant of this culture were found, this species would be likely to be reduced to a subspecies of *M. echinospora* (Luedemann and Brodsky, 1964).

The 30 strains in Cluster 2 shared many properties, the most diagnostic of which were that all were able grow in the presence of a concentration of 2% sodium chloride. All were able to utilise lactate and arbutin, none were able to utilise succinate and melibose as sole sources of carbon.

Cluster 3 comprised 19 strains which were broadly similar in morphology and pigmentation to those in Cluster 2. All produced dark, oily colonies with brown pigmentation. The cluster included four *Micromonospora sagamiensis* strains which were over 90% similar to *M. purpurea* 11-90. Ten *M. inyoensis* strains also featured prominently in

this sub-cluster, and appeared to be approximately 82% similar to the remaining four *Micromonospora purpurea* strains. Again all strains in this sub-cluster produced aminoglycoside antibiotics (Wagman and Weinstein, 1980). *M. sagamiensis* strains are important producers of sagamicin and *M. inyoensis* strains produce sisomicin. The most characteristic feature about this group is that all utilised arbutin and lactate.

Cluster 6 contained 13 strains; a combination of 11 *Micromonospora rosaria*-like strains and 2 gilvocarcin producing strains. *M. rosaria*, along with many *M. chalcea* strains are important producers of rosamycin. Strains in this cluster were morphologically similar, producing reddish orange colonies often turning brown and eventually black on sporulation.

Cluster 7 contained three species of *Micromonospora chalcea*, along with 6 *Micromonospora chalcea*-like strains. All produce a number of different antibiotics, including rosamycin and juvenimicin, macrolide antibiotics; rifamycin, an ansamycin; bottromycin, a peptide and many of the oligosaccharide antibiotics such as everninomycins (Wagman and Weinstein, 1980).

Cluster 10 contained M. sp. SCC 969 and M. sp. SCC 1000 as well as 13 M. sp. strains which have been found to produce myxins and phenazine type compounds. Cluster 10 joined Cluster 9 at the 76% S_{sm} level, and Cluster 11 at the 74% S_{sm} level. One of the most diagnostic features of this group was that none of its members can utilise arbutin.

Cluster 11 contains *M. carbonacea* type strains, ATCC 27114 and ATCC 27115, *M. chalcea* type strains ATCC 27333 and ATCC 21994 and *M. halophytica* type strains SCC 807 and SCC 760. All were incapable of utilising mannitol, rhamnose, glycerol, inositol and D-ribose.

4.5.1 (b) Composition and characteristics of minor and single-member clusters: Phenogram 1

Eleven strains (9.06%) representing five genera were recovered in the six minor clusters (Cluster-groups 4, 5, 8, 12, 13 and 14) defined at the 77.5% S_{sm} S-level. Two of the clusters contained 3 strains, 1 contained 2 strains and the remaining 3 were single member clusters.

Cluster 4 (*M. polytrota*, AR 5, 15 368) shares the closest similarity to the single-member Cluster 5 (*M. capillata Ferm. P*), joining at the 76% S_{sm} S-level. Clusters 4 and 5 separate from the major clusters 1, 2 and 3 at the 71% S_{sm} S-level. Cluster 8 contains 3 known toxic compound producers. The group was characterised mainly by the inability of its members to utilise lactate and to grow in the presence of phenol (0.075%, w/v). It joined Clusters 6 and 7 at the 74% S_{sm} S-level.

4.5.1 (c) Composition and characteristics of all clusters: Phenogram 2

All of the test strains were recovered in 9 Cluster groups, defined at the 75.5% S_{sm} Similarity (S) level. This level was selected, after examination of the dendograms, as they were the highest which gave clear groupings. Clusters defined at this level were assigned to 5 major groups (4 or more strains) and 4 minor ones (less than 4 strains. As expected the clustering of the *Micromonospora* type strains including the 14 *Actinomadura* strains, resulted in the identical grouping of *Micromonospora* shown in phenogram 1.

4.5.2 Phenograms 5 and 6

Fifty five *Micromonospora* strains were investigated representing 29 different species groups and 26 putative *Micromonospora* isolates. The strains were characterised on the basis of 49 traits (**Test Set 2**). The latter included cultural, morphological and physiological characters, as well as parameters including sensitivity to antibiotics. On the basis of the above, 2 phenograms were produced, one using all 55 strains (**Strain Set 2**), the other using just ATCC type strains (**Strain Set 2(Subset A)**).

The taxonomic analysis of phenogram 5 (**Strain Set 2/Test Set 2**) defined clusters at the 74% S_{sm} Similarity level. At this level of similarity 10 clusters were formed and were assigned to 5 major clusters (consisting of 4 or more strains) and 5 minor clusters (consisting of less than 4 strains).

Phenogram 6 (**Strain Set 2(Subset A)/Test Set 2**) showed good cluster formation at the 72.5% S_{sm} S-level. At this level 5 clusters were formed, 3 major ones and 3 minor ones.

4.5.2(a) Composition and characteristics of major clusters: Phenogram 5

Cluster 1 contained 9 strains of which none were wild isolates, fusing with clusters 2 and 3 at the 73% S_{sm} S-level. Cluster 1 contained *M. carbonacea* and *M. echinobrunnea* which fused at the 86% S-level. All strains produced deep orange pigmented substrate mycelium forming dark colonies on sporulation.

Compared to the clustering patterns of Phenograms 1 and 2, the groups formed showed no patterns which reflected antibiotic production profiles. Each of the groups contained a number of aminoglycoside,

macrolide, and oligosaccharide compound producers with little homogeneity.

Cluster 2, at the 76% S_{sm} S-level could be divided into 2 subgroups. Subgroup 2A consisted of 6 *Micromonospora* type strains including *M. chalcea*, *M. globosa* and *M. melanosporea*. In contrast, Subgroup 2B consisted of wild isolate strains only.

Cluster 3 consisted of 12 wild isolates, taken from a variety of environments including soil from a local field site near Warwick University and a permanent pasture soil from Salisbury, in Wiltshire. In addition the cluster contained one type strain *M. globosa*. All strains were resistant to novobiocin, trimethoprim and sulphamethoxazole.

Cluster 6 consisted of 3 type strains, *M. yulogensis*, *M. chalcea var. flavida* and *M. parva* and 4 wild isolates. It joined with Cluster 5 at the 74% S-level. Cluster 8 consisted of *M. echinospora var. pallida*, *M. echinospora var. ferruginea*, *M. viridifaciens* and *M. lacustris*.

4.5.2 (b) Composition and characteristics of minor and single member clusters: Phenogram 5

Ten strains (18.18%) representing 6 genera were recovered in 3 minor clusters and 2 single member clusters. Two of the clusters contained three strains, one contained two strains.

Cluster 4 joined Clusters 1, 2 and 3 at the 70% S_{sm} S-level, and contained just wild isolates. Clusters 5 and 6 were joined at the 74% S_{sm} S-level. Cluster 5 contained only two strains, *M. fulvopurpurea* and *M. narashinoensis*. Clusters 7, 9 and 10, of which the latter two are single member clusters join at the 73% S_{sm} S-level.

4.5.2(c) Composition and characteristics of all clusters: Phenogram 6

All of the type strains were recovered in 5 clusters at the 72.5% S-level. This comprised of 3 major clusters (containing 13, 6 and 4 strains) and 2 minor clusters (containing 2 strains in each one).

The type strains clustered in the same way illustrated in phenogram 5.

4.5.3 A comparison of phenogram 2 and phenogram 6

Phenograms resulting from the two sets of type strains (**Strain Set 1 (Subset A)** and **Strain Set 2 (Subset B)**) revealed contrasting sets of clustering patterns for the 12 *Micromonospora* type strains which were common to both. They were *M. carbonacea, M. chalcea* (3), *M. purpurea, M. echinospora* (2), *M. narashinoensis, M. halophytica, M. purpureochromogenes, M. inyoensis* and *M. zionensis*.

Both phenograms clustered *M. zionensis* and *M. purpurea* together; these strains are prolific producers of aminoglycoside antibiotics; *M. purpurea* produces gentamicin and *M. zionensis* produces the Antibiotic G-52.

However, it was interesting to note that using **Test Set 2** (phenogram 7) *M. purpurea* and *M. echinospora* did not cluster together as they did using **Test Set 1**. This was surprising as *M. purpurea* was widely thought to be a bald naturally occurring mutant of *M. echinospora*. This could possibly be explained by the fact that 179 characterisation tests were used to produce phenogram 2 whilst only 49 tests were used for phenogram 7. In addition **Test Set 1** contained considerably more antibiotic resistance tests than **Test Set 2**.

M. chalcea and *M. halophytica* var. *halophytica* also clustered together in both phenograms. Both shared many features morphologically, producing orange/brown colonies which eventually turned black on sporulation and in addition, both utilised melibiose and raffinose.

4.6 Hierarchical clustering patterns using antibiotic resistance profiles

Many actinomycetes including *Micromonospora* have distinctive antibiotic resistance patterns. These have been applied to both classification (Wellington *et al.*, 1987) and selective isolation (Wellington *et al.*, 1990) studies. In general, however, antibiotic production and resistance is strain specific (Hotta *et al.*, 1983a and b; Okami and Hotta, 1988). It has been shown by characterising resistance profiles to aminoglycoside (Hotta *et al.*, 1983a), macrolide (Fujizawa and Weisblum, 1981), and oligosaccharide antibiotics that many actinomycetes, both type strains and wild isolates, have diverse individual patterns of multiple resistance.

The relationship between biosynthesis and resistance genes have widely been reported (Chater and Bruton, 1985; Skeggs *et al.*, 1987) as it is known that producers of auto-toxic compounds require self-defence mechanisms to secure theire own survival (Cundliffe, 1986, 1989). Resistance mechanisms to certain antibiotics have also been found to exist in strains which are incapable of producing that antibiotic. For example, kanamycin resistance is exhibited by *S. griseus* (Hotta, 1988) and *S. tenebraius* (Cundliffe, 1987) which although produce aminoglycoside compounds, do not produce kanamycin.

Links have been made in organisms between interesting resistance profiles and the production of secondary metabolites. For example indolizamycin was discovered during protoplast fusion experiments revealing that the resistance patterns of the producing strain differed from those of the two parental strains (Yamashita *et al.*, 1985). This indicates that antibiotic resistance profiles could be used to select strains which may be potential producers of biologically active compounds. This hypothesis has been tested by Phillips (1992) and Phillips *et al.* (1992) by evaluating the phenotypic diversity of streptomycete populations based on antibiotic-resistance profiles. Little work has been done, however, to study antibiotic-resistance profiles in *Micromonospora* species, which , next to *Streptomyces*, are second most important producers of antibacterial compounds.

This study aimed to briefly examine antibiotic-resistance patterns in type strains and in wild isolates by using clustering patterns obtained from the NTSYS program, and to examine them in the light of antibiotic production patterns.

4.6.1 Antibiotic resistance profiles: Strain Set 1 and Strain Set 2 (phenograms 3, 4 and 7)

Phenograms 3, 4 and 7 were produced using Strain Sets 1 and 2 using just antibiotic resistance data. Phenogram 3, produced using the largest data set, gave good group formation at the 78% S_{sm} similarity level using the simple matching coefficient and the UPGMA algorithm. Cluster group 1 contained 48 strains and was divided at the 80% S_{sm} level into 3 subgroups. All strains were aminoglycoside producers. Subset A included *M. inyoensis*, producer of sisomicin, *M. grisea*, verdamicin producer and *M. olivasterospora*, fortimicin producer.

Subset B predominantly contained *M. echinospora* strains, all prolific producers of gentamicin. Subset C again included *M. echinospora* strains as well as *M. purpurea* which was to be expected as it is generally accepted that *M. purpurea* is very closely related to *M. echinospora*. All members of cluster group 2 were related to *M. purpurea*, all producers of gentamicin.

The second largest cluster group, Cluster group 3 contained *M. rosaria* and related strains, all produce rosaramicin, a macrolide antibiotic. Cluster group 11, containing 4 strains all related to *M. chalcea*, were all producers of the macrolide antibiotic, neorustmicin. Cluster groups 17 and 18 contain *Actinomadura* strains as expected.

All other cluster groups contain a combination of aminoglycoside, macrolide and ansamycin producers as well as some of the more unusual antibacterial compounds such as gilvocarcin and nocardicin. Similar groupings were achieved in phenogram 4, which used the same set of antibiotic resistance data as above, but only included results obtained from type cultures. As above, the phenogram was produced using the simple matching coefficient and the UPGMA algorithm. Groups formed at the 72.7% S_{sm} similarity level clustered all aminoglycoside producers together (cluster 1, subsets A, B and C). All *Actinomadura* marker strains clustered together as did all *M. chalcea* and *M. carbonacea*.

Phenogram 7 was produced from Strain Set 2 using antibiotic resistance data. Clusters were produced using the simple matching coefficient and the UPGMA algorithm. At the 77% S_{SM} Similarity level 11 cluster groups were formed. Cluster group 2 was the second largest, containing 20 *Micromonospora* including a number of aminoglycoside producers, (*M. sagamiensis*) as well as some producers of macrolide antibiotics (*M. narashinoensis*, *M. chalcea*). Cluster group 8, the largest

group, contained predominantly gentamicin producers including *M. echinospora* and *M. inyoensis*.

Clustering *Micromonospora* on the basis of antibiotic resistance profiles provided some interesting results. Although it did not exclusively cluster *Micromonospora* into groups of antibiotic producers, the pattern which emerged showed that aminoglycoside producers all tended to cluster together, supporting the findings of Phillips (1992).

4.7 Conclusions

The results provided some interesting implications, for example all phenograms emphasised the strong phenetic relationships existing between *Micromonospora echinospora* and *M. purpurea*. Both clustered together in phenograms produced using Strain set 1, the largest data set comprising of a maximum number of 135 strains including *Actinomadura* marker strains. This perhaps was to be expected as, although the taxonomic position of *M. purpurea* is uncertain, it is generally accepted that if a sporulating variant of this culture is found, this species would be likely to be reduced to a subspecies of *M. echinospora* (Kawamoto, 1989; Luedemann and Brodsky, 1964). Additionally, when they were included, all *Actinomadura* clustered together at the species group level (between 70 and 80% similarity).

Strong phenetic relationships also existed between *M. grisea* and *M. olivasterospora*; both shared similar morphological characteristics and both produced aminoglycoside antibiotics.

M. carbonacea, M. halophytica and *M. chalcea* all regularly fell into the same species group; all were closely related morphologically and physiological tests indicated a high level of relatedness.

The S_{sm} coefficient at the 77.5% S_{sm} similarity level ditributed 122 *Micromonospora* strains (phenogram 1) among 14 groups; 5 major clusters (six strains or more) and 4 minor clusters (fewer than six strains) and whilst the species status of the three single member clusters was unaltered many of the smaller cluster groups could be regarded as species groups. Majority of the major cluster groups however were largely heterogeneous and must be considered as including greater than one species type (see Chapter 5 for Phenogram 1 species groups).

Data based on solely antibiotic resistance profiles, as expected, indicated a relationship between biosynthesis and resistance genes, particularly with respect to aminoglycoside producers. This provides interesting implications for pharmaceutical industries as links could be explored between isolates with interesting antibiotic resistance profiles and production of secondary metabolites. Such links have begun to be explored using *Streptomyces* (Phillips, 1992) although little has been done using *Micromonospora*.

Figure 17 Phenogram (1) to show the clustering of Strain Set 1 using Test Set 1

Figure 17 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 14, defined at the 77.5% S_{sm} similarity level, are shown in Table 19.

1. *Micromonospora olivasterospora*
2. *M. echinospora*
3. *M. sagamiensis*
4. *M. polytrota*
5. *M. capillata*
6. *M. rosaria*
7. *M. chalcea*
8. *M. sp.1*
9. *M. purpureochromogenes*
10. *M. sp2*
11. *M. halophytica*
12. *M. sp3*
13. *M. fusca*
14. *M. carbonacea*

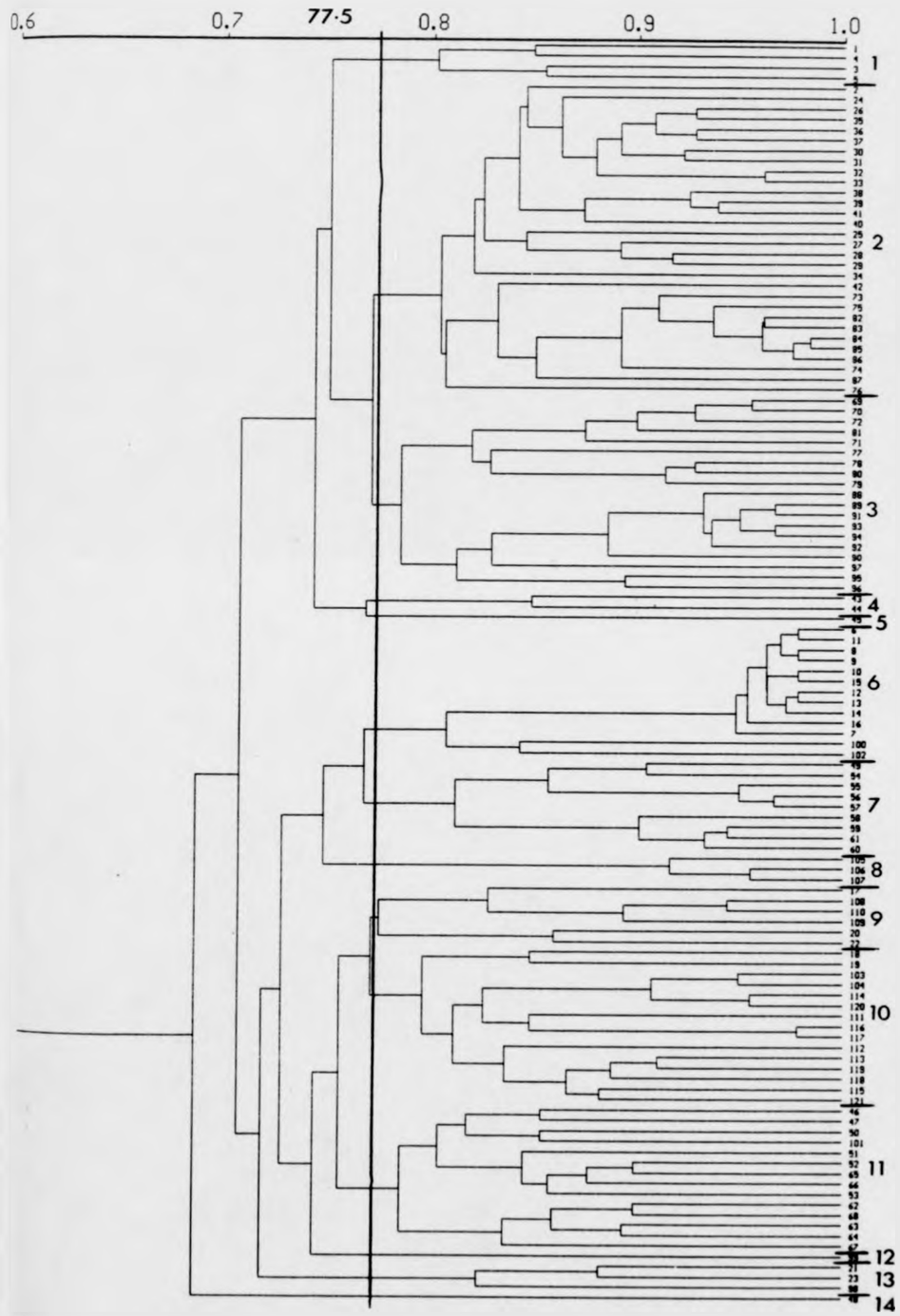


Table 19

Phenogram 1 Strain Set 1 (excluding Actinomadura strains)/Test Set 1

Strains assigned to:

Cluster-group 1

Micromonospora grisea	G55
M. olivasterospora	ATCC 31010
M. olivasterospora	ATCC 21819
M. olivasterospora	ATCC 31009

Cluster-group 2

M. zionensis	G52
M. echinospora var. *echinospora*	817
M. echinospora var *ferruginea*	782
Echinospora-like	10 245
Echinospora-like	11 224
Echinospora-like	11 365
Echinospora-like	799
Echinospora-like	805
Echinospora-like	10-408-C
Echinospora-like	10-408-35
Echinospora-like	12 46
Echinospora-like	12 123
Echinospora-like	12 44
Echinospora-like	12 125
Echinospora-like	TS 8
M. echinospora var. *pallida*	TS 5
Echinospora-like	795
Echinospora-like	774
Echinospora-like	11 89
M. echinospora var *challensensis*	14 261
M. purpurea	NTS 9
M. purpurea	SCC 1038
Purpurea-like	12-33
Purpurea-like	12-34
Purpurea-like	12-35
Purpurea-like	12-36
Purpurea-like	12-37
M. purpurea	ATCC 31119
Micromonospora sp.	NRRL 3543
Purpurea-like	SCC 1039

Cluster-group 3

M. sagamiensis	ATCC 21803
M. sagamiensis	ATCC 21826
M. sagamiensis	ATCC 21949
Purpurea-like	11-90
M. sagamiensis	ATCC 21827

Purpurea-like	71-1225
Purpurea-like	H334-0
Purpurea-like	334-13
Purpurea-like	334-5
M. inyoensis	ATCC 27600
Inyoensis-like	NRRL 5742
Inyoensis-like	1081
Inyoensis-like	10-8
Inyoensis-like	10-9
Inyoensis-like	72-469
Inyoensis-like	6640
Inyoensis-like	71-64
Inyoensis-like	984
Inyoensis-like	990

Cluster-group 4

M. polytrota	AR 5
M. polytrota	15 368

Cluster-group 5

M. capillata Ferm. P	2598

Cluster-group 6

M. rosaria	ATCC 29337
Rosaria-like	9603
Rosaria-like	Q319
Rosaria-like	Q1578
Rosaria-like	4215
Rosaria-like	T5771
Rosaria-like	S2742
Rosaria-like	T816
Rosaria-like	X70
Rosaria-like	T5804
Rosaria-like	S9683
Gilvocarcin producer	13 304
Gilvocarcin producer	12 402

Cluster-group 7

M. chalcea	ATCC 12452
M. chalcea	ATCC 27083
M. chalcea	ATCC 27084
Chalcea-like	R 460
Chalcea-like	R 461
Chalcea-like	69 683
Chalcea-like	1020
Chalcea-like	1063
Chalcea-like	1062

Cluster-group 8

Toxic compound producer	68-742
Toxic compound producer	68-742-12
Toxic compound producer	68-742-1284

Cluster-group 9

M. purpureochromogenes	ATCC 27007
Thiostrepton producer	68-1147
Thiostrepton producer	10-259
Thiostrepton producer	68-1147-134
M. fusca	CBS SCC 821
M. narashinoensis	SCC 939 IFO

Cluster-group 10

Purpureochromogenes-like	SCC 969 (M1021)
Purpureochromogenes-like	SCC 1000(68-I)
Phenazine-type producer	124
Phenazine-type producer	12 414
Myxin producer	13-22
Myxin producer	13-83
Myxin producer	13-16
Myxin producer	13-26
Myxin producer	13-34
Myxin producer	13-17
Myxin producer	13-96
Myxin producer	13-20
Myxin producer	13-40
Myxin producer	13-23
Myxin producer	13-36

Cluster-group 11

M. carbonacea var.carbonacea	ATCC 27114
M. carbonacea var.aurantiaca	ATCC 27115
M. chalcea	ATCC 27333
Gilvocarcin producer	13 335
M. chalcea	ATCC 21994
Chalcea-like	91 372
Halophytica-like	785 (R476)
Halophytica-like	818 (R476)
Chalcea-like	91 1306
M. halophytica var halophtica	SCC 807 SP-30
Halophytica-like	815 (SP14)
M. halophytica var nigra	SCC 760
Halophytica-like	SCC 763
Halophytica-like	737 (SP14)

Cluster-group 12

Nocardicin (22JC) producer	13 859

Cluster-group 13

M. fusca	SCC 900
M. purpureochromogenes	ATCC 10026
Nocardicin (22JC) producer	SCC 895

Cluster-group 14

M. carbonacea var.africana	ATCC 39149

Figure 18 Phenograms to show the clustering of Strain Set 1 using Test Set 1 using different coefficients and algorithms.

A shows a phenogram created using the Dice coefficient and the UPGMA algorithm.

B shows a phenogram created using the Jaccard coefficient and the UPGMA algorithm.

C shows a phenogram created using the simple matching coefficient and the single linkage algorithm.

D shows a phenogram created using the simple matching coefficient and the WPGMA algorithm.

Groups 1 to 14, as defined in Figure 17, are redefined on phenograms A, B, C and D.

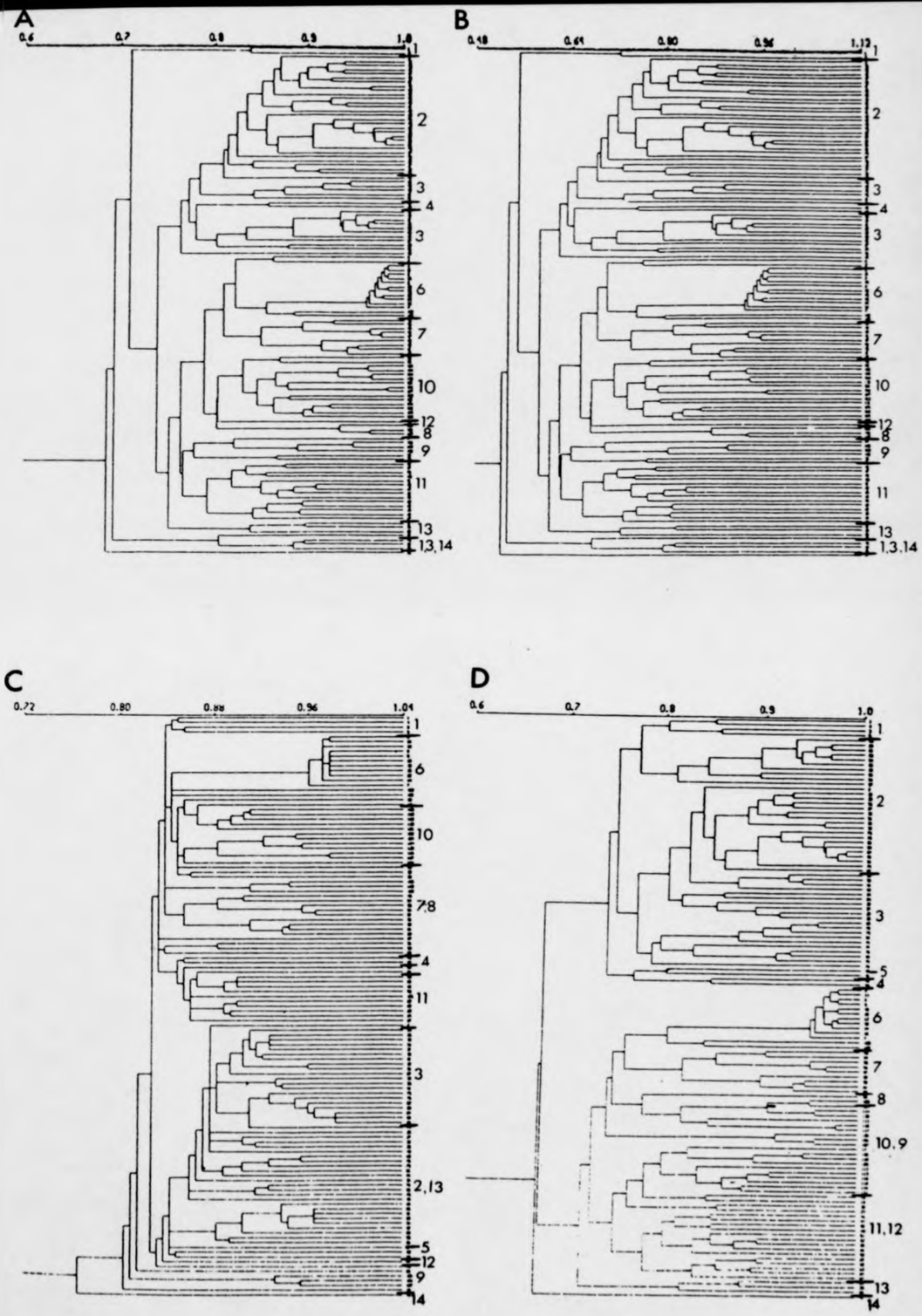


Figure 19 Phenogram (2) to show the clustering of Strain Set 1 (Subset A) using Test Set 1

Figure 19 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 9, defined at the 75.5% S_{sm} similarity level, are shown in Table 20.

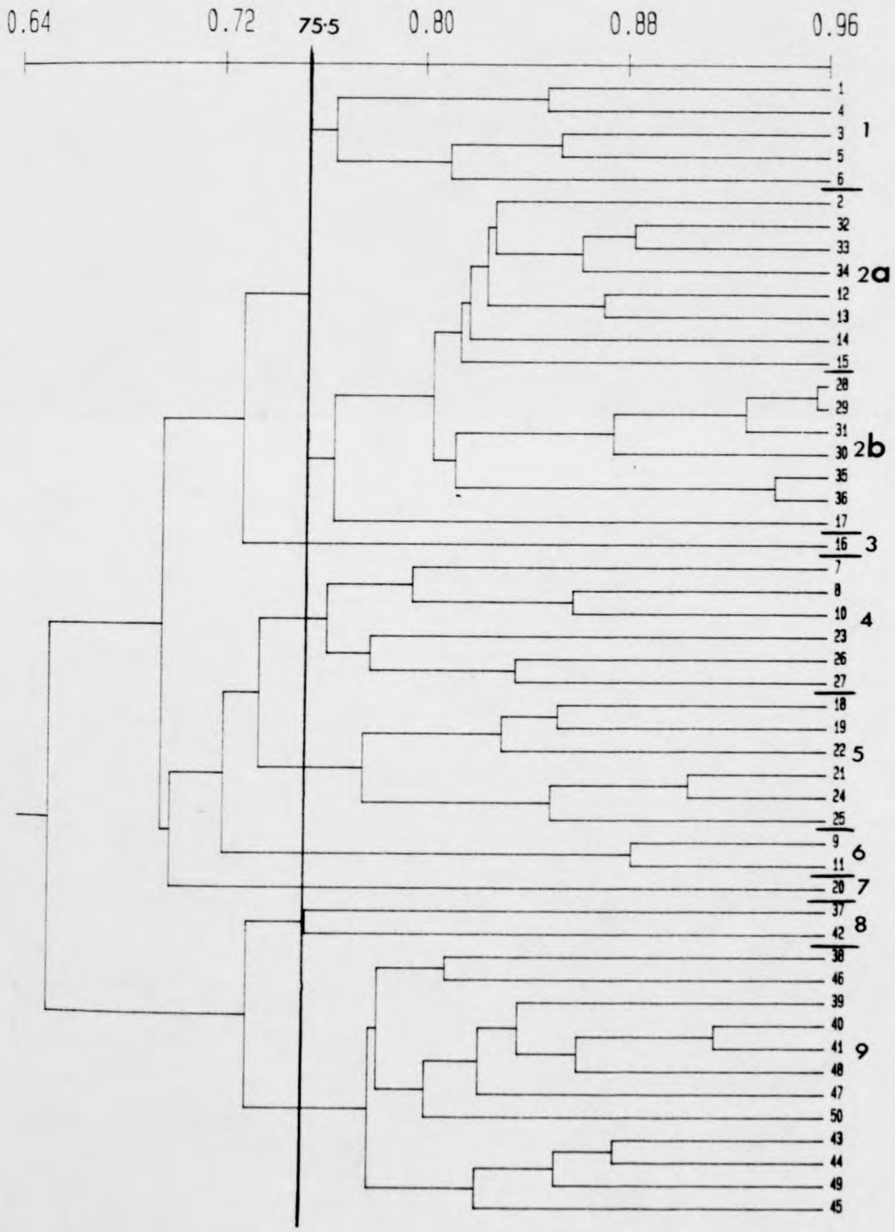


Table 20 Phenogram 2

Strain Set 1 (Subset A)/ Test Set 1

Strains assigned to:
Cluster-group 1

Micromonospora grisea	G55
M. olivasterospora	ATCC 31010
M. olivasterospora	ATCC 21819
M. olivasterospora	ATCC 31009
M. rosaria	ATCC 29337

Cluster-group 2
Subcluster A

M. zionensis	G52
M. purpurea	TS 9
M. purpurea	ATCC 31119
M. purpurea	NRRL 3543
M. echinospora var. echinospora	817
M. echinospora var. ferruginea	782
M. echinospora var. pallida	TS 5
M. echinospora var. challensensis	14 261

Subcluster B

M. sagamiensis	ATCC 21803
M. sagamiensis	ATCC 21826
M. sagamiensis	ATCC 21949
M. sagamiensis	ATCC 21827
M. inyoensis	ATCC 27600
M. inyoensis	NRRL 5742
M. capillata	2598

Cluster-group 3

M. polytrota	15 368

Cluster-group 4

M. purpureochromogenes	ATCC 27007
M. fusca	SCC 821
M. narashinoensis	SCC 939 IFO
M. chalcea	ATCC 21994
M. halophytica var. *halophytica*	SCC 807 SP-30
M. halophytica var. *nigra*	SCC 760

Cluster-group 5

M. carbonacea var. *carbonacea*	ATCC 27114
M. carbonacea var. *aurantiaca*	ATCC 27115
M. chalcea	ATCC 27333
M. chalcea	ATCC 12452
M. chalcea	ATCC 27083
M. chalcea	ATCC 27084

Cluster-group 6

M. fusca	SCC 900
M.purpureochromogenes	ATCC 10026

Cluster-group 7

M. carbonacea var. *africana*	ATCC 39149

Cluster-group 8

Actinomadura	SCC 906
Actinomadura	ATCC 27298

Cluster-group 9

Actinomadura	SCC 918
Actinomadura	ATCC 29553
Actinomadura	ATCC 27295
Actinomadura	ATCC 27296
Actinomadura	ATCC 27297
Actinomadura	AA-6
Actinomadura	13-363
Actinomadura	ATCC 23218
Actinomadura	ATCC 27299
Actinomadura	ATCC 27887
Actinomadura	BL-6
Actinomadura	ATCC 27888

Figure 20 Phenograms to show the clustering of Strain Set 1 (Subset A) using Test Set 1 using different coefficients and algorithms.

A shows a phenogram created using the Dice coefficient and the UPGMA algorithm.

B shows a phenogram created using the Jaccard coefficient and the UPGMA algorithm.

C shows a phenogram created using the simple matching coefficient and the single linkage algorithm.

D shows a phenogram created using the simple matching coefficient and the WPGMA algorithm.

Groups 1 to 9, as defined in Figure 19, are redefined on phenograms A, B, C and D.

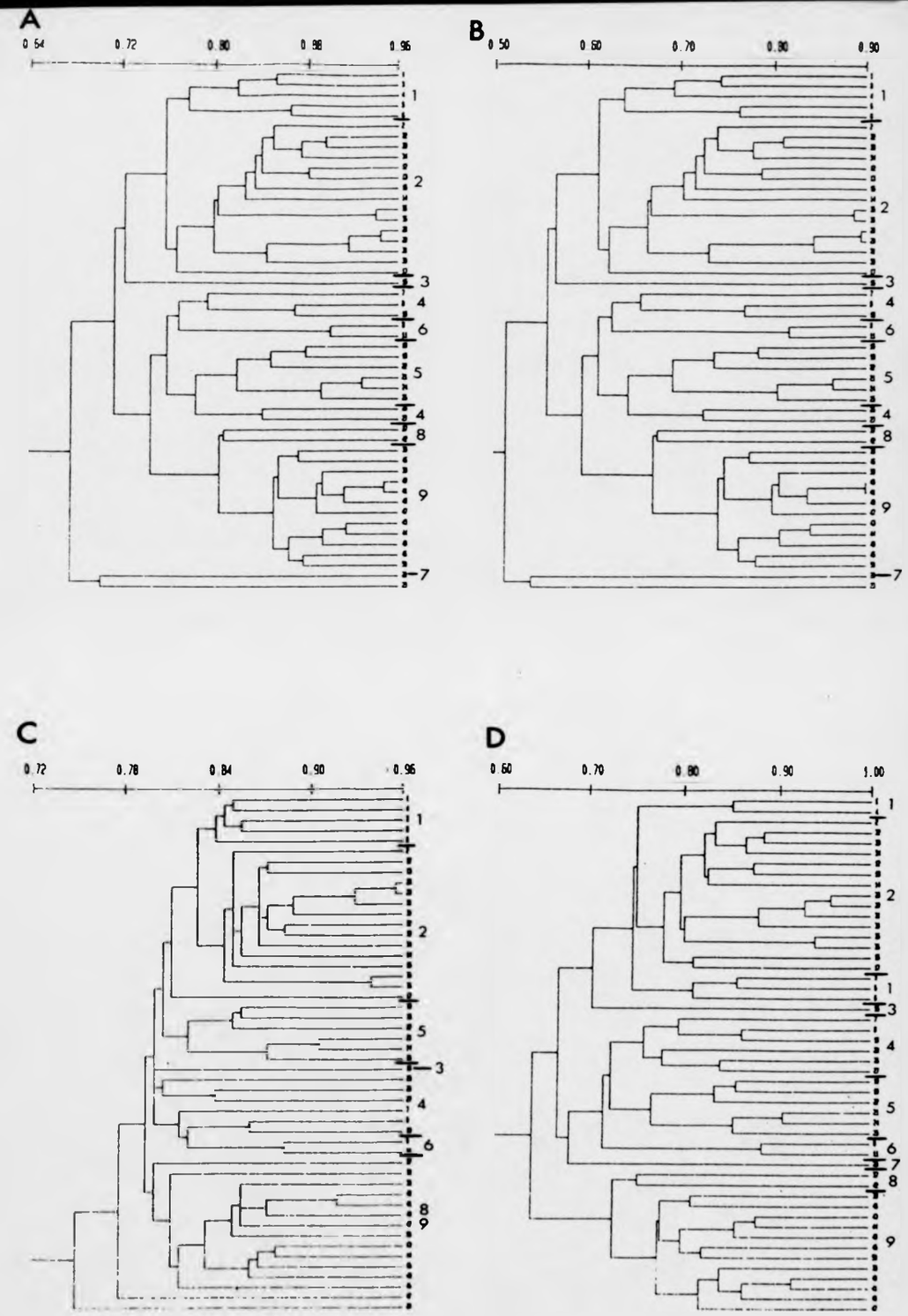


Figure 21 Phenogram (3) to show the clustering of Strain Set 1 using Test Set 1 (Subset A)

Figure 21 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 18, defined at the 78% S_{sm} similarity level are shown in Table 21.

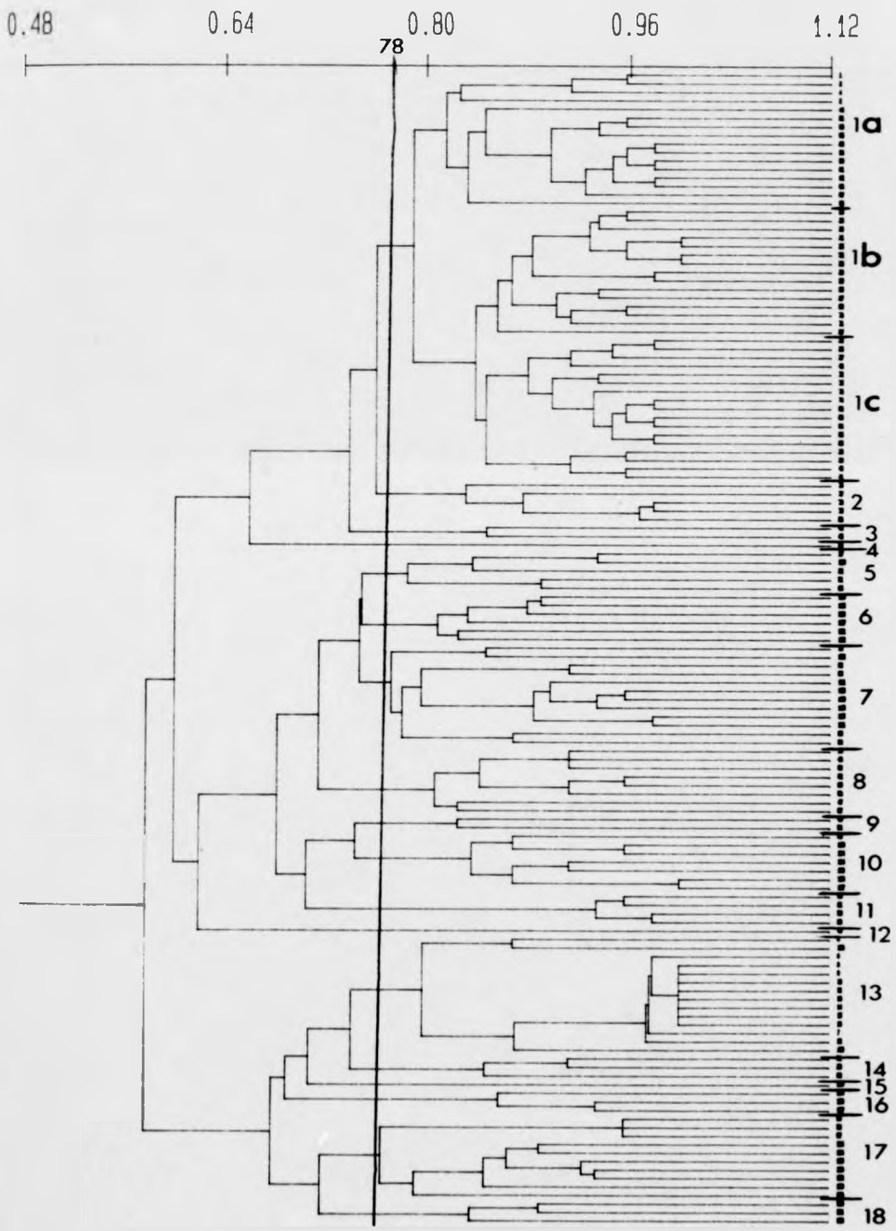


Table 21 Phenogram 3
Strain Set 1/Test Set 1(Subset A)

Strains assigned to:

Cluster-group 1
Subcluster A

Micromonospora grisea	G55
M. olivasterospora	ATCC 21819
M. olivasterospora	ATCC 31010
M. zionensis	G52
M. polytrota	15 368
Purpurea-like	11-90
Inyoensis-like	6640
Inyoensis-like	984
M. inyoensis	ATCC 27600
Inyoensis-like	10-9
Inyoensis-like	NRRL 5742
Inyoensis-like	1081
Inyoensis-like	72-469
Inyoensis-like	10-8
Inyoensis-like	71-64
Inyoensis-like	990

Subcluster B

M. echinospora var. echinospora	817
M. echinospora var ferruginea	782
Echinospora-like	799
Echinospora-like	805
Echinospora-like	10 245
Echinospora-like	10-408-C
Echinospora-like	10-408-35
Echinospora-like	795
Echinospora-like	774
Echinospora-like	TS 8
M. echinospora var. pallida	TS 5
M. sagamiensis	ATCC 21803
M. sagamiensis	ATCC 21826
M. sagamiensis	ATCC 21827
Micromonospora sp.	NRRL 3543

Subcluster C

Echinospora-like	11 89
Echinospora-like	11 224
Echinospora-like	11 365
M. echinospora var challensensis	14 261
M. sagamiensis	ATCC 21949
M. purpurea	SCC 1038
M. purpurea	NTS 9
M. purpurea	ATCC 31119
Purpurea-like	12-33
Purpurea-like	12-34

Purpurea-like	12-35
Purpurea-like	12-36
Purpurea-like	12-37
Echinospora-like	12 46
Echinospora-like	12 123
Echinospora-like	12 125
Echinospora-like	12 44
Cluster-group 2	
Purpurea-like	SCC 1039
Purpurea-like	71-1225
Purpurea-like	H334-0
Purpurea-like	334-5
Purpurea-like	334-13
Cluster-group 3	
M. polytrota	AR 5
M. capillata Ferm. P	2598
Cluster-group 4	
M. fusca	CBS SCC 821
Cluster-group 5	
M. purpureochromogenes	ATCC 27007
Thiostrepton producer	68-1147-134
M. narashinoensis	SCC 939 IFO
M. chalcea	ATCC 21994
Halophytica-like	818 (R476)
Cluster-group 6	
Thiostrepton producer	68-1147
Myxin producer	13-17
Thiostrepton producer	10-259
Actinomadura	A 27298
Myxin producer	13-16
Actinomadura	SCC 906
Cluster-group 7	
Purpureochromogenes-like	SCC 1000 (68-I)
Gilvocarcin producer	12 402
M. halophytica var halophtica	SCC 807 SP-30
Halophytica-like	815 (SP14)
Gilvocarcin producer	13 335
Myxin producer	13-20
Myxin producer	13-40
Myxin producer	13-36
Myxin producer	13-23
Myxin producer	13-9
M. halophytica var nigra	SCC 760
Halophytica-like	SCC 763
Cluster-group 8	

Purpureochromogenes-like	SCC 969 (M1021)
M. carbonacea var.carbonacea	ATCC 27114
M. chalcea	ATCC 27333
Chalcea-like	91 372
Halophytica-like	785 (R476)
Chalcea-like	91 1306
M. carbonacea var.aurantiaca	ATCC 27115
Halophytica-like	737 (SP14)

Cluster-group 9

49. *M. chalcea*	ATCC 12452
54. *M. chalcea*	ATCC 27083

Cluster-group 10

Nocardicin (22JC) producer	13 859
Phenazine-type producer	124
Phenazine-type producer	12 414
Myxin producer	13-22
Myxin producer	13-83
Myxin producer	13-26
Myxin producer	13-34

Cluster-group 11

Chalcea-like	69 683
Chalcea-like	1063
Chalcea-like	1020
Chalcea-like	1062

Cluster-group 12

M. carbonacea var.africana	ATCC 39149

Cluster-group 13

M. olivasterospora	ATCC 31009
Actinomadura	A 27888
M. rosaria	ATCC 29337
rosaria-like	Q319
rosaria-like	Q1578
rosaria-like	4215
rosaria-like	9603
rosaria-like	T816
rosaria-like	X70
rosaria-like	T5771
rosaria-like	T5804
rosaria-like	S9683
rosaria-like	S2742
Gilvocarcin producer	13 304

Cluster-group 14

M. fusca	SCC 900
M. purpureochromogenes	ATCC 10026
Nocardicin (22JC) producer	SCC 895

Cluster-group 15

Actinomadura	A 23218

Cluster-group 16

Toxic compound producer	68-742
Toxic compound producer	68-742-12
Toxic compound producer	68-742-1284

Cluster-group 17

M. chalcea	ATCC 27084
Chalcea-like	R460
Chalcea-like	R461
Actinomadura	A 27295
Actinomadura	BL-6
Actinomadura	A 27296
Actinomadura	A 27297
Actinomadura	13-363
Actinomadura	A 27887
Actinomadura	AA-6

Cluster-group 18

Actinomadura	SCC 918
Actinomadura	A 29553
Actinomadura	A 27299

Figure 22 Phenograms to show the clustering of Strain Set 1 using Test Set 1 (Subset A) using different coefficients and algorithms.

A shows a phenogram created using the Dice coefficient and the UPGMA algorithm.

B shows a phenogram created using the Jaccard coefficient and the UPGMA algorithm.

C shows a phenogram created using the simple matching coefficient and the single linkage algorithm.

D shows a phenogram created using the simple matching coefficient and the WPGMA algorithm.

Groups 1 to 18, as defined in Figure 21, are redefined on phenograms A, B, C and D.

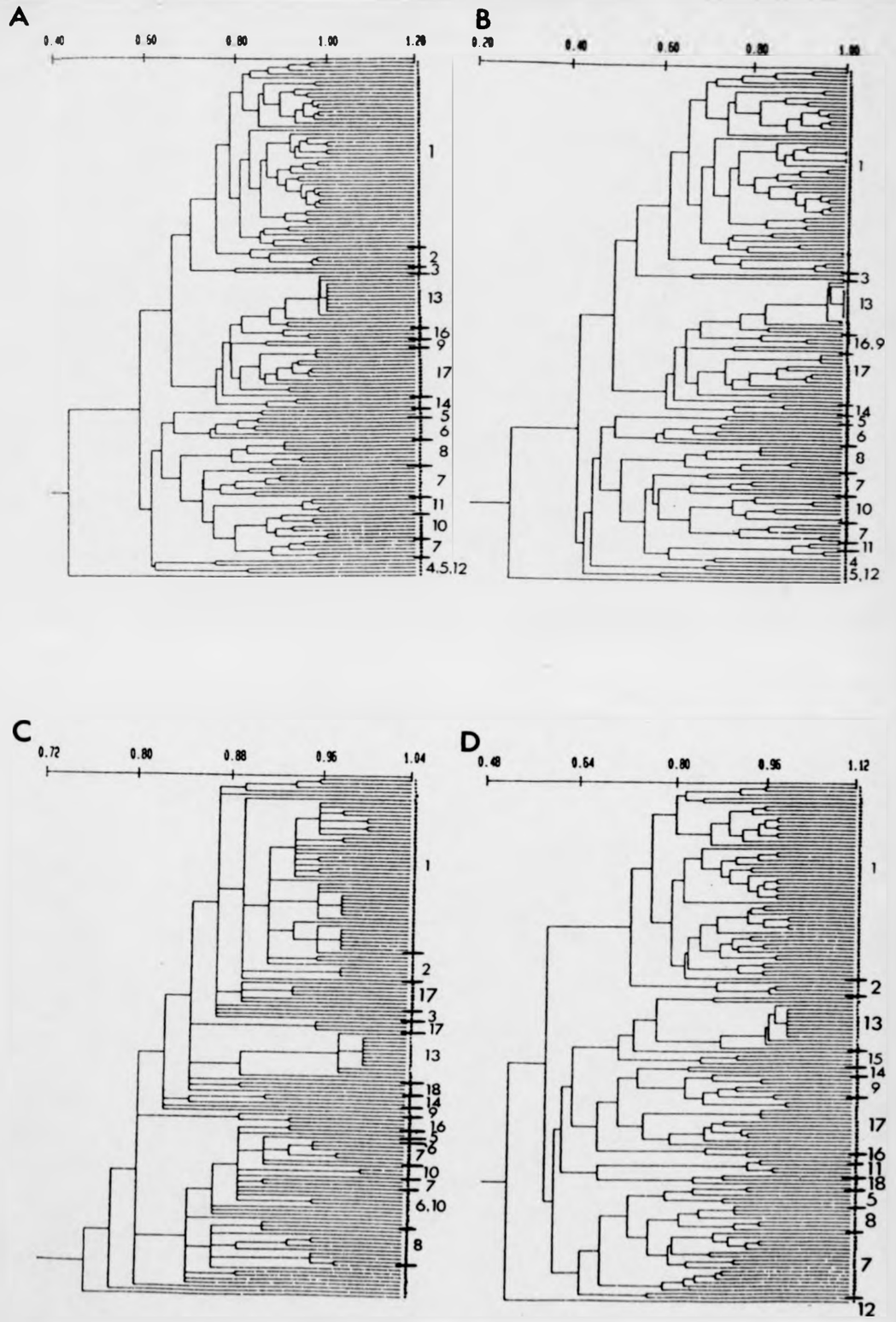


Figure 23 Phenogram (4) to show the clustering of Strain Set 1 using Test Set 1

Figure 23 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 11, defined at the 72.7% S_{sm} similarity level are shown in Table 22.

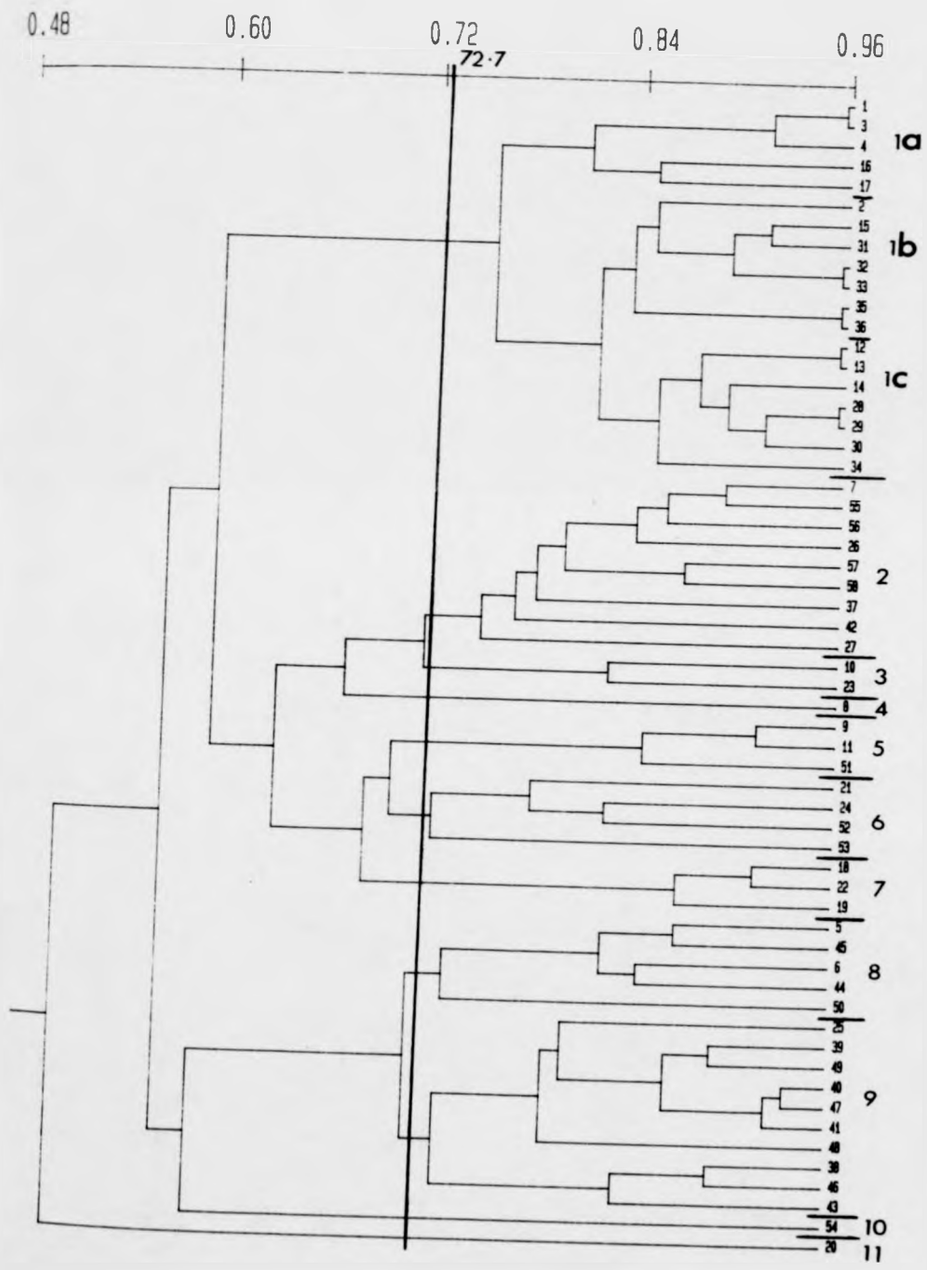


Table 22 Phenogram 4

Strain Set 1 (Subset B)/Test Set 1(Subset A)
Strains assigned to:

Cluster-group 1
Subcluster A

Micromonospora grisea	G55
M. olivasterospora	ATCC 21819
M. olivasterospora	ATCC 31010
M. polytrota	AR 5
M. polytrota	15 368

Subcluster B

M. zionensis	G52
M. echinospora var. *challensis*	14 261
M. sagamiensis	ATCC 21949
M. purpurea	TS 9
M. purpurea	ATCC 31119
M. inyoensis	ATCC 27600
Micromonospora sp.	NRRL 5742

Subcluster C

M. echinospora var. *echinospora*	817
M. echinospora var. *ferruginea*	782
M. echinospora var. *pallida*	TS 5
M. sagamiensis	ATCC 21803
M. sagamiensis	ATCC 21826
M. sagamiensis	ATCC 21827
Micromonospora sp.	NRRL 3543

Cluster-group 2

M. purpureochromogenes	ATCC 27007
Thiostrepton producer	68-1147
Thiostrepton producer	10-259
M. halophytica var. *halophytica*	SCC 807 SP-30
Myxin producer	13-16
Myxin producer	13-17
Actinomadura	SCC 906
Actinomadura	ATCC 27298
M. halophytica var. *nigra*	SCC 760

Cluster-group 3

M. narashinoensis	SCC 939 IFO
M. chalcea	ATCC 21994

Cluster-group 4	
M. fusca	SCC 821
Cluster-group 5	
M. fusca	SCC 900
M. purpureochromogenes	ATCC 10026
Nocardicin producer	SCC 895
Cluster-group 6	
M. chalcea	ATCC 12452
M. chalcea	ATCC 27083
Gilvocarcin producer	13 304
Phenazine-type producer	12 414
Cluster-group 7	
M. carbonacea var. *carbonacea*	ATCC 27114
M. chalcea	ATCC 27333
M. carbonacea var. *aurantiaca*	ATCC 27115
Cluster-group 8	
M. olivasterospora	ATCC 31009
Actinomadura	ATCC 27888
M. rosaria	ATCC 29337
Actinomadura	ATCC 27887
Actinomadura	ATCC 23218
Cluster-group 9	
M. chalcea	ATCC 27084
Actinomadura	ATCC 27295
Actinomadura	ATCC 23218
Actinomadura	ATCC 27296
Actinomadura	AA6
Actinomadura	SCC 918
Actinomadura	ATCC 29553
Actinomadura	ATCC 27299
Actinomadura	SCC 906
Actinomadura	ATCC 27297
Cluster-group 10	
Toxic compound producer	68-742
Cluster-group 11	
M. carbonacea var. africana	ATCC 39149

Figure 24 Phenogram (5) to show the clustering of Strain Set 2 using Test Set 2

Figure 24 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 1O, defined at the 74% S_{sm} similarity level, are shown in Table 23.

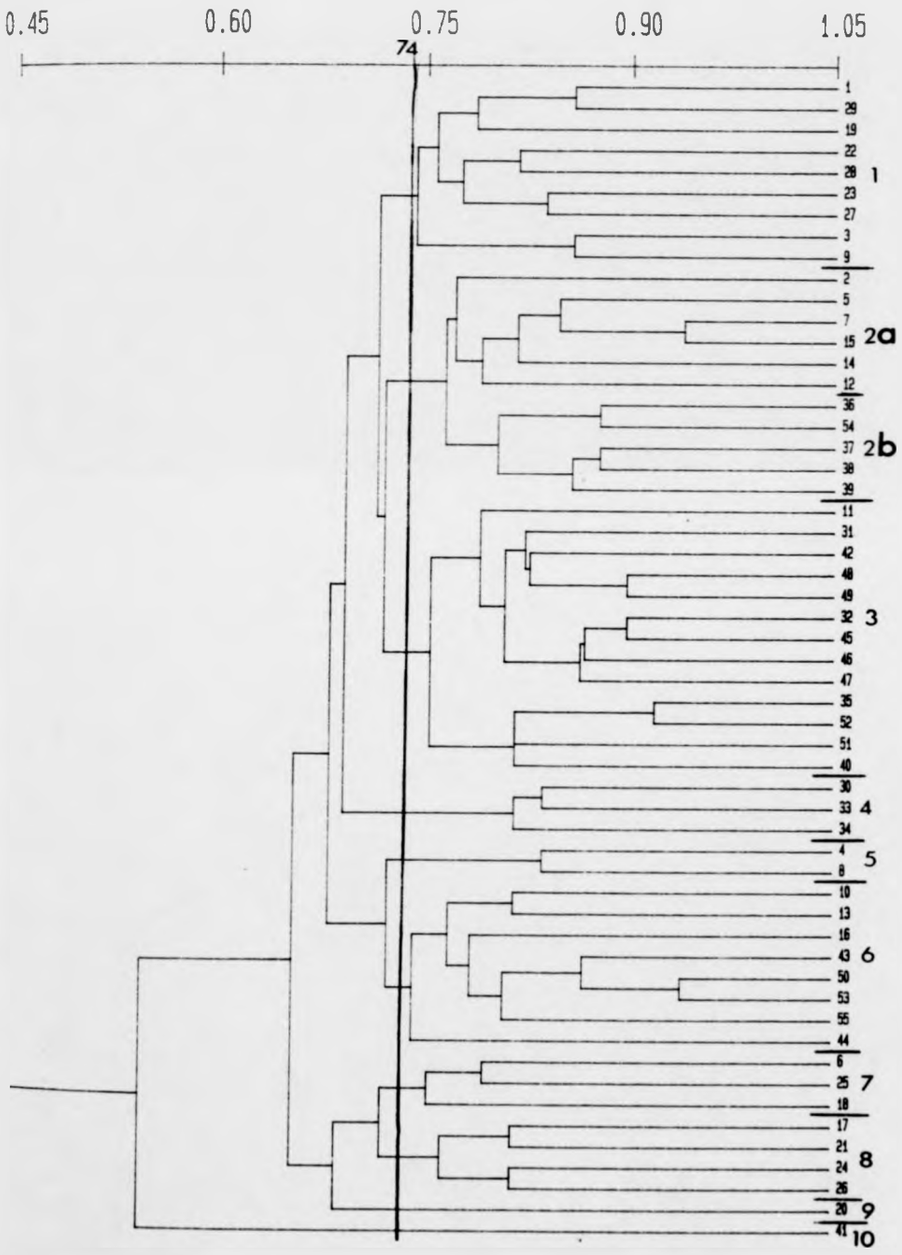


Table 23 Phenogram 5

Strain Set 2/Test Set 2
Strains assigned to:
Cluster-group 1

Micromonospora carbonacea var aurantiaca	ATCC 27115
M. echinobrunnea	JCM 3327
M. chalcea var. izumensis	ATCC 21561
M. zionensis	NRRL 5466
M. purpurea	ATCC 15835
M. halophytica	NRRL 3097
M. fulvoviridis	JCM 3259
M. chalcea	ATCC 27344
M. floridensis	NRRL 8020

Cluster-group 2

Subcluster A

M. chalcea var. chalcea	ATCC 12452
M. echinoaurantiaca	JCM 3257
M. nigra	JCM 3328
M. melanosporea	IFO 12515
M. globosa	NRRL B2673
M. rubra	ATCC 27031

Subcluster B

Wild Isolate G	Regina soil/Heat treatment
Wild Isolate Y	Warwick soil/Heat treatment
Wild Isolate H	Pond Sediment
Wild Isolate I	Bicutan soil/Heat treatment
Wild Isolate J	Italian soil/Antibiotic
treatment	

Cluster-group 3

M. globosa	JCM 3358
Wild Isolate B	Permanent Pasture Soil/Heat
Wild Isolate M	Permanent Pasture Soil/Heat
Wild Isolate S	Warwick soil/Heat treatment
Wild Isolate T	Warwick soil/Heat treatment
Wild Isolate C	Permanent Pasture Soil/Heat
Wild Isolate P	Italian Soil/Antibiotics
Wild Isolate Q	Warwick Soil/Heat treatment
Wild Isolate R	Warwick Soil/Heat treatment
Wild Isolate F	Warwick Soil/Heat treatment
Wild Isolate W	Warwick Soil/Heat treatment
Wild Isolate V	Warwick Soil/Heat treatment
Wild Isolate K	Warwick Soil/Heat treatment

Cluster-group 4

Wild Isolate A	Warwick Soil/Heat treatment
Wild Isolate D	Warwick Soil/Antibiotic
	treatment

Wild Isolate G	Regina Soil/Heattreatment
Cluster-group 5	
M. fulvopurpurea	JCM 5696
M. narashinoensis	IFM 110-76
Cluster-group 6	
M. yulogensis	ATCC 43540
M. chalcea var. flavida	ATCC 27084
M. parva	NRRL B2676
Wild Isolate N	Permanent Pasture Soil/Heat
Wild Isolate U	Warwick Soil/Heat treatment
Wild Isolate X	Warwick Soil/Heat treatment
Wild Isolate Z	Warwick Soil/Heat treatment
Wild Isolate O	Permanent Pasture Soil/Heat
Cluster-group 7	
M. purpureochromogenes	ATCC 27007
M. inyoensis	ATCC 27600
M. megalomicea var. nigra	ATCC 27598
Cluster-group 8	
M. echinospora var. pallida	ATCC 15838
M. echinospora var. ferruginea	NRRL 2995
M. viridifaciens	ATCC 31136
M. lacustris	ATCC 21974
Cluster-group 9	
M. sagamiensis	ATCC 21826
Cluster-group 10	
Wild Isolate L	Bicutan Soil/Heat treatment

Figure 25 Phenograms to show the clustering of Strain Set 2 using Test Set 2 using different coefficients and algorithms.

A shows a phenogram created using the Dice coefficient and the UPGMA algorithm.

B shows a phenogram created using the Jaccard coefficient and the UPGMA algorithm.

C shows a phenogram created using the simple matching coefficient and the single linkage algorithm.

D shows a phenogram created using the simple matching coefficient and the WPGMA algorithm.

Groups 1 to 10, as defined in Figure 24, are redefined on phenograms A, B, C and D.

Figure 26 Phenogram (6) to show the clustering of Strain Set 2 (Subset A) using Test Set 2

Figure 26 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 5, defined at the 72.5% S_{sm} similarity level, are shown in Table 24.

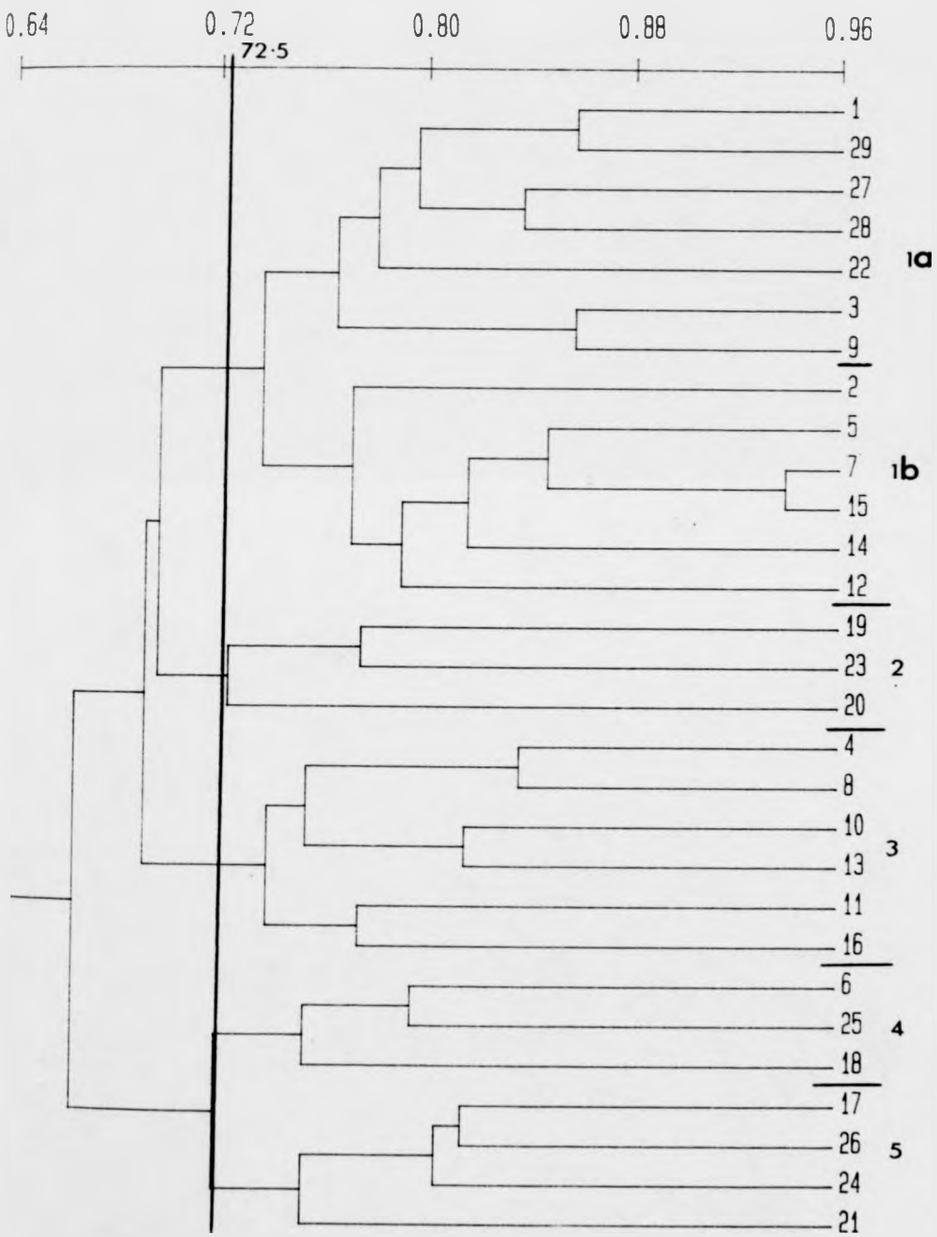


Table 24 Phenogram 6

Strain Set 2 (Subset A)/Test Set 2

Strains assigned to:
Cluster-group 1

Subcluster A

M. carbonacea var. aurantiaca	ATCC 27115
M. echinobrunnea	JCM 3327
M. fulvoviridis	JCM 3259
M. purpurea	ATCC 15835
M. zionensis	NRRL 5466
M. chalcea	JCM 5696
M. floridensis	NRRL 8020

Subcluster B

M. chalcea var. chalcea	ATCC 12452
M. echinoaurantiaca	JCM 3257
M. nigra	JCM 3328
M. melanosporea	IFO 12515
M. globosa	NRRL B2673
M. rubra	ATCC 27031

Cluster-group 2

M. chalcea var. izumensis	ATCC 21561
M. halophytica	NRRL 3097
M. sagamiensis	ATCC 21826

Cluster-group 3

M. fulvopurpurea	JCM 5696
M. narashinoensis	IFM 110-76
M. yulogensis	ATCC 43540
M. chalcea var. flavida	ATCC 27084
M. globosa	JCM 3358
M. parva	NRRL B2676

Cluster-group 4

M. purpureochromogenes	ATCC 27007
M. inyoensis	ATCC 27600
M. megalomicea var. nigra	ATCC 27598

Cluster-group 5

M. echinospora var. pallida	ATCC 15838
M. lacustris	ATCC 21974
M. viridifaciens	ATCC 31146
M. echinospora var. ferruginea	NRRL 2995

Figure 27 Phenogram (7) to show the clustering of Strain Set 2 using Test Set 2 (Subset A)

Figure 27 shows a phenogram created from binary data using the simple matching coefficient (S_{sm}) and the UPGMA algorithm.
The clusters, 1 to 11, defined at the 77% S_{sm} similarity level, are defined in Table 25.

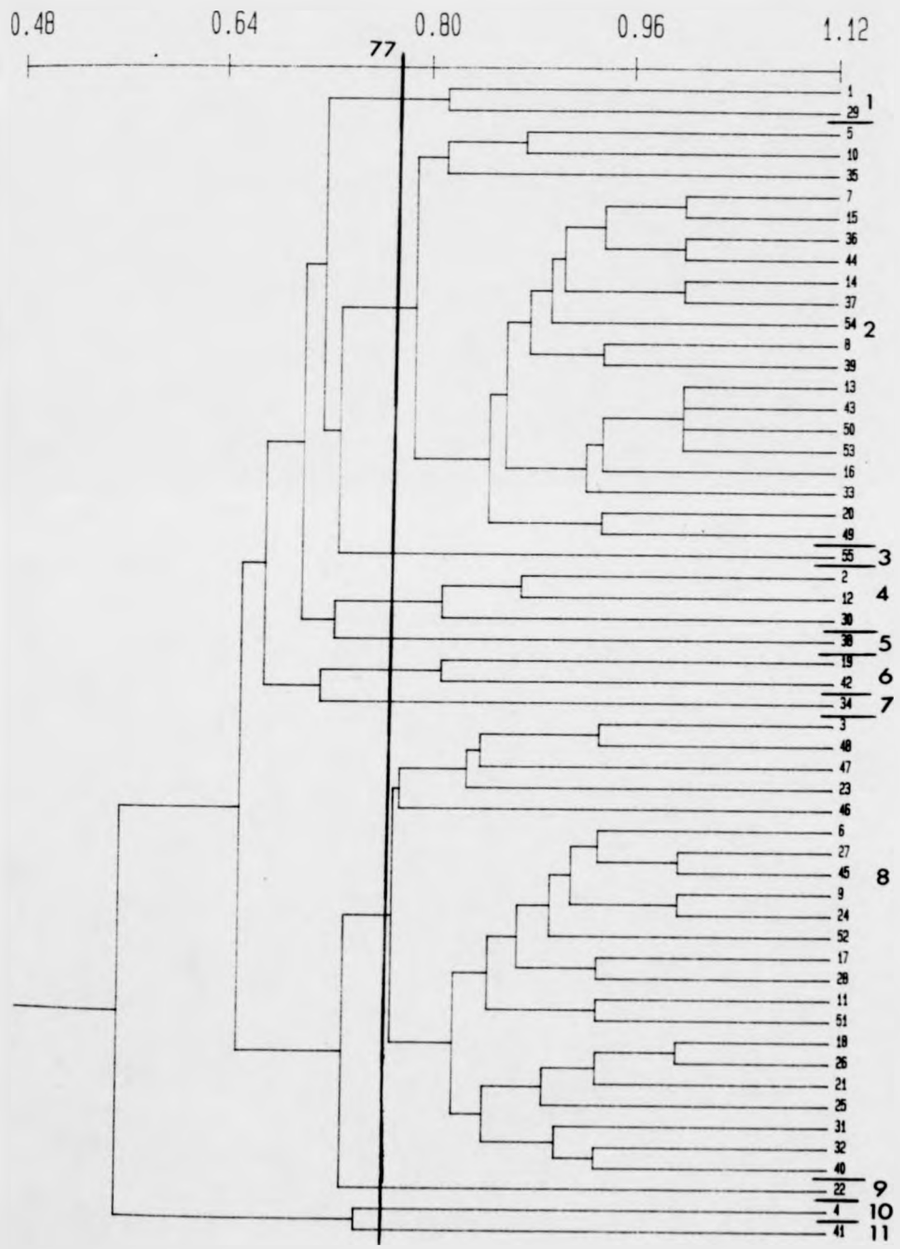


Table 25 Phenogram 7

Strain Set 2/Test Set 2(Subset A)
All strains belong to the *Micromonospora* genus
Strains assigned to:
Cluster-group 1

M. carbonacea var. *aurantiaca*	ATCC 27115
M. echinobrunnea	JCM 3327

Cluster-group 2

M. echinoaurantiaca	JCM 3257
M. yulogensis	ATCC 43540
Wild Isolate F	Warwick soil/Heat treatment
M. nigra	JCM 3328
M. melanosporea	IFO 12515
Wild Isolate G	Regina soil/Heat treatment
Wild Isolate O	Permanent Pasture soil/Heat
M. globosa	NRRL B2673
Wild Isolate H	Pond Sediment
Wild Isolate Y	Warwick soil/Heat treatment
M. narashinoensis	IFM 110-76
Wild Isolate J	Italian soil/Antibiotic treatment
M. chalcea var. *flavida*	ATCC 27084
Wild Isolate N	Permanent Pasture soil/Heat
Wild Isolate U	Warwick soil/Heat treatment
Wild Isolate X	Warwick soil/Heat treatment
M. parva	NRRL B2676
Wild Isolate D	Warwick soil/Antibiotic treatment
M. sagamiensis	ATCC 21826
Wild Isolate T	Warwick soil/Heat treatment

Cluster-group 3

Wild Isolate Z	Warwick soil/Heat treatment

Cluster-group 4

M. chalcea var. chalcea	ATCC 12452
M. rubra	ATCC 27031
Wild Isolate A	Warwick soil/Heat treatment

Cluster-group 5

Wild Isolate I	Bicutan soil/Heat treatment

Cluster-group 6

M. chalcea var. *izumensis*	ATCC 21561
Wild Isolate M	Permanent Pasture soil/Heat

Cluster-group 7

Wild Isolate E	Regina soil/Heat treatment

Cluster-group 8

M. chalcea	ATCC 27344
Wild Isolate S	Warwick soil/Heat treatment
Wild Isolate R	Warwick soil/Heat treatment
M. halophytica	NRRL 3097
Wild Isolate Q	Warwick soil/Heat treatment
M. purpureochromogenes	ATCC 27007
M. fulvoviridis	JCM 3259
Wild Isolate P	Italian soil/Antibiotics
M. floridensis	NRRL 8020
M. viridifaciens	ATCC 31146
Wild Isolate W	Warwick soil/Heat treatment
M. echinospora var. *pallida*	ATCC 15838
M. purpurea	ATCC 15835
M. globosa	JCM 3358
Wild Isolate V	Warwick soil/Heat treatment
M. megalomicea var. *nigra*	ATCC 27598
M. lacustris	ATCC 21974
M. echinospora var. *ferruginea*	NRRL 2995
M. inyoensis	ATCC 27600
Wild Isolate B	Permanent Pasture soil/Heat
Wild Isolate C	Permanent Pasture soil/Heat
Wild Isolate K	Warwick soil\Heat treatment

Cluster-group 9

M. zionensis	NRRL 5466

Cluster-group 10

M. fulvopurpurea	JCM 5696

Cluster-group 11

Wild Isolate M	Permanent Pasture soil\Heat

CHAPTER 5

Construction of Probabilistic Identification Matrices

For the Rapid Identification

of *Micromonospora*

5.1 Introduction

An important application of any stable classification is identification. Identification may be defined as the process of determining to which group or taxon a new organism or operational taxonomic unit (OTU) belongs.

The most reliable methods of microbial identification are computer based. Computer identification programs rely on the formation of an identification matrix, which for bacteria, consists of the selected taxa with their most diagnostic characters, which are usually expressed as the percentage positive states for each character within each taxon. This information can then be used to identify unknown organisms, in addition providing a measure of the likelihood that the identification is correct. Such identification, described as polythetic (Sneath and Sokal, 1973) as no one single characteristic is essential for taxon membership, contrasts with the widely used monothetic sequential keys which tend to be more susceptible to test error (Sneath, 1974). Generally polythetic methods are favoured for the identification of bacteria as often there are not clear-cut distinctions between taxa of lower rank.

Probabilistic identification schemes have been developed for use with a wide range of actinomycetes, with the exception of *Micromonospora*. Arguably the most comprehensive matrix to date is that of Williams *et al.* (1983a) which was based on the classification of 394 type cultures of *Streptomyces* and other actinomycete genera by 139 unit characters. These data were then used to construct a probability matrix for the identification of 23 phena (Williams *et al.*, 1983b). Subsequent probabilistic matrices were developed for *Streptomyces* (Langham *et al.*, 1989) and *Streptoverticillium* (Williams *et al.*, 1985).

Although *Micromonospora* are the second most common of the sporogenous actinomycetes there has yet been no published information concerning the development of a matrix for the identification of this important genus. Consequently our study aimed to tackle this by producing, from the classification data discussed in Chapter 4, a probabilistic identification matrix specifically for this genus.

5.2 Key to computer programmes used and developed throughout this study

The binary data based on **Strain Set 1/Test Set 1** (Section 4.4)(Tables 26 and 27) was stored in the Lotus 123 database. These data were then used to construct a probabilistic identification matrix using the following programs.

5.2.1 The Numerical Taxonomy Package

A program called the Numerical Taxonomy Package (NTP), written by Mr. S. Greensides (Greensides *et al.*, unpublished data), was used to create an identification matrix from the phenogram produced by the NTSYS package (Figure 28). The NTP allows a cut-off level on the phenogram to be chosen based on percentage similarity (S_{SM}) at which a number of clusters are formed (Table 28). An identification matrix is then created containing the percentage of positives for each of the unit characters in each of the clusters. This PERCENT table is then used as input for all of the following programs involved in constructing the final matrix.

The program also plays a part in the crude selection, from the identification matrix, of possible diagnostic characters for each of the

clusters or groups. It performs this role by creating a table which initially scores each unit character - a high score is given to characters which are >85% positive or <15% negative for any of the groups in the matrix. The program then calculates the standard deviation of the percentage values in each of the clusters for each character. If a character is 'useful' as a potential separator of groups it is likely to have a high score along with a high standard deviation.

This program was used in conjunction with CHARSEP and DIACHAR (Sneath, 1979a, 1980a) in the selection of diagnostic characters.

Table 26

Unit characterisation tests for construction of probabilistic identification matrix

The characterisation tests underlined and highlighted indicate those used in the final matrix.

Growth on Carbohydrates
1. Adonitol
2. Amygdalin
3. D-Arabinose
4. L-Arabinose
5. Arbutin
6. Cellobiose
7. Dextrin
8. Dulcitol
9. Erythritol
10. Fructose
11. L-Fucose
12. Galactose
13. Glucose
14. D-2 Glucose
15. a B Glucopyranose
16. Glycerol
17. Glycogen
18. Inositol
19. Inulin
20. Lactose
21. Maltose
22. Mannitol
23. Mannose
24. Melibose
25. Melezotose
26. Raffinose
27. Rhamnose
28. Ribose
29. Salicin
30. Sorbitol
31. Sucrose
32. Trehalose
33. D-Xylose
34. L-Xylose

Utilisation of Organic Acids
35. Acetate
36. Benzoate
37. Butyrate
38. Caprylate
39. Citrate
40. Formate
41. Gluconate

42. Glucuronate
43. Glutamate
44. Lactate
45. Malate
46. Oleate
47. Oxalate
48. Propionate
49. Pyruvate
50. Succinate
51. Tartrate
52. Calcium glycerate
53. Fumarate
54. a-Ketoglutarate

Hydrolysis of:

55. Adenine
56. Hypoxanthine
57. Tyrosine
58. Xanthine
59. Xylan
60. Chitin
61. Casein
62. Starch
63. DNA
64. Gelatin
65. Arbutin
66. Esculin
67. Urea, 7 days
68. Urea, 28 days
69. Allantoin
70. Hippurate
71. Catalase
72. Oxidase
73. Nitrate.

Growth at:

74. pH 5.0
75. pH 6.0
76. pH 7.0
77. pH 8.0
78. pH 9.0
79. pH 10.0
80. 10°C
81. 27°C
82. 35°C
83. 40°C
84. 42°C
85. 45°C
86. 50°C/8 hours

Resistance to:

87. Lysozyme
88. Sodium salicyclate
89. Sodium chloride 1%
90. 1.5%
91. 2%
92. 3%
93. 4%
94. 5%
95. $Na_2S_2O_3$ 1%
96. 2%

97. 3%
98. 4%
99. $(NH_4)SO_4$ 1%
101. 3%
102. 4%
103. Sodium azide 0.001%
104. 0.005%
106. 0.02%
107. 0.03%
108. Phenyl EtOH 0.05%
109. 0.1%
110. 0.2%
111. 0.3%
112. 0.4%
113. $CoCl_2$ 1 x 10^{-4}
114. 5 X 10^{-4}
115. 1 X 10^{-3}
116. 5 X 10^{-3}
117. 1 X 10^{-2}
118. Phenol 0.025%
119. 0.05%
120. 0.075%
121. 0.1%
122. Thallium acetate 0.0001%
123. 0.001%
124. 0.01%
125. Tetrazolium 0.001%
126. 0.005%
127. 0.01%
128. 0.1%
129. Crystal violet 0.000001%
130. 0.0005%
131. 0.0001%
132. 0.005%
133. 0.001%

Antibiotic Resistances:

134. Gentamicin 10mg/ml
135. 50mg/ml
136. Sisomicin 10
137. 50
138. Neomicin 10
139. 50
140. Kanamycin 10
141. 50
142. Erythromycin 10
143. 50
144. Halomicin 10
145. 50
146. Evernomicin 10
147. 50
148. Rosamicin 10
149. 50
150. Novobiocin 10
151. 50
152. Chloroamphenicol 5
153. 10
154. Bacitracin 2
155. 10

156. Nalidixic acid 10
157. 50
158. Cycloserine 10
159. 50
160. Streptomicin 10
161. 50
162. Gantisin 100
163. 150
164. Tetracycline 10
165. 50
166. Penicillin G 10
167. 50
168. Lincomycin 10
169. 50
170. Clindamycin 10
171. 50
172. Genta A 10
173. 50
174. Paromycin 10
175. 50
176. Cephalothin 10
177. 50
178. Sulphadiazin 100
179. 150

Table 27

Strains used for the Construction of the Probabilistic Identification Matrix

1. *Micromonospora grisea*	G55
2. *M. zionensis*	G52
3. *M. olivasterospora*	ATCC 21819
4. *M. olivasterospora*	ATCC 31010
5. *M. olivasterospora*	ATCC 31009
6. *M. rosaria*	ATCC 29337
7. *rosaria*-like	S9683
8. *rosaria*-like	Q319
9. *rosaria*-like	Q1578
10. *rosaria*-like	4215
11. *rosaria*-like	9603
12. *rosaria*-like	S2742
13. *rosaria*-like	T816
14. *rosaria*-like	X70
15. *rosaria*-like	T5771
16. *rosaria*-like	T5804
17. *M. purpureochromogenes*	ATCC 27007
18.	SCC 969 (M1021)
19.	SCC 1000 (68-I)
20. *M. fusca*	CBS SCC 821
21. *M. fusca*	SCC 900
22. *M. narashinoensis*	SCC 939 IFO
23. *M. purpureochromogenes*	ATCC 10026
24. *M. echinospora* var. *echinospora*	817
25.	TS 8
26. *M. echinospora* var *ferruginea*	782
27. *M. echinospora* var. *pallida*	TS 5
28.	795
29.	774
30.	799
31.	805
32.	10-408-C
33.	10-408-35
34.	11 89
35.	10 245
36.	11 224
37.	11 365
38.	12 46
39.	12 123
40.	12 125
41.	12 44
42. *M. echinospora* var *challensensis*	14 261
43. *M. polytrota*	AR 5
44. *M. polytrota*	15 368
45. *M. capillata Ferm. P*	2598
46. *M. carbonacea* var.*carbonacea*	ATCC 27114
47. *M. carbonacea* var.*aurantiaca*	ATCC 27115
48. *M. carbonacea* var.*africana*	ATCC 39149
49. *M. chalcea*	ATCC 12452
50. *M. chalcea*	ATCC 27333
51. *M. chalcea*	ATCC 21994
52.	91 372

53.		91 1306
54.	*M. chalcea*	ATCC 27083
55.	*M. chalcea*	ATCC 27084
56.		R 460
57.		R 461
58.		69 683
59.		1020
60.		1062
61.		1063
62.	*M. halophytica* var *halophtica*	SCC 807 SP-30
63.	*M. halophytica* var *nigra*	SCC 760
64.		SCC 763
65.		785 (R476)
66.		818 (R476)
67.		737 (SP14)
68.		815 (SP14)
69.	*M. sagamiensis*	ATCC 21803
70.	*M. sagamiensis*	ATCC 21826
71.	*M. sagamiensis*	ATCC 21827
72.	*M. sagamiensis*	ATCC 21949
73.	*M. purpurea*	NTS 9
74.	*M. purpurea*	ATCC 31119
75.	*M. purpurea*	SCC 1038
76.		SCC 1039
77.		71-1225
78.		H334-0
79.		334-5
80.		334-13
81.		11-90
82.		12-33
83.		12-34
84.		12-35
85.		12-36
86.		12-37
87.	*Micromonospora sp.*	NRRL 3543
88.	*M. inyoensis*	ATCC 27600
89.		NRRL 5742
90.		6640
91.		1081
92.		72-469
93.		10-8
94.		10-9
95.		984
96.		990
97.		71-64
98.	Nocardicin (22JC) producer	SCC 895
99.	Nocardicin (22JC) producer	13 859
100.	Gilvocarcin producer	13 304
101.	Gilvocarcin producer	13 335
102.	Gilvocarcin producer	12 402
103.	Phenazine-type producer	124
104.	Phenazine-type producer	12 414
105.	Toxic compound producer	68-742
106.	Toxic compound producer	68-742-12
107.	Toxic compound producer	68-742-1284
108.	Thiostrepton producer	68-1147
109.	Thiostrepton producer	68-1147-134
110.	Thiostrepton producer	10-259

111. Myxin producer	13-16
112. Myxin producer	13-17
113. Myxin producer	13-20
114. Myxin producer	13-22
115. Myxin producer	13-23
116. Myxin producer	13-26
117. Myxin producer	13-34
118. Myxin producer	13-36
119. Myxin producer	13-40
120. Myxin producer	13-83
121. Myxin producer	13-96
122. *Actinomadura*	SCC 906
123. *Actinomadura*	SCC 918
124. *Actinomadura*	A 27295
125. *Actinomadura*	A 27296
126. *Actinomadura*	A 27297
127. *Actinomadura*	A 27298
128. *Actinomadura*	A 27299
129. *Actinomadura*	A 27887
130. *Actinomadura*	A 27888
131. *Actinomadura*	A 29553
132. *Actinomadura*	13-363
133. *Actinomadura*	AA-6
134. *Actinomadura*	BL-6
135. *Actinomadura*	A 23218

5.2.1 CHARSEP

A BASIC program termed CHARSEP was used for calculating the usefulness of different characters for separating between the 14 groups in the matrix (Sneath, 1979a). The program recognises that any character that is always positive or always negative in every group is of no help in distinguishing between groups. Similarly, a character whose frequency is consistantly 50% in every group is equally useless. CHARSEP used the PERCENT table from the above program.

For each of the 179 characters CHARSEP calculated the following separation indices: Gyllenberg's Sum of C (Gyllenberg, 1963), Gyllenberg's R, Niemela's index (Niemela *et al.*, 1968), the VSP index (Sneath and Johnson, 1972) and the CSP index. 0Additional quantities that were calculated by CHARSEP were the numbers of groups to which an unknown could be allocated reliably if it was scored positive or negative for a character, the character consistancy within taxa, and the separation potential of each character. In addition characters of poor diagnostic value were determined readily by the program.

5.2.2 DIACHAR

This program determined the most diagnostic character states of each of the 14 groups in the matrix (Sneath, 1980a). The program allows the user to determine several strongly diagnostic properties for each group. The diagnostic scores obtained were based on a principle used by Morse (1974) and were adapted to percentages of positive values of characters.

5.2.3 OVERMAT

OVERMAT calculates the phenetic overlap between the 14 groups of the matrix (Sneath, 1980b), allowing a determination to be made of the extent to which pairs of groups overlapped in the identification matrix. The program represents the 14 taxa as hyperspherical multivariate Gaussian clusters of objects in a hyperspace consisting of m dimensions, one for each character. For each pair of taxa the disjunction index W, and the corresponding nominal overlap V_G, was determined. The latter ranges from 1.0 for complete overlap to 0 for complete disjunction. In addition, the program calculates the significance of the determined overlap using a non-central t-statistic, against a selected critical overlap value (Vo).

5.2.4 MOSTTYP

MOSTTYP evaluates the quality of chosen identification matrices by calculating the best identification scores that an entirely typical example of each group could achieve (Sneath, 1980c). The properties of these examples are compared by the program with a previously prepared identification matrix that contains the percentage of positives for each character in each of the groups.

The program calculates the identification scores of the most typical member of each group (the Hypothetical Median Organism) against all of the groups in the matrix. It offers a choice of five polythetic identification coefficients: (a) Willcox probability, (b) taxonomic distance, (c) standard error of the taxonomic distance, (d) the Gaussian integral corresponding to this standard error and (e) pattern distance. A good identification matrix should ideally give excellent

scores on all four coefficients for an HMO against its own taxon. The Willcox coefficient should be close to 1.0, the taxonomic distance should be typically less than 0.15, the s.e.(d) should be a negative value, and the Gaussian integral should again be close to 1.0. Finally, the second, third and fourth best identities should give much poorer scores on all four coefficients.

5.2.5 MATIDEN

MATIDEN gives an online computer-assisted identification of an unknown sample scored for presence-absence properties (Sneath, 1979b). The properties are compared with a previously prepared identification matrix that contains the percentage of positives for each property in each of the groups to which the unknown might belong. The output consists of identification scores of the unknown to the various groups. Identification with one group is achieved if the first identification score is sufficiently good, and also sufficiently better than any scores against alternative groups. The program calculates the identification scores of the unknown against the groups by using the same principles and polythetic identification coefficients as used in the above program MOSTTYP.

Figure 28. Phenogram based on 179 physiological and biochemical tests, created using S_{sm} and UPGMA.

The diagram illustrates the species-groups which were formed at the 77.54% similarity level. Similarity was calculated using the simple matching coefficient and the UPGMA algorithm.

1. *Micromonospora olivasterospora*
2. *M. echinospora*
3. *M. sagamiensis*
4. *M. polytrota*
5. *M. capillata*
6. *M. rosaria*
7. *M. chalcea*
8. *M. sp.1*
9. *M. purpureochromogenes*
10. *M. sp.2*
11. *M. halophytica*
12. *M. sp.3*
13. *M. fusca*
14. *M. carbonacea*

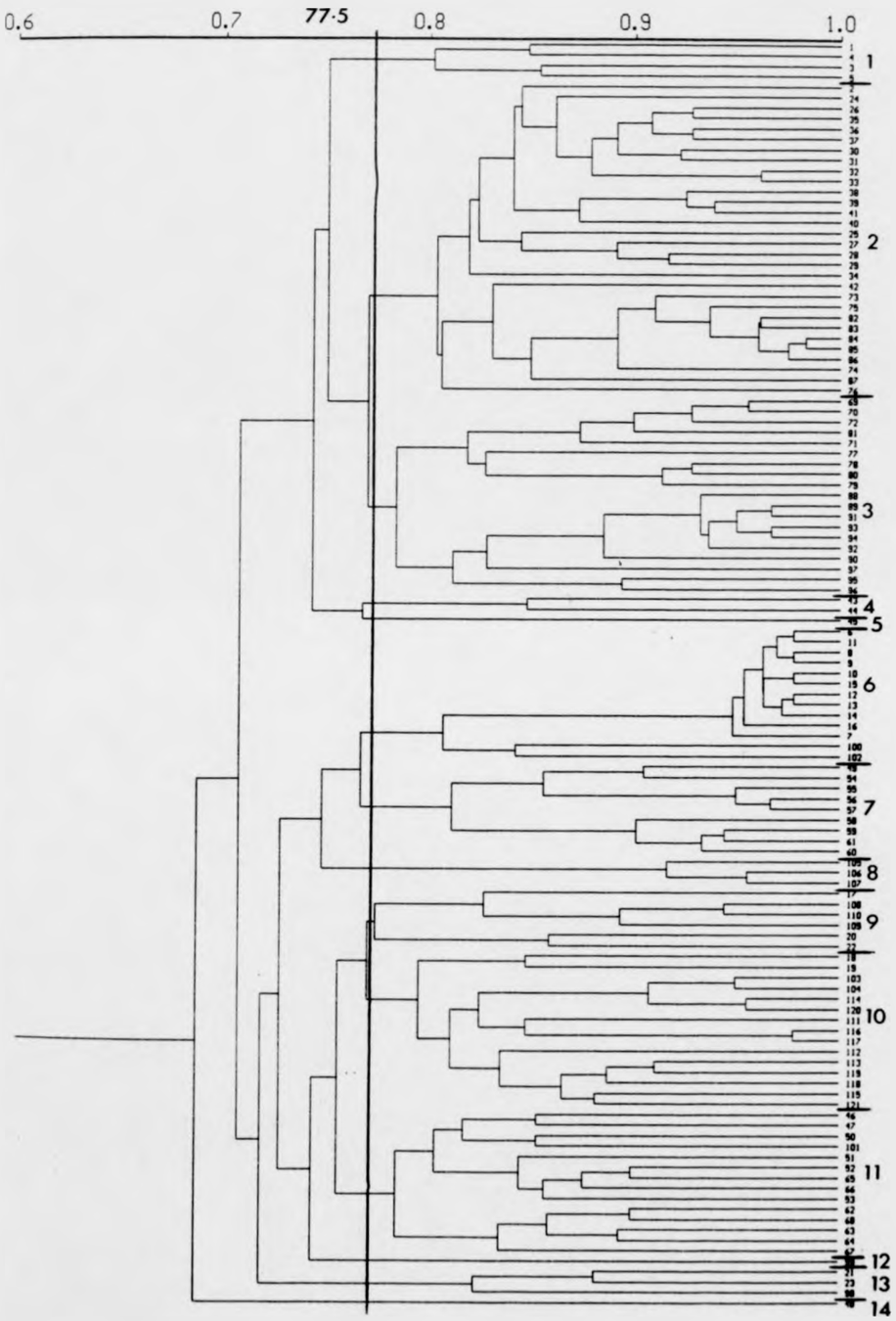


Table 28

Species-Groups Defined at 77% S_{sm} S-Level

Strains assigned to:

Cluster-group 1 ***Micromonospora olivasterospora***

1. *Micromonospora grisea*	G55
4. *M. olivasterospora*	ATCC 31010
3. *M. olivasterospora*	ATCC 21819
5. *M. olivasterospora*	ATCC 31009

Cluster-group 2 ***M. echinospora***

2. *M. zionensis*	G52
24. *M. echinospora* var. *echinospora*	817
26. *M. echinospora* var. *ferruginea*	782
35. *M. sp.*	10 245
36. *M. sp.*	11 224
37. *M. sp.*	11 365
30. *M. sp.*	799
31. *M. sp.*	805
32. *M. sp.*	10-408-C
33. *M. sp.*	10-408-35
38. *M. sp.*	12 46
39. *M. sp.*	12 123
41. *M. sp.*	12 44
40. *M. sp.*	12 125
25. *M. sp.*	TS 8
27. *M. echinospora* var. *pallida*	TS 5
28. *M. sp.*	795
29. *M. sp.*	774
34. *M. sp.*	11 89
42. *M. echinospora* var *challensensis*	14 261
73. *M. purpurea*	NTS 9
75. *M. purpurea*	SCC 1038
82. *M. sp.*	12-33
83. *M. sp.*	12-34
84. *M. sp.*	12-35
85. *M. sp.*	12-36
86. *M. sp.*	12-37
74. *M. purpurea*	ATCC 31119
87. *Micromonospora sp.*	NRRL 3543
76. *M. sp.*	SCC 1039

Cluster-group 3 ***M. sagamiensis***

69. *M. sagamiensis*	ATCC 21803
70. *M. sagamiensis*	ATCC 21826
72. *M. sagamiensis*	ATCC 21949
81. *M. sp.*	11-90
71. *M. sagamiensis*	ATCC 21827
77. *M. sp.*	71-1225
78. *M. sp.*	H334-0

80. *M. sp.*	334-13
79. *M. sp.*	334-5
88. *M. inyoensis*	ATCC 27600
89. *M. sp.*	NRRL 5742
91. *M. sp.*	1081
93. *M. sp.*	10-8
94. *M. sp.*	10-9
92. *M. sp.*	72-469
90. *M. sp.*	6640
97. *M. sp.*	71-64
95. *M. sp.*	984
96. *M. sp.*	990

***Cluster-group 4* M. polytrota**

43. *M. polytrota*	AR 5
44. *M. polytrota*	15 368

***Cluster-group 5* M. capillata**

45. *M. capillata Ferm. P*	2598

***Cluster-group 6* M. rosaria**

6. *M. rosaria*	ATCC 29337
11. *M. sp.*	9603
8. *M. sp.*	Q319
9. *M. sp.*	Q1578
10. *M. sp.*	4215
15. *M. sp.*	T5771
12. *M. sp.*	S2742
13. *M. sp.*	T816
14. *M. sp.*	X70
16. *M. sp.*	T5804
7. *M. sp.*	S9683
100. *M. sp.*	13 304
102. *M. sp.*	12 402

***Cluster-group 7* M. chalcea**

49. *M. chalcea*	ATCC 12452
54. *M. chalcea*	ATCC 27083
55. *M. chalcea*	ATCC 27084
56. *M. sp.*	R 460
57. *M. sp.*	R 461
58. *M. sp.*	69 683
59. *M. sp.*	1020
61. *M. sp.*	1063
60. *M. sp.*	1062

***Cluster-group 8* M. sp1**

105. *M. sp.*	68-742
106. *M. sp.*	68-742-12
107. *M. sp.*	68-742-1284

Cluster-group 9 ***M. purpureochromogenes***

17. *M. purpureochromogenes*	ATCC 27007
108. *M. sp.*	68-1147
110. *M. sp.*	10-259
109. *M. sp.*	68-1147-134
20. *M. fusca*	CBS SCC 821
22. *M. narashinoensis*	SCC 939 IFO

Cluster-group 10 ***M. sp2***

18. *M. sp.*	SCC 969 (M1021)
19. *M. sp.*	SCC 1000(68-I)
103. *M. sp.*	124
104. *M. sp.*	12 414
114. *M. sp.*	13-22
120. *M. sp.*	13-83
111. *M. sp.*	13-16
116. *M. sp.*	13-26
117. *M. sp.*	13-34
112. *M. sp.*	13-17
121. *M. sp.*	13-96
113. *M. sp.*	13-20
119. *M. sp.*	13-40
115. *M. sp.*	13-23
118. *M. sp.*	13-36

Cluster-group 11 ***M. carbonacea***

46. *M. carbonacea* var.*carbonacea*	ATCC 27114
47. *M. carbonacea* var.*aurantiaca*	ATCC 27115
50. *M. chalcea*	ATCC 27333
101. *M. sp.*	13 335
51. *M. chalcea*	ATCC 21994
52. *M. sp.*	91 372
65. *M. sp.*	785 (R476)
66. *M. sp.*	818 (R476)
53. *M. sp.*	91 1306
62. *M. halophytica* var. *halophtica*	SCC 807 SP-30
68. *M. sp.*	815 (SP14)
63. *M. halophytica* var. *nigra*	SCC 760
64. *M. sp.*	SCC 763
67. *M. sp.*	737 (SP14)

Cluster-group 12 ***M. sp3***

99. *M. sp.*	13 859

Cluster-group 13 ***M. fusca***

21. *M. fusca*	SCC 900
23. *M. purpureochromogenes*	ATCC 10026
98. *M. sp.*	SCC 895

Cluster-group 14 ***M. carbonacea***

48. *M. carbonacea var.africana*	ATCC 39149

5.3 Methods

5.3.1 Strains

Details of the origins, cultivation and preservation of strains, together with the definition and composition of clusters were given in Chapter 4 (Figure 28).

5.3.2 Selection of clusters

The clusters for the construction of the identification matrix were the 14 groups defined at the 77.5% S_{sm} level from the phenogram produced by the NTSYS clustering package (Figure 28, Table 28). See Chapter 4.

5.3.3 Selection of the most diagnostic characters

The minimum number of characters required to differentiate between the 14 groups in the matrix was selected from the 179 unit characters used to define the clusters.

An initial selection of characters was made with the assistance of the Numerical Taxononmy Package (NTP) (Greensides *et al.*, unpublished data), which from the original identification matrix containing the percentages of positive values of the 179 characters in each of the 14 groups, formulated a PERCENT table. Tests which were consistantly $>85\%$ positive or $<15\%$ negative for each group, with a high standard deviation of percentages, were given high scores reflecting, to some extent, their separation potential.

A further selection of characters was achieved using the CHARSEP program (Sneath, 1979a). The separation indices, the Gyllenberg's (1963) sum of $C_{(i)}$ and rank measure $R_{(i)}$, the Niemala index (1968), the VSP index (Sneath and Johnson, 1972) and an analogous index, CSP were calculated and the usefulness of each of the 179 characters for separating the 14 groups was determined.

Character selection was then checked by applying the DIACHAR program (Sneath, 1980a), which ranks the diagnostic scores of each character for each of the 14 groups in the matrix and also provides the sum of scores of all characters for each group. High scores are therefore desirable.

5.3.4 Characters included in the identification matrix

Three matrices were developed incorporating 10, 15 and 20 character tests (Table 29). Full practical details for the determination of all tests are given below. The character numbers quoted are those used in the identification matrix. Unless stated, the inoculum consisted of spore and mycelial suspensions stored in 10% (w/v) glycerol at -20°C (Wellington and Williams, 1978). All incubations were at 30-32°C unless otherwise stated.

5.3.4(a) Growth on carbohydrates

Appropriate weights of each carbohydrate (L-arabinose, galactose, glycogen, lactose, melibose, raffinose, salicin, D-xylose) were sterilised in 50ml flasks with loose fitting bungs by covering the solid with diethyl ether which was allowed to evaporate at room temperature in a fume cupboard for 18 hours. Final traces of ether were removed by placing

the flasks in a laminar flow cabinet for 10 minutes. Alternatively carbon sources were sterilised by filtration. Each of the 8 sterilised carbon sources was added, aseptically, to give a concentration of 1% (w/v), to a basal medium containing (l^{-1}): 5g Yeast extract, 1g $CaCO_3$, Agar, 15g; adjusted to pH 7.0 (modified from Pridham and Gottlieb, 1948). Repli dishes were inoculated with 0.1 to 0.2 ml of cells. Cultures were incubated at 30°C-32°C and read at 14, 21 and 28 days. The ability of a strain to use a carbon source was a determined by comparison of its growth with that on the negative control (no carbon source).

5.3.4(b) Utilisation of organic acids

The ability of strains to use gluconate (41), lactate (44), succinate (50) and fumarate (53) as sole carbon sources was examined on Koser's Citrate Agar (Modified)(see Appendix 2). A solution of 0.04% phenol red (20ml/litre) was added to the media. Carbon sources were then added at 0.2% (w/v), except for lactate which was added at the concentration 3.3 ml/litre. The final colour of the agar was a very light orange neutral. Each slant of medium was inoculated with 0.1 to 0.2 ml of washed inoculum and incubated at 30-32°C. Growth was scored at 7, 14, 21 and 28 days. A positive result was indicated by the agar turning a rose pink.

5.3.4(c) Degradative tests

The degradation of hypoxanthine (56) and xylan (59) (0.5 and 0.4%, w/v respectively) were detected in plates containing (per litre) Yeast extract 10g, Dextrose 10g, Agar 15g. Plates were inoculated with cells and incubated at 30-32°C. After 3 weeks plates were examined for

growth; clearing of the insoluble compounds from under and around areas of growth was scored as positive.

Arbutin degradation (65) was studied in tubes containing (l^{-1}): 10g yeast extract (Oxoid), 1g arbutin, 0.5g ferric ammonium citrate, and 15g agar, pH 7.2 (Kutzner, 1976). Controls without arbutin were also inoculated. After 3 weeks, a positive result was indicated by a brown-black pigment. Comparison with a control was necessary to avoid confusion with melanin production.

5.3.4(c) Growth inhibition

Growth inhibition tests were carried out in Repli dishes containing (l^{-1}): 10g yeast extract (Oxoid), 10g Dextrose, 1g $CaCO_3$, and 15g agar, pH 7.3.

Each potential inhibitor was added to this medium before autoclaving to give the appropriate final dilution; inhibitors were sodium chloride, 2% (91), ammonium sulphate, 4% (102), phenyl ethanoate, 0.2% (110) and phenol, 0.075% (120). After 2 weeks growth was compared with that on the medium without the inhibitor; absence of growth, or very weak growth was recorded as negative.

Growth at 42°C (84) was determined in Repli dishes using the same basal medium. Plates were incubated also for 14 days, enclosed in a polythene bag to prevent desiccation, and presence or absence of growth was recorded.

5.3.4(d) Antibiotic resistances

Resistances to gentamicin 50mg/ml (135), kanamicin 10mg/ml (140), novobiocin 50mg/ml (151) and cycloserine 50mg/ml (159) were

Table 29. Characters incorporated into final 3 identification

Matrices, A, B and C

Identification Matrix A - 20 Unit Characters

12. Growth on Galactose
17. Growth on Glycogen
20. Growth on Lactose
24. Growth on Melibiose
26. Growth on Raffinose
29. Growth on Salicin
41. Utilisation of Gluconate
44. Utilisation of Lactate
50. Utilisation of Succinate
53. Utilisation of Fumarate
56. Hydrolysis of Hypoxanthine
59. Hydrolysis of Xylan
65. Hydrolysis of Arbutin
84. Growth at 42°C
91. Resistance to NaCl (2%, w/v)
102. Resistance to NH_4SO_4 (4%, w/v)
110. Resistance to Phenyl ethanoate (0.2%, w/v)
135. Resistance to Gentamicin (50 mg/ml)
140. Resistance to Kanamycin (10 mg/ml)
151. Resistance to Novobiocin (50mg/ml)

Identification Matrix B - 15 Unit Characters

4. Growth on L-Arabinose
17. Growth on Glycogen
24. Growth on Melibiose
29. Growth on Salicin
33. Growth on D-Xylose
44. Utilisation of Lactate
50. Utilisation of Succinate
56. Hydrolysis of Hypoxanthine
65. Hydrolysis of Arbutin
84. Growth at 42°C
91. Resistance to NaCl (2%, w/v)
120. Resistance to Phenol (0.075%, w/v)
135. Resistance to Gentamicin (50 mg/ml)
151. Resistance to Novobiocin (50 mg/ml)
159. Resistance to Cycloserine (50 mg/ml)

Identification Matrix C - 10 Unit Characters

17. Growth on Glycogen
24. Growth on Melibose
29. Growth on Salicin
44. Utilisation of Lactate
50. Utilisation of Succinate
56. Hydrolysis of Hypoxanthine
65. Hydrolysis of Arbutin
84. Growth at 42°C
91. Resistance to NaCl (2%, w/v)
120. Resistance to Phenol (0.075%, w/v)

determined on the same basal medium as above. Each antibiotic was filter sterilised and added to the medium asceptically after autoclaving to the appropriate concentration. Plates were examined for growth after 2 weeks.

5.3.5 Theoretical evaluation of the identification matrix

The three matrices, incorporating 10, 15 and 20 unit characters (reduced from 179 characters) were then assessed in three ways.

5.3.5(a) Assessment of cluster overlap

The probability of significant overlap between the clusters in the three matrices was assessed by the OVERMAT program (Sneath, 1980b). This was achieved by using percentage positive values for character states to calculate a disjunction index (W) and a corresponding nominal overlap (V_G). The significance of the determined overlap was then assessed using a non-central *t*-statistic, against a selected critical overlap value (Vo) of 5%.

5.3.5(b) Determination of identification scores for the Hypothetical Median Organism (HMO) for each cluster

Data from the three matrices were input to the MOSTTYP program (Sneath, 1980c) to obtain the theoretically best identification scores for the most typical member of each cluster (the Hypothetically Median Organism). The matrices consisted of the percentages of positive values of m characters in q taxa or groups (where m = 10, 15 and 20, and q = 14). Small alterations were made to the percentages in the

matrix; all 0 and 100 values were changed to 1 and 99 respectively (Lapage et al., 1970).

MOSTTYP initially converts the percentages in the matrix to proportions, P_{ij} for the ith character of taxon j. The character state values of the most typical possible individual of each taxon (HMO) are then compared with each taxon in turn, identification coefficients are calculated and the best identification scores are given.

Identification coefficients used by MOSTTYP are:

(a) The Willcox Probability : $L_{uj}/E^{q}L_{uj}$, which is the likelihood of *u* against taxon *j* divided by the sum of the likelihoods of *u* against all *q* taxa (Willcox *et al.*, 1973). Ideally, for a good identification matrix, high values of the Willcox probability should be obtained (as close to 1 as possible).

(b) The Taxonomic Distance (d) : $v[E(u_i-P_{ij})^2/m']$, which is the distance of an unknown from the centroid of the group with which it is being compared. Low scores indicate relatedness and are hence desirable.

(c) The Standard Error of the Taxonomic Distance (c) : This is defined as the constant c in this equation: $d = d_j + cs_{dj}$, d_j is the mean distance of OTU's of taxon *j* from the centroid and s_{dj} is the standard deviation of those distances. A good identification matrix should give a negative value of s.e.(d).

(d) The Gaussian integral of s.e.(d), where Gau (s.e.(d)) is <0.5 for negative s.e.(d). The function Gau is obtained from an algorithm cited by Hastings (1955) and from Mill's Ratio (Owen, 1962).

(e) The Pattern Distance dp, $D_P^2 = D_T^2 - D_V^2$, where D_T is d^2, the squared taxonomic distance, and D_V is $(u_i - P_{ij})\ u_i - P_{ij}/m'$.

5.3.5(c) Determination of identification scores for cluster representatives using data from the numerical phenetic classification

A strain from each cluster was randomly selected. The original test data for these strains were input to the three MATIDEN programs, for each of the three matrices (Sneath, 1979b). Using the same coefficients and principles as MOSTTYP, MATIDEN provides the three best identification scores for a known or unknown strain against a matrix. Characters of the strain are then listed which are atypical of the phenon to which it best identifies.

5.3.6 Practical evaluation of the identification matrix

5.3.6(a) Re-determination of character states of cluster representatives

Fourteen randomly selected representatives were taken, one from each group. The character states of the strains (for the characters comprising each of the three matrices) were re-determined and used to obtain identification scores using MATIDEN (Sneath, 1979b). Using the results obtained from Matrix A only (the largest matrix: 20 strains) any discrepancies were noted, and the test variances (S_i^2) calculated from $S_i^2 = d/2t$, where d is the number of strains giving a discrepant result and t is the number of strains tested (Sneath and Johnson, 1972). In addition the average probability of an erroneous test result was calculated using the mean of test variances (Sneath and Johnson, 1972).

5.3.6(b) Identification of unknown isolates

The 20 character state matrix was further evaluated by using it for the identification of isolates obtained from soil and sediment environments. A total of 20 *Micromonospora* were used, isolated from a variety of soil and sediment samples. Character states were determined and, using MATIDEN (Sneath, 1979b), identification scores were obtained. Those isolates with acceptable scores were then given cluster identifications.

5.4 Results

5.4.1 Construction and composition of the matrices

The diagnostic scores provided by the Numerical Taxonomy Package, NTP (Greensides *et al.*, unpublished data) and the CHARSEP program (Sneath, 1979a) allowed an assessment to be made of the diagnostic value of each of the 179 unit characters in the matrix. Examination of the separation indices from both programs provided an initial indication that many of the characters in the original matrix were indeed of limited diagnostic value. Tables 30 and 31 illustrate examples of the output from CHARSEP and NTP showing characters with high diagnostic values chosen for the matrix and those with low values excluded from it. Occasionally tests with high values were not included in the matrix due to practical difficulties in their determination.

The DIACHAR program (Sneath, 1980a) was used to provide an alternative assessment of the three groups of tests containing 10, 15 and 20 unit characters. If the clusters were well defined by the chosen tests, the sums of the character values should be high. For the 10 character

Table 30 **Examples of results obtained by CHARSEP to determine the separation values of characters**

Examples of characters selected for the matrix	No. of clusters which character is predominantly:		Separation indices				
					Gyllenberg (1963)		
	+ve	-ve	VSP (%)	CSP	$EC_{(i)}$	$ER_{(i)}$	Niemela *et al.* (1968) index
Resistance to gentamicin (50 mg/ml)	6	7	92.52	0.92	13.71	576.07	7.44
Growth on glycogen	7	4	69.38	0.74	12.86	360.32	5.79
Growth with NaCl (2%, w/v)	7	4	67.48	0.70	12.59	352.72	5.79
Growth on melibose	5	7	76.24	0.81	13.43	470.26	6.67
Resistance to novobiocin (50 mg/ml)	9	3	52.38	0.66	12.92	348.98	5.39
Resistance to NH_4SO_4 (4%, w/v)	5	6	73.71	0.73	12.62	378.78	6.13
Growth on L-arabinose	8	4	74.77	0.81	13.55	433.77	6.20
Use of succinate	5	8	72.60	0.77	13.31	532.51	7.1601
Use of gluconate	7	6	84.30	0.84	13.34	560.51	7.44
Hydrolysis of xylan	6	5	78.64	0.79	12.94	388.38	6.13

Table 30 continued

Examples of characters rejected for the matrix	No. of clusters which character is predominantly:		Separation indices				
				Gyllenberg (1963)			
	+ve	-ve	VSP (%)	CSP	$EC_{(i)}$	$ER_{(i)}$	Niemela *et al.* (1968) index
Growth on dulcitol	0	14	0	0.048	13.86	0	0
Growth on erythritol	0	14	0	0.04	13.86	0	0
Growth at 10°C	0	10	3.70	0.27	12.43	0	0
Growth at pH 7	14	0	0.10	0.05	13.79	0	0

Table 31 **Examples of results obtained by NTP to determine the separation values of characters**

Examples of selected characters for the matrix	NTP separation value	Standard deviation
Growth on L-arabinose	795	37
Growth on galactose	729	45
Growth on glycogen	706	42
Growth on salicin	602	42
Growth on melibiose	632	47
Utilisation of lactate	557	37
Hydrolysis of xylan	643	44
Resistance to gentamicin (50 mg/ml)	759	51

matrix, these ranged from 2.70 to 6.32, for the 15 character matrix 6.50 to 7.68, and finally for the 20 character matrix the values ranged from 10.08 to 13.67. All of these values were regarded as acceptable, although from the values it could be concluded that some clusters were less defined than others.

The final three identification matrices are shown in Tables 32, 33 and 34 and consist of 14 clusters x 10, 15 and 20 characters respectively.

5.4.2 Theoretical evaluation of the identification matrices

5.4.2(a) Assessment of cluster overlap

In any identification matrix overlap between groups should be minimal. Group overlap in each of the three matrices was assessed by the OVERMAT program (Sneath, 1980b). The critical overlap value (V_O) selected was 5%. No significant overlap was observed between any of the pairs of clusters.

5.4.2(b) Identification scores for the Hypothetical Median Organism of each cluster

The best possible identification scores attainable within each cluster, for each of the three matrices were provided by the MOSTTYP program (Sneath, 1980c) and are shown in Tables 35, 36 and 37. Suitable scores for all coefficients of the HMO against its own taxon

Table 32 **Percentage positive probability matrix A for *Micromonospora* clusters (20 characters)**

	CLUSTER	Galac -tose	Glyco -gen	Lact -ose	Meli -biose	Raffi -nose	Sali -cin	Gluco -nate	Lac -tate	Succi -nate	Fuma -rate	Hypo -xant.	Arbitin
		12	17	20	24	26	29	41	44	50	53	56	59
1.	*M.Micromonospora olivasterospora*	25	99	1	1	1	25	50	50	75	1	1	1
2.	*M.echinospora*	30	60	30	23	13	27	97	90	13	10	3	73
3.	*M.sagamiensis*	16	53	11	1	1	21	5	90	11	1	1	21
4.	*M.polytrota*	1	1	1	1	1	1	1	99	1	99	99	1
5.	*M.capillata*	1	1	1	1	1	99	1	99	1	1	1	99
6.	*M.rosaria*	31	99	99	15	15	99	99	15	15	15	1	99
7.	*M.chalcea*	99	33	99	99	99	33	89	44	1	1	1	99
8.	*M.sp1*	99	99	99	1	99	99	99	1	99	67	1	1
9.	*M.purpureochromogenes*	99	99	99	99	50	67	50	17	99	1	50	
10.	*M.sp2*	99	99	99	93	87	60	99	87	87	1	27	53
11.	*M.halophytica*	99	79	99	99	99	99	64	86	7	7	1	99
12.	*M.sp3*	1	99	1	1	1	1	99	99	1	1	1	1
13.	*M.fusca*	99	99	33	99	99	1	99	33	99	99	1	99
14.	*M.carbonacea*	99	1	99	1	1	99	1	99	99	99	1	99

Table 32 continued

	CLUSTER	Arbutin	42°C	NaCl (2%)	NH_4 SO_4 (4%)	Phenyl EtOH (0.2%)	Gentamicin resistance (50 mg/ml)	Kanamicin resistance (10 mg/ml)	Novobiocin Resistance (50 mg/ml)
		65	84	91	102	110	135	140	151
1.	*Micromonospora olivasterospora*	25	75	25	25	75	99	99	1
2.	*M.echinospora*	87	80	97	80	70	99	99	47
3.	*M.sagamiensis*	90	42	42	47	11	99	99	58
4.	*M.polytrota*	99	50	50	50	1	99	99	50
5.	*M.capillata*	99	99	99	99	99	99	1	1
6.	*M.rosaria*	92	92	99	99	99	85	1	92
7.	*M.chalcea*	22	99	89	99	99	1	78	99
8.	*M.sp1*	1	99	99	67	1	99	1	99
9.	*M.purpureochromogens*	67	1	33	67	99	1	33	99
10.	*M.sp2*	1	87	93	87	99	1	47	99
11.	*M.halophytica*	93	29	64	50	79	1	1	99
12.	*M.sp3*	1	99	1	1	99	1	99	99
13.	*M.fusca*	67	1	99	99	99	1	1	99
14.	*M.carbonacea*	1	1	1	1	1	1	1	99

Table 33 **Percentage positive probability matrix B for *Micromonspora* clusters (15 characters)**

	CLUSTER	Arab-inose	Gly-cogen	Meli-bose	Sali-cin	D. Xylose	Lac-tate	Succi-nate	Hypox-anthine	Arbu-tin	Growth at 42°C	NaCl (2%	Phenol (0.075%
		4	17	24	29	33	44	50	56	65	84	91	120
1.	*Micromonospora olivasterospora*	25	99	1	25	99	50	75	1	25	75	25	75
2.	*M.echinospora*	99	60	23	27	99	90	13	3	87	80	97	73
3.	*M.sagamiensis*	16	53	1	21	68	90	11	1	90	42	42	37
4.	*M.polytrota*	50	1	1	1	1	99	1	99	99	50	50	50
5.	*M.capillata*	99	1	1	99	99	99	1	1	99	99	99	99
6.	*M.rosaria*	99	99	15	99	99	15	15	1	92	92	99	99
7.	*M.chalcea*	99	33	99	33	78	44	1	1	22	99	89	67
8.	*M.sp1*	99	99	1	99	99	1	99	1	1	99	99	1
9.	*M.purpureochromogenes*	83	99	99	67	99	17	99	1	67	1	33	83
10.	*M.sp2*	60	99	93	60	99	87	87	27	1	87	93	99
11.	*M.halophytica*	99	79	99	99	93	86	7	1	93	29	64	71
12.	*M.sp3*	1	99	1	1	99	99	1	1	1	99	1	99
13.	*M.fusca*	99	99	99	1	33	33	99	1	67	1	99	1
14.	*M.carbonacea*	99	1	1	99	99	99	99	1	1	1	1	1

Table 33 continued

	CLUSTER	Gentamcin resistance (50 mg/ml)	Novobiocin resistance (50 mg/ml)	Cycloheximide resistance (50 mg/ml)
1.	*Micromonospora olivasterospora*	99	1	50
2.	*M.echinospora*	99	47	1
3.	*M.sagamiensis*	99	58	16
4.	*M.polytrota*	99	50	1
5.	*M.capillata*	99	1	99
6.	*M.rosaria*	85	92	99
7.	*M.chalcea*	1	99	99
8.	*M.sp1*	99	99	99
9.	*M.purpureochromogenes*	1	99	99
10.	*M.sp2*	1	99	99
11.	*M.halophytica*	1	99	50
12.	*M.sp3*	1	99	99
13.	*M.fusca*	1	99	67
14.	*M.carbonacea*	1	99	1

Table 34 **Percentage positive probability Matrix C for *Micromonospora* clusters (10 characters)**

	Cluster	Glycogen	Melibose	Salicin	Lactate	Succinate	Hypoxanthine	Arbutin	Growth at 42°C	NaCl (2% w/v)	Phenol (0.075%, w/v)
		17	24	29	44	50	56	65	84	91	120
1.	*Micromonospora olivasterospora*	99	1	25	50	75	1	25	75	25	75
2.	*M.echinospora*	60	23	27	90	13	3	87	80	97	73
3.	*M.sagamiensis*	53	1	21	90	11	1	90	42	42	37
4.	*M.polytrota*	1	1	1	99	1	99	99	50	50	50
5.	*M.capillata*	1	1	99	99	1	1	99	99	99	99
6.	*M.rosaria*	99	15	99	15	15	1	92	92	99	99
7.	*M.chalcea*	33	99	33	44	1	1	22	99	89	67
8.	*M.sp1*	99	1	99	1	99	1	1	99	99	1
9.	*M.purpureochromogenes*	99	99	67	17	99	1	67	1	33	83
10.	*M.sp2*	99	93	60	87	87	27	1	87	93	99
11.	*M.halophytica*	79	99	99	86	7	1	93	29	64	71
12.	*M.sp3*	99	1	1	99	1	1	1	99	1	99
13.	*M.fusca*	99	99	1	33	99	1	67	1	99	1
14.	*M.carbonacea*	1	1	99	99	99	1	1	1	1	1

Table 35 **Identification scores for the Hypothetical Median Organism of each cluster provided by the MOSTTYP program (Matrix A - 20 characters)**

	CLUSTER	Identification Scores: Willcox probability	Taxonomic distance	Standard error of taxonomic distance	Gaussion Integral
1.	*Micromonospora olivasterospora*	0.999	0.223	-2.462	0.993
2.	*M.echinospora*	0.999	0.2240	-2.409	0.992
3.	*M.sagamiensis*	0.999	0.238	-1.872	0.969
4.	*M.polytrota*	1.000	0.223	-2.086	0.981
5.	*M.capillata*	1.000	0.010	-5.795	1.000
6.	*M.rosaria*	1.000	0.112	-3.572	0.999
7.	*M.chalcea*	1.000	0.163	-2.504	0.993
8.	*M.sp1*	1.000	0.104	-3.172	0.999
9.	*M.purpureochromogenes*	0.997	0.257	-1.665	0.9521
10.	*M.sp2*	0.999	0.196	02.157	0.984
11.	*M.halophytica*	0.999	0.189	-2.244	0.987
12.	*M.sp3*	1.000	0.0100	5.795	1.000
13.	*M.fusca*	0.999	0.128	-2.999	0.998
14.	*M.carbonacea*	1.000	0.010	-5.795	1.000

Table 36 **Identification socres for the Hypothetical Median Organism of each cluster provided by the MOSTTYP program (Matrix B - 15 characters)**

	CLUSTER	Identification Scores: Willcox probability	Taxonomic distance	Standard error of taxonomic distance	Gaussion Integral
1.	*Micromonospora olivasterospora*	0.999	0.250	-2.027	0.978
2.	*M.echinospora*	0.989	0.210	-1.948	0.974
3.	*M.sagamiensis*	0.999	0.272	-1.562	0.940
4.	*M.polytrota*	0.999	0.288	01.655	0.951
5.	*M.capillata*	0.999	0.010	-4.995	1.000
6.	*M.rosaria*	0.999	0.080	3.445	0.999
7.	*M.chalcea*	0.999	0.204	-1.957	0.974
8.	*M.sp1*	0.999	0.010	-4.935	1.000
9.	*M.purpureochromogenes*	0.996	0.166	-2.417	0.992
10.	*M.sp2*	0.997	0.174	-2.151	0.984
11.	*M.halophytica*	0.996	0.204	-1.894	0.970
12.	*M.sp3*	0.999	0.101	-4.995	1.000
13.	*M.fusca*	0.999	0.169	-2.436	0.992
14.	*M.carbonacea*	1.000	0.010	-4.995	1.000

Table 37 **Identification scores for the Hypothetical Median Organism of each cluster provided by the MOSTTYP program (Matrix C - 10 characters)**

		Identification Scores			
on l	CLUSTER	Willcox probability	Taxonomic distance	Standard error of taxonomic distance	Gaussi Integra
1.	*Micromonospora olivasterospora*	0.899	0.250	-1.774	0.962
2.	*M.echinospora*	0.859	0.210	-1.832	0.966
3.	*M.sagamiensis*	0.977	0.280	-1.133	0.871
4.	*M.polytrota*	0.975	0.274	-1.331	0.908
5.	*M.capillata*	0.960	0.010	-4.041	1.000
6.	*M.rosaria*	0.986	0.089	-2.754	0.997
7.	*M.chalcea*	0.996	0.241	-1.448	0.926
8.	*M.sp1*	0.996	0.010	-3.99	1.000
9.	*M.purpureochromogenes*	0.993	0.196	-1.819	0.966
10.	*M.sp2*	0.994	0.171	-1.940	0.973
11.	*M.halophytica*	0.972	0.192	-1.840	0.967
12.	*M.sp3*	0.965	0.010	-4.041	1.000
13.	*M.fusca*	0.976	0.148	-1.995	0.977
14.	*M.carbonacea*	0.999	0.010	-4.041	1.000

for each of the three matrices were obtained, which included Willcox probabilities of between 0.99 and 1.00, low taxonomic distances of typically less than 0.15, negative values of the standard error of the taxonomic distance and high values, close to 1, of the Gaussian integral.

Additionally, in most cases the second best identity to each group gave much poorer scores on all four coefficients.

5.4.2(c) Identification score determination

Table 38 provides an example of the output from the MATIDEN program (Sneath, 1979b) which was used to determine identification scores.

5.4.2(d) Identification scores for cluster representatives using original data matrices

Each matrix was tested by identifying representatives from each group, using the original phenetic data (Figure 29, Tables 26, 27 and 28). All strains identified to the correct clusters, and whilst the identification scores obtained were not as good as those for the HMO's, most were satisfactory (Tables 39, 40, and 41).

5.4.3 Practical evaluation of the identification matrix

5.4.3(a) Re-determination of character states of selected cluster representatives

The identification scores compared well with those obtained using the original classification data. Overall agreement between the

Table 38 **Example of the output provided by the MATIDEN program to identify an unknown strain against the identification matrix.**

Input the character values of the unknown, reference number of unknown is M12-35. Isolate M12-35 - best identification is *M.echinospora*. Scores for coefficients: 1 (Willcox probability), 2 (Taxonomic distance), 3 (Standard error of taxonomic distance).

	1	2	3
M.echinospora	0.999	0.175	-2.45
M.sagamiensis	0.0002	0.413	3.04
M.olivasterospora	0.140×10^{-4}	0.420	3.15

Characters against *M.echinospora* - none. Additional characters that assist ins eparating *M.echinospora* from

M.sagamiensis - None

M.olivasterospora - None

Table 39 **Identification scores for cluster representatives obtained using classification test data for Matrix A (20 characters)**

Cluster No	Cluster Representative	Identification Score: Willcox probability	Identification Score: Taxonomic distance distance	Identification Score: Standard error of taxonomic
1.	*Micromonospora olivasterospora* ATCC 31010	0.998	0.300	-1.001
2.	*M.echinospora* var. *challensensis* 14261	0.999	0.100	-1.004
3.	*M.sp* 10-8	0.997	0.166	0.500
4.	*M.polytrota* AR5	0.899	0.174	0.001
5.	*M.capillata* Ferm. P 2598	0.994	0.231	-2.400
6.	*M.sp* S2742	0.999	0.240	-2.417
7.	*M.sp* R461	1.000	0.111	-0.187
8.	*M.sp* 68-742-12	0.998	0.250	0.156
9.	*M.purpureochromogenes* ATCC 27007	0.919	0.331	-0.144
10.	*M.sp* 13-96	0.999	0.300	-1.431
11.	*M.carbonacea* var. *aurantiaca* ATCC 27115	0.899	0.299	-1.241
12.	*M.sp* 13859	0.999	0.301	-1.000
13.	*M.fusca* SCC900	1.000	0.291	0.091
14.	*M.carbonacea* var. *africana* ATCC 39149	0.899	0.111	0.098

Table 40 **Identification scores for cluster representatives obtained using classification tests data for Matrix B (15 characters)**

Cluster No	Cluster Representative	Identification Score: Willcox probability	Taxonomic distance distance	Standard error of taxonomic
1.	*Micromonospora olivasterospora* ATCC 31010			
2.	*M.echinospora* var. *challensensis* 14261	0.899	0.301	-0.199
3.	*M.sp* 10-8	0.994	0.199	-0.101
4.	*M.polytrota* AR5	0.912	0.299	0.199
5.	*M.capillata* Ferm. P 2598	0.920	0.241	0.184
6.	*M.sp* S2742	0.899	0.301	0.144
7.	*M.sp* R461	0.910	0.255	-0.101
8.	*M.sp* 68-742-12	0.874	0.311	-0.150
9.	*M.purpureochromogenes* ATCC 27007	0.956	0.321	1.412
10.	*M.sp* 13-96	0.941	0.294	1.210
11.	*M.carbonacea* var. *aurantiaca* ATCC 27115	0.921	0.199	-0.510
12.	*M.sp* 13859	0.850	0.198	1.000
13.	*M.fusca* SCC900	0.84	0.174	-1.000
14.	*M.carbonacea* var. *africana* ATCC 39149	0.899	0.199	-1.240

Table 41 **Identification scores for cluster representatives obtained using classification tests data for Matrix C (10 Characters)**

Cluster No	Cluster Representative	Identification Score: Willcox probability	Taxonomic distance distance	Standard error of taxonomic
1.	*Micromonospora olivasterospora* ATCC 31010			
2.	*M.echinospora* var. *challensensis* 14261	0.864	0.391	0.010
3.	*M.sp* 10-8	0.765	0.409	0.002
4.	*M.polytrota* AR5	0.941	0.456	-0.031
5.	*M.capillata* Ferm. P 2598	0.921	0.347	1.041
6.	*M.sp* S2742	0.899	0.501	1.009
7.	*M.sp* R461	0.791	0.599	1.014
8.	*M.sp* 68-742-12	0.755	0.319	2.010
9.	*M.purpureochromogenes* ATCC 27007	0.841	0.391	1.98
10.	*M.sp* 13-96	0.862	0.400	-0.010
11.	*M.carbonacea* var. *aurantiaca* ATCC 27115	0.810	0.399	-0.030
12.	*M.sp* 13859	0.799	0.491	1.412
13.	*M.fusca* SCC900	0.920	0.301	0.984
14.	*M.carbonacea* var. *africana* ATCC 39149	0.899	0.345	0.871

Table 42. **Assessment of test error of Matrix A (20 characters) by independent re-determination of character states for selected cluster representatives.**

	Character	No. of Discrepancies	Variance
50.	Utilisation of succinate		
17.	Growth on glycogen		
44.	Utilisation of lactate		
53.	Utilisation of fumarate	0	0
65.	Hydrolysis of arbutin		
135.	Resistance to gentamicin (50 mg/ml)		
151.	Resistance to novobiocin (50 mg/ml)		
56.	Hydrolysis of hypoxanthine		
12.	Growth on galactose		
24.	Growth on melibose		
20.	Growth on lactose		
26.	Growth on raffinose		
140.	Resistance to kanamicin (10 mg/ml)	1	0.050
102.	Resistance to NH_4SO_4 (4%, w/v)		
41.	Utilisation of Gluconate		
84.	Growth at 42°C		
59.	Hydrolysis of xylan	2	0.045
29.	Growth on salicin		
110.	Resistance to phenylethanoate (0.2%, w/v)		
91.	Resistance to NaCl (2% w/v)	3	0.079

Total test discrepancies = 6.07%
Mean test variance = 0.032
Mean probability of error = 3.5%

Table 43. Examples of identification scores for unknown isolates

Origin of isolates	Cluster Identification	Identification score: Willcox probability	Taxonomic Distance	Standard error of taxonomic distance
Warwick soil	*M. echinospora*	0.999	0.274	-1.231
Warwick soil	*M. chalcea*	0.999	0.284	-0.567
Pond sediment	*M. echinospora*	0.997	0.341	-0.101
Pond sediment	*M. carbonacea*	0.954	0.356	0.003
Woodland soil	*M. echinospora*	0.94	0.311	0.950
Warwick soil	*M. olivasterospora*	0.892	0.401	1.312
Warwick soil	*M. olivastenosspora*	0.872	0.429	2.145
Pond sediment	*M. sagamiensis*	0.850	0.416	1.738
Warwick soil	Unidentified	0.531	0.426	3.261

Table 44 Identification of unknown isolates

Cluster identification	No of isolates placed in cluster	Willcox probability 0.990-0.999	0.850-0.989
M. echinospora	7	5	2
M.olivasterospora	5	4	1
M. sagoniensis	3	3	0
M. chalcea	1	1	0
M. carbonacea	1	1	0
TOTAL	**17 (85%)**	**14 (70%)**	**3 (15%)**

independantly determined results and those from the classification study was high (Table 39, 40 and 41).

The assessment of intra-laboratory error for Matrix A (20 characterisation tests) provided by comparing the re-determined identification scores of cluster representatives with the original identification results is summarised in Table 42. Results showed satisfactory agreement with 17 discrepancies in 280 determinations (6.07%), a mean variance of 0.032 and a mean probability of error of 3.5%. This is satisfactory as it is below the limit of 5.0% recommended by Sneath and Johnson (1972) for test error within a laboratory. The maximum number of discrepancies was 3, with 15 characters having either 1 or 0.

5.4.3(b) Identification of unknown isolates

In order for an identification to be positive, 3 criteria were adopted:

(a) A Willcox probability greater than 0.850 with low scores for taxonomic distance and its standard error;

(b) First group scores should be significantly better than those against the next best two alternatives;

(c) 'Characters against' should be zero.

Examples to illustrate the range of scores obtained for identified and non-identified isolates are given in Tables 43 and 44. The scores for the identification coefficients were as expected. Scores for the taxonomic distance and standard error increased as Willcox probabilities decreased. Of the 20 *Micromonospora* strains isolated from a range of aquatic and terrestrial environments, 17 (85%) were identified to one of the 14 clusters. Most were placed in the *Micromonospora echinospora* (Cluster

group 2) group which was the largest group containing 30 *Micromonospora* all of which are producers of aminoglycoside antibiotics, and many of which are found to be common inhabitants of natural environments.

5.5 Discussion

The objective grouping of *Micromonospora* using 179 unit characters led to the distribution of 121 *Micromonospora* strains between 14 clusters at the 77.5% S_{SM} S-level.

Such a large database can provide the potential user with many options, for example the formulation of selective isolation procedures. However, all numerical classifications, when based on a large number of phenetic characters, are of the most potential value in the construction of a probabilistic identification system containing the minimum of characters needed to discriminate between the clusters (Hill, 1974). Such schemes contrast favourably with the widely used monothetic sequential keys which are especially susceptible to test ranking and test error (Sneath, 1974).

Probabilistic identification matrices have been constructed for several bacterial genera, including *Streptomyces* (Williams *et al.*, 1983b; Langham *et al.*, 1989), *Streptoverticillium* (Williams *et al.*, 1985) and *Enterobacteriaceae* (Holmes *et al.*, 1986). In the present study a probabilistic identification matrix was constructed for the rapid and reproducible identification of *Micromonospora*.

There are several crucial stages in the production of a probabilistic identification matrix and each one was achieved by using one of six specifically designed computer programs. Selection of a minimum number of diagnostic tests was made by employing the Numerical

Taxonomy Package (Greensides *et al.*, unpublished data), CHARSEP (Sneath, 1979a) and DIACHAR (Sneath, 1980a) as such tasks cannot accurately be achieved by eye. Evaluation of the quality of the matrix was achieved by a further two programs, OVERMAT (Sneath, 1980b) and MOSTTYP (Sneath, 1980c), culminating in the identification of strains, both known and unknown, against the matrix by the program MATIDEN (Sneath, 1979b). All programs used in the study are based on previously prepared identification matrices that contain the percentage of positives for each property in each of the clusters.

The characters most diagnostic of the clusters which were included in the final three matrices were selected mainly from the output of the CHARSEP program which calculated indices for each of the characters in the matrix giving a reflection of their usefulness. It should be emphasized that not all characters with high indices were necessarily useful as many may duplicate others closely and that the proper employment of these indices was to exclude useless characters. It has been determined in bacteria that for most identification systems there must be at least as many good diagnostic characters as there are taxa (Sneath and Chater, 1978); the number of characters reflecting the variation within the clusters. The results of this study indicated that the minimum number of diagnostic tests required for a reliable matrix to distinguish between 14 groups, was 20, however two less reliable matrices were constructed incorporating 10 and 15 tests providing a system for a rapid preliminary identification. The DIACHAR program (Sneath, 1980a) provided a final assessment of the tests selected, by determining the most diagnostic properties of the clusters and summing the values for characters in each cluster. The program is particularly useful because it quickly allows one to determine taxa for which the information is inadequate. A poorly defined taxon, with poor overall

scores, may interfere with all other taxa that are near it, hence the importance of identifying such clusters at an early stage in the study.

The tests selected for the *Micromonospora* matrices covered a wide range of properties, including carbon source utilization, organic acid utilization, degradation, antibiotic resistance and growth inhibition. However, morphological characteristics, which have often been given great emphasis in the grouping of many actinomycetes (Williams *et al.*, 1985; Langham *et al.*, 1989; Williams *et al.*, 1983a,b; Baldacci and Locci, 1974) for the identification of *Micromonospora* were found to be of no diagnostic value as all strains had very similar morphological properties.

A wide range of criteria was provided for evaluation of the quality of the matrix, using the MOSTTYP program (Sneath, 1979c) initially, followed by the MATIDEN program (Sneath, 1979b) for the re-identification or identification of known or unknown strains respectively. The most frequently used criterion for identification has been the Willcox coefficient (Willcox *et al.*, 1973) which is a Bayesian coefficient related to taxonomic distance. When used to identify groups of Gram negative bacteria most workers previously have required a score of greater than 0.9 for a positive identification (Schindler *et al.*, 1979) and in most cases a score of greater than 0.999 has been required (Feltham and Sneath, 1982; Holmes *et al.*, 1986). For the identification of *Streptomyces* (Williams *et al.*, 1983b; Langham *et al.*, 1989) a Willcox probability of as low as 0.850 was accepted as one indication of a positive identification, as the species-groups to which strains were being identified, were significantly less narrowly defined than the bacterial species studied by previous workers. The same principle also applied to the current study in which the taxonomic distance, it's standard error and the Gaussian integral were also taken into account. If the Willcox

coefficient is relied on alone results may be deceptive in situations where the true group to which the unknown belongs is not in the matrix. This is due to the normalisation process in the calculation of this coefficient (Willcox *et al.*, 1980). This problem, however, is not encountered if the taxonomic distance and it's standard error is taken into consideration.

The evaluation of the three *Micromonospora* identification systems by the re-identification of strains included in the matrix, revealed them to be theoretically and practically sound. As expected, identification scores for cluster representatives using data from the classification study, were less impressive than those for the Hypothetical Median Organism (HMO) of each cluster. However, they were generally acceptable. Practical evaluation of the identification matrices by the re-determination of character states of selected cluster representatives revealed that there was little deterioration of the identification scores compared with those obtained using the original classification data.

The practical evaluation of the largest identification matrix (20 characterisation tests) against wild type *Micromonospora*, isolated from a range of environments, proved also to be positive. Using the criteria for identification outlined previously, 85% of these isolates were identified to a cluster or species group. However if Willcox probabilities of higher than 0.85 were required to ascertain group membership then the success rate of identification of unknown isolates would have been slightly lower (Willcox probabilities between 0.990-0.999 - 70% were positive identifications). The incidence of slightly lower success rates of identification at higher probability levels is by no means uncommon. Williams *et al.* (1983b), using a probability matrix for streptomycetes, found that only 42.2% of unknown isolates identified positively at Willcox probability levels of between 0.995-0.995. Such low success rates at higher probability levels was described by Williams *et al.* (1983b)

as being due to the fact that many of the clusters used in their study were sufficiently heterogenous to be regarded as species groups and not all *Streptomyces* species were included in the matrix.

It is hoped that in future studies, the probabilistic identification matrices will be of value in the assessment of the phenotypic diversity of *Micromonspora* populations in the natural environment (Section 6.4).

CHAPTER 6

General Discussion

6.1 Method development

It is widely thought that most actinomycetes, including *Micromonospora*, are present in terrestrial environments as spores. These propagules are of importance as they serve as agents of dispersal and survival and the differential resistance properties of spores have been exploited to allow selective isolation from soil (Hayakawa *et al.*, 1991). In the present study a highly selective method for the direct isolation of *Micromonospora* from soils and sediments was developed exploiting the heat resistance of *Micromonospora* spores compared to other actinomycete propagules and other members of the microbial soil population. Previous methods have included heat treatment of samples at 70^{o}C in a water bath for 10 minutes (Rowbotham and Cross, 1977; Sandrak, 1977) and chlorine treatment with 4μg/l ammonia followed by 2μg/l chlorine for 30 minutes (Burman *et al.*, 1969; Willoughby, 1969). More recent developments have involved the treatment of soil with 1.5% phenol prior to dilution and plating on HV agar supplemented with both tunicamycin and nalidixic acid (Hayakawa *et al.*, 1991).

The present study revealed that heat treating air dried soil samples in a dry oven at 120°C for 60 minutes drastically reduced the number of both bacteria and streptomycetes occurring on the isolation plates. This is an advantage when compared to other methods such as the use of Chelex extraction (Herron and Wellington, 1990) which, although effective in reducing bacterial numbers, allowed proliferation of a great number of streptomycetes. The use of dry heat treatment for selective isolation compliments the work of both Nonomura and Ohara (1969) and Hayakawa *et al.*, (1991) who used dry heating of soil samples for the isolation of *Microbispora*. Further studies enabled assessment of the diversity of *Micromonospora* species isolated. Although many species

were capable of surviving heat treatment, some species were more sensitive than others, highlighting the need for the development of alternative methods of isolation which could be used in conjunction with heat treatment to allow a wider selection of *Micromonospora* species to be isolated.

Incorporation of antibiotics into isolation media facilitated the isolation of a diverse range of *Micromonospora* species. The selective isolation of *Micromonospora* on media containing a number of antibacterial compounds has been well documented (Ivanitskaya *et al.*, 1978; Sveshnikova *et al.*, 1976; Table 7). Tunicamycin and nalidixic acid have been commonly used by many workers (Hayakawa *et al.*, 1991; Orchard, 1980) although the present study found the use of lincomycin and novobiocin to be the most effective for the purposes of selective isolation. In conjunction with dry heat treatment, the use of antibiotic incorporated media allowed a diverse range of *Micromonospora* to be isolated for use in future studies. In the majority of isolation studies a medium comprising of a complex carbon source, yeast extract, and an inorganic nitrogen source, sodium nitrate was used. This medium allowed the recovery of large numbers of *Micromonospora* from soil compared with other currently used media such as AGS and colloidal chitin agar. Furthermore microscopic examination indicated a diverse range of types obtained from using this medium which offered a considerable advantage for the purposes of studying diversity.

A traditional method available for the extraction and enumeration of total actinomycete numbers in soil is that of Wellington *et al.* (1990) based on shaking a given mass of soil with Ringers solution. The percentage recovery of *Micromonospora* using this method was found to vary between 50 and 80%. The method has a number of drawbacks such as variable percentage recovery, perhaps the most significant, however, is

the inability of this method to detect viable but non culturable cells. To its advantage, the method is rapid and easy to use and for the purposes of this study was adequate and convenient to use on a regular basis.

The development of the *Micromonospora* spore extraction method utilised the superior ability of *Micromonospora* spores to withstand heat treatment and enabled enumeration of both the sporing and the mycelial population in soil. It enabled us to definitively prove that heat treatment of soil is selective for the spore population in soil only.

6.2 Growth and survival of *Micromonospora* in soil

Using spore extraction procedures developed in this study the fate of *Micromonospora chalcea* and *M. fulvopurpurea* were tracked in sterile amended and sterile unamended soil. Both species showed a marked germination and sporulation cycle when inoculated as a spore suspension, sharing a similar profile to that shown by streptomycetes in soil (Cresswell *et al.*, 1992; Herron and Wellington, 1990; Wellington *et al.*, 1990). Both species consistantly showed significant germination at Day 1 with spore numbers starting to increase by Day 2 (*ca.* 10^5 spores/g soil recovered for both treatments). Following rapid sporulation at Day 5, a plateau at *ca.* 10^7 to 10^8/c.f.u./g soil was attained. Studies with *Streptomyces* show similar trends although plateaus usually reached a higher spore number (Herron, 1991). Indeed Bleakley and Crawford (1989) found that *Streptomyces lividans* reached a maximum of *ca.* 10^8 to 10^9, and that *S.parvulus* reached levels of *ca.* 10^{10} in sterile amended soil. The reason for the low plateau achieved with *Micromonospora* species, indicating the inability of the soil to support more propagules, is unclear but could be due to nutrient depletion.

Amendment of soil with starch and chitin had little effect on the growth of both *M. chalcea* and *M. fulvopurpurea*. The reason for this is not clear as many studies (Mayfield *et al.*, 1972) found that amendment of soil with chitin caused a significant increase in the percentage of *Streptomyces* spores germinating in soil. Autoclaving soil would provide a source of nutrients by acting to release nutrients as well as those trapped inside dead organisms (Lloyd, 1969; Salonius *et al.*, 1967).

6.3 Cluster analysis and development of a rapid identification procedure for *Micromonospora*

One of the main aims of this project was to develop a rapid and reproducible probabilistic matrix for the identification of *Micromonospora* isolated from the natural environment. Initially numerical bacterial taxonomic techniques (Sneath and Sokal, 1973) were used to cluster many strains comprising of both type cultures and wild isolates. The data consisted of large numbers of phenotypic tests recorded in binary form which were used to obtain polythetic classifications, as no single character was deemed important enough to entitle an organism to group membership. Numerical taxonomic methods were used by Williams *et al.* (1983) to study *Streptomyces*, providing one of the first definitive classification studies of the actinomycetes. The principles used by Williams *et al.* (1981) classification differed quite markedly from the traditional approaches used up until the 1980's in actinomycete taxonomy which involved recognition of taxa on the basis of only a limited number of characters (Gause et al., 1957; Pridham, 1976).

When embarking upon any classification however trivial, all numerical phenetic data must be rigorously tested as strain similarities

can easily be misinterpreted by test error, low test reproducibility and the choice of statistical analyses (Goodfellow *et al.*, 1979; Sneath and Johnson, 1972). In the present study the taxonomic groupings were relatively unaffected by test error (*p*) which was 3.59% (calculated using the largest data set; Strain set 1/Test set 1). This experimental error is well within the 10% limit suggested by Sneath and Johnson (1972) and is close to that calculated in a similar investigation on *Streptomyces* (*p* 3.36%, Williams *et al.*, 1983).

Again, due to the possibility of accepting misleading results, prior to clustering the nature of the data were carefully considered (Austin and Colwell, 1977). Due to the possibility that some species may have been measured as falsely positive for a number of characters it was thought unreliable to use clustering coefficients which take into consideration positive matches only, as the Dice and Jaccard coefficients do. Additionally such errors may exert a greater effect on the clustering when using a coefficient which ignores positive matches. Phenograms were therefore produced using the simple matching coefficient which considers both positive and negative matches. A number of the data sets, in addition, were clustered using all three coefficients and a number of algorithms. In these instances, all phenograms compared favourably, indicating good, stable cluster formation.

Phenogram 1, produced using the largest set of both strains and test data, was perhaps the most pertinant as it was used in later studies as the basis for the production of the probabilistic identification matrix. Taxonomic analysis revealed well defined cluster formation at the 77.5% S_{sm} similarity level giving 14 distinct cluster groups. Comparison of the clusters defined with previous groupings of *Micromonospora* was impeded to some extent by the inconsistant choices of strains and tests, however, a number of comparisons can be drawn. Eight species groups,

based on 18 characters, have been described by Kawamoto (1989) in the ninth edition of *Bergey's Manual of Determinative Bacteriology*. Similar groupings were observed in *Micromonospora* which were common to both studies, for example all *M. echinospora* subspecies clustered together, as did *M. halophytica*, *M. chalcea* and *M. carbonacea*.

The main conclusions to be made, concerning clustering patterns, were with reference to *M. echinospora* and *M. purpurea*. In all phenograms these strains clustered together. Both are prolific producers of gentamicin and, with the exception that *M. purpurea* is asporogenous, both share similar morphological characteristics. Indeed, it is thought (Kawamoto, 1989; Luedmann and Brodsky, 1964) that if a sporulating variant of *M. purpurea* is found, it would be classified as a subspecies of *M. echinospora*. All phenograms grouped species together which shared similar patterns of antibiotic production (e.g. aminoglycoside producers, macrolide antibiotic producers), particularly with the phenograms produced from antibiotic resistance data only.The relationship between biosynthesis and resistance genes has been widely reported (Chater and Bruton, 1985; Phillips, 1992; Phillips *et al.*, 1992; Skeggs *et al.*, 1987), indicating that antibiotic resistance profiles could be used to select strains which may be potential producers of biologically active compounds. Indeed much work has been done on this hypothesis by Phillips (1992) who evaluated the phenotypic diversity of streptomycete populations based on antibiotic resistance-profiles.

Identification, defined as the process of determining to which group or taxon a new organism or operational taxonomic unit (OTU) belongs, is an important application of any stable classification. Probabilistic identification schemes are widely used and have been developed for use with a range of actinomycetes (Langham *et al.*, 1989; Williams *et al.*, 1983b; Williams *et al.*, 1985). Using the extensive set

of classification data obtained for *Micromonospora*, it was possible to demonstrate the application of a number of theoretical concepts in the development of a probabilistic identification matrix specifically for these organisms. There are several essential stages in the development of any such system, each of which were achieved by use of a specially designed computer program. The initial selection of the most diagnostic tests was made with the assistance of the Numerical Taxonomy Package (NTP), developed by Greensides *et al.*, (unpublished data), and the CHARSEP program of Sneath (1979a). NTP was developed throughout the course of this project to complement the programs of Sneath (1979a, 1980a). It gave a simple indication of the 'usefulness' of individual characters as potential separators of groups on the basis of a number of simple calculations. Whilst ineffective if used alone, when used in conjunction with the programs of Sneath it provided a useful means of character selection. Evaluation of the quality of the matrix, which is essential before its application, was achieved by a further two programs (Sneath, 1979b, 1980b). Final identification of strains, both known and unknown, against the matrix, was achieved using MATIDEN (Sneath, 1979b). All programs used were based on previously prepared identification matrices that contained the percentage of positives for each property in each of the clusters. Three matrices were constructed, incorporating 10, 15 and 20 diagnostic tests which covered a wide range of properties, including carbon source utilisation, antibiotic resistance and growth inhibition.

Evaluation of the quality of each matrix was achieved using the MOSTTYP program (Sneath, 1980c) followed by the MATIDEN program (Sneath, 1979b) for the identification of both known and unknown strains. A range of criteria was used by the MATIDEN program the most important being the Willcox coefficient (Willcox *et al.*, 1973). In the present study, a Willcox probability of 0.850 was accepted

as the main indication of a positive result; a somewhat lower value than was acceptable by other workers (Hill *et al.*, 1978; Lapage *et al.*, 1973). This was justified by the fact that the identifications were being made to species-groups which were less narrowly defined than the bacterial species studied by previous workers (Hill *et al.*, 1978).

The evaluation of the three *Micromonospora* identification systems by the re-identification of strains included in the matrix, revealed them to be theoretically and practically sound. It was also necessary, however, to evaluate matrices by using strains not involved in their construction (Sneath and Sokal, 1973). Therefore Matrix A, comprising of 20 characters was finally assessed by using it to identify 20 putative *Micromonospora* isolates from a range of habitats. 85% (17) of these isolates were identified to one of the 14 species groups at the Willcox probability level of 0.85. If higher Willcox probabilities were required to ascertain group membership then the success rate for the identification of unknown *Micromonospora* would have been slightly lower (70% at 0.990-0.999) probably due to the fact that not all *Micromonospora* species groups were included in the matrix.

6.4 Future work

Perhaps the main outcome of this project has been the development of a probabilistic identification matrix allowing the rapid identification of *Micromonospora* isolates. The initial remit of the project was to devise a tool enabling assessments to be made of the phenotypic diversity of *Micromonospora* in the natural environment. Future work will involve isolating a number of *Micromonospora* using methods developed throughout the course of this project, from a number of environments, both aquatic and terrestrial. Assessments of the

phenotypic diversity of these *Micromonospora* populations will then be made using the developed probabilistic identification matrices. Comparisons will also be made between the heat treatment method of selective isolation and the method requiring the use of antibiotic incorporated media.

By taking a more traditional, taxonomic approach this project has provided good grounding for further genotypic studies. Indeed, as a result of the initial classification of *Micromonospora* we have been able to select strains representing each of the 14 species groups (defined in Phenogram 1) for 16S rRNA sequencing. As well as enabling a genotypic assessment to be made, sequence data will also allow construction of species-group specific 16S rRNA probes. It is hoped that these probes will then be used as tools for the development of a direct *in situ* method for assessing the diversity of *Micromonospora* in the natural environment. Such probes will also enable an assessment to be made of the efficacy of the traditional methods of both identification and isolation used throughout the course of this project.

Finally it is hoped that future work will also include an analysis of the anaerobic representatives of the genus *Micromonospora* as little published information is available concerning the phenotypic and genotypic properties of these organisms with reference to their taxonomic position to the main *Micromonospora* genus.

Appendices

Appendix 1 Chemicals used in this study

Chemical	Supplier
Adonitol	Sigma
Agar, Bacto	Difco
a Aminobutyric acid	Sigma
Ammonium sulphate	BDH
Arbutin	Sigma
L-Arginine	Sigma
L-Asparagine	Sigma
Calcium carbonate	Fisons
Calcium chloride	BDH
Cellobiose	Sigma
Chelex-100	Biorad
Chitin (crabshell)	Sigma
Copper chloride	Fisons
Cycloheximide	Sigma
Dipotassium phosphate	BDH
Ferric ammonium citrate	BDH
D-Fructose	Sigma
D-Glucose	BDH
Glycerol	BDH
Glycine	BDH
Glycogen	Sigma
Hydroxyproline	BDH
Inulin	Sigma
Lab lemco	Oxoid

Lincomycin	Sigma
Magnesium chloride	BDH
Magnesium sulphate	BDH
Novobiocin	Sigma
Nystatin	Sigma
NZ Amine-A	Sheffield Products, Morham Ltd.
PEG 6000	BDH
Phenol red	BDH
Potassium dihydrogen sulphate	BDH
Potassium nitrate	BDH
Potassium sulphate	BDH
D-Raffinose	Sigma
L-Rhamnose	Sigma
Sodium azide	BDH
Sodium chloride	BDH
Sodium dihydrogen phosphate	BDH
Sodium hydrogen carbonate	BDH
Sodium hydroxide	BDH
Starch (soluble)	BDH
Sucrose	BDH
Tryptone soya broth	Oxoid
Yeast extract	Oxoid

Appendix 2 **Media**

Medium	Constituents per litre[1] (unless otherwise indicated)	
Arginine Glycerol Salts (AGS)	L-Arginine	1g
	Glycerol	12.5g
	Bacto agar	15 g
	K_2HPO_4	1g
	NaCl	1g
	$MgSO_4$	0.5 g
	$Fe(SO_4)_3.6H_2O$(10 g/l)	1 ml
	$CuSO_4.5H_2O$ (1 g/l)	1 ml
	$ZnSO_4.7H_2O$ (1 g/l)	1 ml
	$MnSO_4.4H_2O$ (1 g/l)	1 ml
	[pH 8.0]	
Bennett's agar	Yeast extract	1 g
	Lab lemco	0.8 g
	Glycerol	10 g
	N-Z Amine-A	2 g
	Bacto agar	15 g
	[pH 7.3]	
Colloidal chitin agar	Colloidal chitin	4 g
	K_2HPO_4	0.7 g
	KH_2PO_4	0.3 g
	$MgSO_4.5H_2O$	0.5 g

	$FeSO_4.7H_2O$	0.5 g
	$FeSO_4.7H_2O$	0.01 g
	$ZnSO_4$	0.001 g
	$MnCl_2$	0.001 g
	Bacto agar	20 g
	[pH 7.0]	
Complex carbon/inorganic		
nitrogen medium	Yeast Extract	5 g
(medium A)	$NaNO_3$	0.5 g
	K_2HPO_4	0.15 g
	NaCl	0.25 g
	$CaCO_3$	1.5 g
	$MgSO_4.7H_2O$	0.5 g
	Agar	15 g
	[pH 7.0]	
Complex carbon/organic	Yeast Extract	5 g
nitrogen medium	NZ Amine-A	5 g
(medium B)	K_2HPO_4	0.15 g
	NaCl	0.25 g
	$CaCO_3$	1.5 g
	$MgSO_4.7H_2O$	0.5 g
	Agar	15 g
	[pH 7.0]	

Simple carbon/inorganic	Glucose	5 g
nitrogen (medium C)	$NaNO_3$	0.5 g
	K_2HPO_4	0.15 g
	NaCl	0.25 g
	$CaCO_3$	1.5 g
	$MgSO_4.7H_2O$	0.5 g
	Agar	15 g
	[pH 7.0]	
Simple carbon/organic	Glucose	5 g
nitrogen (medium D)	N2 Amine-A	5 g
	K_2HPO_4	0.15 g
	NaCl	0.25 g
	$CaCO_3$	1.5 g
	$MgSO_4.7H_2O$	0.5 g
	Agar	15 g
	[pH 7.0]	
Hippurate agar	Sodium hippurate	10 g
	NaCl	5 g
	$NH_4H_2PO_4$	1 g
	K_2HPO_4	1 g
	$MgSO_4.7H_2O$	0.2 g
	Phenol red	2 g
	[pH 6.8]	
Kadota's cellulose	$NaNO_3$	0.5 g
Benzoate medium	K_2HPO_4	1.0 g
	$MgSO_4.7H_2O$	0.5 g

	$FeSO_4.7H_2O$	0.01 g
	Sodium benzoate	20 g
	Bacto agar	20 g
	[pH 7.2]	
Koser's citrate agar	NaCl	1.0 g
(modified)	$MgSO_4.7H_2O$	0.2 g
	$(NH_4)_2HPO_4$	1.0 g
	KH_2PO_4	0.5 g
	Bacto Agar	15 g
	[pH 7.0]	
M3 agar medium	KH_2PO_4	0.466 g
	Na_2HPO_4	0.732 g
	KNO_3	0.10 g
	NaCl	0.29 g
	$MgSO_4.7H_2O$	0.10 g
	$CaCO_3$	0.02 g
	Sodium propionate	0.20 g
	$FeSO_4.7H_2O$	200 μg
	$ZnSO_4.7H_2O$	180 μg
	$MnSO_4.4H_2O$	20 μg
	Bacto Agar	18 g
	Cycloheximide	50 mg
	Thiamine-HCl	4.0 mg
	[pH 7.0]	

Oatmeal agar	Oatmeal	20 g
	Yeast extract	5 g
	Bacto agar	15 g
Tap water agar	Tap water	1000 ml
	Bacto agar	15 g
Tryptone soya broth (TSB)	Tryptone soya broth	30 g
	sucrose	100 g

Appendix 3 Buffers

Buffer	Constituents	
Phosphate buffered saline	NaCl	8g
(PBS)	KCL	0.2 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.2 g
	[pH 7.3]	
1/4 strength Ringer's sol^n	NaCl	2.25 g
	KCl	0.105 g
	$CaCl_2$	0.12 g
	$NaHCO_3$	0.05 g
	SDW to	100 ml

Appendix 4 **Antibiotic stocks**

Antibiotic	Stock Solution (mg/ml)	Final concentration in medium (μg/ml)
[1]cycloheximide	25	50
[2]gentamicin	50	10-40
[2]lincomycin	50	10-40
[2]novobiocin	50	10-40
[3]nystatin	50	50
[2]penicillin	50	10-40

[1]Dissolved in distilled water and sterilised by autoclaving

[2]Dissolved in distilled water sterilized through a 0.22 μm nitrocellulose filter

[3]Dissolved in small volume of sterile 0.1 N NaOH and made up to volume with SDW

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