Polyphasic Taxonomy of Thermophilic Actinomycetes

Thesis submitted in accordance with the requirement of the University of Newcastle upon Tyne, U.K. for the Degree of Doctor of Philosophy

by

Bongcheol Kim

(B.Sc., Seoul National University, Seoul, Korea)

Department of Agricultural and Environmental Science,

University of Newcastle upon Tyne, U.K.

February 1999

An excellent book which
I enjoyed reading.

with best wishes

Megan G Reid

2/4/99

Dedicated to

my loving father and mother

Acknowledgements

I would like to express my sincere gratitude to my supervisor Professor Michael Goodfellow for his guidance, patience and advice during the preparation of the thesis and, most of all, for his care and concern about my life in Newcastle.

I am also grateful to Professor David E. Minnikin for his sound advice on the chemotaxonomic work and to Dr. Alan C. Ward for his time and help with the computing. I am also indebted to Professor Yung Chil Hah for introducing me to microbial systematics and for his interest in my work.

Special thanks go to Dr. Nevzat Sahin whose project provided the basis for much of my research work. I am also very grateful to Drs. Seung Bum Kim and Jongsik Chun for their invaluable advice on my project.

I am grateful to Professor Marian Mordarski and Dr. Jolanta Zakrzewska-Czerwinska for arranging my stay in Wroclaw, Poland; to Maria Guzik and Bashia Lis for their wonderful assistance with the DNA:DNA hybridisation experiments and to Dr. Stefan Rowinski for his friendship and his 'passionate' tours of Poland.

I am also indebted to Professor Reiner M. Kroppenstedt for providing cultures and to Professor Erko Stackebrandt for kindly comparing my 16S rRNA sequences with the Streptomycete 16S rRNA sequence database held at DSMZ.

I would also like to thank my friends, Amira M. Al-Tai, Noelle Brennan, Ann Brown, Rose Brown, Russell Davenport, Elayne V. Ferguson, Kamil Isik, Seong-Gyun Kang, Sam-Bong Kim, Jeongjin Lee, Luis Maldonado, Gilson P. Manfio, Hye-Sook Mheen, Sun-Woo Nam, Gail Payne, Ehsan Rashidian, Jae-Young Rho, Langkah Sembiring, Fiona Stainsby and Ampaitip Sukhoom who shared the 'tough' life in the laboratory.

I gratefully acknowledge receipt of an Overseas Research Student Award.

Finally, to my wife, SuKyung, for her incredible and unconditional moral support and love.

Publications

Part of the work described in this thesis is also included in the following publications:

Kim, B., Sahin, N., Minnikin, D. E., Zakrzewska-Czerwińska, J., Mordarski, M. & Goodfellow, M. (1999). Classification of thermophilic streptomycetes including the description of *Streptomyces thermoalcalitolerans. International Journal of Systematic Bacteriology* **49**, 7-17.

Kim, B, Al-Tai, A. M., Kim, S. B., Somasundaram, P. & Goodfellow, M. (1999) *Streptomyces thermocoprophilus* sp. nov., a cellulase-free endo-xylanase producing streptomycete. *International Journal of Systematic Bacteriology* (submitted).

Kim, B., Sahin, N., Zakrzewska-Czerwinska, J., Mordarski, M. & Goodfellow, M. (1999). *Amycolatopsis eurythermus* sp. nov., a thermophilic member of the genus *Amycolatopsis. International Journal of Systematic Bacteriology* (submitted).

Poster presentation

Kim, B., Sahin, N. & Goodfellow, M. (1997). Taxonomic studies on novel thermophilic streptomycetes. *10th International Symposium on Biology of Actinomycetes*. Beijing, China.

Abstract

Molecular systematic methods were applied in a series of studies designed to resolve the taxonomic relationships of thermophilic actinomycetes known to be difficult to classify using standard taxonomic procedures. The test strains included representatives of clusters defined in an extensive numerical phenetic survey of thermophilic streptomycetes and twelve marker strains. The resultant genotypic data together with the results of corresponding phenotypic studies were used to highlight novel taxa and to improve the circumscription of validly described species.

The most comprehensive study was undertaken to clarify relationships within and between representative alkalitolerant, thermophilic and neutrophilic, thermophilic streptomycetes isolated from soil and appropriate marker strains. The resultant data, notably those from DNA:DNA relatedness studies, supported the taxonomic integrity of the validly described species *Streptomyces thermodiastaticus*, *Streptomyces thermoviolaceus* and *Streptomyces thermovulgaris*. However, the genotypic and phenotypic data clearly show that *Streptomyces thermonitrificans* Desai and Dhala 1967 and *Streptomyces thermovulgaris* (Henssen 1957) Goodfellow *et al.* 1987 represent a single species. On the basis of the priority, *Streptomyces thermonitrificans* is a later subjective synonym of *Streptomyces thermovulgaris*. Similarly, eight out of eleven representative alkalitolerant, thermophilic isolates and three out of sixteen representative neutrophilic, thermophilic isolates had a combination of properties consistent with their classification as *Streptomyces thermovulgaris*. One of the remaining alkalitolerant, thermophilic isolate, *Streptomyces* strain TA56, merited species status. The name *Streptomyces thermoalcalitolerans* sp. nov. is proposed for this strain. A neutrophilic, thermophilic isolate, *Streptomyces* strain NAR85, was identified as *Streptomyces thermodiastaticus*. Four other neutrophilic thermophilic isolates assigned to a numerical phenetic cluster and a thermophilic isolates from poultry faeces were also considered to warrant species status; the names *Streptomyces eurythermophilus* sp. nov. and *Streptomyces thermocoprophilus* sp. nov. are proposed to accommodate these strains. It was also concluded that additional comparative taxonomic studies are required to clarify the relationships between additional thermophilic streptomycete strains included in the present investigation.

A corresponding polyphasic approach was used to clarify the taxonomy of six thermophilic isolates provisionally assigned to either the genera *Amycolatopsis* or *Excellospora*. Two of the isolates, strain NT202 and NT303, had properties consistent with their classification in the genus *Amycolatopsis*. However, the genotypic and phenotypic data also showed that these strains formed a new centre of taxonomic variation for which the name *Amycolatopsis eurythermus* sp. nov. is proposed. Similarly, the four remaining strains formed two new centre of taxonomic variation within the genus *Excellospora*. It is proposed that isolates TA113 and TA114 be designated *Excellospora alcalithermophilus* sp. nov. Similarly, the name *Excellospora thermoalcalitolerans* sp. nov. is proposed for strains TA86 and TA111. An emended description is also given for the genus *Excellospora*.

Contents

Chapter I:

Current Trends in Bacterial Systematics

1. Polyphasic taxonomy

Bacterial classification and identification are data dependant and hence are in a progressive state of development as they are influenced by the introduction and application of new taxonomic concepts and methods. Nomenclature, by ensuring that the current internationally recognised scientific names are given to taxa (classification) and unknown strains (identification), covers both disciplines and, as with classification and identification, is constantly being adjusted and refined. Sound classification is a prerequisite for both stable nomenclature and reliable identification. Classification and identification are core disciplines as they are relevant to both basic and applied research. Nomenclature is central to all aspects of microbiology as microbiologists need to know what organisms they are working with before they can pass on information about them. In other words, an organism's name is the key to its literature, an entry to what is known about it.

Classification and identification of actinomycetes is essentially a two stage process (Goodfellow & O'Donnell, 1989). Reliable taxonomic criteria are needed to assign organisms to genera prior to the selection of diagnostic tests for identification to constituent species. Identification to the genus level and above can usually be achieved either by using a combination of chemical and morphological markers (Lechevalier & Lechevalier, 1970a,b; Goodfellow& Cross, 1984; Williams *et al.*, 1989; Goodfellow *et al.*, 1997a) or by using 16S rDNA/rRNA sequence data (Embley *et al.*, 1988b; Chun *et al.*, 1995; Kroppenstedt *et al.*, 1997; Zhang *et al.*, 1998). In contrast, few reliable and well tested schemes are available for the differentiation of species, the basic taxonomic unit in bacterial systematics. The nature of the species concept remains a source of argument amongst bacteriologists (Goodfellow & O'Donnell, 1993; Goodfellow *et al.*, 1997a; Ward, 1998).

Early definitions of bacterial species were usually based on monothetic groups which were delineated using subjectively selected sets of phenotypic properties. This traditional species concept has several limitations as strains which vary in key characters are liable to be misidentified. Monothetic classifications also tend to lack uniformity as different taxonomic criteria are usually used to delineate species belonging to different genera. This problem can be exemplified by considering the taxonomic status of members of the families *Bacillaceae* and *Enterobacteriaceae*, the former group is markedly underspeciated (Rainey *et al.*, 1993a; White *et al.*, 1993) whereas with the latter different generic and species designations are retained for organisms related at the species level, as exemplified by *Escherichia coli* and *Shigella sonnei* (Brenner *et al.*, 1972, 1973; Brenner, 1984).

There is still no widely accepted definition of the term species in bacteriology. However, it is often useful to distinguish a taxospecies, a group of strains which share a high proportion of phenotypic properties (Sneath, 1989a); from a genospecies, a group of organisms capable of genetic exchange (Ravin, 1961); from a genomic species a group of organisms which have a high degree of DNA:DNA relatedness (Wayne *et al.*, 1987; Murray *et al.*, 1990). Nucleic acid sequence data can be used to generate a hierarchical branching pattern of relationships but the definition of taxonomic borders still relies on the discontinuous distribution of phenotypic characters (Murray *et al.*, 1990; Goodfellow *et al.*, 1997a). There is, therefore, a need for a unified, holistic approach to bacterial systematics based on detailed interdisciplinary taxonomic studies though it is important to remember that species, genera and higher taxonomic ranks are artificial constructions and hence are subjective (Hull, 1997).

Bacterial systematics began as a largely intuitive science but has become increasingly objective due to the introduction and application of chemotaxonomic, molecular systematic and numerical phenetic methods (Goodfellow & O'Donnell, 1993). The new advances, especially in molecular systematics, promoted the need to compare older

and more recent approaches to bacterial classification (Wayne *et al.*, 1987; Murray *et al.*, 1990; Stackebrandt *et al.*, 1997). This exercise led to the view that bacterial classification at all levels in the taxonomic hierarchy should be based on the integrated use of genotypic and phenotypic data (Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997a). This approach, known as polyphasic taxonomy, was introduced by Colwell (1970) to signify successive or simultaneous studies on groups of organisms using a set of taxonomic procedures designed to yield good quality genotypic and phenotypic data. Genotypic information is derived from analyses of nucleic acids (DNA and RNA) and phenotypic data from studies on cultural, chemotaxonomic, nutritional, physiological and other expressed features. Polyphasic taxonomic studies can be expected to yield well defined taxa and a stable nomenclature.

The polyphasic approach to the circumscription of bacterial taxa only became possible due to the availability of rapid data acquisition systems and improved data handling procedures (Canhos *et al.*, 1993; Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997a). It is encouraging that most descriptions of new cultivable bacteria in recent issues of the *International Journal of Systematic Bacteriology* are based on a judicious selection of genotypic and phenotypic data. In the current issues of the journal, for example, several new actinomycete taxa are proposed based on the polyphasic approach. These taxa include the genus *Verrucosispora* (Rheims *et al.*, 1998) and the species *Corynebacterium confusum* (Funke *et al.*, 1998) and *Mycobacterium bohemicum* (Reischl *et al.*, 1998).

Polyphasic taxonomy is now widely practised but little attempt has been made to say which methods should be used to generate genotypic and phenotypic information. At present, polyphasic taxonomic studies tend to reflect the interests of individual research groups and the equipment and procedures which they have at their disposal. It is difficult to say exactly which methods should be applied in polyphasic taxonomic studies as those employed to some extent depend on the rank of the taxa under investigation (Table 1-1). Nevertheless, it is clear that 16S ribosomal (r) RNA sequencing is a powerful method for

Table 1-1. Sources of taxonomic information*

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

* Modified from Priest & Austin (1993) and Vandamme *et al.*(1996).

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

establishing suprageneric relationships between bacteria though it is of less value when the object is to unravel relationships below the genus level (Goodfellow *et al.*, 1997a; Stackebrandt *et al.*, 1997). In contrast, DNA:DNA hybridisation, molecular fingerprinting and phenotypic procedures are preferred when delineating groups at species and infrasubspecific levels (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1996).

The polyphasic taxonomic approach to circumscribing bacterial species can be expected to meet most of the primary challenges facing bacterial systematists, namely, the need to generate well defined species, a stable nomenclature and improved identification systems. In the present study, several alkalitolerant and neutrophilic thermophilic actinomycetes, which were initially assigned to the genera *Actinomadura, Amycolatopsis* and *Streptomyces* using chemotaxonomic and numerical phenetic data (Sahin, 1995), were the subject of comprehensive polyphasic taxonomic studies designed to clarify their taxonomic status.

2. Suprageneric classification: Nucleic acid sequencing

(a) Background

The most specific and informative methods for classifying microorganisms are based on the determination of precise nucleotide sequences of specific regions of the chromosome. Sequencing methods have developed rapidly in recent years so that comparative sequencing of homologous genes is now a standard procedure in molecular systematic studies (Woese, 1987; Kurtzman, 1992; Olsen & Woese, 1993). Conserved genes, such as those coding for 23S rRNA (Ludwig *et al.*, 1992), ATPase subunits (Ludwig *et al.*, 1993), elongation factors (Ludwig *et al.*, 1993), RNA polymerases (Zillig *et al.*, 1989) and citrate synthases (Birtles & Raoult, 1996), have been sequenced to establish the position of organisms in an overall taxonomic scheme. Similarly, less conserved genes have been used to distinguish between closely related organisms (e.g., exotoxin A gene to type toxin

producing strains of *Pseudomonas aeruginosa* [Loutit &Tompkins, 1991], the cholera toxin gene to type toxin producing strains of *Vibrio cholerae* [Alm & Manning, 1990], the DNA gyrase B subunit (gyrB) and RNA polymerase Sigma (70) factor (rpoD) genes to differentiate between *Pseudomonas putida* strains [Yamamoto & Harayama, 1996, 1998] and the outer surface protein rOmpA genes to type rickettsiae [Fournier *et al.*, 1998]).

It is now well known that rRNA genes are essential for the survival of all organisms. These genes are highly conserved in eukaryotes and prokaryotes and are being used to generate a universal tree of life. Two premises underlie this approach, namely, that lateral gene transfer has not occurred between rDNA genes and that the degree of dissimilarity of rRNA sequences between a given pair of organisms is representative of the variation shown by corresponding whole genomes. The good congruence found between phylogenies based on 16S rRNA and those derived from studies on alternative conserved molecules, such as protein-translocating ATPase subunits, elongation factors, 23 rRNA and RNA polymerases, lends substance to this latter point (Ludwig *et al.*, 1992, 1993; Olsen & Woese, 1993; Goodfellow *et al.*, 1997a). It also seems likely that lateral gene transfer between rRNA genes will be rare as these genes are responsible for the maintenance of functional and tertiary structural consistency (Woese, 1987). There is evidence that horizontal gene transfer (Lan & Reeves, 1996) can occur between 16S rRNA genes as exemplified by aeromonads (Sneath, 1993) and rhizobia (Eardley *et al.*, 1996).

Ribosomal RNA operons are transcribed into single pre-rRNA transcripts which contain several components, usually, in the following order (5' to 3'): 16S rRNA, spacer region, (tRNA, variably present), spacer region, 23S rRNA, spacer region and 5S rRNA (Watson *et al.*, 1987; Gürtler & Stanisich, 1996). The 16S rRNA genes are similar in length throughout the bacterial domain (about 1.5 kb) and contain both highly conserved and variable regions. The location of rare changes in the variable regions are specific to the group or species in which they occur (Stackebrandt & Woese, 1981; Woese, 1987; Dams *et*

al., 1988; Stackebrandt *et al.*, 1997).

Southern hybridisation studies between 16S rDNA sequences and genomic DNA digests with restriction enzymes that cut relatively infrequently (e.g., *Eco* RI, *Hid* III) showed that several bands may hybridise with the probe. The number of bands often corresponds to the number of rRNA operons in the genomes (*e.g.*, ten in *Bacillus subtilis* [Loughney *et al.*, 1982] and *Clostridium perfringens* [Garnier *et al.*, 1991], seven in *Escherichia coli* [Srivastava & Schlessinger, 1990]; three in *Saccharomonospora* spp. [Yoon *et al.*, 1996], and one or two in *Mycobacterium* [Bercovier *et al.*, 1986] and *Mycoplasma* spp. [Amikam *et al.*, 1984]).

There are a few examples where more than one rRNA operon per organism have been sequenced. In most of these cases the nucleotide sequences of different operons from the same strain have been found to be either identical or to show a low level of heterogeneity (about 0.1% or a few differences in nucleotide positions; Maden *et al.*, 1987; Dryden & Kaplan, 1990; Heinonen *et al.*, 1990; Ji *et al.*, 1994). However, there are some instances where heterogeneity has been found between different rRNA operons in the same strain. The halophilic archaebacterium, *Haloarcula marismortui*, is exceptional as it has two nonadjacent rRNA operons where the two 16S rRNA coding regions each contain 1472 nucleotides but differ in nucleotide substitutions at seventy-four positions (5% nucleotide sequence dissimilarity; Mylvaganam & Dennis, 1992). These investigators found that each of the rRNA operons were transcribed and that the resultant 16S rRNA molecules were present in intact 70S ribosomes. They also noted that none of the seventy-four heterogeneous nucleotide positions were related to sites seen as functionally important for interactions with tRNA, mRNA or translational factors during protein synthesis. Similarly, *Thermobispora bispora* has two types of 16S rRNA genes which are transcribed and functional but differ at 98 nucleotide positions (6.4% nucleotide sequence dissimilarity; Wang *et al.*, 1997). *Mycobacterium terrae*, unlike other slowly-growing mycobacteria, has

two copies of 16S rDNA operons that differ by 18 nucleotide substitutions (1.2 % nucleotide sequence dissimilarity; Ninet *et al.,* 1996). These degrees of dissimilarity exceed the difference found between 16S rDNA sequences of members of some well-established bacterial species (Portaels *et al.*, 1996).

It is evident from the results of the independent sequencing studies outlined above that nucleic acid sequencing methods which do not include a cloning step may be flawed in cases where rRNA operons are heterogeneous. However, the identification of most taxa should not be affected because multiple copies of 16S rRNA genes found in microorganisms tend to show a high level of homogeneity (Cilia *et al.*, 1996).

The characterisation of 16S rRNA genes is now a well established standard method used for the classification of bacteria (Woese, 1987; Amann *et al.,* 1995). By 1997, 16S rRNA sequences of representatives of over 5487 different bacterial species were available (Van de Peer *et al.*, 1998). The corresponding number of sequences available for the 23S rRNA gene is 170 (De Rijk *et al.*, 1998). Initially, the 5S rRNA gene was widely studied (Stackebrandt & Liesack, 1993) but this macromolecule is now rarely considered due to the ease of sequencing the 16S rRNA gene which contains much more taxonomic information.

The advantages of using 16S rRNA sequencing for the delineation of species far outweigh its deficiencies. In addition, the ability to sequence rRNA from difficult to culture and uncultivable bacteria is helping to unravel the diversity of prokaryotic species (Embley & Stackebrandt, 1997; Pace, 1997). Nucleic acid sequence data can also be used to design probes for *in situ* hybridisation (Schleifer *et al.*, 1993) and thereby facilitate the development of appropriate selective isolation strategies by showing whether environmental samples contain representatives of target taxa.

Another advantage of nucleotide sequencing studies is that the resultant sequence information can be added to nucleotide sequence databases and readily retrieved for comparative studies. 16S rRNA sequence data are held in the GenBank Database (Benson *et*

al., 1998), the European Molecular Biology Laboratories Database (EMBL; Stoesser *et al.*, 1998), the DNA Data Bank of Japan (DDBJ; Tateno *et al.*, 1998), the Small Ribosomal Subunits Database (Van de Peer *et al.*, 1998) and the Ribosomal Database Project (RDP; Maidak *et al.*, 1997). Access to sequence data is provided in most databases by means of user-friendly interfaces, such as World Wide Webs (Appendix 1).

It is not always realised that evolutionary relationships between bacteria need to be interpreted with care as estimates of phylogeny are based on relatively simple assumptions when viewed against the complexities of evolutionary processes (Goodfellow *et al.*, 1997a). All methods of phylogenetic inference are based on assumptions that may be violated by data to a greater or lesser extent (Swofford & Olsen, 1990; Hillis *et al.*, 1993). O'Donnell *et al.* (1993a) also pointed out that potential problems in interpreting nucleotide sequence data include alignment artifacts, non-independence of sites, inequalities in base substitution frequencies between sequences, and lineage-dependent inequalities in rates of change.

(b) 16S rDNA sequence analysis

The development of molecular biological techniques for sequencing 16S rRNA revolutionised bacterial systematics (Lane *et al.*, 1985; Pace *et al.*, 1986a,b; Woese, 1987; Woese *et al.*, 1990; Olsen & Woese, 1993). The introduction of the reverse transcriptase method (Qu *et al.*, 1983; Lane *et al.*, 1985) made it possible to determine almost complete 16S rRNA sequences by using primers complementary to the conserved regions in 16S rRNA molecules. The reliability of nucleotide sequences determined by using the reverse transcriptase method is influenced by strong posttranscriptional base modifications and by the secondary structure of rRNA (Stackebrandt, 1992). DNA sequencing methods which involve the polymerase chain reaction (PCR) were subsequently found to give better quality sequence data than those derived from the application of the reverse transcriptase method. The application of the PCR to 16S rRNA sequencing coupled with the development of automated nucleotide sequencers, notably those employing non-radioactive labelling (e.g.,

Applied Biosystems Prism sequencing kits), and computer-assisted data acquisition popularised this approach to unravelling bacterial relationships at and above the genus level.

Two procedures are commonly used to determine PCR-amplified 16S rRNA gene (rDNA) sequences. In each case, the first step involves amplification of 16S rDNA using the PCR. The amplified rDNA can either be sequenced directly, following a suitable DNA purification step (Böttger, 1989; Embley, 1991), or cloned into a vector followed by sequencing the resultant recombinant (Stackebrandt & Liesack, 1993). The main differences between these two procedures is that the direct sequencing method determines the nucleotide sequences of mixtures of all of the rRNA operons present in genomes whereas only one operon is sequenced with the cloning method thereby avoiding the generation of chimeric nucleotides. However, despite this the two procedures are more or less equally suitable for phylogenetic analyses due to the limited heterogeneity found between rRNA operons from the same organism. Detailed procedures for 16S rRNA/rDNA sequencing are available (Ludwig, 1991; Stackebrandt & Liesack, 1993; Goodfellow *et al.*, 1997a).

Comparative sequencing of 16S rRNA/rDNA is used to derive phylogenies (Woese, 1987; Stackebrandt *et al.*, 1997). It has already been pointed out that evolutionary relationships based on 16S rRNA/rDNA sequence data should be interpreted with care as analyses of nucleotide sequence data are strongly influenced by different treeing algorithms (Goodfellow *et al.*, 1997a). The concept of homology underpins all phylogenetic analyses, namely, that ancestry can only be traced by estimating changes in nucleotide sequences of homologous genes prepared from representative test strains (Sneath, 1989b).

Alignment of sequence data. Complete or partial sequences of 16S rDNA from test strains need to be compared with known sequences. This involves the alignment of the sequence data to a set of sequences of representative reference organisms. Complete alignment may not always be possible due to factors such as differences in terminal length (i.e., some sequences are longer than others), internal length variation (i.e., the presence of base

deletions or insertions), recombination and high mutation rates associated with hypervariable regions and the degree of uncertainty about the 'historical correctness' of the data.

Pairwise and multiple sequence alignments are based on minimising mismatches between sequences by introducing gaps or shifting bases to overcome mismatches, taking into account secondary structural features, that is, base signatures, pairing between distant regions, conserved mismatches, hairpins, gaps and loops. Chun (1995) incorporated base pairing features of 16S rRNA secondary structure in PC software, namely, the AL16S program, designed specifically for the analysis of 16S rRNA sequence data. Unalignable regions are omitted from phylogenetic analyses.

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

(c) Phylogentic trees

Phylogenetic relationships between organisms, expressed as similarity or dissimilarity values, can be visualised as trees which consist of internal and terminal points (nodes) connected by edges (branches). The terminal nodes represent the molecules of the analysed organisms whereas an internal node represents a common stage in the evolution of these molecules. Unrooted trees show interrelationships between organisms as the emphasis is on establishing relationships between neighboring taxa. In rooted trees the position of the common ancestor is indicated hence the perceived order in which the organisms evolved is

displayed. The distances between two nodes (organisms) are measured by the sum of edges between the nodes.

Numerous tree-making methods can be used to infer ancestry once nucleotide sequences have been aligned (Sneath & Sokal, 1973; Nei, 1987; Felsenstein, 1988). The three major types of tree inferring approaches most widely used are the distance (Felsenstein, 1978; Fitch & Margoliash, 1967; Saitou & Nei, 1987), maximum parsimony (Fitch, 1971) and maximum likelihood methods (Felsenstein, 1981).

Distance methods involve two consecutive procedures, namely, transformation of sequence similarity data to evolutionary distances and construction of trees from information in distance matrices (Swofford & Olsen, 1990). The most frequently used method for calculating distances is the one-parameter model proposed by Jukes and Cantor (1969) which assumes that there is independent change at all of the nucleotide positions hence there is an equal probability of ending up with each of the other three bases. This model is also based on the assumption that base composition does not vary over time. Several other distance transformation methods based on this equation are available . The two-parameter model (Kimura, 1980) provides for differences between transition and transversion rates. Jin and Nei (1990) developed a method based on the Kimura model of base substitution where the rate of substitutions was assumed to vary from site to site according to a gamma distribution. Another approach used to estimate evolutionary distances with consideration of gaps was introduced by Van de Peer *et al.* (1990).

Construction of trees from information in distance matrices is carried out by using many of the available tree-making methods which are used to estimate the additive model, these include the neighbour-joining (Saitou & Nei, 1987) and weighted least-squares methods (Fitch & Margoliash, 1967). The **neighbour-joining method** (Saitou & Nei, 1987) is theoretically related to clustering methods, such as the UPGMA, but is not based on the assumption that data are ultrametric and that all lineages have equally diverged. In contrast

to cluster analysis, the neighbour-joining method keeps track of nodes on the tree rather than taxa or clusters of taxa. The evolutionary distance matrix is provided as input data and a modified distance matrix is constructed in which the separation between each pair of nodes is adjusted on the basis of their average divergence from all of the other nodes. This procedure leads to the normalisation of the divergence of each taxon for its average clock rate.

The **least-squares method** fits a given set of pairwise evolutionary distance estimates to an additive tree. A concrete definition of the net disagreement between the tree and the original data, as an objective function to be minimised, is needed. Two procedures are generally required to find the phylogenetic tree with the lowest value of error of fitting distance estimates to a tree (*E* value), namely, optimising the branch lengths given a tree topology and finding a tree topology with the lowest *E* value of all of the possible trees.

The **maximum parsimony method** is used to find the most parsimonious tree among all possible tree topologies (Felsenstein, 1981). With this approach the sum of changes which must have occurred to give the sequences in the alignment are determined. The number of necessary base changes along the edges connecting the terminal nodes are determined for each alignment position. The tree with the minimal overall number of changes needed is the most parsimonious one hence it is taken as the one which reflects evolution most closely. Parsimony methods tend to misplace individuals or groups of organisms when the rate of evolution differs significantly in different lineages (Stackebrandt & Rainey, 1995).

The **maximum likelihood method** is the most statistically sound way of reconstructing phylogeny (Felsenstein, 1988). This approach to phylogenetic inference is used to determine an explicit model of evolution by analysing the sequences on a site by site basis. It is used to evaluate the net likelihood that the given evolutionary model will yield the observed sequences; the inferred trees are those with the highest likelihood. The use of

this method is hampered by its computational cost and by the fact that the number of organisms that can be handled at any one time is normally below twenty (Olsen *et al.*, 1994). The importance of this approach to bacterial systematics has been emphasised by Ludwig and Schleifer (1994).

Factors influencing tree topology. All available treeing methods are based on various premises including the assumption that individual sites evolve independently from one another. However, this assumption may not be true with real data. Furthermore, due to the enormous number of possible tree topologies and the expense in computing time needed to generate them programs are not usually used to perform exhaustive tests of all possible tree topologies. This means that the optimal tree may not be found. In order to make the best use of computing time most programs add and treat data according to their order in an alignment. This practice introduces bias which can be eliminated by performing several runs and by changing the order of entries randomly.

In terms of 16S rRNA sequencing, the newcomer to the field of microbial phylogeny may assume that complete nucleotide sequences provide the maximum of available phylogenetic information accessible from a given sequence. This is true in principle but it implies that the inclusion of all positions in all analyses gives optimal results (Stackebrandt & Rainey, 1995). The choice of regions to select for sequencing depends on the phylogenetic level of relatedness sought between the test strains. Absolutely invariant and highly conserved positions indicate homology and are invaluable for aligning sequences. However, these positions confer little phylogenetic information. On the other hand, highly variable regions, which are subject to multiple changes at a given site and for which homology is difficult to determine, should be excluded from analyses as they lead to an underestimation of remote relationships. The higher the number of conserved nucleotides the closer the degree of relatedness; in such cases nucleotide differences will be found mainly in the variable regions. In other words for highly related organisms the hypervariable

regions are the only regions which may show differences. The influence of hypervariable positions on tree topologies can be tested by performing several analyses which involve successive removal of these positions or regions. It is still a matter of debate whether these regions reflect phylogeny (Stackebrandt & Rainey, 1995).

The statistical significance of the order of particular subtrees in a phylogenetic tree can be tested by resampling methods such as the **bootstrap** procedure (Efron & Gong, 1983; Felsenstein, 1985). This approach involves random resampling of alignment positions with the result that some of them are included more often than others in analyses whereas others are not included at all. The procedure is usually repeated between 100 to 1000 times with alternatively truncated or rearranged data sets. The higher the fraction of runs of recomputation in which the taxa defined by the branching points appear as a monophyletic subtree, the higher is the significance of the individual branching points.

The root of a subtree can be determined by including homologous sequences from phylogenetically moderately related organisms (Swofford & Olsen, 1990). The "outgroup" reference can be represented by a single sequence (Ludwig & Schleifer, 1994) but it is preferable to include entries from members of different genera in order to avoid artifacts due to differences in the evolutionary rate between the outgroup and the organisms under investigation (Stackebrandt & Rainey, 1995).

It should be kept in mind that the addition of any new homologous sequence to an existing data set may influence the topology of the resultant tree. This is due to the fact that each new sequence which shows differences to those in the data set gives rise to a new set of dissimilarity values which causes the algorithm to make adjustments to branch lengths in order to match the binary values most closely. The inclusion of this additional information may improve the tree locally or even globally but may also have a negative influence on the stability of clusters (Stackebrandt & Rainey, 1995). Lineages represented by a single organism often cannot be stably positioned in phylogenetic trees. The addition of related

nucleotide sequences usually stabilises the branching point of the respective lineage. The addition of incomplete or incorrect sequences may reduce the stability of subtrees of related sequences. It is evident that the influence of nucleotide sequences on tree topology needs to be checked carefully.

Another potential source of treeing artifacts is caused by compositional bias. The G+C contents of rRNAs of most prokaryotes vary within a narrow margin, that is, between 50 and 55 mol% (Stackebrandt & Rainey, 1995). These values are usually higher in molecules from thermophilic bacteria (Rainey *et al.*, 1993b). However, transversion analysis is able to compensate for this bias as the purine content is rather stable among bacterial rRNAs (Rainey *et al.*, 1993b).

The restriction of sequence analysis to selected portions of 16S rDNA can cause significant deviation in the branching points of almost all species. The variation of tree topologies is due to significant differences in the similarity values determined for certain regions of the sequence from the same set of organisms. The position of hypervariable regions within the 16S rDNA primary structure differs from taxon to taxon and needs to be determined individually for their use in the determination of relatedness (Stackebrandt *et al.*, 1992, Goodfellow *et al.*, 1997a).

It is obvious from what has been said that partial sequences should not be used to unravel intrageneric phylogeny and that the phylogenetic positions of bacterial taxa should be based on the analysis of complete nucleotide sequences. However, the 3' terminal 900 nucleotides, or the first 450 (5'→3') nucleotides can be used for the rapid allocation of isolates and clone sequences to higher taxa (Stackebrandt & Rainey, 1995). Sequencing of the first 500 bases of an environmental 16S rDNA clone for example can be used to assign the clone to a main line of bacterial descent. This approach can be used to determine the degree of diversity in a particular clone library and to design oligonucleotide probes for screening clone libraries for the abundance of probe-positive clones (Stackebrandt &

Rainey, 1995).

It is evident that there are still several pitfalls which can distort phylogenies based on 16S rRNA sequence data. Examples of these include the assignment of taxa to erroneous taxonomic ranks due to the comparison of 16S rRNA sequences with inadequate reference strains (Stackebrandt & Ludwig, 1994), classifications biased by the use of nucleotide sequences with high numbers of ambiguities from reverse transcriptase sequencing (Bowen *et al.*, 1989), PCR generation of chimeric amplification products (Liesack *et al.*, 1991), omission of data from critical differentiating regions (Ruimy *et al.*, 1994), sequencing of the 'wrong' strains (Stahl & Urbance, 1990; Rainey *et al.*, 1995a) and oversimplified interpretation of phylogenetic analyses when proposing major taxonomic changes (Martinez-Murcia *et al.*, 1992; Embley & Stackebrandt, 1994; Rainey *et al.*, 1995a). It is clear, therefore, that 16S rRNA sequence data must be carefully interpreted. This can be achieved by preparing comprehensive guidelines to bridge the knowledge gaps of statistical assumptions underlying different algorithms used for data analysis and highlighting possible sources of error at various steps of the process.

(d) Suprageneric classification of actinomycetes

The order *Actinomycetales* Buchanan 1917[AL] was proposed as the only member of the class *Actinomycetes* Krassilnikov 1949[AL]. The order, which was introduced to accommodate members of the family *Actinomycetaceae,* was mainly morphological in concept. A somewhat muted challenge to the reliance on morphology came from Gottlieb (1973) who defined actinomycetes as '*varied groups of bacteria whose common feature is the formation of hyphae at some stages of development*' but went on to say that '*in some instance hyphal formation was tenuous and required imagination to believe in it*'. In 1974, Gottlieb defined members of the order *Actinomycetales* as '*bacteria that tend to form branching filaments which in some families developed into a mycelium*' but conceded that filaments might be short, as in members of the families *Actinomycetaceae* Buchanan 1918[AL]

and *Mycobacteriaceae* Chester 1897[AL]. He also noted that in members of certain taxa filaments underwent fragmentation and hence could only be observed at some stages in the growth cycle.

The relatively simple morphology of mycobacteria partly explains why these organisms were sometimes omitted from actinomycete classifications (Waksman, 1961, 1967). Other workers questioned whether actinomycetes formed a natural group and regarded them as a convenient albeit artificial taxon (Sneath, 1970; Prauser, 1970, 1978, 1981; Goodfellow & Cross, 1974). The difficulty of distinguishing between nocardioform actinomycetes and coryneform bacteria was also widely recognised (Williams *et al.*, 1976; Goodfellow & Minnikin, 1981).

The reliance on morphology when describing actinomycetes has been challenged by information derived from the application of chemical and molecular systematic methods. Data from 16S rRNA cataloguing studies showed that morphological features tended to be poor markers of phylogenetic relationships and that the traditional morphological definition of an actinomycete could not be sustained (Stackebrandt *et al.*, 1983; Stackebrandt & Woese, 1981). The morphologically simple corynebacteria were found to be phylogenetically close to the more highly differentiated mycobacteria, nocardiae and rhodococci, an association that was consistent with chemotaxonomic (Minnikin & Goodfellow, 1980, 1981), comparative immunodiffusion (Lind & Ridell, 1976) and numerical phenetic data (Goodfellow & Minnikin, 1981; Goodfellow & Wayne, 1982).

The traditional practice of separating the more highly differentiated actinomycetes from the relatively morphologically simple coryneform bacteria no longer holds as strains of *Actinomyces*, *Oerskovia* and *Promicromonospora* show a closer phylogenetic affinity to members of the genera *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Curtobacterium* and *Microbacterium* than to mycelium-form organisms such as *Nocardia* and *Streptomyces* (Goodfellow & Cross, 1984). In addition, members of the mycelium forming genus

Thermoactinomyces have been reclassified in the family *Bacillaceae* (Park *et al.*, 1993) whereas *Arthrobacter* and *Micrococcus* strains have been considered to be indistinguishable on the basis of 16S rRNA cataloguing data (Stackebrandt & Woese, 1981; Stackebrandt *et al.*, 1980). It is evident from these findings that the possession of branched hyphae should not automatically place a strain with the actinomycetes.

It became increasingly clear from the studies outlined above that the order *Actinomycetales* needed to be formally redefined. Stackebrandt and Woese (1981) assigned Gram-positive bacteria to two major subgroups which corresponded to '*the high G+C (i.e., over 55%) actinomycete-type of organisms and the low G+C (i.e., below 50%) endospore-forming organisms and their asporongenous relatives*'. The genera *Actinomyces*, *Bifidobacterium*, *Corynebacterium*, *Mycobacterium*, *Propionibacterium* and *Streptomyces* and related taxa were assigned to the high G+C group ("*actinomycetes*") and the genera *Bacillus*, *Staphylococcus*, *Streptococcus* and *Thermoactinomyces* to the low G+C group. These workers recommended that the genera *Bifidobacterium* and *Propionibacterium* be omitted from the order *Actinomycetales* as members of these taxa were anaerobic and only loosely associated with aerobic actinomycetes on the basis of 16S rRNA catalogue data. A similar definition of "*actinomycetes*" and the order *Actinomycetales* were given by Stackebrandt and Schleifer (1984).

Goodfellow and Cross (1984) attempted to redefine actinomycetes in phylogenetic terms as '*Gram-positive bacteria with a high G+C content in their DNA (above 55 mol%) which are phylogenetically related from the evidence of 16S rRNA oligonucleotide sequencing and nucleic acid hybridisation studies*', thereby excluding the genera *Bifidobacterium*, *Kurthia* and *Propionibacterium*. Murray (1992) proposed the class *Thallobacteria* for '*Gram-positive bacteria showing a branch habit, the actinomycetes and related organisms*' but did not give a precise definition of the taxon.

Embley and Stackebrandt (1994) considered that the genera *Bifidobacterium* and

Propionibacterium should be included in the order *Actinomycetales* though they excluded several Gram-positive bacteria with DNA rich in G+C, such as *Coriobacterium glomerans* and *Sphaerobacter thermophilus*. A precise definition of actinomycetes based on the G+C content of DNA is complicated by the close relationship found between *Coriobacterium glomerans* and the genus *Atopobium* (Rainey *et al.*, 1994a). Members of the genus *Atopobium* contain DNA which falls within the range 35 to 46 mol%.

Stackebrandt *et al.* (1997) proposed a new hierarchic classification, namely, the class *Actinobacteria*, for the actinomycete line of descent based solely on analyses of small subunit (16S) rRNA and genes coding for this molecule. Their definition of the taxon is given below.

Description of the class *Actinobacteria* classis nov., Stackebrandt *et al.* 1997. *Actinobacteria*, (Ac.ti.no.bac.te'ri.a. Gr. n. *actis, actinis*, a ray, beam; Gr. dim. n. *bakterion*, a small rod; *-ia*, proposed ending to denote class; *Actinobacteria*, actinomycete group of bacteria of diverse morphological properties). The class is definable in phylogenetic terms, as one of the main lines of descent within the domain *Bacteria* (Woese *et al.*, 1990), on the basis of the analysis of macromolecules of universally homologous functions and includes a wide range of morphologically diverse organisms, most of which are Gram-positive. Strains of the class *Actinobacteria* can constantly be recovered as members of the same phylogenetic lineage, revealing > 80 % 16S rRNA/rDNA sequence similarity among each other (Fig. 1-1), and the presence of the following signature nucleotides in the 16S rRNA/rDNA: an A residue at position 906 and either an A or a C residue at position 955 (except for members of the families *Rubrobacteraceae* and *Sphaerobacteraceae* which show U residues at these positions).

It is apparent from Figure 1-1 that the class *Actinobacteria* encompasses six orders, including the order ***Actinomycetales* (Buchanan 1917) emend. Stackebrandt, Rainey and Ward-Rainey 1997.** The order *Actinomycetales* (Ac.ti.no.my.ce.ta'les. M.L. masc. n.

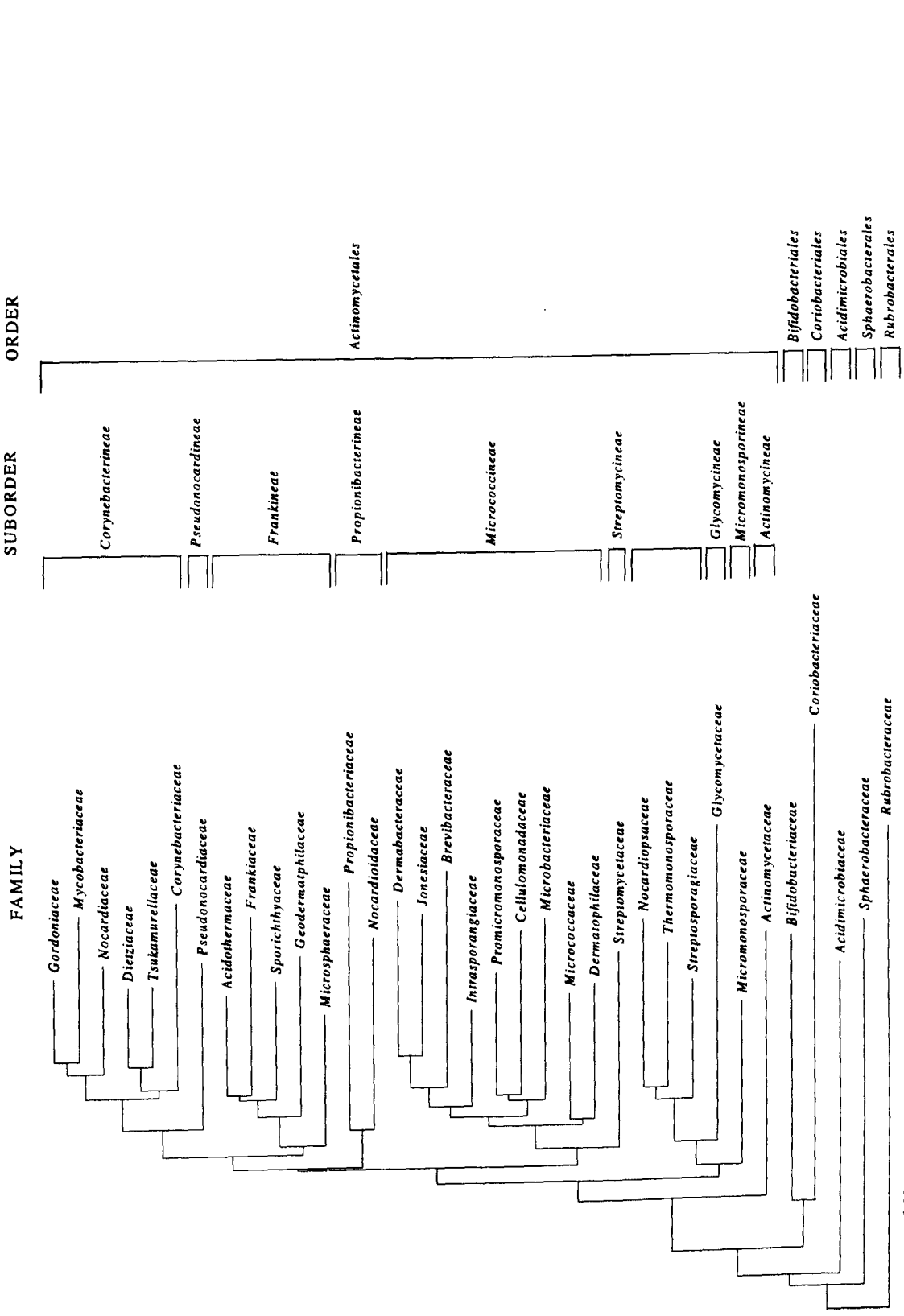


Figure 1-1. Intraclass relatedness of *Actinobacteria* showing the presence of six orders as well as the 10 suborders of the order *Actinomycetales* based on 16S rRNA/rDNA sequence comparison. The scale bar represents 2 nucleotide substitutions per 100 nucleotides.

Table 1-2. Genera, families, orders and subclasses in the class *Actinobacteria*

Family	Genus
Subclass *Acidimicrobidae*	
Order *Acidimicrobiales*	
Acidimicrobiaceae	***Acidimicrobium***
Subclass *Actinobacteridae*	
Order *Actinomycetales*	
Acidothermaceae	***Acidothermus***
Actinomycetaceae	*Arcanobacterium*, ***Actinomyces***, *Mobiluncus*, *Actinobaculum**
Brevibacteriaceae	***Brevibacterium***
Cellulomonadaceae	***Cellulomonas***, *Oerskovia*, *Rarobacter*
Corynebacteriaceae	***Corynebacterium***, *Turicella*
Dermabacteraceae	*Brachybacterium*, *Demetria**, ***Dermabacter***
Dermatophilaceae	***Dermatophilus***, *Dermacoccus*, *Kytococcus*
Dietziaceae	***Dietzia***
Frankiaceae	***Frankia***
Geodermatophilaceae	*Blastococcus*, ***Geodermatophilus***
Glycomycetaceae	***Glycomyces***
Gordoniaceae	***Gordonia***, *Skermania**
Intrasporangiaceae	***Intrasporangium***, *Janibacter**, *Sanguibacter*, *Terrabacter*, *Terracoccus**
Jonesiaceae	***Jonesia***
Microbacteriaceae	*Agrococcus*, *Agromyces*, *Aureobacterium*, *Clavibacter*, *Cryobacterium**, *Curtobacterium*, *Leucobacter**, ***Microbacterium***, *Rathayibacter*
Micrococcaceae	*Arthrobacter*, *Kocuria*, ***Micrococcus***, *Nesterenkonia*, *Renibacterium*, *Rothia*, *Stomatococcus*
Micromonosporaceae	*Actinoplanes*, *Catellatospora*, *Catenuloplanes*, *Couchioplanes*, *Dactylosporangium*, ***Micromonospora***, *Pilimelia*, *Sprilliplanes**, *Verrucosispora**
Microsphaeraceae	***Microsphaera***
Mycobacteriaceae	***Mycobacterium***
Nocardiaceae	***Nocardia***, *Rhodococcus*
Nocardioidaceae	*Aeromicrobium*, ***Nocardioides***
Nocardiopsaceae	***Nocardiopsis***, *Thermobifida**
Promicromonosporaceae	***Promicromonospora***

Propionibacteriaceae	*Friedmanniella*, Luteococcus, Microlunatus, Propioniferax,* ***Propionibacterium***
Pseudonocardiaceae	*Actinokineospora*, Actinopolyspora, Actinosynnema, Amycolatopsis, Kibdelosporangium, Kutzneria, Lentzea, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptoalloteichus, Prauserella*,* ***Pseudonocardia,*** *Thermocrispum*
Sporichthyaceae	***Sporichthya***
Streptomycetaceae	*Kitasatospora*,* ***Streptomyces***
Streptosporangiaceae	*Herbidospora, Microbispora, Microtetraspora, Nonomuria*, Planobispora, Planomonospora,* ***Streptosporangium***
Thermomonosporaceae	*Actinocorallia*, Actinomadura, Excellospora*, Spirillospora,* ***Thermomonospora***
Tsukamurellaceae	***Tsukamurella***
Order *Bifidobacteriales*	
Bifidobacteriaceae	***Bifidobacterium****, Gardnerella*
Subclass *Coriobacteridae*	
Order *Coriobacteriales*	
Coriobacteriaceae	*Atopobium,* ***Coriobacterium***
Subclass *Rubrobacteridae*	
Order *Rubrobacterales*	
Rubrobacteraceae	***Rubrobacter***
Subclass *Sphaerobacteridae*	
Order *Sphaerobacterales*	
Sphaerobacteraceae	***Sphaerobacter***
Genera in search of family	*Bogriella*, Cryptosporangium*, Kineococcus*, Kineosporia*, Micropolyspora*, Thermobispora**

Table based on Stackebrandt *et al.* (1997).

Type genera are highlighted in bold.

*, Taxa which were either not included or described subsequently to Stackebrandt *et al.* (1997).

Actinomyces, type genus of the order; -ales, ending to denote an order; M.L. pl. fem. n. *Actinomycetales*, the *Actinomyces* order) encompasses Gram-positive bacteria with DNA rich in G+C (above 55 mol%) and characteristic nucleotide signature. The 16S rRNA signature pattern consists of nucleotides at positions 122-239 (A-G), 449 (A), 450-483 (G-C), 823-877 (G-C) and 1118-1155 (U-A). The order contains the ten suborders and thirty-five families (Fig. 1-1). The families and genera included in the class are shown in Table 1-2.

3. Classification at genus and species levels

Current bacterial species concepts have tended to reflect the taxonomic methods used to classify individual strains (Goodfellow *et al.*, 1997a). The dramatic impact made by the application of chemotaxonomic, molecular systematic and numerical phenetic procedures on bacterial classification is testimony to this point. Technique driven approaches to the circumscription of bacterial species are sound in an operational sense but do not take into account the fact that species are products of evolutionary processes (Ward, 1998). It is, therefore, the overall pattern of properties shown by cultivable bacteria not the processes which gave rise to them which is currently seen to be paramount in bacterial classification. Views on how bacterial species should be delineated have been greatly influenced by the emergence of chemotaxonomy (Goodfellow & Minnikin, 1985), molecular systematics (Stackebrandt & Goodfellow, 1991) and numerical taxonomy (Sokal & Sneath, 1963; Sneath & Sokal, 1973).

(a) Chemotaxonomy

Chemical data derived from the analysis of cell components can be used to classify bacteria at genus and species levels according to the pattern of distribution of the different compounds within and between members of different taxa (Goodfellow & O'Donnell, 1994). Chemotaxonomic analyses of chemical macromolecules, particularly amino acids and

peptides (e.g., from peptidoglycan and pseudomurein), lipids (lipopolysaccharides), polysaccharides and related polymers (e.g., methanochondroitin, wall sugars), proteins (e.g., bacteriochlorophyll, whole-organism protein patterns), enzymes (e.g., hydrolases, lyases) and other complex polymeric compounds, such as isoprenoid quinones and sterols, all provide valuable chemotaxonomic data. In addition, chemical fingerprints of taxonomic value can be obtained by using analytical chemical techniques, notably, Curie-point pyrolysis mass spectrometry (Goodfellow *et al.*, 1997a). Other approaches which provide valuable data for delineating species include analyses of cellular fatty acids (Stead *et al.*, 1992; Vauterin *et al.*, 1996) and whole-organism proteins (Vauterin *et al.*, 1993; Veríssimo *et al.*, 1996) and the elucidation of enzyme profiles based on chromogenic and fluorogenic substrates (Manafi *et al.*, 1991).

Developments in molecular systematics are sometimes perceived to be a threat to the continued significance of chemosystematics but this is not so as the two approaches are essentially complementary. Phylogenetic data provide a hierarchic framework of relationships among bacteria but do not give reliable information for the delineation of taxa above the species level. In contrast, chemical markers are unevenly distributed across taxa but rarely give information on their hierarchic rank. It is, therefore, very encouraging that good congruence exists between the distribution of chemical markers and the relative position of taxa in phylogenetic trees (Goodfellow & O'Donnell, 1994; Chun *et al.*, 1996). Chemical data are not only employed to evaluate existing phylogenies but can also be used to adjudicate between conflicting phylogenetic trees (Goodfellow *et al.*, 1997a).

The value of different types of chemical markers in bacterial systematics have been considered in detail (Goodfellow & O'Donnell, 1994; Suzuki *et al.*, 1993). Chemical methods that have been shown to be of value in the classification and identification of actinomycetes are cited below.

Specific markers. The most commonly used chemical characters in actinomycete

systematics are derived from analyses of fatty acids, menaquinones, mycolic acids, peptidoglycans, polar lipids and whole-organism sugars (Goodfellow, 1989a; Suzuki *et al.*, 1993). Some of these methods provide quantitative or semi-quantitative data, as in the case of cellular fatty acid and menaquinone analyses, whereas others yield qualitative data, as exemplified peptidoglycan, polar lipid and whole-organism sugar determinations.

Fatty acids. Fatty acids can be defined as carboxylic derivatives of long-chain aliphatic molecules. Most fatty acids are found in the cytoplasmic membrane, as constituents of polar lipids and glycolipids (Kates, 1964), where they form an integral part of the lipid bilayer (Ratledge & Wilkinson, 1988). Taxonomically, fatty acids in the range C_{10} to C_{24} have provided the greatest information and are found in a diverse range of microorganisms. In addition to chain length, the location of double bonds on unsaturated fatty acids and the presence of substituent groups, as found in hydroxylated and 10-methyl branched fatty acids, provide data that are useful both from biosynthetic and taxonomic points of view (Suzuki *et al.*, 1993).

A functional plasma membrane requires the presence of a suitable mix of both relatively fluid and solid fatty acids esterified to polar lipid head groups. Several different types of fatty acid mixtures are found in actinomycetes. At one extreme, straight chain fatty acids occur mixed with monounsaturated compounds such as cyclopropane and tuberculostearic acids, for example, in members of the genera *Actinomyces, Corynebacterium* and *Mycobacterium.* At the other extreme, *Actinomadura, Actinopolyspora, Amycolatopsis* and *Streptomyces* strains contain *iso*-fatty acids as their main relatively solid base, smaller amounts of straight chain fatty acids and *anteiso*-fatty acids provide the fluid element in these organisms. Actinomycetes belonging to the genera *Pseudonocardia, Saccharomonospora, Saccharopolyspora* and *Streptosporangium* contain complex mixtures of fatty acids (Suzuki *et al.*, 1993).

Kroppenstedt (1985) assigned actinomycetes to several groups and subgroups on the

Table 1-3. Fatty acid patterns of actinomycetes according to the classification of Kroppenstedt (1985)

Type	Representative taxa	Fatty acid composition								
		Straight chain		*iso*-14/16/17	*iso*-15/17	*anteiso*-15/17	10-Methyl-		Cyclo-propane	2-Hydroxy
		Saturated	Unsaturated				17	18		
1a	*Corynebacterium, Dermatophilus*	+++	+++	-	-	-	-	-	-	-
1b	*Gordonia, Mycobacterium, Nocardia*	+++	+++	-	-	-	-	++	-	-
1c	*Actinomyces israelii*	+++	+++	-	-	-	-	-	++	-
2a	*Saccharomonospora*	++	+	++++	+	(+)	-	-	-	+
2b	*Geodermatophilus, Intrasporangium*	(+)	+	++	+++	+	-	-	-	-
2c	*Streptomyces*	+	(v)	+++	+	+++	-	-	-	-
2d	*Actinoplanes*	+	+	+++	+++	+++	-	-	-	-
3a	*Actinomadura, Nocardioides, Thermomonospora*	+++	++	+++	(+)	(+)	(+)	+++	-	+
3b	*Micromonospora*	+	+	+++	+++	++	++	(+)	-	-
3c	*Microbispora*	+	+	++	+	+	+++	(+)	-	-
3d	*Nocardiopsis*	+	+	+++	++	+++	(+)	+++	-	-

+ , positive; -, negative; v, variable; ( ), less than 5% of the total fatty acids.

basis of the type and amount of fatty acid components they contained (Table 1-3). Organisms with a type 1 pathway contain predominantly straight-chain saturated and unsaturated fatty acids and sometimes 10-methyl-branched and cyclopropane fatty acids; the 10-methyl-branched and cyclopropane fatty acids are synthesised from the unsaturated components. Strains with a type 2 pathway have mainly terminally-branched fatty acids, that is, *iso*- and/or *anteiso*-branched fatty acids, in addition to minor amounts of straight-chain saturated and unsaturated fatty acids. The final group, type 3, encompasses organisms that have complex branched fatty acid patterns, that is, they contain a high percentage of straight-chain saturated and unsaturated or 10-methyl-branched fatty acids (type 1) in addition to major amount of *iso*- and/or *anteiso*-branched fatty acids (type 2).

Highly standardised conditions are needed to prepare and analyse fatty acids in order to reduce test error. Fatty acid composition is affected by extraction and cultivation conditions. The ratio of cyclopropane acids in *Lactobacillus* strains, for instance, generally increases with culture age whereas the corresponding monounsaturated fatty acids decrease proportionally to this increase (Veerkamp, 1971). A similar phenomenon has been demonstrated with 10-methyl fatty acids in corynebacteria (Suzuki & Komagata, 1983). Cellular fatty acid compostion also varies significantly with growth temperature as cells grown at higher temperatures show a reduction in the ratio of unsaturated to saturated fatty acids (Kawaguchi *et al.*, 1979). Cultivation media often affect cellular fatty acid composition, particularly that of organisms with the branched-chain fatty acid type. This can be demonstrated by comparing the fatty acid composition of coryneform bacteria grown on glucose and peptone-based media (Suzuki & Komagata, 1983; Saddler *et al.*, 1986; Suzuki *et al.*, 1993).

The most commonly used method for the analysis of fatty acids involves gas-chromatographic separation of fatty acid methyl esters using capillary columns; the resultant individual peaks are identified by comparison of their retention times with those of

commercially available fatty acid standards (Embley & Wait, 1994). Fatty acid data can also be analysed quantitatively using a variety of statistical methods (Wold & Sjöström, 1977; O'Donnell, 1985; Saddler *et al.*, 1987; Ninet *et al.*, 1992; Nijhuis *et al.*, 1997).

The MIDI system (Newark, Delaware, U.S.A.), a commercially available identification procedure for bacteria based on GLC analysis of cellular fatty acids, has been widely used (Miller & Berger, 1985; Wallace *et al.*, 1998; Larsson *et al.*, 1989; Stead *et al.*, 1992; McNabb *et al.*, 1997; Muller *et al.*, 1998). In this system the cultivation of bacteria and the preparation of fatty acid methyl ester samples are completely standardised. Samples are automatically injected and analysed and the possible names of species shown on a screen of a connected personal computer together with similarity scores.

Mycolic acids. Mycolic acids are β-hydroxylated fatty acids substituted at the α-position with a moderately long aliphatic chain. They are only found in corynebacteria, dietziae, gordoniae, mycobacteria, nocardiae, rhodococci, skermaniae and tsukamurellae, that is, in some actinomycetes that have an arabinogalactan-based cell wall and peptidoglycan type A1γ. Mycolic acids vary widely in structure and several techniques of varying degrees of complexity have been developed to recognise the different types (Minnikin & Goodfellow, 1980; Minnikin, 1988, 1993; Yassin *et al.*, 1993; Hamid, 1994). Mycolic acids can readily be analysed by using alkaline (Asselineau, 1966) or acid methanolysis (Minnikin *et al.*, 1980) and the resultant derivatives analysed by thin-layer chromatography (Minnikin, 1988) or high performance liquid chromatography (Minnikin, 1988; Butler *et al.*, 1987).

Isoprenoid quinones. These compounds are found in the membranes of most prokaryotes where they have a role as carriers in electron transport systems (Ingledew & Poole, 1984). Several types of isoprenoid quinones have been detected in bacteria (Collins, 1994) but menaquinones (MK) are the most common type found in actinomycetes (Kroppenstedt, 1985: Suzuki *et al.*, 1993; Collins, 1994). These molecules have a chemical structure analogous to that of vitamin K2 (MK-7; unsaturated menaquinone with seven isoprene

units) and are classified according to the number of isoprene units, which can vary from one up to fifteen, and the degree of saturation or hydrogenation (Collins & Jones, 1981).

Menaquinones with seven to twelve isoprene units with various degrees of hydrogenation have been found to be useful in the classification of actinomycetes. The structure and composition of bacterial menaquinones are determined either semi-quantitatively by mass spectrometry or quantitatively by high-performance liquid chromatography (Kroppenstedt, 1985; Collins, 1994). The position or point of hydrogenation in isoprenoid side-chains can be very specific and hence of taxonomic value (Collins, 1994). Sophisticated techniques, such as silver-phase high-performance liquid chromatography (Kroppenstedt, 1985) and tandem mass spectrometry (Collins *et al.*, 1988; Ramsey *et al.*, 1988) are needed to determine the points of hydrogenation in isoprene units. Such studies have provided valuable information for the classification of *Actinomadura* (MK9[II,III,VIII-H6]), *Microtetraspora* (MK9[II,VIII,IX-H6]) and *Streptomyces* strains (MK9[II,III,IX-H6]) [Yamada*et al.*, 1982; Collins *et al.*, 1988c; Kroppenstedt *et al.*, 1990].

Peptidoglycan composition. Peptidoglycans of Gram-positive walls consist of long glycan chains (up to 100 units) composed of β-1,4-linked disaccharides of N-acetylglucosamine and N-acetylmuramic acid. These chains are covalently cross-linked in three dimensions by oligopeptides which interconnect 3-O-lactoyl groups of muramic acid residues in the glycan chains (Hancock, 1994). Two systems have been proposed to classify peptidoglycans. The first was introduced by Ghuysen (1968) and the second by Schleifer and Kandler (1972). In each case the mode of cross-linkage is central to the classification, but the system proposed by Schleifer and Kandler (1972) is more comprehensive, using a tridigital code consisting of either the letter A or B to represent the position of the cross-linkage, a number to indicate the presence and type of interpeptide bridge and a small Greek letter to identify the amino acids in position 3 of the peptide subunit (Table 1-4).

Phospholipids. Phospholipids are the most common polar lipids found in bacterial

Table 1-4. Classification of peptidoglycans*

Position of cross-link	Peptide bridge	Amino acid at position 3
Peptidoglycan A:	1. None	α. L-lysine
Cross-linkage between		β. L-ornithine
position 3 and 4 of two		γ. *meso*-diaminopimelic acid
peptide subunits		
	2. Polymerised subunits	α. L-lysine
	3. Monocarboxylic L-amino	α. L-lysine
	acid or glycine or	β. L-ornithine
	oligopeptide thereof	γ. LL-diaminopimelic acid
	4. Contains a dicarboxylic	α. L-lysine
	amino acid	β. L-ornithine
		γ. *meso*-diaminopimelic acid
		δ. L-diaminobutyric acid
	5. Contains lysine and a	α. L-lysine
	dicarboxylic amino acid	β. L-ornithine
Peptidoglycan B:	1. Contains a L-amino acid	α. L-lysine
Cross-linkage between		β. L-homoserine
position 2 and 4 of two		γ. L-glutamic acid
peptide subunits		δ. L-alanine
	2. Contains a D-amino acid	α. L-ornithine
		β. L-homoserine
		γ. L-diaminobutyric acid

* Modified from Rogers *et al.* (1980), Schleifer & Kandler (1972) and Schleifer & Seidl (1985).

Table 1-5. Major phospholipids found in actinomycetes*

Name	Polar head group substituent	Overall charge
Phosphatidylglycerol	Glycerol	1+
Diphosphatidylglycerol	Phosphatidylglycerol	2+
Phosphatidylbutanediol	Butane-2,3-diol	1+
Phosphatidylinositol	Inositol	1+
Phosphatidylinositol mannosides	Acylated mannosylinositols	1+
Phosphatidylethanolamine	Ethanolamine	0
Phosphatidylcholine	Choline	0
Phosphatidylmethylethanolamine	Methylethanolamine	0

*, Modified from Minnikin and O'Donnell (1984).

Table 1-6. Classification of actinomycete phospholipids*

Type	Characteristics
I	Nitrogenous phospholipids absent, phosphatidylglycerol variable
II	Only phosphatidylethanolamine
III	Phosphatidylcholine, phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidylglycerol variable; phospholipids containing glucosamine absent
IV	Phospholipids containing glucosamine with phosphatidylethanolamine and phosphatidylmethylethanolamine variable
V	Phospholipids containing glucosamine, phosphatidylglycerol with phosphatidylethanolamine variable

* Categories recognised by Lechevalier *et al.* (1977, 1981); most actinomycetes contain phosphatidylinositol (Suzuki *et al.*, 1993).

cytoplasmic membranes (Suzuki *et al.*, 1993). Amphipathic polar lipids consist of a hydrophilic head group linked to two hydrophobic, aliphatic chains. The aliphatic chains show considerable variability and when cleaved from the hydrophilic head group and methylated yield fatty acid methyl esters which may be analysed as described earlier to provide taxonomic information. Less variability is found in the polar or hydrophilic head group but when present can provide valuable information for actinomycete taxonomy. The major phospholipids found in actinomycetes are shown in Table 1-5.

Phospholipids extracted from actinomycetes by using organic solvent systems (Minnikin *et al.*, 1984) can be separated by two dimensional thin-layer-chromatography (e.g., Embley *et al.*, 1983) and detected using non-specific (5 %, w/v, ethanolic molybdophosphoric acid) or specific spray reagents (Suzuki *et al.*, 1993). The latter can be used to detect α-glycols (periodate-Schiff; Shaw, 1968), amino groups (0.2 % ninhydrin, w/v, in water-saturated butanol; Consden & Gordon, 1948), choline (Dragendorff reagent; Wagner *et al.*, 1961), lipid phosphates (Dittmer & Lester, 1964) and sugars (α-naphthol; Jacin & Mishkin, 1965). Lechevalier *et al.* (1977, 1981) classified actinomycetes into five phospholipid groups based on *'semi-quantitative'* analyses of major phospholipid markers found in whole-organism extracts (Table 1-6).

In general, members of the same actinomycete genus have a common phospholipid pattern. Phospholipid patterns can be important for the recognition of actinomycete genera (Goodfellow, 1989a; Williams *et al.*, 1989) and have proved to be of value in the recognition of the genera *Aeromicrobium* (Tamura & Yokota, 1994) and *Dietzia* (Rainey *et al.*, 1995b). *Amycolatopsis* strains have phophatidylethanolamine and phosphatidylglycerol as major polar lipids with diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides variably present (phospholipid type II *sensu* Lechevalier *et al.*, 1977). Similarly, *Actinomadura* strains contain phosphatidylinositol and diphosphatidylglycerol as major polar lipids with phosphatidylinositol mannosides and

phosphatidylglycerol variably present (phospholipid type I *sensu* Lechevalier *et al.*, 1977), and *Streptomyces* strains diphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides, but not glucosamine-containing phospholipids (phospholipid type II *sensu* Lechevalier *et al.*, 1977).

Sugars. Neutral sugars, which are major components of actinomycete cell envelopes, are useful taxonomic markers. Sugar composition can be determined by simple paper chromatography (Schaal, 1985) or by using gas-liquid chromatography (Saddler *et al.*, 1991) following full hydrolysis of purified cell walls or whole-organisms (Hancock, 1994). In the latter case, quantitative sugar profiles can be analysed using multivariate statistical methods (St-Laurent *et al.*, 1987). The location and function of sugars in the bacterial cell wall is not fully understood (Hancock, 1994). Information derived from analyses of sugar composition provides fairly crude data for understanding cell wall structure as the presence of a particular sugar in different strains does not necessarily mean that it is derived from the same macromolecule. More detailed analyses of cell wall polysaccharide polymers, such as so-called linkage analyses, can be used to provide additional information on the structure and function of actinomycete envelopes (Daffé *et al.*, 1993).

Actinomycetes can be assigned to five groups on the basis of the discontinuous distribution of major diagnostic sugars (Lechevalier & Lechevalier, 1970a,b); group A, presence of arabinose and galactose; group B, presence of madurose (3-O-methyl-D-galactose), and group D, presence of arabinose and xylose. Group E was added by Labeda *et al.* (1984) to denote the presence of major amount of galactose and rhamnose. Organisms which contain *meso*-A_2pm but lack any of the diagnostic sugars are assigned to group C.

A number of 'rare' sugars have been shown to be diagnostic for members of some actinomycete taxa, notably, the occurrence of tyvelose in members of the genus *Agromyces* (Maltsev *et al.*, 1992) and 3-O-methyl-rhamnose in *Catellatospora* strains (Asano *et al.*, 1989). Sugar patterns of actinomycetes are usually determined by gas- chromatographic/

Table 1-7. Cell wall chemotypes of some actinomycete taxa*

Wall chemotype	Major components [a]	Family/Genus
I	LL-diaminopimelic acid and glycine	*Streptomycetaceae*
II	*meso*-diaminopimelic acid and/or hydroxy-diaminopimelic acid and glycine	*Micromonosporaceae*
III A	*meso*-diaminopimelic acid and madurose	*Dermatophilaceae* *Frankiaceae* *Streptosporangiaceae*
III B	*meso*-diaminopimelic acid	*Brevibacteriaceae* *Thermomonosporaceae*
IV A	*meso*-diaminopimelic acid, arabinose, galactose and mycolic acids	*Corynebacterineae*
IV B	*meso*-diaminopimelic acid, arabinose and galactose	*Pseudonocardiaceae*
V	lysine and ornithine	*Actinomyces israelii*
VI	lysine (aspartic acid and galactose) [b]	*Microbacterium* *Oerskovia*
VII	diaminobutyric acid and glycine (lysine)	*Agromyces* *Clavibacter*
VIII	ornithine	*Curtobacterium* *Cellulomonas*

*, Table modified from Lechevalier & Lechevalier (1970a,b) and Goodfellow & O'Donnell (1989).

[a], All wall preparations contain major amounts of alanine and glutamic acid.

[b], Variable components.

mass-spectrometric analyses of derivatised preparations from whole-organism hydrolysates (Saddler *et al.*, 1991; Hancock, 1994) though semi-quantitative thin-layer chromatographic procedures can be used (Ruan *et al.*, 1994).

Amycolatopsis strains contain arabinose and galactose (group A *sensu* Lechevalier & Lechevalier, 1970a,b), *Actinomadura* strains madurose (group B *sensu* Lechevalier & Lechevalier, 1970a,b), and *Streptomyces* strains no characteristic sugars (group C *sensu* Lechevalier & Lechevalier, 1970a,b).

Wall chemotypes. The introduction of wall chemotypes by Lechevalier and Lechevalier (1970a,b, 1980) provided a much needed practical way of assigning actinomycetes to a number of groups using qualitative chemical data. The system is based on the discontinuous distribution of major diagnostic amino acids and sugars in whole-organism hydrolysates (Table 1-7). The diagnostic amino acids can readily be detected by their characteristic chromatographic behaviour and staining properties on thin-layer chromatography (Staneck & Roberts, 1974) or by gas chromatography (O'Donnell *et al.*, 1982). Similarly, diagnostic sugars in whole-organism hydrolysates can be analysed by thin-layer chromatographic or gas chromatographic separation of derivatised molecules (Drucker, 1981; Hancock, 1994).

Ribosomal proteins. Ochi (1989) found that ribosomal protein patterns contained information that could be used for classification and identification. He and his colleagues showed that the ribosomal protein AT-L30 varied amongst actinomycetes and could thereby be used to identify actinomycetes to genera on the basis of 2-D electrophoretic patterns (Ochi, 1992, 1995; Ochi & Yoshida, 1991; Ochi & Miyadoh, 1992; Ochi & Hiranuma, 1994).

Chemical characters as phylogenetic markers. It is encouraging that good congruence exists between the distribution of certain chemical markers and classifications based on 16S rRNA sequence data (Goodfellow, 1989a; Embley & Stackebrandt, 1994; Goodfellow *et al.*, 1997a). Members of the family *Microbacteriaceae*, for example, have the uncommon type B

peptidoglycan, branched-chain fatty acids (fatty acid type 2c), unsaturated menaquinones with 9 to 12 isoprene units, a type I phospholipid pattern and DNA within the range of 64 to 75 mol% G+C. The assignment of these organisms to a common family is also supported by 5S rRNA (Park *et al.*, 1993) and 16S rRNA sequence data (Stackebrandt *et al.*, 1997).

Actinomycetes with *meso*-A_2pm, arabinose and galactose in the wall peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970a,b) fall into two distinct suprageneric groups (Goodfellow, 1992). Wall chemotype IV actinomycetes which contain mycolic acids belong to the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania* and *Tsukamurella* (Goodfellow, 1992; Rainey *et al.*, 1995b; Chun *et al.*, 1996, 1997; Goodfellow *et al.*, 1998) and the their mycolateless counterparts to the family *Pseudonocardiaceae* (Embley *et al.*, 1988a,b; McVeigh *et al.*, 1994; Warwick *et al.*, 1994). The mycolic acid-containing wall chemotype IV actinomycetes, together with members of *Turicella otitidis* which lack mycolic acids but are morphologically related to corynebacteria, were currently assigned to suborder *Corynebacterineae* on the basis of 16S rRNA sequence data (Stackebrandt *et al.*, 1997).

(b) Numerical taxonomy

Early bacterial systematists relied on tests that were based on morphology and a number of other selectively weighted phenotypic features. Strains were assigned to groups on the basis of morphology, staining properties, ability to produce acid from sugars, motility, nutritional requirements, pigmentation and spore formation. In contrast, the primary objective of early numerical taxonomic studies was to assign individual bacterial strains to homogeneous groups or clusters, which could be equated with taxospecies, using large sets of phenotypic data. The resultant quantitative data on numerically defined taxospecies were used to design improved identification schemes (Priest & Williams, 1993).

The theoretical basis of numerical taxonomy is well documented (Sneath & Sokal,

1973; Goodfellow *et al.*, 1985; Sackin & Jones, 1993; Goodfellow, 1998) and hence is not considered in detail here. Numerical taxonomic data are usually stored and managed using computer systems due to the widespread availability of specialised software and the need to study large numbers of strains and properties (Canhos *et al.*, 1993; Sackin & Jones, 1993).

Numerical taxonomic procedures have been applied to most groups of cultivable bacteria in order to revise existing taxonomies and to classify unknown strains isolated from diverse environmental habitats (Goodfellow & Dickinson, 1985; Sackin & Jones, 1993). It is evident from such studies that this approach to classification provides an effective way of delineating taxospecies. The method has been less successful in generating higher taxonomic ranks, but this is almost certainly due to the types of data used rather than to fundamental flaws in numerical methods. Thus, representative strains from diverse genera may have different metabolisms and growth requirements which can make studies across generic boundaries difficult. Numerical taxonomic surveys have been widely used to circumscribe taxospecies, including those encompassed in taxonomically complex actinomycete taxa such as *Mycobacterium* (Wayne *et al.*, 1996) and *Streptomyces* (Williams *et al.*, 1983a, Kämpfer *et al.*, 1991; Manfio *et al.*, 1995).

Taxonomic clusters or taxospecies are 'operator unbiased' representations of natural relationships between strains though group composition may be influenced by the choice of strains and tests, experimental procedures, test error and data handling methods (Sackin & Jones, 1993; Goodfellow *et al.*, 1997a). It is, therefore, essential to evaluate the taxonomic integrity of taxospecies by examining representative strains using independent taxonomic criteria derived from the application of chemotaxonomic and molecular systematic methods.

The ability to delineate taxospecies has had a marked influence on the way bacteria are classified and identified. Any tendency to see numerical phenetic taxonomy as a method with a long past and an uncertain future should be resisted as improved methods and

automated data acquisition systems can be expected to facilitate the generation of high-quality phenotypic databases for a variety of purposes. It can, for example, be anticipated that with the developing interest in bacterial species diversity these databases, which may reflect the functional diversity of a habitat, will be put to even more fundamental uses.

(c) Molecular systematics

It has already been pointed out that the driving force in bacterial systematics owes much to developments in molecular biology, notably, nucleic acid sequencing studies. However, several other molecular systematic methods provide valuable data for classification of bacterial species, notably, the estimation of the mean overall base composition of DNA and indirect comparisons of nucleotide sequences by DNA:DNA hybridisation.

DNA base composition. DNA base composition is expressed as the mole percent of guanosine plus cytosine (mol% G+C). The mol% G+C content of bacteria ranges from 25 to 80 with the value being constant for a given organism (Johnson, 1989; Tamaoka, 1994). However, DNA base composition data cannot be used as a direct measure of relatedness though such studies do provide supportive evidence for taxonomic work as organisms that differ markedly in DNA base composition cannot be considered to be closely related. The converse is not necessarily true as organisms with similar mol% G+C ratio may be unrelated (Stackebrandt & Liesack, 1993) as such values do not take into account the linear arrangement of nucleotides in the DNA.

DNA base composition is usually considered to be one of the characteristics required to characterise the genome and should form part of the minimal descriptions of species and genera (Lévy-Frébault & Portaels 1992). In practice, DNA base composition studies provide additional information for assigning and/or confirming the placement of bacterial strains to broad taxonomic groups, such as the high and low G+C Gram-positive phyletic lines. Such studies may also be used to distinguish between members of taxa that have a similar

morphology, such as *Micrococcus* and *Staphylococcus*, but are genetically different (Colwell & Mandel, 1964; Silvestri & Hill, 1965). The DNA base composition values of strains from well circumscribed species do not usually differ by more than 3 mol% G+C (Mandel, 1966; Tamaoka, 1994). Similarly, members of species within a genus should not differ from one another by more than about 10 mol% G+C. However, there are no firm guidelines set for the range of DNA base compositions that can be encompassed at these taxonomic ranks.

Two relatively simple methods are available for determining DNA base composition: direct chromatographic separation of enzymetically hydrolysed nucleotides using HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984; Tamaoka, 1994) and indirect estimation of mol% G+C content from thermal denaturation curves using spectrophotometry (Marmur & Doty, 1962). DNA base composition data need to be interpreted with care as there is evidence that DNA preparation, choice of analytical methods and experimental conditions greatly influence results (De Ley, 1970; Tamaoka & Komagata, 1984; Tamaoka, 1994).

DNA:DNA hybridisation. A unique property of DNA and RNA is their ability for reassociation or hybridisation. The complementary strands of DNA, once denatured, can, under appropriate experimental conditions, reassociate to reform native duplexes. The specific pairings are between the base pairs, adenine with thymine and guanine with cytosine; the overall pairing of the nucleic acid fragments is dependent on similar linear arrangements of these bases along the DNA. When comparing nucleic acids from different organisms, the amount of molecular hybrid and its thermal stability provide an average measurement of nucleotide sequence similarity.

Information from DNA:DNA pairing studies has been extensively used to delineate bacterial species (Krieg, 1988; Stackebrandt & Goebel, 1994). The *Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics* (Wayne *et al.*, 1987) proposed a formal molecular definition of bacterial species, namely, that a species should generally include strains with approximately 70 % or more DNA-DNA relatedness with 5 °C or less

divergence values (ΔTm). Values from 30 % to 70 % reflect a moderate degree of relationship, but values become increasingly unreliable once they fall below the 30% level as they can be attributed to experimental artefacts. These guidelines have been used to clarify species relationships in diverse bacterial genera (Stackebrandt & Goebel, 1994; Goodfellow *et al.*, 1997a).

Experimental procedures for estimating DNA relatedness are based on two key properties of DNA molecules, namely, specificity of base pairing and denaturation-renaturation kinetics at specific temperatures (Marmur & Doty, 1961). Double stranded DNA dissociates into single-stranded DNA either at its melting temperature (Tm) or under highly alkaline conditions and reassociates at temperatures 15 °C to 30 °C below the Tm value at neutral pH. Single-stranded (ss) DNA from one organism will hybridise with ssDNA from another organism under appropriate experimental conditions to form heterologous molecules or duplexes. The extent of hybridisation can be directly quantified by monitoring the rate of the reassociation kinetics or by using labelled reference DNA. The amount of mismatch in heterologous duplexes can also be determined by comparing Tm values with those of corresponding homologous duplexes, the smaller the difference between the thermal stabilities (ΔTm), the fewer the mismatches in the hybrid. The stringency of hybridisation reactions can be varied by altering experimental parameters, notably, temperature, salt concentration and added reaction components (Johnson, 1991).

The procedures currently used to measure DNA sequence similarity values either involve "immobilised DNA" or "solution reassociation" assays (Stackebrandt & Goebel, 1994). Most methods, apart from optical determinations of hybrid formation in solution hybridisation, use radioactively labelled reference ssDNA but colorimetric DNA:DNA pairing methods using nonradioactive labelling with either biotin or digoxigenin are safer and increasingly applied alternatives (Ezaki *et al.*, 1989; Jahnke, 1994; Hirayama *et al.*, 1996).

The rationale for using DNA reassociation as the gold standard for species delineation originates from the results of numerous studies where a high degree of correlation was found between DNA similarity and chemotaxonomic, genomic, serological, and numerical phenetic data (Stackebrandt & Goebel, 1994; Goodfellow *et al.*, 1997a). These studies were based on the original finding that single-stranded DNA from two different strains will reassociate to a measurable extent and form a DNA hybrid if the strands contain less than 15 % base mispairing (Ullmann & McCarthy, 1973). In general, organisms which have 70 % or greater DNA similarity show at least 96 % DNA sequence identity (Stackebrandt & Goebel, 1994). Therefore, DNA:DNA pairing does not provide a satisfactory resolution for establishing relationships between organisms at the genus level and above due to the limited rate of hybridisation imposed by sequence divergence hence comparisons are limited to species and sub-species levels.

DNA-rRNA hybridisation. This method involves the use of labelled rRNA as a probe for determining relationships between strains at taxonomic levels at and above the generic level (De Smedt & De Ley, 1977). The experimental procedure which underpins DNA-rRNA hybridisation studies is similar to that for direct binding DNA-DNA homology assays with the difference that the denatured immobilised DNA on membrane filters is hybridised with either 16S or 23S labelled rRNA at optimal reassociation temperatures (25-35 °C below *T*m). Relatedness between strains is measured by the amount of rRNA probe bound and the stability of the resultant duplexes. Homology determinations may be strongly biased by the number of rRNA operons in test strains and by self-renaturation of probe rRNA due to the stable secondary structure of rRNA (Kilpper-Bälz, 1991). Once individual species have been defined, they can then be arranged into genera and families on the basis of overall similarity to form a hierarchic system.

Few DNA-rRNA hybridisation studies have involved actinomycetes, though ribosomal RNA cistron similarity data showed that acid-fast actinomycetes are

phylogenetically close (Mordarski *et al.*, 1980, 1981). Similarly, it has been shown that sporoactinomycetes fall into at least three major phylogenetic clades which correspond to the genera *Actinoplanes, Ampullariella* and *Micromonospora*; to the genera *Planobispora, Planomonospora* and *Streptosporangium*; and to the genus *Streptomyces* (including *Chainia, Elytrosporangium, Kitasatoa, Microellobosporia* and *Streptoverticillium*; Stackebrandt *et al.*, 1981).

16S rDNA sequencing. This method provides valuable data for the circumscription of genera and can also be used to highlight new species. The circumscriptions of several actinomycete genera including *Corynebacterium* (Pascual *et al.*, 1995), *Kitasatospora* (Zhang *et al.*, 1997), *Microtetraspora* (Wang *et al.*, 1996a; Zhang *et al.*, 1998), *Microbispora* (Wang *et al.*, 1996a), *Micromonospora* (Koch *et al.*, 1996), *Mycobacterium* (Rogall *et al.*, 1990; Pitulle *et al.*, 1992), *Nocardia* (Chun & Goodfellow, 1995), *Nocardiopsis* (Rainey *et al.*, 1996), *Thermomonospora* (Zhang *et al.*, 1998), *Saccharomonospora* (Kim, S. B. *et al.*, 1995), *Streptomyces* (Witt & Stackebrandt, 1990; Kim, D. et al, 1996), *Streptosporangium* (Ward-Rainey *et al.*, 1996) have been strengthened as a results of 16S rDNA sequence studies. 16S rDNA sequencing data also highlighted the distinctiveness of the genera *Nonomuria* (Zhang *et al.*, 1998), *Skermania* (Chun *et al.*, 1997), *Prauserella* (Kim, S. B. *et al.*, 1998), *Thermobifida* (Zhang *et al.*, 1998) and *Thermobispora* (Wang *et al.*, 1996b), and provided vital information for the recognition of novel actinomycetes species as exemplified by *Nocardia flavorosea* (Chun *et al.*, 1998b), *Saccharopolyspora spinosporotrichia* (Zhou *et al.*, 1998), *Pseudonocardia asaccharolytica* and *Pseudonocardia sulfidoxydans* (Reichert *et al.*, 1998).

Fox *et al.* (1992) pointed out that 16S rRNA molecules from members of closely related species may be so conserved that they cannot be used to differentiate between strains at species level. This important observation means that strains of related species with identical, or almost identical, 16S rRNA/rDNA sequences may belong to different genomic

species. This is the case with species of *Aeromonas* (Martinez-Murcia *et al.*, 1992), *Bacillus* (Fox *et al.*, 1992), *Legionella* (Fry *et al.*, 1991) and *Tsukamurella* (Yassin *et al.*, 1995b, 1996, 1997).

It is clear from the observations noted above that the sensitivity of DNA:DNA hybridisation is greater than that of 16S rRNA/rDNA sequence analyses. Nevertheless, even in this context 16S rRNA/rDNA sequence data can be used to select appropriate reference strains for the more exacting DNA:DNA relatedness studies. The terms rRNA species complex and rRNA superspecies have been proposed for organisms which have virtually identical 16S rRNA sequences but can be distinguished using DNA:DNA relatedness data (Fox *et al.*, 1992).

The correlation between 16S rRNA sequence and DNA relatedness data is not linear though rRNA similarity values below 97 % invariably correspond to DNA relatedness values below 60 % (Stackebrandt & Goebel, 1994; Goodfellow *et al.*, 1997a).

4. Classification at and below the species level

The accurate circumscription of subtypes within a species is assuming greater importance in all branches of microbiology. Microbial typing is important in diagnostic bacteriology (Oyarzabal *et al.*, 1997), ecological studies which involve the release and monitoring of novel microorganisms in natural habitats (Frothingham & Wilson, 1993; Stapleton *et al.*, 1998) and in search and discovery programmes designed to detect new microbial products (Goodfellow & O'Donnell, 1989; Bull *et al.*, 1992). Advances in molecular biology provide highly specific methods for typing isolates based on nucleic acid sequence data (Towner & Cockayne, 1993). The various molecular typing methods have advantages and disadvantages when applied to specific situations. In addition to the ability to discriminate between strains within target species is the ease of performance and interpretation of the tests and the availability of reagents. The usefulness of typing systems

varies depending on the specific organisms of interest. Several molecular typing systems have been found to be helpful in delineating actinomycetes below the species level (Grimont & Grimont 1986; Lévy-Frébault *et al.*, 1989; Welsh & McClelland, 1990; Klijn *et al.*, 1991; Frothingham & Wilson, 1993; Poulet & Cole, 1995; Gürtler & Stanisich,1996; Janssen *et al.*, 1996).

(a) Nucleic acid fingerprinting

Restriction enzyme based techniques. Most if not all bacteria have genomic regions that are highly variable. This variability can be seen when chromosomal DNA is purified and cleaved into thousands of pieces with an appropriate restriction endonuclease, that is, an enzyme that cuts DNA at a constant position within a specific recognition site that is usually composed of 4 to 6 bp. It is often possible to distinguish between differences in banding patterns by visual examination when DNA from two different strains is cleaved into fragments (which usually range in size from 20,000 to 1,000 bp) and separated on an agarose gel. Differences in the length of restriction fragments are due to sequence rearrangements, insertions or deletions in DNA, or to base substitutions within restriction enzyme cleavage sites (Swaminathan & Matar, 1993).

The technique outlined above was originally called **restriction fragment length polymorphism** (RFLP; Towner & Cockayne, 1993). Conventional RFLP analyses have been used to assess the genetic relatedness of bacteria but do not allow strain-specific identification as the large number of restriction fragments do not allow the recognition of specific banding patterns (Collins, 1984, 1985; Ross *et al.*, 1991; Swaminathan & Matar, 1993; Roberts *et al.*, 1998).

Infrequent-cutting restriction endounucleases (e.g., *Apa* I, *Not* I, *Sfi* I, *Sma* I) cut chromosomal DNA into a few **large restriction fragments** (LRFs; Lévy-Frébault *et al.*, 1989), require the application of pulsed-field gel electrophoresis (PFGE; Frothingham & Wilson, 1993) as they cannot be separated by conventional agarose electrophoresis. Pulsed-

field gel electrophoresis allows the generation of simplified chromosomal restriction fragment patterns without the need to resort to probe hybridisation methods. However, several factors limit the application of PFGE to subtyping representatives of bacterial species, notably, the long and tedious procedures required for the isolation and cleavage of genomic DNA, the requirements for expensive enzymes and reagents, and the complexity of experimentally optimising electrophoretic conditions (Swaminathan & Matar, 1993; Roiz *et al.*, 1995).

Other approaches have been developed to simplify genomic RFLP analyses by decreasing the number of DNA fragments that need to be compared. One such approach involves treating chromosomal DNA restriction fragments with one or more labelled probes (Swaminathan & Matar, 1993). Identical or closely related strains have a large number of hybridising fragments in common whereas unrelated strains have few, if any, such fragments.

Restriction fragments of total chromosomal DNA separated on agarose gels can be transferred to nitrocellulose or nylon membranes and then hybridised with labelled probes. Initially, probes were almost always labelled with radioisotopes but the use of nonradioactive reporter molecules, such as biotin or digoxigenin, is now widely used. Probes used for subtyping include randomly cloned genomic DNA fragments (Grimont *et al.*, 1992) and those derived from genes coding for putative virulence factors (Loutit & Tompkins, 1991), insertion sequences (Strässle *et al.*, 1997), bacteriophage DNA (Wei *et al.*, 1992) and rRNA (Laurent *et al.*, 1996).

A generalised typing method based on the procedure outlined above was introduced by Grimont and Grimont (1986). This method, which is commonly known as **ribotyping,** is based on the fact that rRNA genes are highly conserved and scattered throughout the chromosome of most bacteria and hence produce polymorphic restriction endonuclease patterns when probed with rRNA or the corresponding genes. The term "ribotyping" was

coined by Stull *et al.* (1988) to describe RFLP typing in molecular epidemiological studies of diverse bacterial species.

Ribotyping has been applied to a wide range of microorganisms that includes members of the genera *Actinomyces* (Barsotti *et al.*, 1994), *Corynebacterium* (Soto *et al.*, 1991; De Zoysa *et al.*, 1995), *Mycobacterium* (Chiodini, 1990; Kanaujia *et al.*, 1991; Domenech *et al.*, 1994), *Nocardia* (Laurent *et al.*, 1996), *Rhodococcus* (Lasker *et al.*, 1992), *Saccharomonospora* (Yoon *et al.*, 1996), *Streptomyces* (Doering-Saad *et al.*, 1992) and *Tsukamurella paurometabola* (Auerbach *et al.*, 1992). However, this technique is of limited value for typing microorganisms which only contain a single ribosomal operon, notably, slowly-growing mycobacteria (Chiodini, 1990; Arbeit *et al.*, 1993).

Several other typing procedures are based on the use of different probes. **Randomly cloned probes**, that is, randomly cloned DNA fragments of unknown function, can be evaluated in initial experiments to select those which recognise stable chromosomal regions and hence have discriminatory value. Such probes have been shown to be more discriminatory than rRNA probes for subtyping *Brucella* species (Grimont *et al.*, 1992) and *Legionella pneumophila* (Tram *et al.*, 1990). The main disadvantage of this approach is that a new set of probes is required for each bacterial species. In addition, useful probes can only be selected after a large number of clones from a genomic DNA library have been screened.

Restriction fragment length polymorphism techniques have also been used for species delineation and typing members of the genera *Mycobacterium* (Telenti *et al.*, 1993; Steingrube *et al.*, 1995; Swanson *et al.*, 1996, 1997), *Nocardia, Rhodococcus, Streptomyces,* and *Tsukamurella* (Steingrube *et al.*, 1995, 1997) by making use of polymorphisms in the gene coding for the **65-kDa heat shock protein.** Steingrube *et al.*(1995) observed that amplicons of the 65-kDa HSP gene from *Nocardia* isolates did not contain a *Bst* EII recognition site thereby differentiating these organisms from most *Mycobacterium* species as the latter typically produce amplicons which contain one or more *Bst* EII recognition sites

(Steingrube *et al.*, 1995; Telenti *et al.*, 1993). Several investigators have distinguished between mycobacterial species and subgroups by PCR-based RFLP of the 65-kDa HSP gene (Plikaytis *et al.* 1992; Telenti *et al.*, 1993; Steingrube *et al.*, 1995; Swanson *et al.*, 1996, 1997).

Repeated DNA elements have been isolated from an assortment of eukaryotic and prokaryotic organisms (Poulet & Cole, 1995). Distinct families of insertion sequences appear to be widely spread in the prokaryotic world based upon their genetic organisation and host range (Poulet & Cole, 1995). The polymorphism observed when separated endonuclease cleaved fragments of genomic DNA were hybridised with cloned repetitive DNA sequences has been found to be species specific (McAdam *et al.*, 1994). Several insertion elements (IS) have been described in mycobacteria, such as IS*900*, IS*901*, IS*1110*, IS*1141* and IS*1245* in members of the *Mycobacterium avium- intracellulare complex* (Moss *et al.*, 1992; Guerrero *et al.*, 1994; Roiz *et al.*, 1995). The insertion sequences IS*6110*, which is specific to members of the *Mycobacterium tuberculosis* complex, is useful for tracing the global distribution of *Mycobacterium tuberculosis* strains (McAdam *et al.*, 1994; Poulet & Cole, 1995; Strässle, 1997).

DNA probes based on specific virulence factors derived from nucleotide sequences coding for putative virulence factors or from sequences upstream or downstream of virulence associated structural genes have also been used to subtype bacteria (Swaminathan & Matar, 1993). A fragment of the gene coding for exotoxin A has been used to type *Pseudomonas aeruginosa* (Loutit & Tompkins, 1991). Similarly, a probe consisting of the cholera toxin gene was used to type toxin producing strains of *Vibrio cholerae* (Alm & Manning, 1990). This method is not universally applicable as probes need to be developed for each bacterial species and the selection of suitable subtyping probes has to be done empirically by screening several nucleotide sequences.

Polymerase chain reaction based techniques. The introduction of the PCR for

amplification of specific nucleic acid sequences (Saiki *et al.*, 1985) led to the development of several typing methods for the analysis of amplification products (Welsh & McClelland, 1990; Klijn *et al.*, 1991; Versalovic *et al.*, 1991; Smith-Vaughan *et al.*, 1995; Janssen *et al.*, 1996). The simplest of these methods involves the use of gel electrophoresis to identify the size of the reaction products though better specificity and sensitivity can be obtained by analysing the reaction products by hybridisation with specific probes. Polymerase chain reaction methods offer several advantages over other nucleic acid-based subtyping procedures, notably, the need for only a few cells of the microorganism, speed and a protocol which consists of relatively few steps.

Two types of PCR-based methods can be recognised, namely, PCR-RFLP and PCR ribotyping. The PCR-RFLP method involves the use of the PCR technique to amplify a particular region of the genome coupled with restriction endonuclease analyses of PCR products from different strains on agarose or polyacrylamide gels. This method has significant advantages over conventional DNA restriction analyses as it requires only a small amount of chromosomal DNA for digestion and is less involved due to the elimination of the Southern blotting and hybridisation steps. Another important advantage of PCR-RFLP over genomic DNA restriction is that problems of poor restriction of genomic DNA due to DNA base modifications (methylation) are not encountered. The PCR-RFLP technique has been used to clarify relationships below the species level of actinomycetes belonging to the genera *Mycobacterium* (Telenti *et al.*, 1993) and *Nocardia* (Lungu *et al.*, 1994). It has been also used to clarify relationships of members of clinically significant actinomycetes (Steingrube *et al.*, 1997) and the members of *Nocardia asteroides* complex (Laurent *et al.*, 1996).

PCR ribotyping, an alternative to traditional ribotyping, involves the use of PCR to detect polymorphisms in genes or intergenic spacer regions associated with rRNA or tRNA (Klijn *et al.*, 1991). Fingerprinting methods based on 16S-23S rRNA spacer regions

have been used to differentiate between closely related organisms, typically those at and below species rank since these regions show more variability than the corresponding rRNA coding regions (Frothingham & Wilson, 1993; Postic *et al.*, 1994). Intergenic spacer regions often differ in length and show high nucleotide sequence dissimilarity between operons. In some cases, only certain rRNA operons have the tRNA gene(s) in the intergenic spacer region between the 16S and 23S rRNA genes (e.g., East *et al.*, 1992). This procedure has been successfully used to type members of several actinomycete taxa, notably, *Mycobacterium* (Steingrube *et al.*, 1995), *Nocardia* (Lungu, 1994) and *Streptomyces* species (Hain *et al.*, 1997).

Conventional PCR assays can be used to amplify DNA sequences that are characteristic of a particular strain or species (Hackel *et al.*, 1990; Zolg & Philippi-Schulz, 1994; Soini & Viljanen, 1997; Swanson *et al.*, 1996, 1997). However, this approach has the drawback that specific oligonucleotide primers are required; this means that knowledge of the DNA sequence of the test organism is essential. Analysis of **randomly amplified polymorphic DNA fingerprints (RAPD)**, also known as **arbitrarily primed PCR (AP-PCR)**, removes this requirement by using a primer(s) chosen without regard to the sequence of the genome that is to be fingerprinted (Welsh & McClelland, 1990; Williams *et al.*, 1990).

The RAPD procedure involves enzymatic amplification of template DNA directed by one or more arbitrary oligonucleotide primers to produce a characteristic spectrum of polymorphic products. The method is based on the observation by Welsh and McClelland (1990) that a single arbitrarily chosen primer combined with two cycles of PCR at low stringency and many cycles at high stringency generated a discrete and reproducible set of amplification products characteristic of particular genomes. Several variations of the original procedure have been developed (Williams *et al.*, 1990; Caetano-Anollés *et al.*, 1991; Bassam *et al.*, 1992), each showing differences in DNA amplification conditions, the

length of primers used, and the resolution of the resultant amplification products.

Analysis of randomly amplified polymorphic DNA fingerprints is fast and independent of prior biochemical and genetic knowledge of the target organism (Welsh & McClelland, 1990; Williams *et al.*, 1990; Bassam *et al.*, 1992). This means that the method can be applied to all species from which DNA can be extracted. The existence of polymorphisms among the amplification products can be detected and used as genetic markers for the fast and accurate identification of bacterial isolates in ecological, epidemiological and taxonomic studies (Bassam *et al.*, 1992; Brousseau *et al.*, 1993; Stephan *et al.*, 1994). The method has been used to determine interstrain relationships between diverse bacteria, including actinomycetes such as actinomadurae (Trujillo & Goodfellow, 1997), mycobacteria (Palittapongarnpim *et al.*, 1993; Abed *et al.*, 1995; Matsiota-Bernard *et al.*, 1997), nocardiae (Exmelin *et al.*, 1996) and streptomycetes (Anzai *et al.*, 1994).

(b) Chemical fingerprinting

Pyrolysis is a chemical process that involves the thermal breakdown of complex organic material, such as whole organisms or cell fractions, in an inert atmosphere or vacuum to produce a series of volatile, lower molecular weight molecules, the 'pyrolysate' (Irwin, 1982). The breakdown of test material is reproducible under controlled conditions and the resultant fragments are characteristic of the original material. The volatile fragments are ionized and separated by mass spectrometry on the basis of their mass-to-charge ratio (*m/z*) to give a pyrolysis mass spectrum which can be taken as a 'chemical fingerprint' of the original material. The resultant data are complex and need to be analysed using suitable statistical routines (Gutteridge, 1987; Magee, 1993, 1994; Goodfellow *et al.*, 1997b).

One of the major advantages of pyrolysis mass spectrometry (PyMS) over comparable taxonomic methods, such as conventional chemotaxonomic procedures (Suzuki *et al.*, 1993; Embley & Wait, 1994; Pot *et al.*, 1994) and nucleic acid probing (Schleifer *et*

al., 1993; Amman *et al.*, 1995), is that it is rapid with respect to single and multiple samples. Pyrolysis techniques, notably Curie-point pyrolysis mass spectrometry, are currently being introduced to diagnostic and industrial screening laboratories (Sanglier *et al.*, 1992; Goofellow *et al.*, 1997b; Goodacre *et al.*, 1998).

To date, the most important application of Curie point pyrolysis mass spectrometry has been in microbial epidemiology (Goofellow *et al.*, 1997b). Pyrolysis mass spectrometry is not a typing method *per se* as a permanent type designation is not assigned to test strains but it has proved to be a quick and effective method for inter-strain comparisons of bacteria that commonly cause outbreaks of disease. This conclusion is based on studies of clinically significant bacteria, recent examples include *Acinetobacter calcoaceticus* (Freeman *et al.*, 1997), *Bacteroides* spp. (Sultana *et al.*, 1995), *Campylobacter jejuni* (Orr *et al.*, 1995), *Clostridium difficile* (Al-Saif *et al.*, 1998), *Legionella pneumoniae* (Sisson *et al.*, 1991), *Listeria monocytogenes* (Freeman *et al.*, 1991a; Low *et al.*, 1992), *Pseudomonas cepacia* (Corkhill *et al.*, 1994), *Streptococcus pneumoniae* (Freeman *et al.*, 1991b), *Staphylococcus aureus* (Gould *et al.*, 1991) and *Xanthomonas maltophilia* (Orr *et al.*, 1991).

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

Pyrolysis mass spectrometry has also been used to classify and identify industrially significant actinomycetes (Saddler *et al.*, 1988; Sanglier *et al.*, 1992). In this latter study, members of representative actinomycete genera were pyrolysed in order to determine the

effects of medium design, incubation time and sample preparation on experimental data; it was concluded that reproducible results could be obtained given rigorous standardisation of growth and pyrolysis conditions. Sanglier and his colleagues also showed that PyMS data could be used to objectively select strains for pharmacological screens, as unknown or putatively novel actinomycetes appeared as outliers on ordination diagrams. They were also able to distinguish between actinomycetes at and below the species level. In particular, representatives of three closely related *Streptomyces* species, namely *Streptomyces albidoflavus* (subcluster 1A; Williams *et al.*, 1983a), *Streptomyces anulatus* (subcluster 1B; Williams *et al.*, 1983a) and *Streptomyces halstedii* (subcluster 1C; Williams *et al.*, 1983a), were distinguished. These worker also used the procedure to compare *Streptomyces hygroscopicus* isolates and *bona fide* represenatives of *Streptomyces violaceusniger* (cluster 32; Williams *et al.*, 1983a). The separation of these numerically circumscribed streptomycete species indicated that PyMS can provide a rapid way of establishing the taxonomic integrity of established or putatively novel actinomycete species.

Chun *et al.* (1993a,b) demonstrated the value of artificial neural networks (ANN) in the analysis of pyrolysis data for the identification of streptomycetes. Pyrolysis profiles derived from representatives of several actinomycetes taxa, including the genera *Actinomadura, Mycobacterium, Nocardia, Nocardiopsis, Saccharopolyspora, Streptosporangium* and *Streptomyces,* were used to train an ANN to recognise the characteristic pyrolysis profiles of representatives of *Streptomyces* groups A, B and C. Successful identification of members of the three Streptomyces species-groups was also achieved when data from several different PyMS runs were carried out over a twenty-month period (Chun *et al.*, 1997c). These findings demonstrate the potential of ANN analyses in the circumscription of *Streptomyces* species.

PAGE
MISSING
IN
ORIGINAL

Chapter II:

Classification of Novel Thermophilic *Amycolatopsis* and *Excellospora* Strains

Introduction

1. Thermophiles

Thermophiles can be loosely defined as organisms which live at high temperatures. Thermophilic microorganisms are of interest from both fundamental and applied perspectives. Basic research focuses on their ecology, evolution, genetics, molecular biology, origins and taxonomy whereas from the applied aspect they are sources of thermostable enzymes and fine chemicals, and are active agents in high temperature fermentations, mineral leaching and waste-treatment processes.

There is no universally accepted definition of the term thermophile partly because of the varying temperature requirements of thermophilic organisms (Table 2-1). It is also the case that for most taxa only members of a few species are able to live close to the upper temperature limit for the group. Consequently, Brock (1986) defined a thermophile as '*an organism capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part.*' This definition has the advantage that it emphasises thermophily within the context of different groups of organisms. The temperature ranges of representatives of the domain *Bacteria* are cited in Table 2-2.

Many of the other definitions of thermophily which have been used are ill-defined. Bacteria which grow over a fairly wide range of temperatures are considered to be *eurythermal* whereas those which live under relatively constant temperature regimes are

Table 2-1. Limits for growth of selected thermophilic organisms

Group	Upper temperature limit (°C)
Animals [a]	
Fish and other aquatic vertebrates	38
Insects	45-50
Ostracods (crustaceans)	49-50
Plants [a]	
Mosses	50
Vascular plants	45
Eucaryotic microorganisms [a]	
Algae	55-60
Fungi	60-62
Protozoa	56
Bacteria [b]	
Aquifex pyrophylus	95
Bacillus	80
Cyanobacteria	70-74
Thermotoga	90
Archaea [b]	
Methanopyrales	110
Pyrodictiaceae	113
Thermoplasmales	67
Thermoproteaceae	104

Data from [a] Brock (1986) and [b] Kristjansson *et al.* (1998).

Table 2-2. Growth temperatures of selected taxa classified in the domain *Bacteria* [a]

Taxa	Temperature range (°C) [b]
Actinomycetes	
Actinokineospora	23-41
Actinomadura	10-60
Dactylosporangium	15-42
Dermatophilus	(37) [c]
Excellospora	40-60
Jonesia	10-40
Kibdelosporangium	15-45
Kitasatospora	15-42
Mycobacterium	-55
Micromonospora	18-40
Microtetraspora	(20-37) [c]
Nocardiopsis	10-45
Nonomuria	20-55
Pilimelia	10-38
Planobispora	28-40
Planomonospora	20-40
Pseudonocardia	-60
Saccharomonospora	24-60
Saccharopolyspora	20-63
Streptomyces	4-65
Streptosporangium	-55
Terrabacter	10-35
Thermobifida	35-60
Thermomonospora	-65 (45-55) [c]
Chemolithotrophic bacteria	
Thermothrix	40-80
Thiobacillus	(20-50) [c]
Endospore-forming Gram-positive bacteria	
Bacillus	-80
Clostridium	-91 (10-65) [c]
Thermoactinomyces	29-70
Facultatively anaerobic Gram-negative rods	
Aeromonas	(22-28) [c]
Escherichia/ Klebsiella/ Salmonella/ Shigella	(37) [c]
Vibrio	-30
Gliding bacteria	
Myxococcus	-40
Stigmatella	18-37
Gram-negative aerobic bacteria	
Acidothermus	37-70 (50-60) [c]
Aquifex	67-95 (85) [c]

Brucella	20-40
Pseudomonas	1-45
Psychrobacter	5-37
Thermomicrobium	45-80
Thermus	-85 (70-75)[c]
Gram-negative anaerobic bacteria	
Thermobacteroides	35-80
Thermotoga	-90 (70-80)[c]
Gram-positive cocci	
Staphylococcus	10-50
Streptococcus	10-50
Helical or vibrioid bacteria	
Bdellovibrio	10-37 (28-30)[c]
Helicobacter	30-42
Oxygenic phototrophic bacteria	
Chlorogloeopsis	-64
Cyanothece	-43
Sheathed bacteria	
Haliscomenobacter	8-30
Leptothrix	10-35
Sulphate- or sulphur-reducing bacteria	
Desulfobacterium	(20-30)[c]
Thermodesulfobacteium	(65-70)[c]

[a] Data taken from *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989), *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994) and Kristjansson *et al.* (1998).

[b] Temperature ranges cover members of all species in the genus.

[c] Temperature ranges in brackets denote optimal temperatures.

considered to be *stenothermal.* Brock (1986) set the thermophile boundary at 50 to 60 °C on the basis of ecological and evolutionary considerations. Temperatures lower than 60 °C are widespread on earth, for example, in composts whereas temperatures greater than 50 to 60 °C are rare; higher temperatures are almost exclusively associated with geothermal habitats. The upper temperature limit for eucaryotic life is 60 °C (Table 2-1). In contrast, members of several procaryotic taxa can grow above 60 °C.

The terms *obligately (extremely) thermophilic, facultatively (moderately) thermophilic* and *thermotolerant (thermoduric)* are defined with respect to the *thermophile boundary.* Obligate thermophiles cannot grow below the thermophile boundary whereas facultative thermophiles and thermotolerant organisms are able to grow at both sides of the boundary. Facultative thermophiles have optimal temperatures higher than the thermophile boundary whereas thermotolerant organisms have optimal temperatures lower than the thermophile boundary. However, such definitions are arbitrary as organisms grow over a range of temperatures and hence tend to form a continuum. This means that the precise temperature range of thermophilic microorganisms under prescribed culture conditions must be given.

The primary natural habitats of many thermophilic microorganisms are found in geothermal regions (Brock, 1978; Kristjansson & Stetter, 1992, 1998). These areas are found in all parts of the globe associated with tectonic activity and are usually concentrated in well defined geological regions. The latter are mainly of two types, the low pH and alkaline pH types, which reflect the geothermal heat source. The low pH type, which is found in solfatara fields, is characterised by acidic hot springs, acidic soils, boiling mud pots and the production of hydrogen sulphide and sulphur and the alkaline pH type by geysers and freshwater hot springs (Kristjansson & Stetter, 1992). Groundwater percolates into hot areas heated by extinct deep lava flows or by dead magma chambers, warms up and returns to the surface containing dissolved minerals such as silica and some dissolved

gases, mainly carbon dioxide (CO_2). However, on the surface the CO_2 is blown away and silica is precipitated resulting in increased pH.

Other naturally occurring hot places are usually more transient, such as composts and solar-heated ponds and soils. The organisms found in these transient ecosystems are mainly rapidly growing, spore-forming bacteria (Edwards, 1990). Man-made, long-term hot environments of the neutral-alkaline type include hot water pipelines in houses and factories and thermophilic waste treatment plants. Similarly, many processes, for example, in the chemical and food industries, run at high temperatures. All such systems are inhabited by thermophilic microorganisms (Perttula *et al.*, 1991; Kristjansson *et al.*, 1994).

Representatives of a number of thermophilic prokaryotes have been isolated and extensively characterised using modern taxonomic methods, notably chemotaxonomic, molecular systematic and numerical phenetic procedures. These organisms include *Aquifex pyrophilus* (Huber *et al.*, 1992), *Thermotoga maritima* (Huber *et al.*, 1986) and *Thermus aquaticus* (Brock & Freeze, 1969). However, in general, relatively little attention has been paid to the taxonomy of thermophilic microorganisms, including thermophilic actinomycetes.

Small subunit (16S) rRNA sequence analysis is now widely used as the universal method of establishing hierarchical relationships of extant bacteria at and above the species level (Woese, 1987; Goodfellow & O'Donnell, 1993; Stackebrandt *et al.*, 1997; Fig. 1-1, page 21). The universal phylogenetic tree derived from such studies is taken to depict the evolutionary histories of organisms and allows inferences to be drawn about the origin and evolution of cellular machinery and metabolic pathways (Woese, 1987; Olsen & Woese, 1993; Dijkhuizen, 1996; Gray & Spencer, 1996). The systematics of thermophilic prokaryotes is closely linked to questions on the origin and early evolution of life. It is interesting that many thermophilic microorganisms form 'old' lineages that are deeply rooted in phylogenetic trees and striking that seven out of the twelve major bacterial phyla

contain thermophiles. Members of four taxa, the *Aquifex-Hydrogenobacter*, the *Thermotoga*, the green non-sulphur bacteria, including *Thermus*, and the *Thermodesulfobacter* group, form the deepest phylogenetic branches in the bacterial phylogenetic tree.

2. Thermophilic actinomycetes

Thermophilic actinomycetes form part of the autochthonous microflora of habitats where decomposition of organic material takes place at elevated temperatures and under aerobic or semiaerobic conditions. Numerous genera of the order *Actinomycetales* consist of organisms that grow well within the temperature range of 50-60 °C which is regarded as the thermophilic boundary (Brock, 1986); such organisms are considered as thermophilic actinomycetes (Cross, 1968).

Korn-Wendisch and Kutzner (1992) noted that most thermophilic actinomycetes grow within the temperature range 25 to 55 °C. However, Greiner-Mai *et al.* (1987) has assigned actinomycetes to three groups based on temperature requirements: the mesophilic (20-45 °C), moderately thermophilic (28-55 °C) and euthermophilic (37-65 °C) groups. Members of these groups showed considerable overlap and temperature maxima and minima varied on different media thereby showing that the temperature range was of only limited taxonomic value. On the other hand, the optimal temperature for growth was the same for all of the strains of a particular species.

Members of several genera classified in the order *Actinomycetales* contain thermophilic strains (Table 2-2), an observation which raises the question of the evolutionary origin of thermophily in these organisms. There are two hypotheses, the common ancestor of the actinomycetes may have arisen in a high-temperature environment with descendants of this line still evident in a broad range of taxa or thermophily may have evolved with members of several taxa having become adapted to high-temperature

environments. More comparative studies on the biology of thermophilic strains are needed to resolve this question.

It is difficult to apply the term thermophile to actinomycetes in a universal sense as members of different taxa have different temperature requirements (Table 2-2). Nevertheless, it is possible to distinguish between taxa that contain thermophilic strains, notably, the genera *Excellospora, Saccharomonospora, Thermobifida, Thermobispora, Thermocrispum* and *Thermomonospora*, and members of other genera, such as *Streptomyces,* which contain relatively few thermotolerant (growing up to 45 °C) and thermophilic (growing between 28 and 55 °C) organisms. *Streptomyces thermoautotrophicus* is unusual as it is an obligate chemolithoautotrophic organism which grows between 40 and 65 °C (Gadkari *et al.*, 1990).

Relatively little attention has been paid to the taxonomy of thermophilic actinomycetes. Species descriptions of these organisms have mainly been based on incomplete morphological criteria with the consequence that many of the early described species were not included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980; Moore *et al.*, 1985). However, within the last two decades considerable progress has been made in the classification of *Actinobacteria* mainly due to the application of chemical, molecular systematic and numerical phenetic procedures (Williams *et al.*, 1989; Embley & Stackebrandt, 1994; Stackebrandt *et al.*, 1997). Nevertheless, taxonomic studies on thermophilic actinomycetes have continued to lag behind those carried out on their mesophilic counterparts though progress has been made in clarifying the classification of thermophilic organisms belonging to the families *Pseudonocardiaceae* (Embley *et al.*, 1988b; Stackebrandt *et al.* 1997), *Streptomycetaceae* (Goodfellow *et al.*, 1987; Kim, D. *et al.*, 1996, Kim, S. B. *et al.*, 1998) and *Thermomonosporaceae* (Cross & Goodfellow 1973; Zhang *et al.*, 1998). The taxonomy of thermophilic streptomycetes is considered in Chapter 3 and that of the families *Pseudonocardiaceae* and *Thermomonosporaceae* below.

The family *Pseudonocardiaceae*. This taxon was proposed by Embley *et al.* (1988b) for organisms belonging to the genera *Actinopolyspora* Gochnauer *et al.* 1975[AL], *Amycolatopsis* Lechevalier *et al.* 1986, *'Faenia'* Kurup & Agre 1983, *Pseudonocardia* Henssen 1957a[AL], *Saccharomonospora* Nonomura & Ohara 1971[AL] and *Saccharopolyspora* Lacey & Goodfellow 1975[AL]. The taxon was designed to encompass aerobic, Gram-positive, non-acid-fast, catalase positive actinomycetes which exhibited a broad range of morphological and physiological properties, and formed extensively branched vegetative and aerial hyphae. Smooth, spiny, or hairy spores were produced either singly, in pairs, or in chains of variable length, or in sporangium-like structures, on aerial hyphae. Representatives of some taxa produced spores on vegetative hyphae whereas others did not form spores or aerial hyphae. Fragmentation of the mycelium occured but was generally not pronounced. Neither endospores nor sclerotia were formed. The organisms were generally chemoorganotrophic though some were facultatively autotrophic. Members of some taxa were halophilic. Diverse compounds were used as sole carbon and energy sources for growth. Some genera assigned to the family *Pseudonocardiaceae* contained both mesophilic and thermophilic species whereas others only encompassed thermophilic organisms (Table 2-3, pages 66 and 67).

Whole-organism hydrolysates of members of the family *Pseudonocardiaceae* contained *meso*-A_2pm, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970a, b) and acetylated muramic acid (Uchida & Aida, 1977, 1984). Members of the taxon were also shown to have an A1γ peptidoglycan (Schleifer & Kandler, 1972). The distribution of madurose (3-O-methyl-galactose) was seen to vary (Mordarski *et al.*, 1986; Shearer *et al.*, 1986) but mycolic acids were absent. The cell envelope contained major amounts of mainly mono-methyl branched acids of the *iso*- and *anteiso*- series (fatty acid types 2 or 3 *sensu* Kroppenstedt, 1985) though 10-methyl branched, *iso*-10-methyl branched, and straight chain saturated and unsaturated acids were

found as minor components in some strains (Embley *et al.*, 1988a); hydroxy fatty acids were detected in members of the genera *Amycolatopsis, Pseudonocardia* and *Saccharomonospora* (Kroppenstedt, 1985; Embley *et al.*, 1988a). The major menaquinones were tetrahydrogenated with eight, nine or ten isoprene units (Labeda *et al.*, 1984; Kroppenstedt, 1985; Collins *et al.*, 1988; Embley *et al.*, 1988a). Diphosphatidylglycerol and phosphatidylinositol were universally present; phosphatidylethanolamine or derivatives thereof were found in members of all genera, apart from *Actinopolyspora.* Phosphatidylcholine was synthesised by representatives of the genera *Actinopolyspora, Pseudonocardia* and *Saccharopolyspora.* The G plus C ratio of the DNA fell within the range 63 to 79 mol%.

Members of the family *Pseudonocardiaceae* have been isolated from diverse habitats, notably soil and vegetable matter. Some strains cause hypersensitivity disease, others are the source of bioactive compounds (Embley, 1992).

The type genus of the family is *Pseudonocardia* Henssen 1957a.

Other amycolate wall chemotype IV actinomycetes, namely, the genera *Amycolata* Lechevalier *et al.* 1986 and *Kibdelosporangium* Shearer *et al* 1986, were not included in the family *Pseudonocardiaceae* as defined by Embley *et al.* (1988). However, these taxa were classified in the family *Pseudonocardiaceae* by Bowen *et al.* (1989) mainly on the basis of 16S rRNA sequence and chemical data. The genus *Amycolata* was subsequently reduced to a synonym of the genus *Pseudonocardia* using a wealth of genotypic and phenotypic data (Warwick *et al.*, 1994). Bowen *et al.* (1989) did not included the genus *Saccharothrix* in the family *Pseudonocardiaceae* as members of this taxon were considered to have a distinct chemical profile.

Members of the genus *Pseudoamycolata* Akimov *et al.* 1989 have chemical and morphological properties consistent with their classification in the family *Pseudonocardiaceae.* This taxon was reduced to a synonym of *Pseudonocardia* mainly on

the basis of 16S rDNA sequence data despite the presence of phophatidylcholine in the type strain of *Pseudomycolata halophobica* (McVeigh *et al.*, 1994). Similarly, the genus *Faenia* Kurup and Agre 1983 was reduced to a synonym of the genus *Saccharopolyspora* Lacey and Goodfellow 1975[AL] by Korn-Wendisch *et al.* (1989).

The taxonomic position of the genera *Actinokineospora* Hasegawa 1988, *Actinosynnema* Hasegawa *et al.* 1978[AL], *Kutzneria* Stackebrandt *et al.* 1994, *Lentzea* Yassin *et al.* 1995, *Saccharothrix* (Labeda *et al.* 1984) Labeda and Lechevalier 1989, *Streptoalloteichus* Tomita *et al.* 1987 and *Thermocrispum* Korn-Wendisch *et al.* 1995 is equivocal for while these taxa are closely related to the family *Pseudonocardiaceae* on the basis of 16S rRNA sequence data they have a wall chemotype III, that is, they contain *meso*-A_2pm but lack characteristic sugars.

Warwick *et al.* (1994) considered that the genera *Actinosynnema*, *Saccharothrix* and *Streptoalloteichus* might form a sister group to the family *Pseudonocardiaceae*. This now seems to be the case as Labeda (1998b) has suggested that the genera *Actinokineospora*, *Actinosynnema*, *Lentzea* and *Saccharothrix* be assigned to a putatively new taxon, the family *Actinosynnemaceae*, on the basis of chemical and 16S rDNA sequence data. 16S rDNA sequence data also suggest that the genera *Kutzneria* and *Streptoalloteichus* belong to this group (Kim & Goodfellow, 1999). If accepted, these proposals would leave the family *Pseudonocardiaceae* as a relatively homogeneous group. The genus *Prauserella*, which was proposed for organisms previously misclassified as *Amycolatopsis rugosa* Lechevalier *et al.* 1986, also belongs to the family *Pseudonocardiaceae* (Kim & Goodfellow, 1999).

Stackebrandt *et al.* (1997) emended the description of the family *Pseudonocardiaceae* using 16S rDNA data. The revised family encompassed the genera *Actinopolyspora*, *Actinosynnema*, *Amycolatopsis*, *Kibdelosporangium*, *Kutzneria*, *Lentzea*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*,

Streptoalloteichus and *Thermocrispum* but not the genus *Actinokineospora* the suprageneric position of which was considered to be equivocal. However, members of the emended family are markedly heterogeneous with respect to chemical properties. Further comparative studies are also needed to resolve the suprageneric relationship of the genus *Actinobispora.* This taxon was proposed by Jiang *et al.* (1991) for a morphologically unique actinomycete strain which had chemical properties similar to those of members of the family *Pseudonocardiaceae sensu* Warwick *et al.* (1994).

The characteristic features of members of the genera assigned to the family *Pseudonocardiaceae* (Embley *et al.*, 1988a,b; Warwick *et al.*, 1994; Kim & Goodfellow, 1998), and to the putatively novel family *Actinosynnemaceae* (Labeda, 1998b), are shown in Table 2-3. The thermophilic members of these taxa are highlighted in Table 2-4. The taxonomy of the genera *Amycolatopsis* and *Excellospora* are considered below as these taxa are the focus of the present study.

The genus *Amycolatopsis.* *Amycolatopsis* (A.my.co.la.top'sis. M.L. fem. n. *Amycolata* genus belonging to the order *Actinomycetales*; Gr. n. *opsis* appearance; M.L. fem n. *Amycolatopsis* that which appears similar to *Amycolata*).

The genus *Amycolatopsis* was proposed by Lechevalier *et al.* (1986) for mycolateless, wall chemotype IV actinomycetes that had previously been classified in the genus *Nocardia*. Members of the genus are aerobic, Gram-positive, non-acid fast, non-motile organisms which form a branched substrate mycelium (0.5-2 μm in diameter) that fragments into squarish elements. When formed, aerial hyphae may be sterile or differentiate into long chains of smooth-walled, squarish to ellipsoidal spore-like structures. Chains of spores are also produced on vegetative hyphae. Some strains are

Table 2-3. Characteristic features of members of the genera classified in the families *"Actinosynnemaceae"* and *Pseudonocardiaceae*

Genera	Cell wall chemotype [a]	Characteristic sugars [b]	Major menaquinones: MK- [c]	Polar lipids [d]	Substrate mycelium		Spores on aerial hyphae	G+C content of DNA (mol%)	Temperature requirement
					fragmentation	spores			
Family *Actinosynnemaceae*									
Actinokineospora	IV	Ara, Gal, Rha	$10H_2$, $9H_4$	DPG,PE PI	v	-	Motile zoospores	69-72	Mesophilic
Actinosynnema	III	Gal, Man	$9H_4$, $9H_6$	DPG, PE, PI, PIMs	v	v	Chains of spores; motile zoospores	70-74	Mesophilic
Kutzneria	III	Gal, Rha	$9H_4$	DPG, PE, PE-OH, PI	-	+	Spores in sporangia	70-73	Mesophilic
Lentzea	III	No characteristic sugars [e]	$9H_2$	DPG, PE, PG, PI	-	-	Fragmentation spores	69	Mesophilic
Saccharothrix	III	Gal, Man, Rib	$9H_4$,$10H_2$	DPG, PE, PG, PI	+	+	Long chains of spores	70-73	Mesophilic
Streptoalloteichus	III	Gal, Man, Rha	$9H_6$,$10H_6$	Phospholipid type II [e]	-	+	Long chains of spores	ND	Thermophilic
Family *Pseudonocardiaceae*									
Actinopolyspora	IV	Ara, Gal	$9H_4$ $10H_4$	DPG, PC, PG, PI	-	-	Long chains of spores	64-68	Mesophilic
Amycolatopsis	IV	Ara, Gal, Mad [(v)]	$9H_2$, $9H_4$	DPG, PE, PG, PI, PME	+	+	Sterile or long chains of spores	66-74	Mesophilic, thermophilic
Kibdelosporangium	IV	Ara, Gal, Mad [(v)]	$9H_4$	DPG, PE, PI	+	-	Long chains of spores; pseudosporangia	66	Mesophilic
Prauserella	IV	Ara, Gal	$9H_2$, $9H_4$	DPG, PE	+	-	No spores	67-69	Mesophilic
Pseudonocardia	IV	Ara, Gal, Xyl [(v)]	$8H_2$, $8H_4$	DPG, PC, PE, PG, PI	+	+	Spore chains of variable length	69-74	Mesophilic, thermophilic

Saccharomonospora	IV	Ara, Gal	$8H_4$, $9H_4$	DPG, PE, PG, PI, PME	-	-	Single spores	66-70	Mesophilic, thermophilic
Saccharopolyspora	IV	Ara, Gal	$9H_4$, $10H_4$	DPG, PC, PE, PG, PI	+	v	Long chains of spores	70-77	Mesophilic, thermophilic
Thermocrispum	III	Ara	$9H_4$	DPG, PE, PI	-	-	Spores in pseudosporangia	69-73	Thermophilic
Genus in search of a family									
Actinobispora	IV	Ara, Gal, Xyl	$7H_2$, $9H_2$	PE, PME, UG	-	+	Single or longitudinal pairs of spores	71	Mesophilic

Data taken from: Henssen & Schäfer (1971), Nonomura & Ohara (1971), Gochnauer *et al.* (1975), Lechevalier *et al.* (1970a, b, 1986), Shearer *et al.* (1986, 1989), Labeda (1986), Labeda *et al.*(1984), Embley *et al.* (1988a), Henssen *et al.* (1987), Greiner-Mai *et al.* (1988), Hasegawa (1988), Runmao *et al.* (1988), Akimov *et al.* (1989), Grund & Kroppenstedt (1989), Goodfellow *et al.* (1989), Korn-Wendisch *et al.* (1989, 1995), Kothe *et al.* (1989), Jiang *et al.* (1991), Stackebrandt *et al.* (1994), Korn-Wendisch *et al.* (1995), Yassin *et al.* (1995) and Kim & Goodfellow (1998).

+, Present or positive; -, absent or negative; v, variable.

[a] Cell wall chemotypes: III, *meso*-A_2pm; IV, *meso*-A_2pm, arabinose and galactose (Lechevalier & Lechevalier, 1970a,b, 1980).

[b] Whole-organism sugars: Ara, arabinose; Gal, galactose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose and Xyl, xylose.

[c] Abbreviations exemplified by MK-9(H_4), menaquinones having four of the nine isoprene units hydrogenated.

[d] Characteristic polar lipids: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol and PME, phosphatidylmethylethanolamine.

[e] Details were not given.

Table 2-4. Thermophilic taxa classified in the families *"Actinosynnemaceae"* and *Pseudonocardiaceae*

Taxa	Previous name	Growth temperature (°C)	References
Family *"Actinosynnemaceae"*			
Streptoalloteichus hindustanus		20-54 (45)	Tomita *et al.* (1987)
Family *Pseudonocardiaceae*			
Actinopolyspora mortivallis		10-50 (45)	Yoshida *et al.* (1991)
Amycolatopsis fastidiosa	*Pseudonocardia fastidiosa*	20-60 (28-36)	ex Celmer *et al.* (1977), Henssen *et al.* (1987)
Amycolatopsis methanolica		20-50	de Boer *et al.* (1990)
Pseudonocardia thermophilia		28-60 (40-50)	Henssen (1957)
Saccharomonospora glauca	*Saccharomonospora viridis*	37-60	Greiner-Mai *et al.*, 1988
Saccharomonospora viridis	*Thermomonospora viridis*	28-60	Schuurmans *et al.* (1956), Nonomura & Ohara (1971)
Saccharopolyspora hirsuta	*Nocardia hirsuta*	25-50	Lacey & Goodfellow (1975)
Saccharopolyspora hordei		20-60	Goodfellow *et al.* (1989)
Saccharopolyspora rectivirgula	*Faenia rectivirgula*, *Micropolyspora rectivirgula*	30-63	Krassilnikov & Agre (1964), Korn-Wendisch *et al.* (1989)
Thermocrispum agreste		20-62.5	Korn-Wendisch *et al.* (1995)
Thermocrispum municipale		20-62.5	Korn-Wendisch *et al.* (1995)

Optimal temperatures given in brackets.

facultative autotrophs. The organism contains *meso*-A_2pm as the major diamino acid of an A1γ wall peptidoglycan, arabinose and galactose as major whole-organism sugars, is rich in fatty acids of the *iso-* and *anteiso-* branched series, has di-, tetra-, and hexahydrogenated menaquinones with nine isoprene units as predominant isoprenologues, and phophatidylethanolamine and phosphatidylglycerol as major polar lipids with diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides variably present (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The mol% G plus C content of the DNA ranges from 66 to 74 %.

Members of the genus have been isolated from soil, vegetative matter and clinical specimens.

The type species is *Amycolatopsis orientalis* (Pittenger and Brigham 1956) Lechevalier *et al.* 1986.

The genus *Amycolatopsis* contains ten validly described species which form a phyletic line within the evolutionary radiation occupied by the family *Pseudonocardiaceae* (Fig. 2-1). Members of three out of the ten species, namely, *Amycolatopsis fastidiosa* (ex Celmer *et al.*, 1977) Henssen *et al.* 1987, *Amycolatopsis methanolica* De Boer *et al.* 1990 and *Amycolatopsis thermoflava* Chun *et al.* 1998 grow well between 50 and 60 °C and hence can be considered as thermophilic actinomycetes (Cross, 1968; Brock, 1986). Members of the remaining species, namely, *Amycolatopsis alba* Mertz & Yao 1993, *Amycolatopsis azurae* (Omura *et al.* 1983) Henssen *et al.* 1987, *Amycolatopsis coloradensis* Labeda 1995, *Amycolatopsis japonica* Goodfellow *et al.* 1997c, *Amycolatopsis mediterranei* (Margalith & Beretta 1960) Lechevalier *et al.* 1986, *Amycolatopsis orientalis* (Pittenger & Brigham 1956) Lechevalier *et al.* 1986 and *Amycolatopsis sulphurea* (ex Oliver & Sinclair 1964) Lechevalier *et al.* 1986 are mesophilic and do not grow at above 45 °C. The mesophilic *Amycolatopsis* species form a

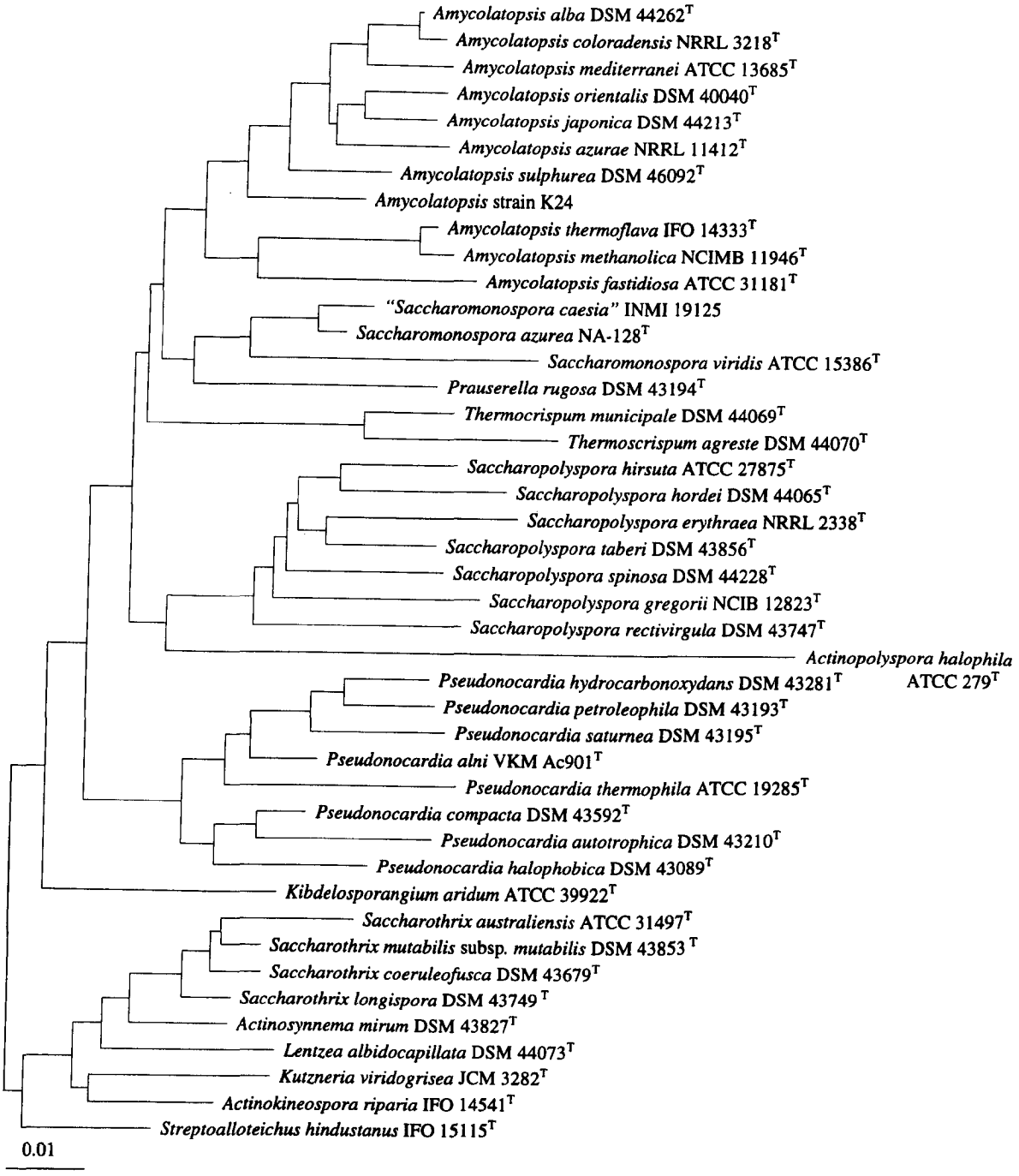


Figure 2-1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16S rRNA sequences showing relationships between representatives of the families "*Actinosynnemceae*" and *Pseudonocardiaceae*. The 16S rRNA sequence of *Streptomyces violaceoruber* A3(2) (Y00411) was used as the outgroup. The scale bar indicates 0.01 substitutions per nucleotide position.

recognisable clade in the phylogenetic tree whereas *Amycolatopsis fastidiosa*, *Amycolatopsis methanolica* and *Amycolatopsis thermoflava* fall into two distinct clades at the periphery of the evolutionary radiation occupied by members of the genus (Fig. 2-1).

The genus *Amycolatopsis* can be distinguished from members of other genera classified in the family *Pseudonocardiaceae* (*sensu* Warwick *et al.*, 1994) using a combination of chemical and morphological markers (Table 2-3). Similarly, members of the validly described species of *Amycolatopsis* can be separated using a combination of phenotypic properties (Table 2-16, page 140-142).

The family *Thermomonosporaceae*. This family was proposed as a taxon of convenience for actinomycetes that formed heat sensitive spores, singly, in pairs or in short-chains on aerial hyphae (Cross & Goodfellow, 1973). The founder members of the family were the genera *Actinomadura* Lechevalier and Lechevalier 1970[AL], *Excellospora* Agre and Guzeva 1975[AL], *Microbispora* Nonomura and Ohara 1957[AL], *Microtetraspora* Thiemann *et al.* 1968[AL], *Nocardiopsis* Meyer 1976[AL] and *Thermomonospora* Henssen 1957a[AL], members of which had walls that contained *meso*-A_2pm without characteristic sugars (wall chemotype III *sensu* Lechevalier & Lechevalier, 1970 a, b). *Saccharomonospora* was also assigned to this group even though representatives of this genus had a wall chemotype IV. It was, therefore, evident from the onset that the family *Thermomonosporaceae* encompassed markedly diverse taxa.

In 1984, Goodfellow and Cross proposed the concept of 'aggregate groups' to provisionally accommodate a number of poorly circumscribed sporoactinomycetes that were thought to need taxonomic revision. One of these artificial groups, the 'maduromycetes' (Goodfellow & Cross, 1984; Goodfellow, 1989) encompassed the genera *Actinomadura* Lechevalier and Lechevalier 1970[AL], *Excellospora* Agre and Guzeva 1975[AL], *Microbispora* Nonomura and Ohara 1957[AL], *Microtetraspora* Thiemann *et al.* 1968[AL], *Planobispora* Thiemann and Beretta 1968[AL], *Planomonospora* Thiemann *et al.*

1967 [AL], *Spirillospora* Couch 1963[AL] and *Streptosporangium* Couch 1955 [AL]. Members of this taxon were defined as aerobic, Gram-positive actinomycetes which formed a branched substrate mycelium that did not carry spores but bore aerial hyphae that differentiated either into short chains of arthrospores or into spore vesicles (sporangia) that contained one to many spores. Constituent strains of the taxon had a peptidoglycan that contained *meso*-A_2pm but lacked characteristic sugars, produced major proportions of straight- and branched-chain fatty acids and partially hydrogenated menaquinones with nine isoprene units as major isoprenologs. They also had DNA rich in G and C.

The genera *Microbispora*, *Microtetraspora*, *Planobispora*, *Planomonospora*, *Spirillospora* and *Streptosporangium* were subsequently classified in the family *Streptosporangiaceae* Goodfellow *et al.* 1990 and the genera *Saccharomonospora* and *Saccharothrix* in the families *Pseudonocardiaceae* (Embley *et al.*, 1988b) and *"Actinosynnemaceae"* (Labeda, 1988b), respectively. These proposals left the genera *Actinomadura* and *Thermomonospora* as the sole constituents of the family *Thermomonosporaceae* (Cross & Goodfellow 1973) emend. Kroppenstedt & Goodfellow 1992.

The emended family *Thermomonosporaceae* encompassed aerobic, Gram-positive, non-acid-alcohol fast, chemoorganotrophic actinomycetes which produced a branched substrate mycelium bearing aerial hyphae that differentiated into single or short chains of arthrospores. Constituent strains contained *meso*-A_2pm in an A1γ peptidoglycan (Schleifer & Kandler, 1972), N-acetylated muramic acid (Uchida & Aida, 1977), lacked characteristic sugars (Lechevalier & Lechevalier, 1970a,b), but possessed mixtures of straight and branched chain fatty acids, hydrogenated menaquinones with nine isoprene units as predominant isoprenologues, and major amounts of phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides (Kroppenstedt, 1985, 1987; Kroppenstedt *et al.*, 1990). Whole-organism hydrolysates of actinomadurae usually contain

madurose. The G+C content of the DNA was found to be within the range of 66 to 72 mol% (Fischer *et al.*, 1983; Poschner *et al.*, 1985; Miyadoh *et al.*, 1987).

Stackebrandt *et al.* (1997) emended the family *Thermomonosporaceae* solely on the basis of 16S rDNA sequence data (Fig. 1-1, page 21; Table 1-2, pages 22-23). The revised taxon encompassed the genera *Actinomadura* Lechevalier and Lechevalier 1970[AL], *Spirillospora* Couch 1963[AL], and *Thermomonospora* Henssen 1957a[AL]. The taxonomic positions of *Actinocorallia* Iinuma *et al.* 1994 and *Excellospora* Agre and Guzeva 1975[AL], which are closely related to the members of the family *Thermomonosporaceae* (Zhang *et al.*, 1998), were not considered.

The genus *Excellospora.* *Excellospora* (Ex.cel'lo.spo.ra. M.L. fem. adj. from *excellens*, L. pr. part of *excello* prominent; Gr. n. *spora* seed [referring to the special structure of the spores]).

The genus *Excellospora* was proposed by Agre and Guzeva (1975) for thermophilic actinomycetes that were distinguished from actinomadurae primarily by differences in fatty acid composition. The genus contained three species, *Excellospora viridilutea* Agre and Guzeva 1975, the type species, which was cited in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980), and *Excellospora rubrobrunea* (Krassilnikov *et al.* 1968b) Agre and Guzeva 1975 and *Excellospora viridinigra* (Krassilnikov *et al.* 1968b) Agre and Guzeva 1975 which had previously been classified in the genus *'Micropolyspora'* as *'Micropolyspora rubrobrunea'* and *'Micropolyspora viridinigra'*, respectively. Neither *Excellospora rubrobrunea* nor *Excellospora viridinigra* were included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980).

The genus *Excellospora* Agre and Guzeva 1975[AL] encompasses aerobic, Gram-positive, non-acid fast actinomycetes which form extensively branched, nonfragmenting substrate and aerial mycelia. Spores are borne in hooked or spiral chains, singly or in pairs, on both aerial and substrate hyphae; the sporulating hyphae tend to undergo autolysis.

Table 2-5. Cellular fatty acid composition of the type strains of the *Excellospora viridilutea*[a] and *Actinomadura* species[b] and *Actinomadura rubrobrunea* DSM 43751[b].

Taxa	Strains (DSM)	iso-14:0	14:0	iso-15:0	ante iso-15:0	15:0	iso-16:1	iso-16:0	cis-16:1	16:0	10-Meth 16:0	iso-17:0	ante iso-17:0	cis 9 17:1	iso-16:0 2OH	17:0	2-OH 16:0	10-Meth 17:0	iso-18:0	cis-9 18:1	18:0	10-Meth 18:0
E. viridilutea	43934^T							+[c]				+[c]							+[c]			-[c]
A. rubrobrunea	43750^T	0.4		2.7	0.3	4.4		27.7		2.8		19.2	3.7	1.0		18.5		6.8	9.1		1.2	0.3
A. rubrobrunea	43751	0.8	0.5	4.0	0.5	5.7		22.6		7.2		18.2	3.2	1.0		22.9		1.8	5.7		3.9	
A. atramentaria	43919^T					2.6		19.0		12.8				3.6	3.9	4.5	3.9	9.0	7.0	7.9	4.3	21.5
A. aurantiaca	43924^T	0.3	1.0	0.4		1.2	0.7	17.0	8.7	21.1	2.0	1.8	2.3	3.0		2.9		4.4	1.6	16.5	5.9	8.9
A. citrea	43461^T		2.3			0.6		5.4	1.9	43.3	2.4	0.4		0.3		1.4		1.0	0.7	9.5	5.4	25.5
A. coerulea	43675^T		0.8			1.2		10.6	4.0	34.2	1.0			1.5		3.5		1.8	2.5	15.1	3.0	20.8
A. crema	43676^T		2.4			1.5		6.6	2.8	34.5	2.0			1.0		3.3		1.9	1.1	9.4	4.9	28.7
A. echinospora	43163^T	0.4				2.7	0.5	32.0	1.6	8.0	0.6			8.8		9.8		8.9	8.7	11.4	1.4	5.5
A. flavescens	43923^T		3.3			0.6		0.6	1.4	41.5	3.6							0.7		1.0	4.1	43.3
A. kijianata	43764^T	0.7	1.1			5.8		19.5	2.4	11.6	0.6		0.5	10.3		11.1		9.5	4.2	9.3	3.8	10.0
A. libanotica	43554^T		2.0	1.1		4.0		11.6	8.9	21.8	1.3	2.7	1.2	6.5		7.8		3.8	1.4	17.3	5.2	4.1
A. livida	43677^T	0.5	1.1			7.5		19.0	0.9	13.3	0.6			10.8		14.4		12.2	2.4	5.5	5.1	6.9
A. luteofluorescens	40398^T	0.7	1.1			2.3		21.2	5.2	23.2	1.5			5.4		3.9		10.4	2.0	8.1	2.7	12.5
A. macra	43862^T	0.5	3.2			3.9		5.1	2.1	41.6	0.7			2.3		6.6		1.8	0.8	10.3	8.3	12.8
A. madurae	43067^T	1.1	1.6			0.8		19.6	3.4	34.0	0.5			1.4		2.1		3.0	3.2	17.6	3.4	8.5
A. malachitica	43462^T	0.8	1.2			1.9		20.1	1.8	22.0	1.3			2.7		6.7		5.6	6.3	12.9	6.1	13.0
A. oligospora	43930^T	1.2	2.4			2.6		20.4	7.8	25.8	2.9			3.3		6.4		4.9	0.8	12.9	1.8	12.0
A. pelletieri	43383^T					1.2		49.0		14.8				1.0		2.9		3.0	7.6	10.9	2.3	7.2
A. spadix	43459^T	0.8	0.4	0.9	1.4	1.2	2.3	48.2		3.3	2.3	1.6	16.3			4.2		4.9	1.0		4.5	5.5
A. umbrina	43927^T	0.3				0.6	1.1	38.1	1.0	4.9	1.0	0.6	0.8	1.9		2.2		10.9	19.3	2.9	4.8	9.6
A. verrucosospora	43358^T	0.5	0.5			1.3		34.4	1.4	15.8	1.6	0.2		3.2		3.7		13.5	5.0	5.7	2.5	10.7
A. vinacea	43765^T		1.8			0.6		1.2	5.9	34.9				1.0		1.8		0.6		21.9	13.0	17.2
A. yumaensis	43931^T		2.8			1.8		5.1	2.7	54.2	2.3					1.6		1.3	2.0		1.8	24.5

Data taken from Agre & Guzeva (1975)[a] and from Kroppenstedt *et al.* (1990)[b] who grew the test strains in shake flasks of tryptic soy broth at either 28 °C (mesophilic strains) or 45 °C (thermophilic strains)

[c], Detailed data were not given; '+' indicates abundant components and '-' minor components.

[d], Abbreviations: 14:0, tetradecanoic acid; *iso*-14:0, 12-methyltridecanoic acid; 15:0, pentadecanoic acid; *anteiso*-15:0, 12-methyltetradecanoic acid; *iso*-15:0, 13-methyltetradecanoic acid; 16:0, hexadecanoic acid; *iso*-16:0, 14-methylpentadecanoic acid; *iso*-16:1, monounsaturated 14-methylpentadecanoic acid; *iso*-16:0 2OH, 2-hydroxy-14-methylpentadecanoic acid; *cis* 16:1, monounsaturated hexadecanoic acid; 10-Meth 16:0, 10-methylhexadecanoic acid; *anteiso*-17:0, 14-methylhexadecanoic acid; *iso*-17:0, 15-methylhexadecanoic acid; *cis*-9 17:1, monounsaturated *cis*-9,10-heptadecanoic acid; 10-Meth 17:0, 10-methylheptadecanoic acid; *iso*-18:0, 16-methylheptadecanoic acid; *cis*-9 18:1, monounsaturated *cis*-9,10-octadecanoic acid (oleic acid) and 10-Meth 18:0, 10-methyloctadecanoic acid (tuberculosteric acid).

Excellosporae share a number of morphological and physiological characteristics but can be differentiated into species on the basis of the colour of their aerial and substrate mycelia. The organism contains *meso*-A_2pm in an A1γ peptidoglycan, madurose, 14-methylpentadecanoic acid (*iso*-16:0), 15-methylhexadecanoic acid (*iso*-17:0) and 16-methylheptadecanoic acid (*iso*-18:0) as major fatty acids and minor proportions of 10-methyloctadecanoic acid (10-meth 18:0) (Table 2-5). The temperature range for growth is between 37 and 65 °C. Excellosporae have been isolated from desert and salty soil samples collected from Uzbekistan (Agre & Guzeva, 1975) and from soils under maize and rice in Egypt (Krassilnikov *et al.*, 1968b).

Excellosporae are difficult to separate from actinomadurae. It is not surprising, therefore, that the genus was listed as a *genus incertae sedis* in *Bergey's Manual of Systematic Bacteriology* (Meyer, 1989). *Excellospora rubrobrunea* and *Excellospora viridinigra* strains were subsequently shown to have many properties in common both with one another and with *Actinomadura madurae* and related species (Greiner-Mai *et al.*, 1987; Meyer, 1989; Kroppenstedt *et al.*, 1990). It was noted that they could be separated from actinomadurae as they contain relatively high proportions of *iso*-branched fatty acids (high melting point) and low amounts of 10-methyl branched acids (low-melting point) (Table 2-5) but these differences were merely attributed to the thermophilic nature of these organisms (Kroppenstedt *et al.*, 1990).

Kroppenstedt *et al.* (1990) proposed that *Excellospora viridinigra* be recognised as a synonym of *Excellospora rubrobrunea* and that the latter be transferred to the genus *Actinomadura* as *Actinomadura rubrobrunea*. These authors did not consider the taxonomic position of *Excellospora viridilutea*. It is now evident that *Excellospora viridilutea* IFO 14480^T falls towards the periphery of the evolutionary radiation occupied by *Actinomadura* and related taxa (Fig. 2-2). It is not possible to comment on the phylogenetic relationship of *Actinomadura rubrobrunea* and *Excellospora viridilutea* as

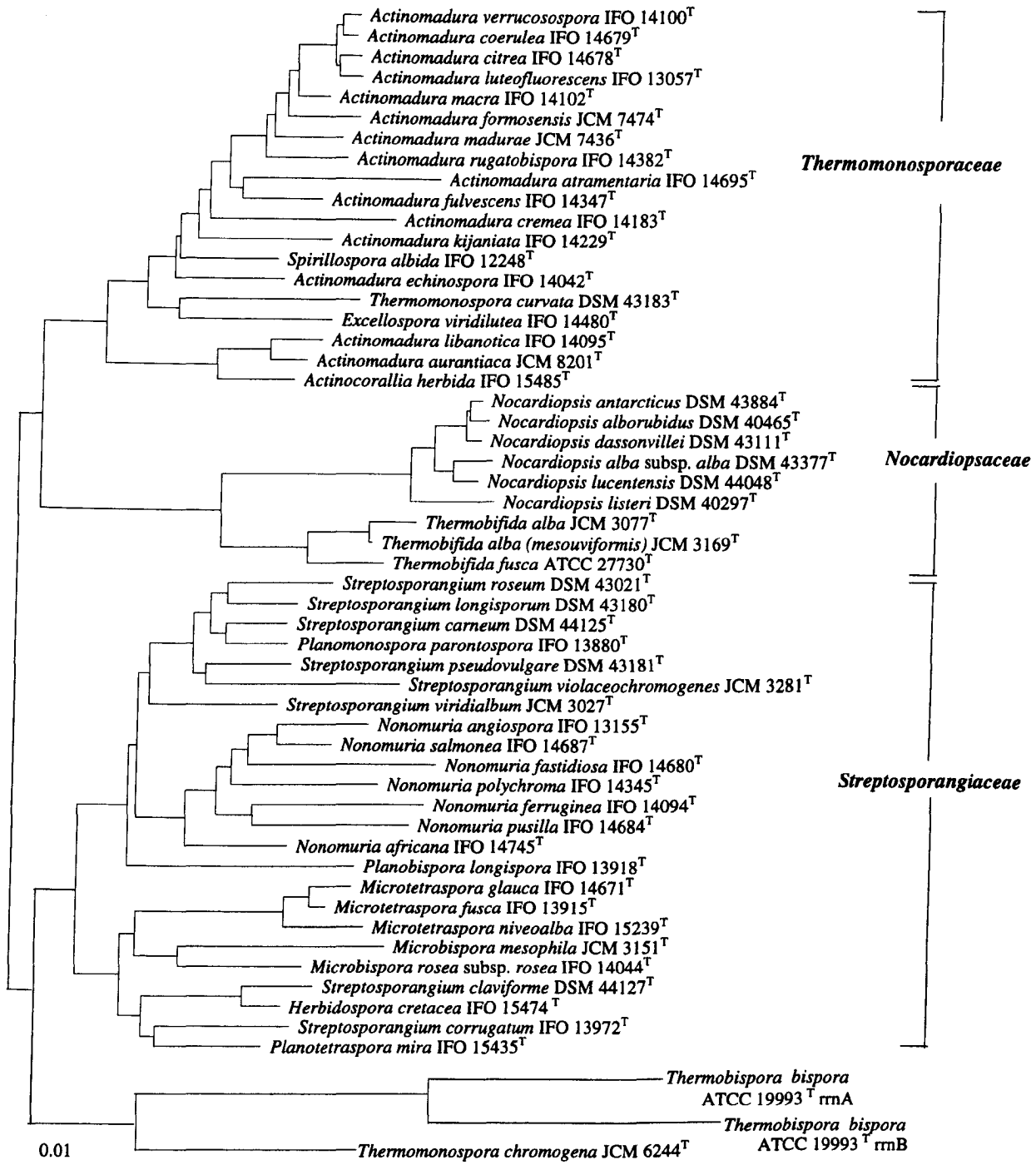


Figure 2-2. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16S rRNA sequences showing relationships between representatives of the families *Nocardiopsaceae, Streptosporangiaceae, Thermomonosporaceae* and related taxa. The 16S rRNA sequence of *Streptomyces violaceoruber* A3(2) (Y00411) was used as outgroup. The scale bar indicates 0.01 substitutions per nucleotide position.

representatives of the former have not been the subject of 16S rDNA sequence studies.

The genus *Actinomadura.* *Actinomadura* (Ac.ti.no.ma.du'ra. Gr. n. *actis* a ray; *Madura* name of province in India; L. fem. n. *Actinomadura* referring to a microorganism first described as the causative agent of "Madura" foot disease)

The genus *Actinomadura* was proposed by Lechevalier and Lechevalier (1970c) for aerobic, Gram-positive, non-acid-fast actinomycetes that formed branched nonfragmenting mycelia and aerial hyphae that carried up to fifteen spores and had cell walls containing *meso*-A_2pm without characteristic sugars (wall chemotype III *sensu* Lechevalier & Lechevalier, 1970a). The taxon initially encompassed *Actinomadura dassonvillei* Brocq-Rousseau 1904, *Actinomadura madurae* Vincent 1894, the type species, and *Actinomadura pelletieri* Laveran 1906.

The founder members of the genus had a long and unsettled taxonomic history (Lacey *et al.,* 1978). The organism now known as *Actinomadura madurae* was first described in 1894 by Vincent as *Streptothrix madurae* for strains isolated from an Algerian case of Madura foot. *Actinomadura dassonvillei* and *Actinomadura pelletieri* had similar taxonomic histories though *Actinomadura pelletieri* was classified in the genus *Micrococcus* by Laveran (1906) as *Micrococcus pelletieri* because hyphae of this organism were considered to fragment into cocci. *Actinomadura madurae* was subsequently transferred to the genus *Nocardia* as *Nocardia madurae* (Vincent 1894) Blanchard 1896, then to the genus *Streptomyces* as *Streptomyces madurae* (Vincent 1894) González-Ochoa and Sandoval 1956. Similarly, *Actinomadura pelletieri* was classified first as *Nocardia pelletieri* (Laveran 1906) Pinoy 1912 and then as *Streptomyces pelletieri* (Laveran 1906) Waksman and Henrici 1948. The taxonomic status of all of these organisms remained controversial until Becker *et al.* (1965) found that whole-organism hydrolysates of representative strains contained *meso*-A_2pm and a characteristic sugar subsequently identified as madurose (Lechevalier and Gerber, 1970). *Actinomadura dassonvillei* was

assigned to the genus primarily on the basis of chemical properties.

The genus *Nocardiopsis* was subsequently proposed to accommodate *Actinomadura dassonvillei* as strains of this taxon lacked the characteristic sugar madurose and formed spores in a characteristic zig-zag formation on aerial hyphae (Meyer, 1976). Subsequent chemical (Collins *et al.*, 1977: Lechevalier *et al.*, 1977; Minnikin *et al.*, 1977; Fischer *et al.*, 1983; Athalye *et al.*, 1984) and numerical phenetic data (Alderson & Gooodfellow, 1979; Goodfellow *et al.*, 1979; Goodfellow & Pirouz, 1982; Athalye *et al.*, 1985) strongly supported the recognition of the genus *Nocardiopsis* with *Nocardiopsis dassonvillei* as the type species.

16S rDNA sequence data confirmed the separation between *Nocardiopsis dassonvillei* and *Actinomadura madurae* and suggested that the genus *Nocardiopsis* was most closely related to the genera *Microtetraspora* and *Streptomyces* (Goodfellow *et al.*, 1988; Kroppenstedt *et al.*, 1990). *Saccharothrix australiensis* Labeda *et al.* 1984, which has chemotaxonomic and morphological properties in common with *Nocardiopsis dassonvillei*, was found to be closely related to members of the family *Pseudonocardiaceae* (Bowen *et al.*, 1989; Warwick *et al.*, 1994; Stackebrandt *et al.*, 1997).

Six out of the eight validly described *Nocardiopsis* species which were subsequently recognised were later considered to be missclassified (Grund & Kroppenstedt, 1989, 1990). *Nocardiopsis coeruleofusca* (Preobrazhenskaya and Sveshnikova 1974) Preobrazhenskaya and Sveshnikova 1985, *Nocardiopsis flava* (Gauze *et al.* 1974) Gauze and Sveshnikova 1985, *Nocardiopsis longispora* (Preobrazhenskaya and Sveshnikova 1974) Preobrazhenskaya and Sveshnikova 1985, *Nocardiopsis mutabilis* Shearer *et al.* 1983 and *Nocardiopsis syringae* Gauze *et al.* 1985 were reclassified in the genus *Saccharothrix* as *Saccharothrix coeruleofusca* (Preobrazhenskaya and Sveshnikova 1974) Grund and Kroppenstedt 1990, *Saccharothrix flava* (Gauze *et al.* 1974) Grund and Kroppenstedt 1990, *Saccharothrix longispora* (Preobrazhenskaya and Sveshnikova 1974)

Grund and Kroppenstedt 1990, *Saccharothrix mutabilis* (Shearer *et al.* 1983) Labeda and Lechevalier 1989 and *Saccharothrix syringae* (Gauze *et al.* 1985) Grund and Kroppenstedt 1990, respectively. Another species, *Nocardiopsis africana* (Preobrazhenskaya and Sveshnikova 1974) Preobrazhenskaya *et al.* 1985, was assigned to the genus *Microtetraspora* as *Microtetraspora africana* (Preobrazhenskaya and Sveshnikova 1974) Kroppenstedt *et al.* 1991. The two remaining species, *Nocardiopsis alba* and *Nocardiopsis dassonvillei,* were retained in the genus *Nocardiopsis.*

The genus *Nocardiopsis* currently encompasses *Nocardiopsis alba* Grund and Kroppenstedt 1990, *Nocardiopsis dassonvillei* (Brocq-Rousseau 1904) Meyer 1976, *Nocardiopsis halophila* Al-Tai and Ruan 1994, *Nocardiopsis listeri* Grund and Kroppenstedt 1990, *Nocardiopsis lucentensis* Yassin *et al.* 1993b, *Nocardiopsis prasina* (Grund and Kroppenstedt 1990) Yassin *et al.* 1997b and *Nocardiopsis synnemataformans* Yassin *et al.* 1997b. Members of the genus form a distinct phyletic line in the 16S rRNA tree (Fig. 2-2).

In the meantime, additional species had been assigned to the genus *Actinomadura* primarily on the basis of morphological and chemotaxonomic data. Even so, the genus *Actinomadura* was listed as a *genus incertae sedis* in the eight edition of *Bergey's Manual of Determinative Bacteriology* (McClung, 1974), but was included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) where twenty-one species were recognised.

Numerical phenetic analyses of the genus *Actinomadura* and related taxa (Goodfellow *et al.* , 1979; Alderson & Goodfellow, 1979; Goodfellow & Pirouz, 1982) confirmed the separation between the genera *Actinomadura* and *Nocardiopsis* and suggested that the *Actinomadura madurae* and the *Actinomadura pelletieri* were heterogeneous species. In comprehensive chemical and numerical phenetic studies members of the genus *Actinomadura* were assigned to two aggregate taxa, the *Actinomadura madurae* and the *Actinomadura pusilla* groups (Athalye *et al.*, 1984, 1985).

Actinomadura madurae and *Actinomadura pelletieri* were again considered to be heterogeneous species.

Fischer *et al.* (1983) provided compelling evidence that the genus *Actinomadura* was heterogeneous when they assigned representative strains to two aggregate groups defined on the basis of chemical and nucleic acid pairing data. Ribosomal RNA partial oligonucleotide sequence (Fowler *et al.*, 1985; Goodfellow *et al.*, 1988), menaquinone (Athalye *et al.*, 1984), polar lipid (Lechevalier *et al.*, 1977), numerical taxonomic (Goodfellow & Pirouz, 1982; Athalye *et al.*, 1985) and DNA:DNA relatedness data (Poschner *et al.*, 1985) underlined this heterogeneity. *Actinomadura madurae* and related species were seen to have a closer affinity to *Thermomonospora curvata* than to *Actinomadura pusilla* and allied taxa, the latter were found to be related to *Streptosporangium roseum* (Fowler *et al.*, 1985).

The division of the genus *Actinomadura* into two separate groups was formally recognised by Kroppenstedt *et al.* (1990) who proposed that the genus *Actinomadura* Lechevalier and Lechevalier 1970c be retained for *Actinomadura madurae* and related species and that the *Actinomadura pusilla* group be reclassified in the redefined genus *Microtetraspora* Thiemann *et al.* 1968; this division was supported by other molecular systematic data, including those from polyacrylamide gel electrophoresis analyses of the ribosomal protein AT-L30 (Ochi *et al.*, 1991).

The revised genus *Actinomadura* (Lechevalier & Lechevalier 1970c) Kroppenstedt *et al.* 1990 accommodated twenty-six validly described species, the members of which characteristically formed non-fragmenting, extensively branched substrate mycelia and aerial hyphae that carried up to fifteen arthrospores. Spore chains were straight, hooked (open loops), or irregular spirals (1-4 turns), and spore surfaces folded, irregular, smooth, spiny or warty. The organisms grew within the temperature range 10 to 60 °C. In addition, they contained major proportions of hexahydrogenated menaquinones with nine isoprene

units saturated at sites II, III, and VIII, and complex mixtures of fatty acids with hexadecanoic, 14-methylpentadecanoic, and 10-methyloctadecanoic acids predominating (Fischer *et al.*, 1983; Athalye *et al.*, 1984; Miyadoh *et al.*, 1989). Whole-organism hydrolysates contained galactose, glucose, mannose, ribose, and madurose, the latter sometimes in trace amounts (Lechevalier & Lechevalier, 1970b).

Trujillo and Goodfellow (1997) examined representative *Actinomadura madurae* and *Actinomadura pelletieri* strains together with markers of validly described species of *Actinomadura* in a polyphasic taxonomic study designed to determine whether species containing clinically significant actinomadurae were heterogeneous, as suggested by the results of earlier studies (Goodfellow, 1979; Alderson & Goodfellow, 1979; Fischer *et al.*, 1983). The *Actinomadura pelletieri* strains were recovered in two distinct numerical phenetic groups the composition of which was supported by Curie-point pyrolysis mass spectrometric and DNA:DNA relatedness data. The name *Actinomadura pelletieri* was retained for the group containing the type strain in accordance with the rule of priority (Sneath, 1992), the epithet *Actinomaura latina* was proposed for the second group.

The genus *Actinomadura* now accommodates thirty-one validly described species, including *Actinomadura rubrobrunea* (Table 2-6). The *Actinomadura* species that were transferred to the genus *Microtetraspora* by Kroppenstedt *et al.* (1991) have recently been assigned to a new taxon, the genus *Nonomuria,* mainly on the basis of 16S rDNA sequence data (Zhang *et al.*, 1998). The reclassification of these and related actinomycete species is outlined in Table 2-7.

Most of the members of the redefined genus *Actinomadura* are mesophilic organisms which grow optimally between 28 and 30 °C. *Actinomadura rubrobrunea* is currently the only thermophilic member of the genus, as *Actinomadura fastidiosa* Soina *et al.* 1975 and *Actinomadura flexuosa* (ex Cross and Goodfellow 1973) Meyer 1989, which encompass also thermophilic organisms, are now classified in the genus *Nonomuria* as

Table 2-6. Validly described species and subspecies of genera classified in the families *Nocardiopsiaceae* and *Thermomonosporaceae*

Taxa	Authors	Type strain
Family *Thermomonosporaceae*		
Genus *Actinocorallia* Iinuma *et al.* 1994		
A. herbida	Iinuma *et al.* 1994	IFO 15485
Genus *Actinomadura* Lechevalier & Lechevalier 1970c		
A. atramentaria	Miyadoh *et al.* 1987	DSM 43919
A. aurantiaca	Lavrova & Preobrazhenskaya 1975	DSM 43924
A. carminata	Gauze *et al.* 1973	DSM 44170
A. citrea	Lavrova *et al.* 1972	DSM 43461
A. coerulea	Preobrazhenskaya *et al.* 1975	DSM 43675
A. cremea subsp. *cremea*	Preobrazhenskaya *et al.* 1975	DSM 43676
A. cremea subsp. *rifamycini*	Gauze *et al.* 1987	DSM 43936
A. echinospora	(Nonomura & Ohara 1971) Kroppenstedt *et al.* 1991	DSM 43163
A. fibrosa	Mertz & Yao 1990	NRRL 18348
A. formosensis	(Hasegawa *et al.* 1986) Zhang *et al.* 1998	IFO 14204
A. fulvescens	Terekhova *et al.* 1987	DSM 43923
A. glomerata	Itoh *et al.* 1996	JCM 9376
A. hibisca	Tomita *et al.* 1991	ATCC 53557
A. kijaniata	Horan & Brodsky 1982	DSM 43764
A. latina	Trujillo & Goodfellow 1997	DSM 43382
A. libanotica	Meyer 1981	DSM 43554
A. livida	Lavrova & Preobrazhenskaya 1975	DSM 43677
A. longicatena	Itoh *et al.* 1996	JCM 9377
A. luteofluorescens	(Shinobu 1962) Preobrazhenskaya *et al.* 1975	DSM 40398
A. macra	Huang 1980	DSM 43862
A. madurae	(Vincent 1894) Lechevalier & Lechevalier 1970c	DSM 43067
A. nitritigenes	Lipski & Altendorf 1995	DSM 44137
A. oligospora	Mertz & Yao 1986	NRRL 15878
A. pelletieri	(Laveran 1906) Lechevalier & Lechevalier 1970c	DSM 43383
A. rubrobrunea	Kroppenstedt *et al.* 1991	DSM 43750
A. rugatobispora	Miyadoh *et al.* 1991	IFO 14382
A. spadix	Nonomura & Ohara 1971	DSM 43459
A. umbrina	Galatenko *et al.* 1987	DSM 43927
A. verrucosospora	Nonomura & Ohara 1971	DSM 43358
A. vinacea	Lavrova & Preobrazhenskaya 1975	DSM 43765
A. viridis	(Nonomura & Ohara 1971) Miyadoh *et al.* 1989	DSM 43175
A. yumaensis	Labeda *et al.* 1985	NRRL 12515

Genus *Excellospora* Agre & Guzeva 1975		
E. viridilutea	Agre & Guzeva 1975	DSM 43934
Genus *Spirillospora* Couch 1963		
S. albida	Couch 1963	DSM 43034
Genus *Thermomonospora* Henssen 1957, emend. Zhang *et al.* 1998		
"T". chromogena	McCarthy & Cross 1984	DSM 43794
T. curvata	Henssen 1957	DSM 43183
Family *Nocardiopsiaceae*		
Genus *Nocardiopsis* (Brocq-Rousseau 1904) Meyer 1976		
N. alba subsp. alba	Grund & Kroppenstedt 1990	DSM 43377
N. dassonvillei	(Brocq-Rousseau 1904) Meyer 1976	DSM 43111
N. halophila	Al-Tai & Ruan 1994	IQ-H3
N. listeri	Grund & Kroppenstedt 1990	DSM 40297
N. lucentensis	Yassin *et al.* 1993	DSM 44048
N. prasina	(Grund & Kroppenstedt 1990) Yassin *et al.* 1997	DSM 43845
N. synnemataformans	Yassin *et al.* 1997	DSM 44143
Genus *Thermobifida* Zhang *et al.* 1998		
T. alba	(Locci *et al.* 1967) Zhang *et al.* 1998	DSM 43310
T. fusca	(McCarthy & Cross 1984) Zhang *et al.* 1998	DSM 43792
Genus in search of family		
Genus *Thermobispora* Wang *et al.* 1996		
T. bispora	(Henssen 1957) Wang *et al.* 1996	DSM 43833

ATCC, American Type Culture Collection, Rockville, MD., U.S.A; DSM, Deutsche Sammlung von Microorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan; NRRL, US Department of Agriculture Regional Research Laboratory Collection, Peoria, Illinois, USA.

Table 2-7. Reclassification of actinomycetes once assigned to the genera *Actinobifida, Actinomadura, Excellospora, Micromonospora, Micropolyspora, Microtetraspora, Nocardiopsis, Saccharothrix, Streptomyces, Thermomonospora* and *Thermopolyspora*

Original assignment	Type strain (DSM)	Other designation	Current Designation
Genus *Actinobifida*			
A. alba (Locci *et al.*, 1967)	43795	*Thermomonospora alba* (Cross & Goodfellow, 1973)	*Thermobifida alba* (Zhang *et al.*, 1998)
A. *chromogena* (Krassilnikov & Agre, 1965)	43794		*Thermomonospora chromogena* (McCarthy & Cross, 1984)
Genus *Actinomadura*			
A. africana (Preobrazhenskaya & Sveshnikova, 1974)	43748	*Nocardiopsis africana* (Preobrazhenskaya *et al.*, 1982), *Microtetraspora africana* (Kroppenstedt *et al.*, 1990)	*Nonomuria africana* (Zhang *et al.*, 1998)
A. coeruleofusca (Preobrazhenskaya & Sveshnikova, 1974)	43679	*Nocardiopsis coeruleofusca* (Preobrazhenskaya *et al.*, 1982)	*Saccharothrix coeruleofusca* (Grund & Kroppenstedt, 1990)
A. coeruleoviolacea (Preobrazhenskaya *et al.*, 1987)	43935		*Saccharothrix coeruleoviolacea* (Kroppenstedt *et al.*, 1991)
A. dassonvillei (Lechevalier & Lechevalier, 1970c)[a]	43111	*Nocardiopsis dassonvillei* subsp. *dassonvillei* (Miyashita *et al.*, 1984; Meyer, 1976)	*Nocardiopsis dassonvillei* (Grund & Kroppenstedt, 1990)
A. fastidiosa (Soina *et al.*, 1975)	43674	*Microtetraspora fastidiosa* (Kroppenstedt *et al.*, 1990)	*Nonomuria fastidiosa* (Zhang *et al.*, 1998)
A. ferruginea (Meyer, 1981)	43563	*Microtetraspora ferruginea* (Kroppenstedt *et al.*, 1990)	*Nonomuria ferruginea* (Zhang *et al.*, 1998)
A. flava (Gauze *et al.*, 1974)	43885	*Nocardiopsis flava* (Preobrazhenskaya *et al.*, 1982)	*Saccharothrix flava* (Grund & Kroppenstedt 1990)
A. helvata (Nonomura & Ohara, 1971)	43142	*Microtetraspora helvata* (Kroppenstedt *et al.*, 1990)	*Nonomuria helvata* (Zhang *et al.*, 1998)
A. longispora (Preobrazhenskaya & Sveshnikova, 1974)	43749	*Nocardiopsis longispora* (Preobrazhenskaya *et al.*, 1982)	*Saccharothrix longispora* (Grund & Kroppenstedt 1990)
A. polychroma (Galatenko *et al.*, 1987)	43925	*Microtetraspora polychroma* (Kroppenstedt *et al.*, 1990)	*Nonomuria polychroma* (Zhang *et al.*, 1998)
A. pusilla (Nonomura & Ohara, 1971)	43357	*Microtetraspora pusilla* (Kroppenstedt *et al.*, 1990)	*Nonomuria pusilla* (Zhang *et al.*, 1998)
A. recticatena (Terekhova *et al.*, 1987)	43937	*Microtetraspora recticatena* (Kroppenstedt *et al.*, 1990)	*Nonomuria recticatena* (Zhang *et al.*, 1998)
A. roseola (Lavrova & Preobrazhenskaya, 1975)	43767	*Microtetraspora roseola* (Kroppenstedt *et al.*, 1990)	*Nonomuria roseola* (Zhang *et al.*, 1998)
A. roseola (Nonomura & Ohara, 1971)	43144	*Microtetraspora roseola* Kroppenstedt *et al.*, 1990)	*Nonomuria roseola* (Zhang *et al.*, 1998)
A. salmonea (Preobrazhenskaya *et al.*, 1975)	43678	*Microtetraspora salmonea* (Kroppenstedt *et al.*, 1990)	*Nonomuria salmonea* (Zhang *et al.*, 1998)
A. spiralis (Meyer, 1981)	43555	*Microtetraspora spiralis* (Kroppenstedt *et al.*, 1990)	*Nonomuria spiralis* (Zhang *et al.*, 1998)

A. turkmeniaca (Terekhova *et al.*, 1987)		*Microtetraspora turkmeniaca* (Kroppenstedt *et al.*, 1990)	*Nonomuria turkmeniaca* (Zhang *et al.*, 1998)
Genus *Microbispora*			
M. bispora (Henssen, 1957; Lechevalier, 1965)	43833		*Thermobispora bispora* (Wang *et al.*, 1996)
M. echinospora (Nonomura & Ohara, 1971b)	43163		*Actinomadura echinospora* (Kroppenstedt *et al.*, 1990; Miyadoh *et al.*, 1989)
Genus *Micromonospora*			
M. rubra (Sveshnikova *et al.*, 1969)	43768	*Actinomadura rubra* (Meyer & Sveshnikova, 1974), *Microtetraspora rubra* (Kroppenstedt *et al.*, 1990)	*Nonomuria rubra* (Zhang *et al.*, 1998)
Genus *Micropolyspora*			
M. rubrobrunea (Krassil'nikov *et al.*, 1968)	43750	*Excellospora rubrobrunea* (Agre & Guzeva, 1975)	*Actinomadura rubrobrunea* Kroppenstedt *et al.*, 1991
M. viridinigra (Krassil'nikov *et al.*, 1968)	43751	*Excellospora viridinigra* (Agre & Guzeva, 1975)	*Actinomadura rubrobrunea* Kroppenstedt *et al.*, 1991
Genus *Microtetraspora*			
M. viridis (Nonomura & Ohara, 1971a)	43175		*Actinomadura viridis* (Miyadoh *et al.*, 1989)
Genus *Streptomyces*			
S. luteofluorescens (Shinobu, 1962)	40398		*Actinomadura luteofluorescens* (Preobrazhenskaya *et al.*, 1975a)
Genus *Thermomonospora*			
T. formosensis (Hasegawa *et al.*, 1986)	43997		*Actinomadura formosensis* (Zhang *et al.*, 1998)
T. fusca (McCarthy & Cross, 1984)	43792		*Thermobifida fusca* (Zhang *et al.*, 1998)
T. mesophila (Nonomura & Ohara, 1971c)	43048		*Microbispora mesophila* (Zhang *et al.*, 1998)
Genus *Thermopolyspora*			
T. flexuosa (Krassil'nikov & Agre, 1964)	43186	*Actinomadura flexuosa* (Meyer, 1989), *Microtetraspora flexuosa* (Kroppenstedt *et al.*, 1990)	*Nonomuria flexuosa* (Zhang *et al.*, 1998)

[a] Previously classified as *Streptothrix dassonvillei* (Brocq-Rousseau, 1904) and *Nocardia dassonvillei* (Liegard & L&rieu, 1911).

Nonomuria fastidiosa and *Nonomuria flexuosa*, respectively (Zhang *et al.*, 1998).

The genus *Thermomonospora.* *Thermomonospora* (Ther.mo.mon'o.spo.ra. Gr. n. *therme* heat; Gr. adj. *monos* single, solitary; Gr. fem. n. *spora* seed; M.L. fem. n. *Thermomonospora* the heat [-loving] single-spored). The genus *Thermomonospora* was proposed by Henssen in 1957a for thermophilic actinomycetes growing on composted horse manure. The genus contained three species the members of which formed single spores on aerial hyphae. Members of all of the species produced colourless-to-pale yellow colonies and white aerial hyphae and were distinguished from one another by the morphology of their aerial mycelia and the type of substrate hyphal branching. *Thermomonospora curvata*, the only species isolated and maintained in pure culture, was later designated the type species of the genus (Henssen & Schnepf, 1967). The description of the remaining two species, *Thermomonspora fusca* and *Thermomonspora lineata,* was based on morphological properties detected in contaminated preparations. Neither *Thermomonspora fusca* nor *Thermomonspora lineata* were included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) even though *Thermomonspora fusca* had been isolated in pure culture and well described (Crawford, 1975; Crawford & Gonda, 1977).

Mesophilic monosporic actinomycetes were subsequently assigned to the genus *Thermomonspora* as *Thermomonspora mesophila* Nonomura and Ohara 1971c and *Thermomonspora mesouviformis* Nonomura and Ohara 1974. A third mesophilic species, *Thermomonspora formosensis,* was described by Hasegawa *et al.* (1986) but cited as a *species incertae sedis* in *Bergey's Manual of Systematic Bacteriology* (McCarthy, 1989).

Krassilnikov and Agre (1964b) proposed the genus *Actinobifida* for actinomycetes that formed single spores on dichotomously branched sporophores. They recognised a single species, *Actinobifida dichotomica*, but failed to mention that dichotomous branching had previously been observed both in the genus *Thermomonospora* (Henssen, 1957a) and in species of the genus *Micromonospora* (Jensen, 1930, 1932; Krassilnikov, 1941).

Krassilnikov and Agre (1965) proposed a second species, *Actinobifida chromogena*, and suggested that all actinomycetes showing dichotomous branching be transferred to this genus. A third species, *Actinobifida alba*, was proposed by Locci *et al.* (1967).

All of the taxa mentioned above were subsequently reclassified. *Actinobifida dichotomica* Krassilnikov and Agre 1964, the type species of the genus, was transferrred to the genus *Thermoactinomyces* as *Thermoactinomyces dichotomica* due to its ability to produce endospores and *Actinobifida alba* Locci *et al.* 1967 was reclassified in the genus *Thermomonospora* as *Thermomonospora alba* as it formed heat-sensitive spores on substrate and aerial hyphae (Cross & Goodfellow, 1973). Similarly, *Actinobifida chromogena* was assigned to the genus *Thermomonospora* as *Thermomonospora chromogena* McCarthy and Cross 1984. In contrast, *Thermomonospora viridis* (Küster & Locci, 1963) was transferred to the genus *Saccharomonospora* as *Saccharomonospora viridis* (Nonomura & Ohara, 1971).

A comprehensive numerical taxonomic survey of the genus *Thermomonospora* and related taxa confirmed the taxonomic status of *Thermomonospora chromogena* and provided strong evidence for the formal recognition of *Thermomonospora fusca* (McCarthy & Cross, 1984). In contrast, *Thermomonospora mesouviformis* Nonomura and Ohara 1974 was considered to be a synonym of *Thermomonospora alba* Locci *et al.* 1967. These taxa, which were called the "white *Thermomonospora* group" because of their white aerial mycelia, were sharply distinguished from *Thermomonospora chromogena* (Krassilnikov & Agre, 1965; McCarthy & Cross, 1984), "*Thermomonospora falcata*" (Henssen, 1970) and similar actinomycetes from mushroom compost (McCarthy & Cross, 1981). The "chromogena" strains with reddish-brown colonies and light-brown aerial hyphae had provisionally been included in the genus *Thermomonospora* on the basis of wall composition and morphology (Cross, 1981). These developments left the genus *Thermomonospora* as a repository for wall chemotype III, thermophilic actinomycetes

which formed single, heat-sensitive spores either on aerial hyphae or on both aerial and substrate hyphae. This reliance on morphological properties, while understandable, was unfortunate as Cross and Lacey (1970) had reported an almost continuous range of morphological variation among monosporic thermophilic actinomycetes.

The discontinuous distribution of chemical markers underlined the heterogeneous nature of the genus *Thermomonospora* (Greiner-Mai *et al.*, 1987) and led to the constituent species being assigned to three distinct groups (Kroppenstedt & Goodfellow, 1992; Kudo, 1997). The first group, which contained *Thermomonosora curvata* and *Thermomonosora formosensis,* had chemotaxonomic properties similar to members of the genus *Actinomadura* (Greiner-Mai *et al.,* 1987), a relationship supported by 16S rRNA cataloguing and sequence data (Fowler *et al.,* 1985; Kroppenstedt *et al.,* 1990). Similarly, chemical data supported the transfer of *Thermomonospora chromogena* and *Thermomonospora mesophila* to the genus *Microtetraspora* as defined by Kroppenstedt *et al.* 1990. These proposals left *Thermomonospora alba* (including *Thermomonospora mesouviformis*) and *Thermomonospora fusca* as related species that were considered to merit generic status.

Zhang *et al.* (1998) clarified the taxonomy of the genera *Actinomadura, Microtetraspora* and *Thermomonospora* in a comprehensive 16S rRNA sequence study, the results of which are shown in Fig. 2-2. These authors proposed that *Thermomonospora formosensis* Hasegawa *et al.* 1986 be transferred to the genus *Actinomadura* as *Actinomadura formosensis* and *Thermomonospora mesophila* Nonomura and Ohara 1971 be reclassified in the genus *Microbispora* as *Microbispora mesophila.* In addition a new taxon, the genus *Thermobifida*, was proposed to accommodate the thermophilic actinomycetes, *Thermomonospora alba* (Locci *et al.* 1967) Cross and Goodfellow 1973 and *Thermomonospora fusca* McCarthy and Cross 1984. The taxonomic position of *Thermomonospora chromogena* was considered to be equivocal. These proposals left the

genus *Thermomonospora* as a monospecific taxon containing *Thermomonospora curvata.*

The revised genus *Thermomonospora* encompasses aerobic, Gram-positive actinomycetes which form branching substrate and aerial mycelia. Single spores are borne at the tips of short sporophores branching from aerial or substrate hyphae. The optimal temperature for growth is 45 to 55 °C. Thermomonosporae lack diagnostic sugars in whole-organism hydrolysates, contain *meso*-A_2pm as the diamino acid in the wall peptidoglycan, have MK-9(H_4) and -9(H_8) as predominant menaquinones, are rich in complex mixtures of straight and branched-chain fatty acids (fatty acid type 3a *sensu* Kroppenstedt, 1985), and contain diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides as major polar lipids (phospholipid type I *sensu* Lechevalier *et al.*, 1977). Thermomonosporae are closely related to actinomadurae on the basis of 16S rDNA sequence data and are common in overheated substrates such as bagasse, composts, fodder and manures.

The type species of the genus *Thermomonospora* is *Thermomonospora curvata* Henssen 1957a^{AL}.

The genus *Actinocorallia*. *Actinocorallia* (Ac.ti.no.co.ral'li.a. Gr. n. *actis* a ray; Gr. n. *corallium* coral; Gr. n. *Actinocorallia* referring to a microorganism that forms sporophores resembling coral). Zhang *et al.* (1998) showed that the monospecific genus *Actinocorallia* is closely related to the members of the family *Thermomonosporaceae*, notably *Actinomadura aurantiaca* JCM 8201^T and *Actinomadura libanotica* IFO 14095^T (Fig. 2-2). *Actinocorallia herbida* was proposed by Iinuma *et al.* (1994) to accommodate a novel isolate, strain IFO 15485^T, which formed coralloid sporophores on substrate hyphae and very occasionally coremia on solid media. The organism is an aerobic, Gram-positive actinomycete which produces non-fragmenting branched substrate hyphae and long chains of nonmotile spores on the tips of sporophores carried on the substrate mycelium. The temperature range for growth is 12 to 38 °C. The organism contains *meso*-A_2pm as the

major diamino acid of the wall peptidoglycan, has N-acetylated muramic acid, lacks characteristic sugars (wall chemotype III *sensu* Lechevalier & Lechevalier, 1970a, b), contains straight-chain saturated and monounsaturated fatty acids (fatty acid type 1a *sensu* Kroppenstedt *et al.*, 1985), but not mycolic acids, has MK-9(H_4) and MK-9(H_6) as predominant menaquinones and phosphatidylethanolamine as the diagnostic polar lipid (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The G+C content of the DNA is 73 mol%.

The type species of the genus is *Actinocorallia herbida* Iinuma *et al.* 1994.

The genus *Spirillospora*. *Spirillospora* (Spi.ril.lo.spo'ra. Gr. n. *speira* coil; Gr. n. *spora* a seed, spore; M.L. fem. n. *Spirillospora* an organism with spores in spirals). Members of the genus *Spirillospora* have chemical profiles that are consistent with their assignment to the family *Thermomonosporaceae* (Zhang *et al.*, 1998). The genus *Spirillospora*, which was proposed by Couch (1963), contains aerobic, Gram-positive, mesophilic, chemo-organotrophic actinomycetes which produce spherical to vermiform spore vesicles (5-24 μm in diameter) on aerial hyphae. The spore vesicles enclose numerous spores that are arranged in coiled and branched spore chains. The spores are rod-shaped or curved (0.5-0.7 × 2.0-6.0 μm) and motile by means of one to seven subpolarly inserted flagella. The colour of the substrate mycelium is white to pale yellow or pale buffy pink to red; the aerial mycelium is usually white. The temperature range for growth is 18 to 35 °C.

The peptidoglycan of the cell wall contains *meso*-A_2pm, and madurose is the characteristic sugar of whole-organism hydrolysates (cell wall chemotype III, sugar pattern B *sensu* Lechevalier & Lechevalier (1970a, b). The organism contains tetra- and hexahyrogenated menaquinones with nine isoprene units as the predominant isoprenologues (Collins *et al.*, 1984), major proportions of *iso*- and *anteiso*-branched fatty acids (Kroppenstedt & Kutzner, 1978), and diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol dimannosides as major polar lipids with

phosphatidylethanolamine variably present (phospholipid type I *sensu* Lechevalier *et al.*, 1977). The G+C content of the DNA is 71.0 to 73.0 mol%. Members of genus *Spirillospora* have been isolated from soil but only infrequently.

The type species of the genus is *Sprillospora albida* Couch 1963[AL].

The family *Thermomonospora sensu novo.* This family currently encompasses the genera *Actinocorallia, Actinomadura, Excellospora, Spirillospora* and *Thermomonospora.* Members of the constituent taxa share many properties in common but can be distinguished using a combination of chemical and morphological markers (Table 2-8). The taxonomic histories of thermophilic actinomycetes classified in the families *Nocardiopsiaceae, Thermomonosporaceae* and *Streptosporangiaceae* are shown in Table 2-9.

3. Present study

The primary aim of the current aspect of the present study was to establish the taxonomic status of two thermophilic, neutrophilic actinomycetes, namely, strains NT202 and NT303, and four alkalitolerant, thermophilic organisms, that is, strains TA86, TA111, TA113 and TA114. All of these organisms were isolated from arid and tropical soil samples after 5 days of incubation at 55°C using starch casein agar supplemented with cycloheximide and rifampicin and adjusted to pH 7.0 and pH 10.5 , respectively (Sahin, 1995). The test strains were assigned to two distinct clusters in a numerical phenetic survey designed to determine the taxonomic diversity shown by thermophilic organisms provisionally assigned to the genus *Streptomyces* (Sahin, 1995). However, all six strains contained *meso*-A_2pm as the major diamino acid of the wall peptidoglycan and hence could not be classified in the genus *Streptomyces.* Strains NT202 and NT303 were then provisionally assigned to the genus *Amycolatopsis,* and strains TA86, TA111, TA113 and TA114 to the genus *Excellospora,* on the basis of chemical and morphological properties. The organisms were the subject of a polyphasic taxonomic study, which involved

phenotypic, chemotaxonomic and molecular systematic analyses, in order to clarify their taxonomic positions.

Table 2-8. Morphological and chemical profiles of genera classified in the family *Thermomonosporaceae* [a]

	Actinocorallia	*Actinomadura*	*Excellospora* [b]	*Spirillospora*	*Thermomonospora*
Morphological characteristics					
Fragmentation of substrate mycelium	-	-	-	-	-
Aerial mycelium	+	+	+	+	+
Spores	> 30	2-15	1-20	many	one
Spores in spore vesicles	-	-	-	+	-
Spore motility	-	-	-	+	-
Temperature range	Mesophilic	Mesophilic	Thermophilic	Mesophilic	Thermophilic
Chemical characteristics					
Wall chemotype [c]	III	III	III	III	III
Peptidoglycan type [d]	A1γ	A1γ	A1γ	A1γ	A1γ
Whole-cell sugars [e]:	C	B	B	B	C
Galactose	+	+ [e]	+	ND	ND
Madurose	-	+	+	+	-
Mannose	+	+	-	ND	ND
Ribose	+	+	-	ND	ND
Fatty acids [f]	1a	3a	3a	3a	3a
Menaquinones [g]	MK-9(H_4,H_6)	MK-9(H_4,H_6,H_8)	MK-9(H_4,H_6,H_8)	MK-9(H_4,H_6)	MK-9(H_4,H_6,H_8)
Phospholipid type [h]	II (PE, PG and others)	I (PI, PIM^{Y}, PG^{Y}, DPG)	I (PI, PIM, PG, DPG)	I (PI, PIM, PG, DPG)	I (PI, PIM, PG, DPG)
G+C ratio of DNA	73	66-72	ND	71-73	ND

+, present; -, absent.

[a] Data taken from Kroppenstedt *et al.* (1990), Kroppenstedt & Goodfellow (1992), Goodfellow (1992) and Iinuma *et al.* (1994).

[b] Includes data on *Actinomadura rubrobrunea,* as well as on *Excellospora viridilutea.*

[c] Wall chemotypes (Lechevalier & Lechevalier, 1970a, b): I, LL- A_2pm; II, *meso*-A_2pm and glycine; III, *meso*-A_2pm, and IV, *meso*-A_2pm, arabinose and galactose.

[d] $A1_{\gamma}$: A, cross-linkage between positions 3 and 4 of adjacent peptide subunits; 1, peptide bridge absent; γ, *meso*-A_2pm at position 3 of the tetrapeptide subunits (Schleifer & Kandler, 1972).

[e] Sugar type: B, presence of madurose; C, absence of any of the diagnostic sugars. Whole-organism hydrolysates of *Actinomadura madurae* DSM 43067^T, *Actinomadura oligospora* DSM 43930^T and *Actinomadura spadix* DSM 43459^T do not contain galactose.

[f] Fatty acid types according to Kroppenstedt (1985): type 1a contains major proportions of hexadecanoic acid (16:0), octadecanoic acid (18:0) and monounsaturated octadecanoic acid (18:1); type 3a major proportions of 14-methyl pentadecanoic acid (*iso*-16:0), monounsaturated heptadecanoic acid (17:1), 10-methyl heptadecanoic acid (10-methyl 17:0), monounsaturated octadecanoic acid (18:1) and 10-methyl octadecanoic acid (10-methyl 18:0).

[g] Abbreviations exemplified by MK-9(H_4), menaquinones having four of the nine isoprene units hydrogenated.

[h] Polar lipid types according to Lechvalier *et al.* (1977). Characteristic polar lipids: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol and PI, phosphatidylinositol.

Table 2-9. Taxonomic history and temperature range for growth of selected actinomycetes classified in the families *Thermomonosporaceae, Streptosporangiaceae* and *Nocardiopsiaceae* and related taxa

Current assignment	DSM numbers	Other names	Original assignment	Optimal growth temperature	References
Family *Thermomonosporaceae*					
Actinomadura formosensis	43997^T	*Thermomonospora formosensis*	*Thermomonospora formosensis*	**23-42**	Hasegawa *et al.* (1986), Zhang *et al.* (1998)
Actinomadura rubrobrunea	43750^T	*Excellospora rubrobrunea*	*Micropolyspora rubrobrunea*	**50-55**	Agre & Guzeva (1975), Kroppenstedt *et al.* (1991)
Actinomadura rubrobrunea	43751	*Excellospora viridinigra*	*Micropolyspora viridinigra*	**50-55**	Agre & Guzeva (1975), Kroppenstedt *et al.* (1991)
Excellospora viridilutea	43934^T		*Excellospora viridilutea*	**50-55**	Agre & Guzeva (1975)
Thermomonospora curvata	43183^T		*Thermomonospora curvata*	**50**	Henssen (1957)
Family *Streptosporangiaceae*					
Microbispora mesophila	43048^T	*Thermomonospora mesophila*	*Thermomonospora mesophila*	**28**	Nonomura & Ohara (1971), Zhang *et al.* (1998)
Microbispora rosea subsp. *aerata*	43176^T	*Microbispora aerata*	*Waksmania aerata*	**50**	Gerber & Lechevalier (1964), Miyadoh *et al.* (1991)
Microbispora rosea subsp. *aerata*	43166	*Microbispora aerata*	*Microbispora thermodiastatica*	**45**	Miyadoh *et al.* (1991)
Nonomuria angiospora	43173^T	*Microtetraspora angiospora, Actinomadura angiospora*	*Micropolyspora angiospora*	**28**	Zhukova *et al.* (1968), Zhang *et al.* (1998)
Nonomuria flexuosa	43186^T	*Microtetraspora flexuosa, Actinomadura flexuosa*	*Thermopolyspora flexuosa*	**50-55**	Meyer (1989), Zhang *et al.* (1998)
Family *Nocardiopsiaceae*					
Thermobifida alba	43185	*Thermomonospora alba*	*Thermomonospora mesouviformis*	**45**	Locci *et al.* (1967), Zhang *et al.* (1998)
Thermobifida alba	43795^T	*Thermomonospora alba*	*Actinobifida alba*	**37**	Locci *et al.* (1967), Zhang *et al.* (1998)
Thermobifida fusca	43792^T	*Thermomonospora fusca*	*Thermomonospora fusca*	**50**	McCarthy & Cross (1984), Zhang *et al.* (1998)
Related taxa					
Thermobispora bispora	43038^T	*Microbispora bispora*	*Thermopolyspora bispora*	**50**	Henssen (1957), Wang *et al.* (1996)
Thermomonospora chromogena	43794^T		*Actinobifida chromogena*	**50**	McCarthy & Cross (1984)

Optimal temperature data were taken from Greiner-Mai *et al.* (1987) and *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989).

Materials and Methods

1. Strains and cultivation

The test strains (Table 2-10) were cultivated on inorganic salt-starch agar (ISP medium 4, Difco; Shirling & Gottlieb, 1966), oatmeal agar (ISP medium 3; Shirling & Gottlieb, 1966) and modified Bennett's agar plates (Jones, 1949) at 45 °C and maintained as suspensions of mycelial fragments in glycerol (20%, v/v) at - 20 °C (Wellington & Williams, 1978). The frozen glycerol suspensions served as a convenient means of long term preservation and as a ready source of inoculum. Inocula were obtained by thawing suspensions at room temperature for approximately 15 minutes. After use, the glycerol suspensions were immediately stored at –20 °C.

Biomass for the chemical and molecular systematic analyses was obtained by growing the strains in 50 ml trypticase soy broth (Difco Laboratories, Detroit, USA) in 250 ml shake flasks (*ca.*150 rpm) for 3 days at 45 °C when growth was checked for purity by subculturing onto inorganic salt-starch agar or modified Bennett's agar plates. The flasks were inoculated with single colonies taken from agar plates and biomass harvested by centrifugation at 6,000 rpm for 10 minutes. The cells used for the chemical studies were washed in distilled water and freeze dried; those required for the molecular systematic investigations were washed in sterile NaCl-EDTA buffer (0.1 M EDTA, pH 8.0; 0.1 M NaCl) and stored at –20 °C until needed.

2. Acquisition of phenotypic data

Morphology and pigmentation. The isolates were examined for aerial spore mass colour, substrate mycelium colour and soluble pigment production following incubation on modified Bennett's agar (Jones, 1949), Czapek Dox agar (Oxoid, Basingstoke, Hampshire,

Table 2-10. Designations, sources and methods used to examine the test strains

Laboratory number	Taxa	Strain histories	Phenotypic properties	Morphology	16S rDNA sequencing	DNA:DNA pairing	Menaquinone profiles	Polar lipid profiles
239^T	*Amycolatopsis methanolica*	NCIB 11946^T; soil, New Guinea	✓	✓		✓	✓	
N1165^T	*Amycolatopsis thermoflava*	J. Chun; "*Nocardia thermoflava*" IFO 19333^T	✓				✓	
NT202	*Amycolatopsis* sp.	N. Sahin; scrubland soil, Madurai, India	✓	✓	✓	✓	✓	
NT303	*Amycolatopsis* sp.	N. Sahin; arid soil, Van, Turkey	✓	✓	✓	✓		
TA86	*Excellospora* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia		✓	✓			
TA111	*Excellospora* sp.	N. Sahin, garden soil, Yogyakarta, Indonesia		✓	✓		✓	✓
TA113	*Excellospora* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia		✓	✓		✓	✓
TA114	*Excellospora* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia		✓	✓			

UK), glucose yeast extract agar (GYEA; Gordon & Mihm, 1962), glycerol asparagine agar (ISP medium 5, Difco; Shirling & Gottlieb, 1966), inorganic salts-starch agar (ISP medium 4, Difco; Shirling & Gottlieb, 1966) and oatmeal agar (ISP medium 3; Shirling & Gottlieb, 1966), and for the production of melanin pigments on peptone yeast extract iron (ISP medium 6, Difco; Shirling & Gottlieb, 1966) and tyrosine agars (ISP medium 7, Difco; Shirling & Gottlieb, 1966). All of the plates were incubated at 45 °C for 5 days.

Spore chain morphology and spore ornamentation of organisms grown on Czapek Dox (Weyland 1969) and oatmeal agar plates (ISP medium 3; Shirling & Gottlieb, 1966) were examined by light and scanning electron microscopy (SEM). Spore chain morphology was observed using a Nikon Optiphot binocular light microscope fitted with long working distance objectives; spore chains were assigned to the morphological categories proposed by Pridham *et al.* (1958). Spore surface ornamentation was determined on SEM preparations using a Joel JSM-51 scanning electron microscope; the spore surface ornamentation categories of Tresner *et al.* (1961) were used.

Degradation and nutritional tests. The degradation and growth tests were carried out using the media and methods described by Williams *et al.* (1983a). Inoculated plates were incubated at 45 °C for 7 days, apart from some of the temperature tests. Growth at 10 °C was detected after 15 days; the remaining temperature tests were read after 7 days (Table 2-16, page 140-142). Clearing of the insoluble compounds from under and around areas of growth was scored as a positive result in the degradation tests (Table 2-16).

The organisms were also examined for their ability to use 49 compounds as sole sources of carbon for energy and growth, and 23 compounds as sole sources of nitrogen for growth (Sahin, 1995). The various compounds were prepared as aqueous solutions, sterilised by filtration using disposable filters (0.45 µm; Acrodisc, Gelman Sciences, 600 South Wagner Road, Ann Arbor, Michigan, USA) and added to a molten basal medium (Boiron *et al.*, 1993; Appendix 4). When scoring results, growth on the test medium was

compared with that on the positive and negative control plates. The positive control plate contained glucose as the sole carbon source and glucose plus yeast extract as the sole carbon and nitrogen source; the negative control plates lacked a carbon source and a carbon plus nitrogen source, respectively. Strains were scored positive if growth on the test plate was greater than that on the negative control plate. Conversely, negative results were recorded where growth was less than or equal to that on the negative control plate. Acid production from sugars was determined after Gordon *et al.* (1974) using Replidishes. The basal inorganic nitrogen medium (Appendix 4) contained 1 %, w/v carbohydrate.

3. Chemotaxonomy

(a) Analysis of menaquinones

Isolation of isoprenoid quinones. The method described by Minnikin *et al.* (1984) was used to extract and purify isoprenoid quinones from the test strains. Dried biomass (*ca.* 50 mg) was placed in a test tube fitted with a Teflon-lined screw cap and 2 ml of aqueous methanol (10 ml of 3% ,w/v aqueous sodium chloride in 100 ml of methanol) and 2 ml of petroleum ether (b.p. 60-80 °C) added. The contents of the tube were mixed for 15 minutes using a tube rotator then centrifuged for 5 minutes at low speed. The upper organic phase, which contained the isoprenoid quinones, was transferred to a small glass vial and dried under nitrogen at room temperature. The preparations were stored in the dark at -20 °C as isoprenoid quinones are susceptible to strong light and high temperatures (Collins, 1994).

Preparative thin-layer-chromatography of isoprenoid quinones. The extracts containing the isoprenoid quinones were resuspended in 50 µl of petroleum ether (b.p. 60-80 °C) and applied as 2 cm bands on plastic-backed silica gel plates (10 cm × 10 cm; Merck 5735; Merck, Darmstadt, Germany). The thin-layer-chromatographic plates were developed in petroleum ether/acetone (95/5, v/v) and the single bands containing the

menaquinones visualised and located under UV light at 254 nm; a standard menaquinone (MK-4; Sigma Chemical Company, Dorset, UK) was co-migrated with the samples to help identify the position of the extracted menaquinones. The latter were detected as dark brown bands on a fluorescent yellow-green background. The bands were scraped from the plastic plates, deposited in 1.5 ml tubes containing 1 ml of diethyl ether, the preparations mixed thoroughly by vortexing and centrifuged at 13,000 rpm for 5 minutes. The supernatants were transferred to small vials, dried under nitrogen and stored in the dark at -20 °C.

Analysis of isoprenoid quinones by high-performance liquid chromatography. The purified menaquinones were resuspended in 50 µl of *n*-hexane and 10 µl of each sample injected into a HPLC instrument (Pharmacia LKB, Uppsala, Sweden) fitted with a reverse-phase column (Spherisorb octadecylsilane [ODS] 5 µm; Jones Chromatography Ltd., Mid Glamorgan, Wales, UK). Acetonitrile-isopropanol (75:25, v/v) was used as the mobile phase and the samples were detected at 254 nm. Retention times and peak areas were determined using an integrator (HP3396A; Hewlett Packard Ltd., Nine Mile Ride, Wokingham, Berkshire, England, UK).

(b) Analysis of polar lipids

Polar lipids extracted from the test strains were examined by two dimensional TLC and identified using published procedures (Minnikin *et al.*, 1984). The purified polar lipid extracts were dissolved in chloroform-methanol (2:1, v/v), and 10 µl samples applied to the corners of six silicagel aluminium sheets (10 by 10 cm; Merck Kieselgel 60 F254 no. 5554). Chromatography was carried out using chloroform-methanol-water (65:25:4, v/v) in the first direction and chloroform-acetic acid-methanol-water (40: 7.5:6:2, v/v) in the second direction. The following differential stains were used to determine the type of lipids present on the chromatograms.

Molybdophosphoric acid spray for the detection of all polar lipids (Gunstone & Jacobsberg, 1972; Suzuki et al., 1993). The first plate was sprayed with

molybdophosphoric acid (5 %, w/v in ethanol) and heated at 120 °C for 15 minutes to detect the presence of all lipids. The latter appeared as dark green/blue spots on a light green background.

Ninhydrin reagent for the detection of lipids containing free amino groups (Consden & Gordon, 1948). A second plate was sprayed with ninhydrin (0.2 %, w/v in water saturated butan-1-ol) and heated at 100 °C for 10 minutes. Phosphatidylethanolamine (PE) and phosphatidylmethylethanolamine (PME), which contain amino groups, appeared as pink spots which were gently ringed with pencil before lightly spraying with Zinzadze reagent.

Zinzadze reagent for the detection of phosphorus containing lipids (Dittmer & Lester, 1964). The Zinzadze reagent used to spray the second plate was prepared as follows: molybdenum trioxide (40.1 g) was added to 1 litre of 25 N sulphuric acid and the mixture boiled gently until all of the residue dissolved (Solution A). Powdered molybdenum (1.5 g) was added to 500 ml of solution A, and the mixture boiled gently for 15 minutes and left to cool (Solution B). Equal volumes of solutions A and B were mixed and the resultant solution diluted with two volumes of distilled water for use. After spraying, phosphatides appeared immediately on the plates as blue spots on a white background.

Periodate-Schiff reagent for the detection of lipids containing vicinal hydroxyl groups (Shaw, 1968). The third plate was treated with an aqueous solution of sodium metaperiodate (1 %, w/v) until it was saturated. It was then left for 10 minutes at room temperature to complete the oxidation and decolourised with sulphur dioxide gas (B.D.H., Poole, UK) to remove excess periodate. The decolourised plate was sprayed very lightly with Schiff's reagent (prepared by decolourisng a 1 %, w/v aqueous solution of pararosaniline hydrochloride with sulphur dioxide gas) before being treated with sulphur dioxide gas again. Phosphatidylglycerol (PG) gives a bright purple/pink spot immediately; phosphatidylinositol (PI) gives a brown colour, characteristic of compounds giving a

malondialdehyde residue on periodate oxidation, in a few minutes but glycolipids often require several hours before the characteristic blue-purple colour develops. The identities of PI and PG were verified by using this reagent.

α-Naphthol reagent for the detection of glycolipids (Jacin & Mishkin, 1965). A fourth plate was sprayed with α-naphthol reagent which consists of a 15 % w/v, α-naphthol solution in 9.5 %, v/v ethanol; 10.5 ml of this solution is then mixed with concentrated sulphruric acid (6.5 ml), 40.5 ml of 9.5 %, v/v ethanol and 4 ml water. The sprayed plate was heated at 100 °C for 10 minutes when phosphatidylinositol dimannosides (PIDM) and glycolipids (GL) were revealed as brown spots.

Draggendorff reagent (Wagner et al., 1961; Beiss, 1964). Another dried chromatogram was sprayed with Draggendorff reagent which contains 0.11 M potassium iodide and 0.6 mM bismuth nitrate in 3.5 M acetic acid. This reagent was used to detect phosphatidylcholine (PC), PE and PME which appear immediately as yellow-orange spots on a yellow background.

(c) DNA base composition

The base composition of genomic DNA isolated from *Amycolatopsis* strains NT202 and NT303 was determined by using the reverse phase HPLC method described by Tamaoka (1994) and the HPLC conditions outlined by Gerke *et al.* (1984). The analyses were performed on a Supelcosil LC-18S column (Supelco Inc., Bellefonte, PA, U.S.A.) with 5 μm particle size and column dimensions of 15 cm × 4.6 mm (internal diameter). Molar G plus C ratios were calculated using the methods described by Mesbah *et al.* (1989).

4. Sequencing and analysis of 16S rDNA

The procedure used to obtain and sequence the 16S rDNA of the test strains is outlined in

Fig. 2-3.

(a) Small scale preparation of genomic DNA

The guanidine thiocyanate DNA extraction procedure of Pitcher *et al.* (1989) was used with specific modifications to optimise the isolation of DNA from the test strains. Treatment of cells with proteinase K (100 µg/ml) and sodium dodecyl sulphate (SDS, final concentration 1%, w/v) was found to greatly facilitate the susceptibility of cells to the standard digestion and extraction procedure of Pitcher *et al.* (1989).

Solutions:

Guanidine thiocyanate solution (5 M guanidine thiocyanate; 100 mM EDTA; 0.5 %, v/v sarkosyl)

Guanidine thiocyanate ..... 60 g

Milli-Q water (autoclaved) ..... 20 ml

0.5 M EDTA, pH 8.0 ..... 20 ml

The guanidine thiocyanate was dissolved with constant stirring at 65°C and the resultant solution cooled to room temperature before adding:

N-laurylsarcosine 10% (v/v, autoclaved) ..... 5 ml

The final volume was made up to 100 ml with Milli-Q water and the solution filtered through a 0.45 µm membrane. The guanidine thiocyanate solution was stored at room temperature in a dark bottle.

7.5 M Ammonium acetate

Ammonium acetate ..... 57.81 g

Milli-Q water ..... to 100 ml

Figure 2-3. Protocol for PCR amplification, purification and sequencing of 16S rDNA

	Genomic DNA (50-100 ng) ↓	
27f and 1525r primers (0.4 µM each)	→ **Vortex and collect reaction mixture by centrifugation** ←	- PCR mix (100 µl) dNTPs: 200 mM each *Taq* buffer
	Denaturation	- 98 °C, 2 minutes - Ice, 1 minute
	Addition of *Taq* DNA polymerase	- 2 U *Taq* polymerase (0.5 µl) - 2 drops mineral oil - Vortexing and short pulse centrifugation (5 seconds)
	PCR (30 cycles)	- Denaturing: 94°C, 2 minutes - Annealing: 55°C, 1 minute - Extension: 72°C, 3 minutes - Final extension: 72°C, 10 minutes
λ *EcoR I*/*Hind* III → or λ *BstE* II DNA MW markers	**Check the size of amplified fragments** (5 µl of PCR product)	- Electrophoresis: 1%, w/v agarose, 0.5 µg ml-1 ethidium bromide, 100 V, 1 hour, 0.5 × TEB - Photography (optional)
If product ~ 1.4 kb →	**Gel purification of 16S rRNA fragment**	- Preparative electrophoresis - Centrifugation through Millipore filter units
16S rDNA reverse → and forward primers	**Automated sequencing**	- *Taq* dye-deoxy cycle sequencing (Applied Biosystems)

Final volume 100 ml. The solution was sterilised by autoclaving and stored at 4°C.

Chloroform-*iso*-amyl alcohol

Chloroform ..... 24 ml

Iso-amyl alcohol ..... 1 ml

Stored at 4°C in a sealed bottle.

Phenol-chloroform-*iso*-amyl alcohol

This reagent was purchased as a pre-mixed formulation (Sigma) of molecular biology grade phenol-chloroform-*iso*-amyl alcohol (25:24:1, v/v) saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0). The reagent was stored at 4 °C in a sealed dark bottle.

TE buffer (pH 8.0)

0.5 M EDTA, pH 8.0 ..... 2 ml

1 M Tris, pH 8.0 ..... 10 ml

Milli-Q water .....to 1 litre

If necessary, the pH of the solution was adjusted to 8.0 before making up to 1 litre. The buffer was sterilised by autoclaving and stored at room temperature.

RNase A stock solution

Dissolve pancreatic RNase (RNase A, Sigma) at a concentration of 10 mg/ml in 10 mM Tris-HCl,15 mM NaCl or distilled water. Heat to 100 °C for 15 minutes then cool to room temperature. Store at –20 °C.

Protease K stock solution

Dissolve powder of protease type I (Sigma) at a concentration of 10 mg/ml in 10 mM Tris-HCl, 10 mM NaCl and self-digest by incubating for 2 hours at 37 °C. Store at –20 °C.

Procedure:

1. Approximately 50 -100 mg wet weight biomass (one loopfull or rice grain size) of test strain growth from the surface of plates or pellet formed by centrifugation of liquid culture was transferred to an Eppendorf microfuge tube.
2. Biomass was resuspended in 100 µl of TE buffer (pH 8.0) containing 50 mg ml-1 dissolved lysozyme (Sigma) and incubated at 37 °C for 30 minutes.
3. Proteinase K (20 µl; 2 mg ml-1) and SDS (20 µl; 10%; final concentration 1%, w/v) were added to the preparation which was left at 45°C for an hour.
4. Lysis was accomplished by adding 500 µl of guanidine thiocyanate solution to the preparation followed by brief mixing and incubation at room temperature for 10 minutes.
5. The lysate was cooled on ice, 250 µl of 7.5 M ammonium acetate was added and the resultant preparation mixed by gently inverting the tube several times.
6. The lysate was incubated on ice for a further 10 minutes prior to the addition of 500 µl of chloroform-*iso*-amyl alcohol (24:1, v/v). The phases were emulsified by shaking by hand and separated by centrifugation at 13,000 rpm for 10 minutes.
7. The aqueous supernatant phase (upper layer) was transferred to a clean microfuge tube using a disposable plastic pipette tip and the DNA precipitated by the addition of a 0.54 volume of cold *iso*-propanol. The tube was inverted several times to mix the solutions or until a visible white fibrous precipitate was seen. DNA was pelleted by centrifugation at 6,500 rpm for 20 seconds.
8. The DNA pellet was washed twice with 70 % ethanol (v/v) and dried under vacuum. Short centrifugation pulses (30 to 60 seconds) were applied in case the pellet detached from the bottom of the tube during the washes.
9. The DNA pellet was redissolved in 90 µl of TE buffer (pH 8.0); RNA was removed by the addition of 10 µl of RNase A (10 mg ml-1 stock; Sigma) and the preparation

incubated for more than 1 hour at 37 °C.

10. Protein was removed from the preparation by the addition of 1 volume of phenol-chloroform-*iso*-amyl alcohol. After mixing thoroughly, the two phases were separated by centrifugation at 13,000 rpm for 3 minutes. The upper aqueous layer was transferred into a clean Eppendorf tube with 1 volume of chloroform-*iso*-amyl alcohol (24:1, v/v). After mixing thoroughly, the two phases were separated by centrifugation as described earlier. The upper aqueous layer was transferred to a clean Eppendorf tube.
11. DNA was precipitated by the addition of 2-3 volumes of cold absolute ethanol with 10 µl of 8 M lithium chloride (LiCl2), the phases mixed by hand (do not vortex) and centrifuged at 6,500 rpm for 3 minutes.
12. Remove the supernatant carefully and wash the pellet once with 70 % ethanol (v/v) prior to drying under vacuum. DNA was resuspended in 30 µl of Milli-Q water and stored at 4°C until needed.

Purity and quantitation of DNA samples. The purity and quantitation of the DNA samples was determined by taking spectrophotometric readings of diluted samples at 230, 260 and 280 nm. The measurement at 260 nm gave an estimate of the DNA content, the corresponding reading at 280 nm gave an indication of protein contamination, and the reading at 230 nm measured any contamination with small molecules, e.g., EDTA, guanidine or polysaccharide. A ratio $A_{260}/A_{230} > 2.0$ was indicative of a carbohydrate-free sample while a ratio $A_{260}/A_{280} > 1.8$ signified protein-free samples.

The DNA concentration was estimated as follows:

Reading × Dilution × 50 µg ml^{-1} = DNA µg ml^{-1}

(a reading of 1 at 260 nm is equivalent to 50 µg ml^{-1} of double stranded DNA (Sambrook *et al.*, 1989).

DNA integrity check. DNA samples were checked by agarose gel electrophoresis in a

checking minigel in order to ensure that they were not excessively sheared or fragmented as a result of the isolation procedure. One µl of each DNA sample was mixed with gel loading buffer (1µl, Sigma) and loaded onto the gel.

(b) PCR amplification of 16S rDNA

The 16S rRNA genes of the test strains were amplified by PCR using conserved primers (Lane, 1991).

Oligonucleotide primers used in PCR amplification and sequencing of 16S rDNA. Oligonucleotide DNA fragments used as primers (Table 2-11) for the PCR amplification and sequencing of almost complete 16S rDNA fragments were synthesised using a Beckman DNA synthesiser (Beckman Instruments Inc., Fullerton, USA) at the 200 pmol scale, according to the manufacturer's protocol. The synthesised oligonucleotides, which were received as dry partially purified precipitated DNA (200 pmol), were resuspended in 1 ml of autoclaved Milli-Q water and allowed to dissolve completely at 4°C overnight. Insoluble material was precipitated by centrifugation at 13,000 rpm and the supernatants transferred to 1.5 ml screw-cap cryogenic storage tubes (Sigma).

The DNA concentrations of the primer suspensions were determined by measuring A_{260} of 1:10 dilutions of the dissolved oligonucleotides in Milli-Q water using a 500 µl cuvette. Accurate concentration of the oligonucleotide primers was determined by using PRIMER software (Chun, 1995) which automatically calculated the molar concentration of the oligonucleotide fragments taking into account the number of nucleotide bases, GC content and the proximity effect of different base linkages, as recommended by Rychlik and Rhoads (1989).

The primers used for PCR amplification of the 16S rDNA were diluted to 20 µM and those for DNA sequencing to 1.6 pmol μl^{-1}. These master stocks were stored in 1.5 ml screw-cap cryogenic storage tubes (Sigma) at -20°C together with the remaining undiluted original primer suspensions. In order to minimise the risk of contamination of primer

Table 2-11. Oligonucleotide primers used in the PCR amplification and sequencing of 16S rDNA

Primer name	Sequence (5' to 3')[a]	Size	Binding site[b]		Usage[c]		Source
			5'	3'	PCR	Seq	
27f[c]	AGAGTTTGATCMTGGCTCAG	20	8	27	√		Lane (1991)
MG2f	GAACGGGTGAGTAACACGT	19	107	125		√	Chun (1995)
MG3f	CTACGGGRSGCAGCAG	16	342	357		√	Chun (1995)
MG4f	AATTCCTGGTGTAGCGGT	18	675	692		√	Chun (1995)
782r	ACCAGGGTATCTAATCCTGT	20	801	782		√	Chun (1995)
MG5f	AAACTCAAAGGAATTGACGG	20	907	926		√	Chun (1995)
MG6f	GACGTCAAGTCATCATGCC	19	1190	1208		√	Chun (1995)
1525r[c]	AAGGAGGTGWTCCARCC	17	1544	1525	√		Lane (1991)
M13f	GTTTTCCCAGTCACGAC	17	–[d]	–		√	Promega (1993)
M13r	CAGGAAACAGCTATGAC	17	–[e]	–		√	Promega (1993)

[a] Degeneracies according to Lane (1991): K= G:T; M= A:C; Y = C:T; R= A:G; S,=C:G; W,=A:T.

[b] Binding site on the 16S rRNA molecule; numbering according to the *Escherichia coli* numbering system (Brosius *et al.*, 1978).

[c] PCR, primers used in the PCR amplification of 16S rDNA; Seq, primers used in the dye-deoxy cycle sequencing of cloned 16S rDNA.

[d] Binding at positions 2944 to 2960 (5' to 3') of the pGEM-T plasmid vector (Promega Corporation, 1993), approximately 23 base pairs upstream from the cloning site.

[e] Binding at positions 177 to 161 (5' to 3') of the pGEM-T plasmid vector (Promega Corporation, 1993), approximately 80 base pairs downstream from the cloning site.

stocks with foreign DNA, a set of diluted primer stocks for PCR and sequencing were prepared by dispensing 100 µl aliquots of the master stocks in 0.5 ml Eppendorf tubes. The latter were stored in a separate container box at –20 °C.

PCR amplification of 16S rDNA. PCR amplification of 16S rDNA was carried out in a HybAid Omnigene automated thermocycler (HybAid, Teddington, UK) using 0.8 ml PCR microfuge t*ubes*. Taq DNA polymerase and reaction buffer were from Hoeffer (Bio*Taq*; Hoeffer Scientific Instruments, Newcastle-under-Lyme, UK). Deoxyribonucleosides (dATP, dCTP, dGTP and dTTP, lithium salts; Boehringer Mannheim) were mixed in a master stock in equimolar ratios; the final concentration of individual deoxyribonucleosides was 25 mM.

The following procedure was used for PCR amplification of 16S rDNA fragments:

1. Stock solutions of primers, dNTPs and 10 × *Taq* buffer were defrosted and kept on ice. *Taq* DNA polymerase was kept at -20° C until needed.
2. The necessary volume of reaction mix was prepared in a 0.8 ml PCR tube (or 1.5 ml Eppendorf tube), **kept on ice** and dispensed into individual PCR tubes. Usually 100 µl PCR reactions were performed but the protocol also works for 50 µl reactions.
3. The reagents for one PCR tube were mixed in the following order (volumes for one reaction):

27f primer (20 µM)	2 µl
1525r primer (20 µM)	2 µl
dNTP mix (25 mM each dNTP)	0.8 µl
10 × *Taq* polymerase buffer (Hoeffer)	10 µl
Milli-Q water	to 95 µl

The reagents were mixed by vortexing and collected at the bottom of the tube by short pulse centrifugation (5 seconds). The tubes were kept in ice and 95 µl of the PCR mix dispensed into each of the PCR reaction tubes.

4. Approximately 50 to 500 ng of genomic DNA dissolved in a 5 µl volume Milli-Q water was added to each 95 µl of reaction mix.
5. The contents of the tubes were thoroughly mixed by vortexing and collected at the bottom of the tube by short pulse centrifugation (5 seconds). Two drops of mineral oil were then added to the top of the reaction mixture.
6. The tubes were heated at 98 °C for 5 minutes on a PCR block and immediately cooled in ice for 5 minutes.
7. The *Taq* DNA polymerase was taken from storage at -20°C and put on ice just prior to being dispensed; 2.5 U (0.4 µl) of *Taq* DNA polymerase was added to the aqueous layer.
8. Tubes were kept in ice until ready for PCR.
9. The PCR reaction was performed according to the following conditions:

Initial denaturation	**Amplification**			**Final Extension**	**Cooling**
	Denaturation	**Annealing**	**Extension**		
94°C	94°C	55°C	72°C	72°C	25°C
2 minutes	1 minute	1 minute	3 minute	10 minutes	1 minute
1 cycle		30 cycles		1 cycle	1 cycle

10. The PCR reactions were kept at -20°C and the amplification products checked by electrophoresis on agarose gels (1%, w/v) in 1 × Tris-borate buffer (TBE; 89 mM Tris-borate and 2.5 mM EDTA, pH 8.0) containing 0.5 µg ml-1 ethidium bromide. A mixture of 5 µl of the PCR products and 1 µl of gel loading buffer (Sigma) were used. Gels were run at 100 V for 1 hour and the size of the amplified fragments estimated by comparison with λ *Eco* RI / *Hind* III or λ *Pst* I molecular size markers (Sigma).

Purification of PCR-amplified 16S rDNA. PCR-amplified 16S rDNA was purified by

preparative agarose gel electrophoresis (Sambrook *et al.*, 1989) followed by elution of the DNA fragments by centrifugation through Ultrafree-MC microfiltration units (reference UFC3 0HV 25; Millipore [UK] Ltd., Watford, UK).

The following procedure was used for purification of the PCR-amplified 16S rDNA fragments:

1. The complete volume of the PCR reaction (*ca.* 100 µl) was mixed with 20 µl of gel loading buffer (Sigma) and loaded into the well of a preparative agarose mini-gel (1 %, w/v) containing ethidium bromide (0.5 µg ml-1). The mini-gel was run in 0.5 × TEB for 1 hour at 100 V. DNA molecular size markers λ *Eco* RI / *Hind* III or λ *Pst* I (Sigma) were used as standards for the determination of DNA fragment sizes.
2. After electrophoresis, the gel was visualised using a UV transilluminator. The rDNA fragment was located by its molecular weight size (approximately 1.5 kb) and a gel slice containing the rDNA was cut using a clean glass coverslip. Exposure to UV light was kept to a minimum to avoid photo-nicking of the DNA fragments.
3. The agarose slice containing the rDNA fragment was transferred to a Millipore microfiltration unit.
4. The gel slices (held inside the tubes) were frozen at –70 °C for 30 minutes and allowed to defrost at room temperature.
5. The tubes were centrifuged at 6,000 rpm for 20 minutes at room temperature. The presence of DNA in the extracted solution collected in the lower tube was verified by an orange glow in the solution from the fluorescence of the ethidium bromide-DNA complex under UV light.
6. The DNA fragments were extracted once with 1 volume of phenol-chloroform-*iso*-amyl alcohol (25:24:1, v/v) and once with 1 volume of chloroform-*iso*-amyl alcohol (24:1, v/v), as described previously (small scale DNA extraction).
7. A 0.1 volume of 8M $LiCl_2$ was added and mixed well prior to the addition of 2 - 3

volumes of cold ethanol. The solutions were mixed thoroughly by inversion of the tubes and kept at –20 °C for 2 hours or at –70 °C for 30 minutes.

8. DNA was pelleted by centrifugation at 13,000 rpm for 10 minutes.
9. The pellet was washed once with cold ethanol (70%, v/v) and dried under vacuum.
10. The rDNA pellet was redissolved in 60 µl of Milli-Q water and stored at –20 °C until needed. DNA samples (3 µl) were checked by agarose gel electrophoresis to estimate the DNA concentration by comparison with DNA standards of known concentration (Sambrook *et al.*, 1989).

(c) Dye-Deoxy™ terminator *Taq* cycle sequencing of 16S rDNA

Sequencing of 16S rDNA fragments was performed using the Dye-Deoxy™ terminator *Taq* cycle sequencing protocol (Applied Biosystems, 1993) and oligonucleotide primers specifically designed for hybridising to conserved sites in the 16S rRNA molecule (Lane, 1991). Several forward and reverse sequencing primers were used (Table 2-11)

Sequencing reactions were prepared and run as recommended by the *Taq* Dye-Deoxy™ Terminator Cycle Sequencing Kit protocols (Applied Biosystems, 1993). A reaction premix containing the components of the sequencing reaction, except the DNA sample and primer, was made as follow (volume for 4 reactions):

5 × TACS buffer	16 µl
dNTP mix	4 µl
DyeDeoxy A terminator	4 µl
DyeDeoxy T terminator	4 µl
DyeDeoxy G terminator	4 µl
DyeDeoxy C terminator	4 µl
Ampli*Taq* DNA polymerase	2 µl

The reagents were mixed thoroughly by gentle vortexing and collected at the bottom of the tube by short pulse centrifugation (5 seconds). The mixture was dispensed into 0.8 ml PCR tubes (9.5 µl per tube). The premix was stored at -20°C for up to one month.

Approximately 1 µg of purified 16S rDNA (dissolved in Milli-Q water) and 3.2 pmol of primer (2 µl of the sequencing primer stock) were added to 9.5 µl of sequencing premix in a PCR tube kept on ice. The final volume was brought up to 20 µl with Milli-Q water and the reaction mixture overlaid with a drop of mineral oil. The contents were mixed thoroughly by gentle vortexing and collected at the bottom of the tube by short pulse centrifugation (5 seconds).

The thermal cycling was performed in a HybAid Omnigene automated thermocycler (HybAid, Teddington, UK). The PCR program was initiated immediately after placing the last PCR tube in the thermal cycler preheated to 96°C. The cycling consisted of 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, in a total of 25 cycles. The extension products were purified by using Centri-Sep spin columns to remove the excess DyeDeoxy terminators, according to recommended protocols (Applied Biosystems, 1993). Clean DNA samples were dried under vacuum and resuspended in 4 µl of a sequencing loading buffer (5 µl deionised formamide and 1 µl 50 mM EDTA, pH 8.0) by vigorous vortexing. The suspensions were collected at the bottom of the tubes by short pulse centrifugation (5 seconds) and denatured by heating at 90°C for 2 minutes followed by incubation in ice for a few minutes. Samples were loaded onto an Applied Biosystems 373A DNA sequencer and electrophoresed, according to the manufacturer's instructions (Applied Biosystems Inc., Warrington, UK) using a 6 % (w/v) polyacrylamide-urea gel.

(d) Analysis of 16S rRNA sequence data

Data from the automated sequencing of 16S rDNA in text file format were transferred into the AL16S program (Chun, 1995). Entries were identified by the strain name and primer used to generate the sequence. The 16S rDNA sequences of *Amycolatopsis* strains NT202 and NT303 were aligned manually with the nucleotide sequences of related taxa (Table 2-12) retrieved from the Ribosomal Database Project (RDP; Maidak *et al.*, 1997) and EMBL-GenBank databases (Benson *et al.*, 1998).

Table 2-12. *Amycolatopsis* and related strains used in phylogenetic studies together with their nucleotide sequence accession numbers

Taxa	Strain	GenBank /EMBL	References
Family *"Actinosynnemaceae"*			
Actinokineosporia riparia	IFO 14541^T	X76953	Warwick *et al.* (1994)
Actinosynnema mirum	DSM 43827^T	X84447	Yassin *et al.* (1995)
Kutzneria viridogrisea	JCM3282^T	U58530	Wang *et al.* (1996b)
Lentzea albidocapillata	IMMIB D-958^T	X84321	Yassin *et al.* (1995)
Saccharothrix australiensis	DSM 43800^T	X53193	Bowen *et al.* (1989)
Saccharothrix coeruleofusca	DSM 43679^T	X76963	Warwick *et al.* (1994)
Streptoalloteichus hindustanus	IFO 15115^T	D85497	Tamura *et al.* (1997)
Family *Pseudonocardiaceae*			
Actinopolyspora halophila	NCIMB 11472^T	X54287	Embley *et al.* (1988)
Amycolatopsis alba	DSM 44262^T	AF051340	Chun *et al.* (1998)
Amycolatopsis azurae	NRRL 11412^T	X53199	Embley *et al.* (1988)
Amycolatopsis coloradensis	NRRL 3218^T	AF051341	Chun *et al.* (1998)
Amycolatopsis fastidiosa	ATCC 31181^T	X53200	Embley *et al.* (1988)
Amycolatopsis japonica	DSM 44213^T	X77959	Goodfellow *et al.* (1997)
Amycolatopsis mediterranei	DSM 13685^T	X76957	Warwick *et al.* (1994)
Amycolatopsis methanolica	NCIB 11946^T	X54274	de Boer *et al.* (1990)
Amycolatopsis orientalis subsp. *orientalis*	DSM 40040^T	X76958	Warwick *et al.* (1994)
Amycolatopsis sulphurea	DSM 46092^T	AF051343	Chun *et al.* (1998)
Amycolatopsis thermoflava	IFO 14333^T	AF052390	Chun *et al.* (1998)
Amycolatopsis strain	K24		Zhou *et al.* in preparation
***Amycolatopsis* strain**	**NT202**	**AJ000285**	**This study**
Kibdelosporangium aridum subsp. *aridum*	DSM 43828^T	X53191	Bowen *et al.* (1989)
Prauserella rugosa	DSM 43194^T	AF051342	Kim & Goodfellow (1998)
Pseudonocardia alni	DSM 44104^T	X76954	Warwick *et al.* (1994)
Pseudonocardia autotrophica	DSM 43210^T	X54288	Embley *et al.* (1988)
Pseudonocardia compacta	DSM 43592^T	X76959	Warwick *et al.* (1994)
Pseudonocardia halophobica	DSM 43089^T	Z14111	McVeigh & Embley unpublished
Pseudonocardia hydrocarbonoxydans	DSM 43281^T	X76955	Warwick *et al.* (1994)
"Pseudonocardia nitrificans"	DSM 46012	X55609	Wersing *et al.*, unpublished
Pseudonocardia petroleophila	DSM 43193^T	X55608	Wersing *et al.*, unpublished

Pseudonocardia saturnea	DSM 43195^T	X76956	Warwick *et al.* (1994)
Pseudonocardia thermophila	ATCC 19285^T	X53195	Embley *et al.* (1988)
Saccharomonospora azurea	K161=NA128^T	Z38017	Kim *et al.* (1995)
"Saccharomonospora caesia"	INMI 19125	X76960	Warwick *et al.* (1994)
Saccharomonospora viridis	ATCC 15386^T	X54286	Embley *et al.* (1988)
Saccharopolyspora erythraea	NRRL 2338^T	X53198	Embley *et al.* (1988)
Saccharopolyspora gregorii	NCIB 12823^T	X76962	Warwick *et al.* (1994)
Saccharopolyspora hirsuta subsp. *hirsuta*	ATCC 27875^T	X53196	Embley *et al.* (1988)
Saccharopolyspora hordei	DSM 44065^T	X53197	Embley *et al.* (1988)
Saccharopolyspora rectivirgula	DSM 43747^T	X53194	Embley *et al.* (1988)
Thermocrispum agreste	DSM 44070^T	X79183	Korn-Wendisch *et al.* (1995)
Thermocrispum municipale	DSM 44069^T	X79184	Korn-Wendisch *et al.* (1995)
Thermomonospora curvata	DSM 43183^T	X97893	Rainey *et al.* (1996)

ATCC, American Type Culture Collection, Rockville, MD., U.S.A.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute of Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan; NCIMB, National Collection of Industrial and Marine Bacteria, St. Machar Drive, Aberdeen, Scotland, UK; NRRL, Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

Table 2-13. *Excellospora* and related strains used in phylogenetic studies together with their nucleotide sequence accession numbers

Taxa	Strain	GenBank /EMBL	References
Family *Thermomonosporaceae*			
Actinocorallia herbida	IFO 15485^T	D85473	Tamura *et al.* (1997)
Actinomadura atramentaria	IFO 14695^T	U49000	Wang (1996)
Actinomadura aurantiaca	JCM 8201^T	D50669	Itoh *et al.* (1995)
Actinomadura citrea	IFO 14678^T	U49001	Wang (1996)
Actinomadura coerulea	IFO 14679^T	U49002	Wang (1996)
Actinomadura cremea	IFO 14183^T	U49003	Wang (1996)
Actinomadura echinospora	IFO 14042^T	U49004	Wang (1996)
Actinomadura formosensis	JCM 7474^T	AF002263	Zhang *et al.* (1998)
Actinomadura fulvescens	IFO 14347^T	U49005	Wang (1996)
Actinomadura kijaniata	IFO 14229^T	U49006	Wang (1996)
Actinomadura libanotica	IFO 14095^T	U49007	Wang (1996)
Actinomadura luteofluorescens	IFO 13057^T	U49008	Wang (1996)
Actinomadura macra	IFO 14102^T	U49009	Wang (1996)
Actinomadura madurae	JCM 7436^T	U58527	Wang (1996)
Actinomadura rugatobispora	IFO 14382^T	U49010	Wang (1996)
Actinomadura verrucosospora	IFO 14100^T	U49011	Wang (1996)
Excellospora viridilutea	IFO 14480^T	D86943	Tamura *et al.* (1997)
***Excellospora* strain**	**TA111**	********	**This study**
***Excellospora* strain**	**TA113**	********	**This study**
Spirillospora albida	IFO 12248^T	D85498	Tamura *et al.* (1997)
Thermomonospora chromogena	JCM 6244^T	AF002261	Zhang *et al.* (1998)
Thermomonospora curvata	DSM 43183^T	X97893	Rainey *et al.* (1996)
Family *Nocardiopsiaceae*			
Nocardiopsis alba	DSM 43377^T	X97883	Rainey *et al.* (1996a)
Nocardiopsis dassonvillei	DSM 43111^T	X97886	Rainey *et al.* (1996a)

Nocardiopsis listeri	DSM 40297^T	X97887	Rainey *et al.* (1996a)
Thermobifida alba	JCM 3077^T	AF002260	Zhang *et al.* (1998)
Thermobifida fusca	ATCC 27730^T	AF002264	Zhang *et al.* (1998)
Family *Streptosporangiaceae*			
Herbidospora cretacea	IFO 15474^T	D85485	Tamura *et al.* (1997)
Microbispora mesophila	JCM 3151^T	AF002266	Zhang *et al.* (1998)
Microbispora rosea subsp. *aerata*	ATCC 15448^T	U48984	Wang *et al.* (1996a)
Microbispora rosea subsp. *rosea*	IFO 14044^T	D86936	Tamura *et al.* (1997)
Microtetraspora fusca	IFO 13915^T	U48973	Wang *et al.* (1996a)
Microtetraspora glauca	IFO 14671^T	U48974	Wang *et al.* (1996a)
Microtetraspora niveoalba	IFO 15239^T	U48976	Wang *et al.* (1996a)
Nonomuria africana	IFO 14745^T	U48842	Wang *et al.* (1996a)
Nonomuria angiospora	IFO 13155^T	U48843	Wang *et al.* (1996a)
Nonomuria fastidiosa	IFO 14680^T	U48844	Wang *et al.* (1996a)
Nonomuria ferruginea	IFO 14094^T	U48845	Wang *et al.* (1996a)
Nonomuria polychroma	IFO 14345^T	U48977	Wang *et al.* (1996a)
Nonomuria pusilla	IFO 14684^T	U48978	Wang *et al.* (1996a)
Nonomuria salmonea	IFO 14687^T	U48982	Wang *et al.* (1996a)
Planobispora longispora	IFO 13918^T	D85494	Tamura *et al.* (1997)
Planomonospora parontospora	IFO 13880^T	D85495	Tamura *et al.* (1997)
Planotetraspora mira	IFO 15435^T	D85496	Tamura *et al.* (1997)
Streptosporangium carneum	DSM 44125^T	X89938	Ward-Rainey *et al.* (1996)
Streptosporangium claviforme	DSM 44127^T	X89940	Ward-Rainey *et al.* (1996)
Streptosporangium corrugatum	IFO 13972^T	U48991	Wang (1996)
Streptosporangium longisporum	DSM 43180^T	X89944	Ward-Rainey *et al.* (1996)
Streptosporangium pseudovulgare	DSM 43181^T	X70428	Kemmerling *et al.* (1993)
Streptosporangium roseum	DSM 43021^T	X89947	Ward-Rainey *et al.* (1996)
Streptosporangium violaceochromogenes	JCM 3281^T	U48997	Wang (1996)
Streptosporangium viridialbum	JCM 3027^T	U48998	Wang (1996)

Thermobispora			
Thermobispora bispora operon rrnA	ATCC 19993 ^{T}	U83909	Wang *et al.* (1997)
Thermobispora bispora operon rrnB	ATCC 19993 ^{T}	U83910	Wang *et al.* (1997)

ATCC, American Type Culture Collection, Rockville, MD., U.S.A.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute of Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan.

Similarly, the 16S rDNA sequences of *Excellospora* strains TA86, TA111, TA113 and TA114 were aligned with corresponding sequences of related taxa drawn from the databases (Table 2-13). In all cases alignment of the nucleotide sequences took into account the secondary structural features of the 16S rRNA molecule using functions incorporated in the AL16S program (Chun, 1995). The sequence ends, which usually contained poor-quality data (ambiguities and duplicated bases), were deleted at this stage of the analysis. A consensus sequence was derived by juxtaposition of several partial sequences obtained by using the different sequencing primers (Table 2-11). Ambiguities were tentatively resolved by referring to the corresponding sequence printouts and by checking with the stable secondary structure. Where necessary, additional sequencing reactions were performed or else the base was identified as an 'N'. The G+C ratio of the 16S rRNA sequences was calculated by using the AL16S program.

For each group of strains, evolutionary trees were inferred by using three treeing algorithms, namely, the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining methods (Saitou & Nei, 1987). Evolutionary distance matrices for the least squares and neighbour-joining methods were generated following established procedures (Jukes & Cantor, 1969; Kimura, 1980). The PHYLIP software package (Felsenstein, 1993) was used for all of the phylogenetic analyses. The resultant unrooted tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method data based on 1000 re-samplings using the SEQBOOT and CONSENSE programs in the PHYLIP package. The root positions were estimated by using outgroup organisms, as described by Swofford and Olsen (1990).

Treefiles obtained from the PHYLIP analyses were used as input files into the TREEVIEW program (Page, 1996) for displaying, changing the order of trees and saving phylogenetic trees in graphic format. Microsoft Word software (Microsoft Corporation, Redmond, WA, USA) was used to edit and change the names of strains to italicised

typographical types prior to printing.

5. DNA:DNA hybridisation

The DNA relatedness assays (Table 2-10) were performed by using the direct binding filter hybridisation method following a well established procedure (Gillespie & Spiegelman, 1965; Denhardt, 1966; Mordarski *et al.*, 1976; Meyer & Schleifer, 1978).

(a) Large scale isolation of DNA

Large scale DNA preparations were used to obtain yields in the range of several milligrams of DNA, as required for the DNA relatedness experiments. The protocol used was a modification (Mordarski *et al.*, 1976; Hopwood *et al.*, 1985) of the well established lysozyme and phenol-chloroform procedure of Saito and Miura (1963).

Solutions:

P-buffer (25 mM Tris-HCl, pH 8.0; 25 mM EDTA; 0.3 M sucrose; pH 8.0)

1 M Tris-HCl, pH8.0.....2.5 ml

500 mM EDTA, pH 8.0.....5 ml

10.3 % sucrose (w/v, autoclaved).....92.5 ml

Water up to a final volume of 100ml

Sterilise by autoclaving at 121 °C for 20 minutes.

Buffer for phenol solution (0.1 M Tris-HCl; 1 %, w/v SDS; 0.1 M NaCl; pH 9.0)

1 M Tris-HCl, pH 9.0.....10 ml

20 %, w/v SDS.....0.5 ml

5 M NaCl.....2 ml

Water up to final volume 100 ml.

Sterilise by autoclaving at 121 °C for 20 minutes.

Phenol solution 1 (always use freshly made solution)

10 N NaOH.....0.8 ml

Phenol (distilled)..... 85 ml

Buffer for phenol..... 15 ml

Phenol solution 2 (always use freshly made solution)

10 N NaOH.....0.8 ml

Phenol (distilled)..... 85 ml

H_2O.....15 ml

Crystalline phenol should be redistilled to remove oxidation products.

Commercial liquified phenol can be used without redistillation.

20 x SSC solution (saline-sodium citrate buffer; pH 7.0)

NaCl.....175.3 g

Sodium citrate, trisodium salt... .....88.2 g

Sterilise by autoclaving.

Sodium acetate solution (3 M sodium acetate, 1 mM EDTA; pH 7.0)

3 M sodium acetate, pH 7.0.....99.8 ml

0.5 M EDTA.....0.2 ml

Sterilise by autoclaving.

RNaseA stock solution

Dissolve pancreatic RNase (RNase A; Sigma) at a concentration of 10 mg ml^{-1} in 10 mM Tris-HCl,15 mM NaCl solution or distilled water. Heat to 100°C for 15 minutes. Cool to room temperature and store at -20°C.

Protease K stock solution

Dissolve protease type I powder (Sigma) at a concentration of 10 mg ml^{-1} in 10 mM Tris-HCl, 10 mM NaCl solution and self-digest by incubating for 2 hours at 37 °C. Store at –20 °C.

Procedure:

1. Resuspend biomass in P-buffer (3-5 ml g^{-1} wet weight of biomass), add lysozyme (2 mg lysozyme / ml P-buffer) and mix well. Usually 1 mg of pure DNA can be extracted from a few grams of wet biomass.
2. Incubate for 20 to 30 minutes at 37 °C until protoplasts can be observed under the microscope.
3. Add 20 %, w/v SDS to give a final concentration of 2 % and incubate at room temperature until the solution becomes transparent.
4. Add an equal volume of phenol solution 1 and mix well and carefully for 15 minutes at 4 °C.
5. Centrifuge for 10 minutes at 10,000 rpm at 4 °C and transfer the upper phase to an Erlenmeyer flask.
6. Add 1/10 volume of sodium acetate solution and 2 volumes of absolute ethanol, spool DNA on a glass rod and then dissolve in 7 ml of 0.1 x SSC (overnight).
7. Add RNase A to give a final concentration of 100 µg ml^{-1} and incubate at 37 °C for 30 minutes.
8. Add protease K to give a final concentration of 50 µg ml^{-1} and incubate at 45 °C for 60 minutes.
9. Repeat steps 4-5 with neutral solution 2 and transfer the upper phase to an Erlenmeyer flask.
10. Add a 1/10 volume of sodium acetate solution and 2 volumes of absolute ethanol, spool DNA on a glass rod and dissolve in 0.1 X SSC (overnight).
11. Add a 1/10 volume of sodium acetate solution and 1 volume isopropanol, spool DNA on a glass rod, wash with 70 % ethanol and dissolve DNA in 0.1 x SSC overnight (for immobilisation of DNA on nitrocellose filter; for radio-labelling and restriction analysis DNA should be dissolved in TE buffer).
12. Check the purity and concentration of DNA solutions by using the spectrophotometry as

described earlier (see small scale preparation of genomic DNA). Check residual phenol by spectrophotometry at 272 nm; if the purity is lower than the required value, repeat step 11 (Sambrook, 1989; Hopwood, 1985).

13. Check the molecular weight of the extracted DNA by agarose gel electrophoresis (0.7 %, w/v).

(b) Loading DNA on nitrocellulose filters

Nitrocellulose membrane filters (Sartorius AG, Göttingen, Germany; type number, 11407-50-N; white with black grid; 50 mm diameter; 0.2 μm pore size) were thoroughly soaked in 6 x SSC at 4 °C and then placed into clean glass vacuum filter holders (Sartorius; type number, SM16316; 47/50 mm diameter; 250 ml volume) with PTFE-coated stainless steel filter supports. Gentle vacuum pressure was applied and 50ml of 6 x SSC was filtered through the system to prime the membranes. Meanwhile purified DNA stocks were diluted to 150 μg ml^{-1} in 1 x SSC. The DNA concentration of the diluted DNA solutions was confirmed by reading absorbance at 260nm. For each filter, 5 ml of the diluted DNA sample was alkali-denatured by mixing with an equal volume of 1 M NaOH in a clean glass flask and the preparation incubated at room temperature for 20 minutes. The preparation was then neutralised by thoroughly mixing with 4 volumes (40 ml) of neutralisation solution (0.25 M Tris-acetate; 0.5 M NaCl; 0.25 M HCl) and the denatured DNA (single-stranded DNA) solution kept at 4 °C throughout the loading procedure. An initial 10 ml aliquot of denatured DNA solution was applied to the filter unit without vacuum suction and the A_{260} of the filtrate used to estimate the amount of DNA retained by the membrane. The subsequent aliquots (40-50 ml) were applied under mild vacuum pressure, the latter was increased as the filter became saturated with DNA. Blank membrane filters were prepared by filtering an equivalent mixture lacking DNA. After final washing with 50 ml of 6 x SSC, the loaded membrane filters were carefully labelled at the edge with a soft pencil and allowed to dry thoroughly at room temperature overnight. The

DNA was heat-fixed by baking at 80 °C for 2 hours. The membranes were kept in glass Petri dishes in a desiccator at 4 °C.

(c) Preparation of labelled reference chromosomal DNA

Reference DNA in TE buffer was labelled by nick-translation (Rigby *et al.*, 1977), using a standard protocol and commercial nick-translation kit (Amersham life Science, Little Chalfont, Buckinghamshire, UK). Deoxy [1',2,'5-^{3}H] cytidine 5'-triphosphate (^{3}H dCTP) supplied at the specific activity of 1 μCi μl^{-1} was dissolved in ethanol / water (1:1, v/v; Amersham Life Science). Tritium cannot be monitored directly because of its low beta-energy and hence special care is needed to handle it. Tritium compounds can be absorbed through the skin and DNA precursors, for example ^{3}H dCTP, are more toxic than tritiated water, partly because activity is concentrated into cell nuclei.

Procedure:

1. Add 17.5-20 μl of ^{3}H-dCTP solution (17.5-20 μCi) to a 1.5 ml Eppendorf tube and dry under nitrogen gas in a safety fumehood. The tubes containing dry ^{3}H-dCTP are stored at –20 °C until needed.
2. Add the following components to Eppendorf tubes containing dry ^{3}H-dCTP and mix well.

   DNA.....2-3 μg

   Nucleotide buffer.....20 μl

   Enzymes (DNase I and Klenow DNA polymerase).....10 μl

   H_2O up to a final volume of 100 μl.

3. Incubate at 14 to 15 °C for 1.5 hour and stop labelling reaction by adding 4 μl of 0.5 M EDTA (pH 8.0). Reaction solution stored at 4 °C.
4. Rinse ELUTIP-D column (Reference number NA010/2; Schleicher & Schuell GmbH, Dassel, Germany) with 1 to 2 ml of a high salt solution (1.0 M NaCl; 20 mM Tris-HCl

[pH 7.4]; 1.0 mM EDTA) and prime column with 5 ml of a low salt solution (0.2 M NaCl; 20 mM Tris-HCl [pH 7.4]; 1.0 mM EDTA).

5. Slowly load column with labelling reaction solution and wash column with 2 to 3 ml of low salt solution.
6. Elute DNA from the column with 0.4 ml of the high salt solution into an Eppendorf tube.
7. Measure the radioactivity of the DNA solution (5 µl eluate + 2 ml liquid scintillation cocktail) using a liquid scintillation counter (Beckman, USA) and store labelled DNA at 4 °C.

(d) Pre-hybridisation

For the hybridisation experiments, duplicated 5 mm diameter disks were cut, using a calibrated cork-borer, from each of the membrane filters corresponding to a test DNA and from the blank membrane. Filters with this diameter contained approximately 12 µg of DNA. However, filters of other diameters (4 to 6 mm) were sometimes employed to ensure that the same amount of DNA was used for all hybridisations. The disks were placed into labelled clean glass bijoux bottles (5 ml volume capacity) fitted with rubber-sealed screw caps. Individual filters were pre-hybridised for 3 to 5 hours in a shaking bath at 60 °C with 0.5 ml of a pre-hybridisation solution that consisted of 5 x Denhardt solution (v/v; Denhardt, 1966) and 4 x SSC (v/v). The Denhardt solution was prepared as a 50 x Denhardt stock solution containing 1 %, w/v bovine serum albumin (Fraction V, Sigma), 1 %, w/v Ficoll (type 400, Pharmacia) and 1 %, w/v polyvinylpyrrolidone in sterile water and stored at –20 °C.

(e) Hybridisation

The probe DNA (0.4 ml) prepared using the procedure described above was thoroughly mixed with hybridisation buffer (3 x SSC and 35 % deionised formamide, v/v) at room temperature and the mixture immediately dispensed into Eppendorf tubes (0.2 ml per

tubes), including an extra tube for scintillation counting. Formamide (Sigma) was deionised using an ion exchange resin (AG® 501-X8, analytical grade mixed bed resin; Bio-RAD). Probe activity was estimated by scintillation counting using 25 µl of the hybridisation solution in 2 ml of Bray's liquid scintillation cocktail. Using clean tweezers, the membrane filter disks were transferred from the pre-hybridisation solution into Eppendorf tubes which contained 0.2 ml of the hybridisation solution and approximately 50,000 cpm of labelled DNA. The filters were carefully placed at the bottom of the tubes with the DNA side facing up. The Eppendorf tubes were transferred to a floating rack and hybridisation carried out at 60 °C for 24 hours in a water bath. After hybridisation, the filter disks were washed with 5 ml of 3 x SSC and dried overnight on filter paper. The filter disks were then baked at 80 °C for 50 minutes prior to scintillation counting.

The concentrations of SSC and formamide and the hybridization temperature were designed to achieve optimal hybridization conditions, that is, 25 °C below the melting temperature (*T*m) assuming that the mean DNA base composition of *Streptomyces* strains is 70 mol % G plus C.

(f) Scintillation counting

The dried filters were transferred to plastic scintillation vials containing 2 ml of 206 scintillation liquid and the vials carefully capped and placed inside clean glass holders. The 206 scintillation cocktail consisted of 0.25 g of 1,4,bis-2-(5-phenyloxazolyl-2)-benzene (POPOP) and 3 g of 2,5-diphenyloxazole (PPO) per litre of toluene (Kilpper-Bälz, 1991). Counting was performed in a LKB-Wallac 1409/11 LSC (Version 1.6) scintillation counter (Pharmacia-LKB, Uppsala, Sweden). The counting mode was expressed as counts per minute (cpm) for ^{3}H isotopes (*Beta* spectrum). Background samples (blank filters) were placed into positions 1 and 2 of the sample holder with a counting time of 300 seconds and the average values automatically subtracted from all of the sample values. Filters from the homologous hybridisation reaction were placed in positions 3 and 4 and these values were

used as the 100% reference for direct calculation of the percent homology of the remaining samples.

The amount of DNA probe that hybridised by specific base-pairing between similar sequences to DNA immobilised to filters is estimated from the radioactivity values of the filters from the homologous and heterologous hybridisation reaction. DNA homology is calculated as follows:

$$\mathbf{H = (r/R) \times 100\ (\%)}$$

where H is the DNA relatedness, R(cpm) the radioactivity of the filter with reference DNA and r(cpm) the radioactivity of the filter with the test DNA.

The degree of relatedness between two DNA preparations may differ depending on which DNA preparation is labelled. Such results can be expected as the hybridisation capacities of each filter are affected by the amount, purity, and length of the labelled DNA. In such a case the degree of relatedness between two DNA preparations can be determined by using the following equation:

$$\mathbf{H = \sqrt{(H_A \times H_B)}}$$

where H_A is the apparent DNA homology between DNA A and DNA B when A is radiolabelled, H_B is the apparent DNA homology between DNA A and DNA B when B is radiolabelled, and H is the estimated DNA homology between DNA A and DNA B.

If C_A and C_B are the hybridisation capacities of filters A and B (in ideal condition $C_A = C_B$)

$H_A = H \times (C_B/C_A)$ ----- equation 1

$H_B = H \times (C_A/C_B)$ ----- equation 2

equation1 $\times$ equation2

$H^2 = H_A \times H_B$

$\mathbf{H = \sqrt{(H_A \times H_B)}}$

DNA relatedness values were depicted either in a similarity matrix or in a UPGMA dendrogram.

Results and Discussion

1. Classification of thermophilic *Amycolatopsis* strains

The almost complete 16S rRNA sequence obtained for strain NT202 (1,477 nucleotides) was compared with the corresponding sequences of representatives of the family *Pseudonocardiaceae* and related taxa. It was apparent that the organism fell within the range of variation encompassed by the genus *Amycolatopsis* (Table 2-14; Figs. 2-4, 2-5 and 2-6). It is clear that strain NT202 forms a distinct clade with *Amycolatopsis methanolica* NCIMB 11946^T and *Amycolatopsis thermoflava* IFO 14333 ^{T} within the *Amycolatopsis* tree. This relationship is supported by the results obtained by using the least-squares (Fig. 2-4) and maximum-likelihood (Fig. 2-5) treeing algorithms and by the 100 % bootstrap value recorded using the neighbour-joining method (Fig. 2-6).

The 16S rDNA sequence similarities between strain NT202 and *Amycolatopsis methanolica* NCIMB 11946^T and *Amycolatopsis thermoflava* IFO 14333 ^{T} are 99.2 % and 98.8 % respectively, these similarity values correspond to 10 and 17 differences out of 1,328 nucleotide positions. A comparable scale of difference exists between members of several validly described species of *Amycolatopsis*, for instance, between *Amycolatopsis alba* DSM 44262^T and *Amycolatopsis coloradensis* NRRL 3218^T (99.2 % sequence similarity which corresponds to 11 nucleotide differences out of 1,339 nucleotides; Chun *et al.*, 1998a). Representatives of these species have been shown to have a relatively low DNA:DNA relatedness value of 27 %, that is, well below the 70 % cut-off point recommended for the recognition of genomic species (Wayne *et al.*, 1987).

16S rRNA sequence similarities between the representatives of the validly described *Amycolatopsis* species fall between 93.3 % and 99.2 % (Table 2-14) with an average value of 95.8 %. The *Amycolatopsis* strains share less than 94.7 % 16S rDNA nucleotide similarity values with members of other genera classified in the family

Table 2-14. The nucleotide sequence similarities and G+C content of the 16S rRNA of representatives of *Amycolatopsis* species and related taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	G+C ratio (mol %)
1. *Amycolatopsis* strain NT202	**100**																		59.1
2. *A. alba*	**95.1**	100																	58.7
3. *A. azurae*	**93.9**	97.6	100																58.3
4. *A. coloradensis*	**94.7**	99.2	97.4	100															58.6
5. *A. fastidiosa*	**94.4**	94.3	94.8	94.3	100														59.0
6. *A. japonica*	**94.4**	98.0	97.2	98.1	94.1	100													58.8
7. *A. mediterranei*	**94.3**	97.4	96.0	97.1	93.3	96.5	100												58.8
8. *A. methanolica*	**99.2**	94.6	93.9	94.5	94.4	93.9	93.8	100											58.8
9. *A. orientalis*	**94.5**	97.7	97.4	97.8	94.5	98.4	96.2	94.2	100										58.5
10. *A. sulphurea*	**95.8**	97.1	96.5	97.0	94.9	96.9	95.9	95.2	97.2	100									58.3
11. *A. thermoflava*	**98.8**	94.8	93.8	94.5	94.2	94.1	94.1	99.0	93.9	95.3	100								59.2
12. *Amycolatopsis* strain K24	**95.4**	96.1	95.4	95.9	93.8	95.3	95.3	95.3	95.3	97.2	95.8	100							58.8
13. *Prauserella rugosa*	**93.2**	94.2	93.7	94.0	92.9	94.0	93.3	93.2	93.4	94.1	93.4	94.0	100						58.6
14. *Kibdelosporangium aridum*	**92.3**	93.2	92.7	93.2	92.9	93.2	92.5	92.5	93.0	93.3	92.0	93.8	91.9	100					59.1
15. *Pseudonocardia thermophilus*	**90.9**	91.3	91.2	91.5	91.7	90.8	90.4	91.2	91.2	91.5	91.3	91.7	91.2	91.4	100				58.9
16. *Saccharomonospora viridis*	**92.1**	91.8	92.2	91.8	92.7	91.5	91.8	91.8	91.7	92.9	91.8	92.4	92.8	92.0	90.7	100			60.9
17. *Saccharopolyspora hirsuta*	**92.8**	92.7	92.9	92.7	93.1	92.1	92.4	92.7	93.2	93.5	92.2	93.5	91.6	93.0	92.3	91.3	100		57.8
18. *Thermocrispum municipale*	**93.5**	93.0	93.3	92.8	92.8	92.8	93.0	93.6	92.5	94.0	93.5	94.7	93.1	91.7	91.3	91.4	92.4	100	58.8

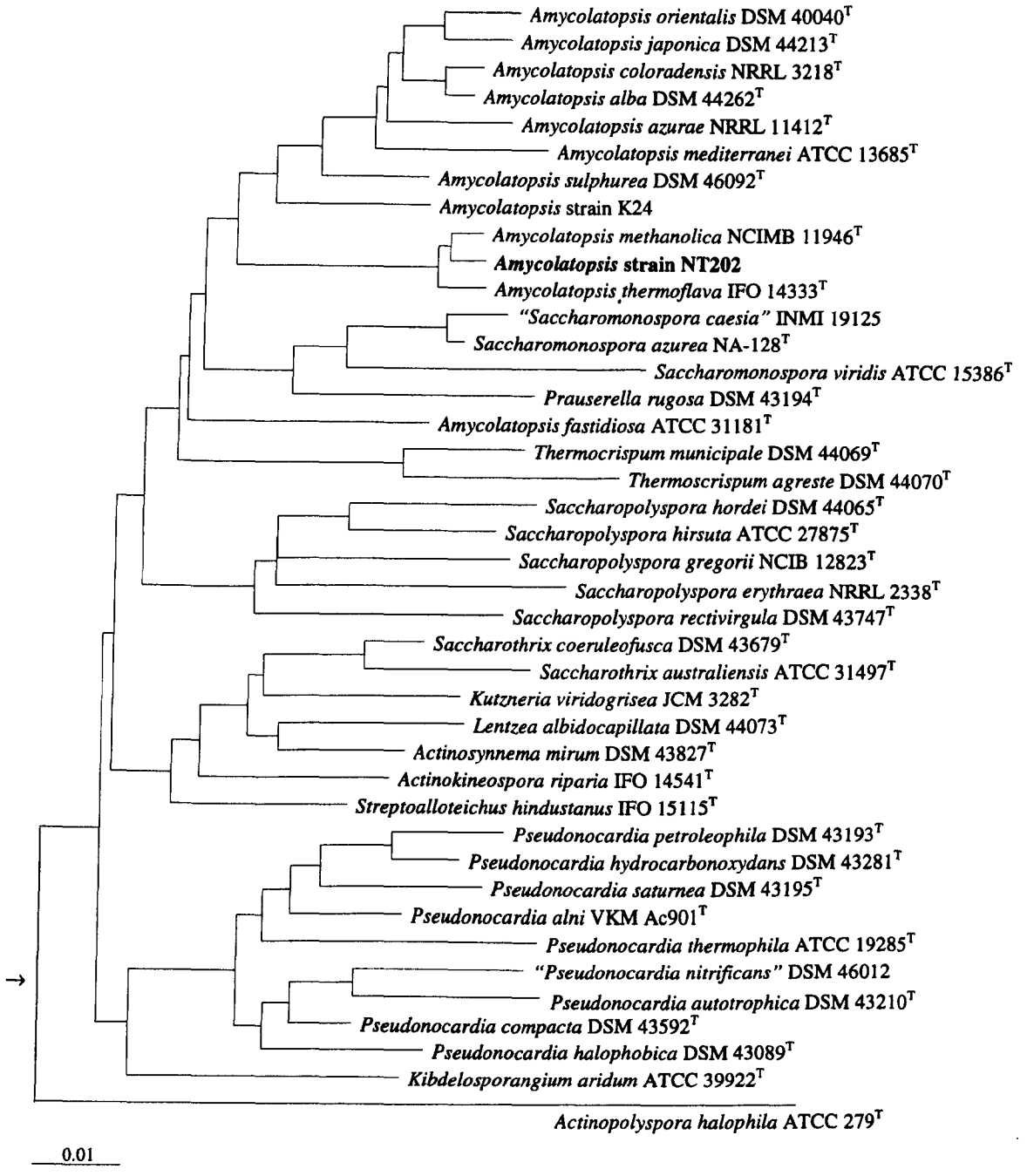


Figure 2-4. Least squares tree (Fitch & Margoliash, 1967) based on almost complete 16S rRNA sequences showing relationships between *Amycolatopsis* strain NT202 and representatives of the families *"Actinosynnemaceae"* and *Pseudonocardiaceae*. The corresponding 16S rRNA sequences of *Thermomonospora curvata* DSM 43183^T (X97893) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The arrow points position of the root. The scale bar indicates 0.01 substitutions per nucleotide position.

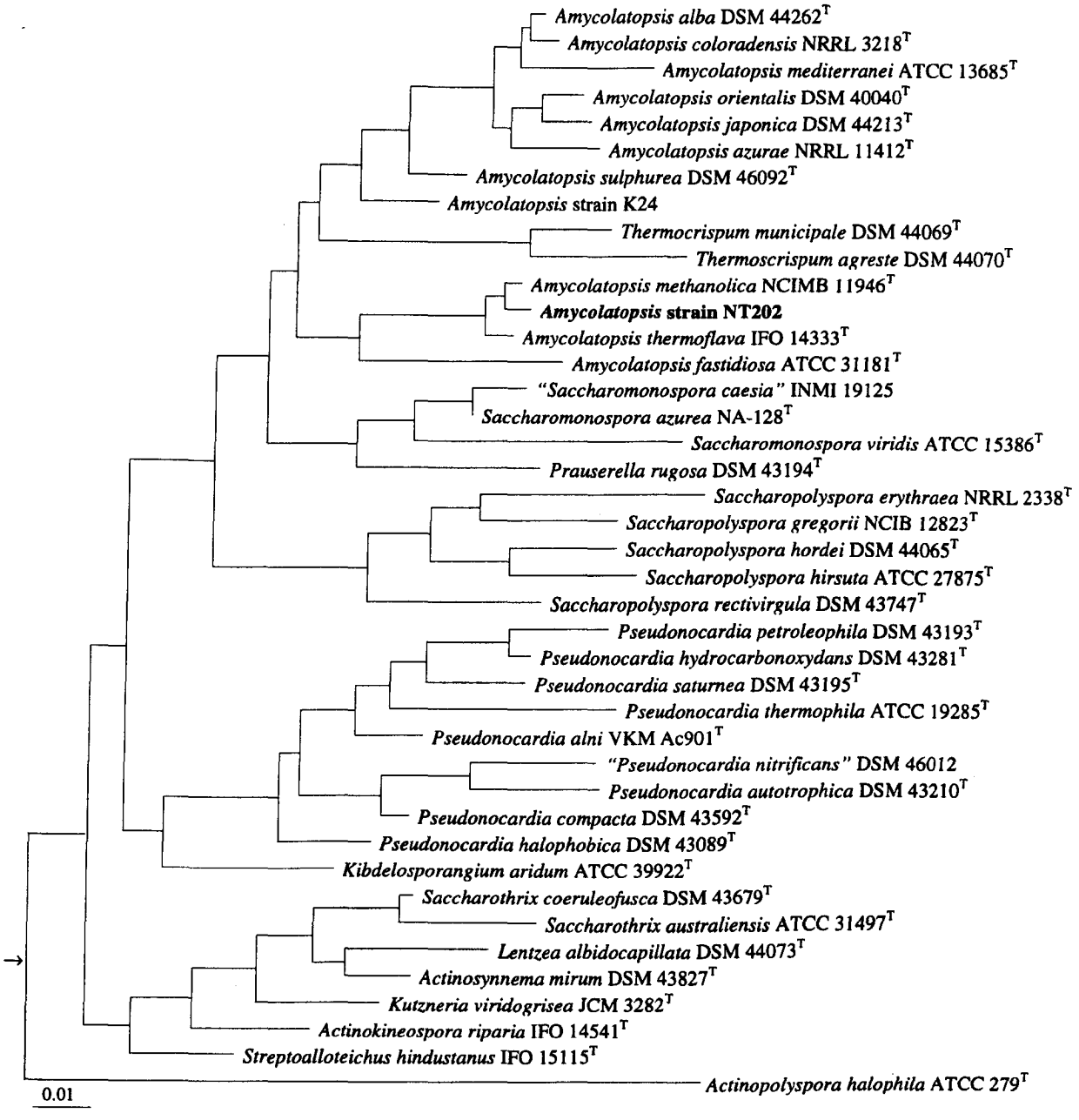


Figure 2-5. Maximum likelihood tree (Felsenstein, 1981) based on almost complete 16S rRNA sequences showing relationships between *Amycolatopsis* strain NT202 and representatives of the families "*Actinosynnemaceae*" and *Pseudonocardiaceae*. The corresponding 16S rRNA sequences of *Thermomonospora curvata* DSM 43183^T (X97893) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The arrow points position of the root. The scale bar indicates 0.01 substitutions per nucleotide position.

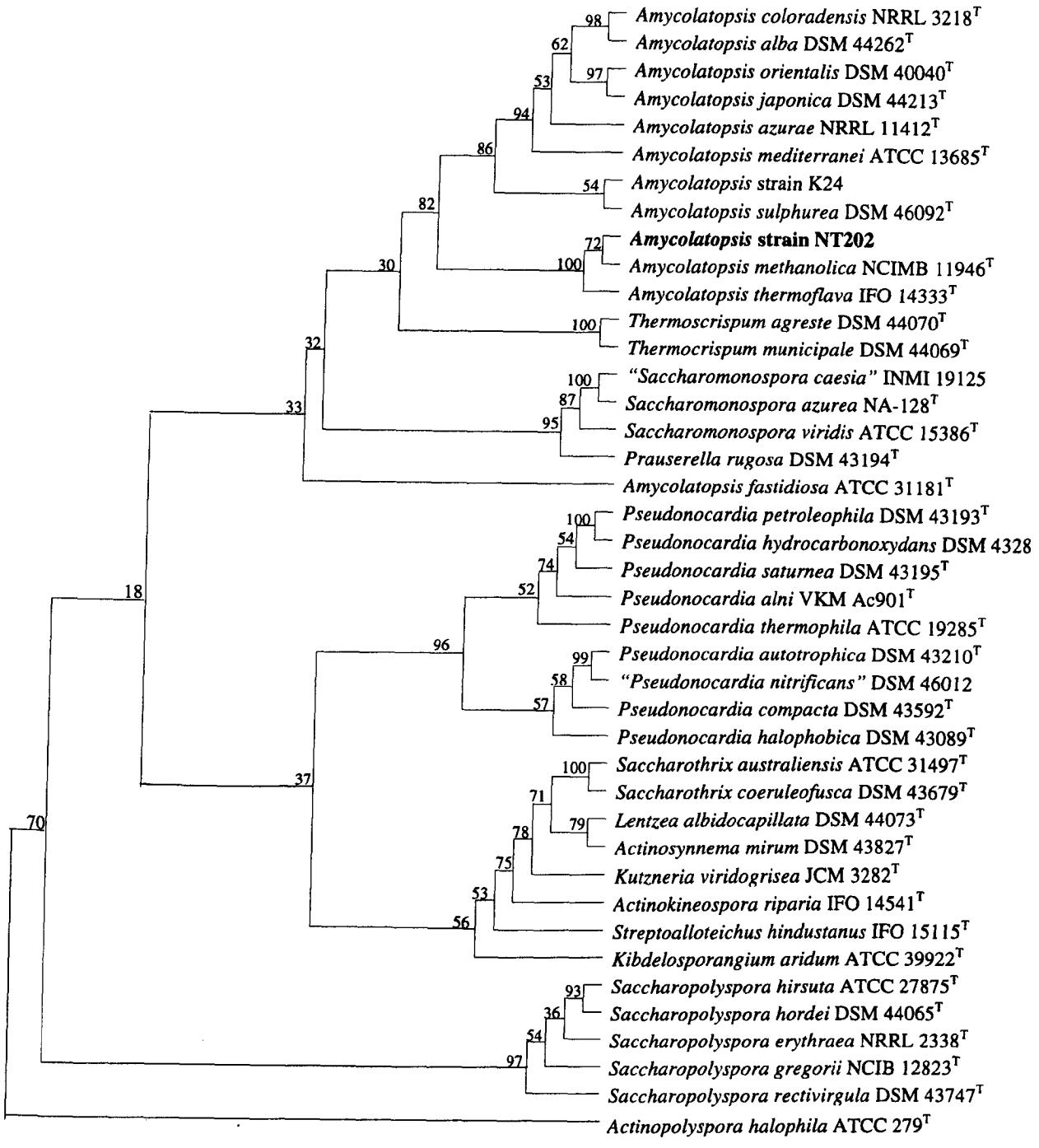


Figure 2-6. Consensus tree derived from bootstrap analysis (Felsenstein, 1985) of 1,000 re-sampled neighbour-joinig trees (Saitou & Nei, 1987) based on almost complete 16S rRNA sequences showing relationships between *Amycolatopsis* strain NT202 and representatives of the families "*Actinosynnemaceae*" and *Pseudonocardiaceae*. The 16S rRNA sequences of *Thermomonospora curvata* DSM 43183^T (X97893) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The numbers (%) at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled data sets.

Pseudonocardiaceae. The corresponding value for members of the genera classified in the family *Actinosynnemaceae* is 94.4 %.

It is apparent from the 16S rDNA sequencing studies that the *Amycolatopsis* strains fall into two well defined clades in the trees based on the least-squares and neighbour-joining treeing algorithms. The larger clade encompasses the mesophilic organisms, including *Amycolatopsis orientalis*, the type species of the genus. The taxonomic integrity of the *Amycolatopsis orientalis* clade was supported by the results obtained using all three treeing algorithms and by the very high bootstrap value (99.5 %) recorded for the neighbour-joining analysis. These results are in good agreement with those of earlier 16S rRNA sequencing study (Warwick *et al.*, 1994)

The second clade encompasses the three thermophilic strains, *Amycolatopsis methanolica* NCIMB 11946^T, *Amycolatopsis thermoflava* IFO 14333 ^{T}. and *Amycolatopsis* strain NT202, all of which grow well at 50 °C. Strain NT303 also belongs to this clade as it was found to have an identical 16S rDNA nucleotide sequence with strain NT202 in a comparison of over 800 nucleotides (16S rDNA nucleotide positions from 44 to 905 according to the *Escherichia coli* numbering system; Brosius *et al.*, 1978) covering several highly variable regions (Neefs *et al.*, 1993). The remaining thermophilic strain, *Amycolatopsis fastidiosa* ATCC 31181^T, was loosely associated with the *Amycolatopsis methanolica* clade in the maximum-likelihood tree but formed a single membered clade in the analyses based on least-squares and neighbour-joining methods (Fig. 2-4 and 2-6). The detailed relationship of *Amycolatopsis fastidiosa* ATCC 31181^T to the *Amycolatopsis methanolica* clade needs to be the subject of additional taxonomic studies.

The mesophilic and the thermophilic *Amycolatopsis* strains were also distinguished by the G+C content of their 16S rDNA sequences. *Amycolatopsis fastidiosa* ATCC 31181^T, *Amycolatopsis methanolica* NCIMB 11946^T, *Amycolatopsis thermoflava* IFO 14333 ^{T} and *Amycolatopsis* strain NT202 have 16S rDNA sequences with a higher G+C

range (58.8 to 59.2 mol%) than those of *Amycolatopsis orientalis* NRRL 2450^T and the related mesophilic strains (58.3 to 58.8 mol%; Table 2-14).

The *Amycolatopsis* clades recovered in the 16S rDNA trees were separated from one another by long branches and low bootstrap values. Similar findings were reported by Warwick *et al.* (1994) who concluded that there was no clear indication that the genus *Amycolatopsis* was heterogeneous given the many chemotaxonomic and phenotypic characteristics shared by members of this taxon. However, it is clear from the present study that further comparative taxonomic studies are needed to determine whether the genus *Amycolatopsis* is heterogeneous or merely encompasses a wide range of taxonomic variation.

DNA:DNA relatedness data are increasingly being used to help resolve the finer taxonomic relationships between species found to be closely associated on the basis of 16S rRNA sequence data (Stackebrandt & Goebel, 1994; Goodfellow *et al.*, 1997a; Kim, S. B. *et al.*, 1998). It has already been pointed out that genomic species encompass organisms which show 70 % or more DNA:DNA relatedness under stringent experimental conditions (Wayne *et al.*, 1987). In the present study DNA:DNA relatedness values were determined between *Amycolatopsis* strains NT202 and NT303 and *Amycolatopsis methanolica* NCIMB 11946^T using the nitrocelluose filter method. It is evident from the resultant data (Table 2-15) that *Amycolatopsis* strains NT202 and NT303 belong to a single genomic species which is closely related, albeit distinct, from *Amycolatopsis methanolica* NCIMB 11946^T. Similarly, *Amycolatopsis methanolica* NCIMB 11946^T and *Amycolatopsis thermoflava* IFO 14333 ^{T}, which share a 16S rRNA similarity value of 99.0 %, have been found to have low DNA:DNA relatedness values (5 % to 21 %; Chun *et al.*, 1998a).

The assignment of strains NT202 and NT303 to the genus *Amycolatopsis* is also supported by the phenotypic data, notably by the distribution of chemotaxonomic and morphological markers. The two isolates are aerobic, nonmotile, Gram-positive, non-acid-

Table 2-15.. Mean levels of DNA relatedness (%) found between *Amycolatopsis* strains NT202 and NT303 and *Amycolatopsis methanolica* NCIMB 11946$^{T\ a}$

Test strains	Labelled DNA		
	A. methanolica	*Amycolatopsis* strains	
	NCIMB 11946 ^{T}	NT202	NT303
A. methanolica NCIMB 11946 ^{T}	100		
Amycolatopsis strain NT202	60	100	
Amycolatopsis strain NT303	58	93	100
Streptomyces thermovulgaris DSM 40444 $^{T\ b}$	5	2	0

^{T}, Type strain.

^{a}, The pairwise DNA relatedness values in the table were obtained by averaging measurements from two sets of hybridisations.

^{b}, Phylgenetically distant control strain.

alcohol fast actinomycetes which produce aerial and substrate hyphae that fragment into squarish, rod-like elements (*ca.* 0.4-0.5 × 0.7-1.6 μm; Fig. 2-7). In addition, strain NT202 has a phospholipid pattern consisting of DPG, PE (taxonomically significant polar lipid), PG, PI, PIDM, PME and an unidentified polar lipid (Fig. 2-8); a fatty acid profile consisting of 14-methylpentadecanoic acid (*iso*-16:0; 45.6 ± 1.8 % of the total cellular fatty acid composition), hexadecanoic acid (16:0; 23.0 ± 2.4%), 14-methylhexadecanoic acid (*anteiso*-17:0; 9.2 ± 0.6%), 15-methylhexadecanoic acid (*iso*-17:0; 6.1 ± 0.1 %), 13-methyltetradecanoic acid (*iso*-15:0; 3.8 ± 0.6 %), heptadecanoic acid (17:0; 3.4 ± 0 %) and octadecanoic acid (18:0; 3.4 ± 0.2 %) (Sahin, personal communication), and di- and tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenologues, but does not contain mycolic acids. The G + C content of the DNA of the isolates is 73.9 ± 0.9 mol%.

The properties outlined above serve to distinguish strains NT202 and NT303 from members of all mycolateless, wall chemotype IV genera, apart from the genera *Amycolatopsis* (Holt *et al.*, 1994; Kim & Goodfellow, 1998). The strains can also be distinguished from representatives of all of the validly described species of *Amycolatopsis* using a combination of growth, morphological and physiological features (Table 2-16).

It is evident from the genotypic and phenotypic data that strains NT202 and NT303 form a new centre of taxonomic variation within the genus *Amycolatopsis*. It is, therefore, proposed that these organisms be classified in the genus *Amycolatopsis* as a new species, namely, *Amycolatopsis eurythermus*.

Description of *Amycolatopsis eurythermus* sp. nov. (eur.y.ther'mus; Gr. pref. *eury* many; Gr. n. *therme* heat; M. L. *eurythermus* wide, heat).

The description is based on data taken from this and an earlier study (Sahin, unpublished). Aerobic, Gram-positive, non-acid-alcohol fast, non-motile actinomycetes which form an

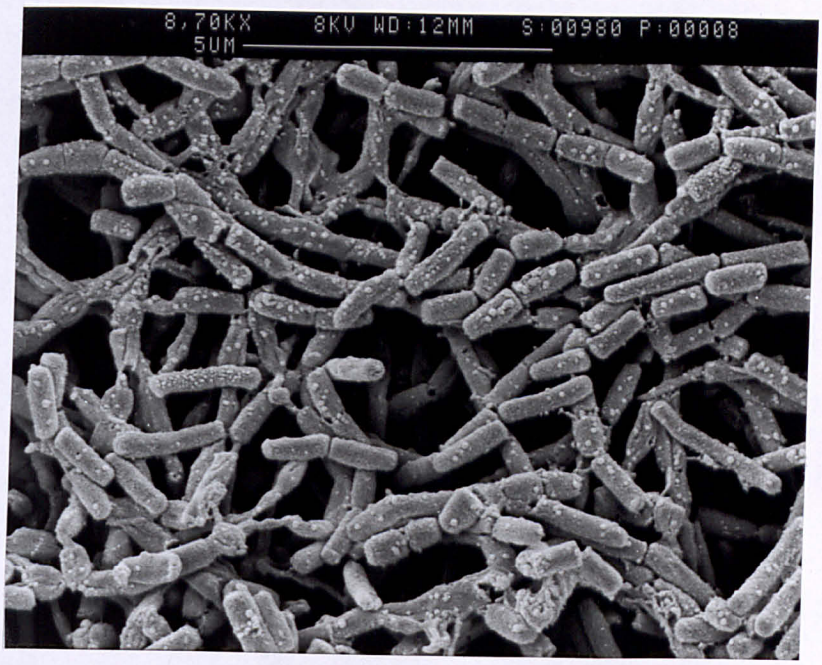


Figure 2-7. Scanning electron micrograph of *Amycolatopsis* strain NT202 showing hyphae that fragment into squarish, rod-like elements. The organism was grown on inorganic salt-starch agar (ISP medium 4) at 45 °C for 5 days. Bar = 5 μm.

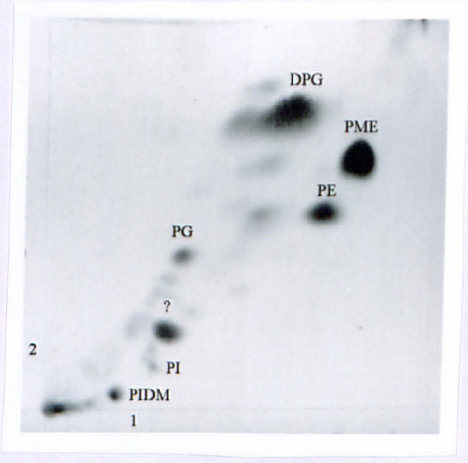


Figure 2-8. Two dimensional thin layer chromatography of polar lipids of *Amycolatopsis* strain NT202. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannoside; PME, phosphatidylmethylethanolamine; ?, unidentified phospholipid. Numbers indicate the order of chromatographic developments.

Table 2-16. Characters separating the test strains from members of validly described species classified in the genus *Amycolatopsis*

Characters	***Amycolatopsis* strains NT202 and NT303**	*A. alba* NRRL 18532^T	*A. azurea* NRRL 11412^T	*A. coloradensis* NRRL 3218^T	*A. fastidiosa* NRRL B-16697^T	*A. japonica* DSM 44213^T	*A. mediterranei* ATCC 13685^T	*A. methanolica* NCIMB 11946^T	*A. orientalis* NRRL 2450^T	*A. sulphurea* NRRL B-2822^T	*A. thermoflava* IFO 14323^T
Colour of aerial mycelium:											
Blue	-	-	+	-	-	-	-	-	-	-	-
Purple	-	-	-	-	+	-	-	-	-	-	-
White	+	+	-	-	+	+	+	+	+	+	+
White to olive buff	-	-	-	+	-	-	-	-	-	-	-
Yellowish green	-	-	-	-	-	-	-	-	-	+	-
Production of diffusible pigment	-	-	+	+	+	-	-	-	-	-	+
Acid production from:											
Adonitol	+	+	+	-	-	+	-	+	+	-	+
L-Arabinose	+	+	+	-	+	+	+	-	+	-	+
D-Cellobiose	+	+	+	+	+	+	+	+	+	-	+
Dextrin	w	-	+	+	+	+	+	-	+	+	-
meso-Erythritol	+	+	+	-	-	+	-	+	+	-	+
D-Galactose	+	+	+	+	-	+	+	+	+	+	+
meso-Inositol	+	+	+	+	-	+	+	-	+	+	-

D-Lactose	w	+	+	-	-	+	+	-	+	-	+
D-Mannitol	+	+	+	+	-	+	+	+	+	+	+
Melibiose	-	+	-	-	-	+	-	-	-	-	+
D-Melezitose	-	-	-	-	-	-	-	-	+	-	-
D-Raffinose	-	+	-	-	-	+	+	-	-	-	-
L-Rhamnose	+	-	-	-	-	-	+	+	-	-	-
Salicin	-	+	+	+	+	+	+	-	+	-	+
D-Sorbitol	+	-	-	-	-	-	-	+	-	-	+
Sucrose	-	-	+	-	+	+	+	-	+	-	-
D-Xylose	+	+	+	+	+	-	+	+	+	-	+
Decomposition of:											
Casein	+	+	+	+	+	+	+	-	+	-	+
Esculin	-	+	+	+	+	+	+	-	+	-	+
Gelatin	+	+	+	+	-	+	+	+	+	+	-
Hypoxanthine	v	+	+	+	-	+	+	+	+	-	+
Starch	-	+	-	+	-	+	-	-	+	-	-
Xanthine	v	+	+	-	-	+	-	+	-	-	+
Growth at:											
10 °C	-	-	+	+	-	+	+	-	+	-	-
25 °C	+	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+	+	+	+	+
45 °C	+	-	-	-	+	+	-	+	-	-	+

50 °C	+	-	-	-	+	-	-	+	-	-	+
55 °C	+	-	-	-	+	-	-	-	-	-	+
60 °C	-	-	-	-	+	-	-	-	-	-	-
Production of											
Amylase	-	+	-	+	-	+	-	-	+	-	-
Nitrate reduction	+	-	+	+	+	-	-	+	+	-	+
Urease	**ND**	+	+	-	+	-	+	-	+	-	+

+, positive or present; -, negative or absent; w, weak positive; v, variable; ND, not determined.

Source of data on validly described *Amycolatopsis* species: Henssen *et al.* (1987), De Boer *et al.* (1990), Embley (1992), Mertz & Yao (1993), Labeda (1995), Goodfellow *et al.* (1997a) and Chun *et al.* (1998).

extensively branched substrate mycelium which fragments into squarish elements (*ca.* 0.4-0.5 × 0.7-1.6 μm). Sparse, white, sterile aerial hyphae are formed. A yellow substrate mycelium is produced on glucose yeast extract and modified Bennett's agars but distinct substrate mycelium colours are not formed on Czapek Dox, glycerol asparagine or oatmeal agars; diffusible pigments are not evident on any of these media. Melanin pigments are not formed on peptone yeast extract iron or tyrosine agars. Growth occurs between 25 °C and 55 °C, and between pH 6.0 and 9.0, but not at either pH 5.0 or 10.0.

Casein, elastin, gelatin, L-tyrosine and xylan are degraded but no activity is shown against adenine, arbutin, chitin, esculin, guanine or starch. The organisms grow on adonitol, L-arabinose, arabitol, D-cellobiose, D-fructose, D-galactose, *meso*-inositol, D-lactose, D-mannitol, D-mannose, melibiose, D-melezitose, α-L-rhamnose, D-ribose, D-sorbitol, starch, sucrose, D-trehalose, D-turanose, xylitol and D-xylose as sole carbon and energy sources, but not on D-raffinose. Strains produce acid from adonitol, L-arabinose, D-cellobiose, dextrin, *meso*-erythritol, D-galactose, *meso*-inositol, D-lactose, D-mannitol, L-rhamnose, D-sorbitol and D-xylose, but not from melibiose, D-melezitose, D-raffinose, salicin or sucrose.

The strains are sensitive to amikacin (4, 8 and 16 μg ml^{-1}), bacitracin (32 μg ml^{-1}), doxycyline (16, 32 and 64 μg ml^{-1}), fusidic acid (8 μg ml^{-1}), gentamycin sulphate (64 μg ml^{-1}), lividomycin A (16 μg ml^{-1}), neomycin sulphate (32 μg ml^{-1}), novobiocin (16 and 32 μg ml^{-1}), streptomycin sulphate (64 μg ml^{-1}), ticarcillin (16 and 32 μg ml^{-1}), and tyrothricin (16 and 32 μg ml^{-1}), but are resistant to ampicillin (8, 16 and 32 μg ml^{-1}), bacitracin (16 μg ml^{-1}), carbenicillin (12 μg ml^{-1}), cefoxitin (16 and 32 μg ml^{-1}), cephaloridine (32, 64 and 128 μg ml^{-1}), cephradine (8, 16 and 32 μg ml^{-1}), doxycyline hydrochloride (4 μg ml^{-1}), ethionamide (16 μg ml^{-1}), fusidic acid (4 μg ml^{-1}), gentamycin sulphate (32 μg ml^{-1}), gramicidin (8 μg ml^{-1}), lincomycin hydrochloride (32, 64 and 128 μg ml^{-1}), isoniazid (16

μg ml^{-1}), lividomycin A (4 and 8 μg ml^{-1}), nalidixic acid (32 μg ml^{-1}), neomycin sulphate (8 μg ml^{-1}), novobiocin (4 μg ml^{-1}), oleandomycin phosphate (16, 32, 64 and 128 μg ml^{-1}), penicillin G (5, 10 and 15 i.u.), polymyxin B sulphate (16 and 32 μg ml^{-1}), rifampicin (16, 32 and 64 μg ml^{-1}), spiramycin (10 μg ml^{-1}), streptomycin sulphate (4, 8, 16, 32 and 64 μg ml^{-1}), tetracyline hydrochloride (4, 8, 16 and 32 μg ml^{-1}), vancomycin (16, 32 and 64 μg ml^{-1}) and viomycin sulphate (20 μg ml^{-1}).

The G plus C ratios of the DNA of the two strains is 73.1 ± 0.9 mol%. Additional chemical features of the organisms have been mentioned earlier.

The organisms were isolated from a scrubland soil sample collected in Madurai, India (strain NT202) and from an arid soil sample collected in Van, Turkey (strain NT303).

The type strain of *Amycolatopsis eurythermus* is NT202 (= DSM 44348).

It is interesting that in the present study the representatives of the genera *Actinokineospora, Actinosynnema, Kutzneria, Lentzea, Saccharothrix* and *Streptoalloteichus* formed a distinct clade in the phylogenetic trees, a result consistent with the view that these taxa be assigned to the putative new family *Actinosynnemaceae* (Labeda, 1998b; Kim & Goodfellow, 1998). However, it is evident from the bootstrap analysis of the neighbour-joining tree that the inclusion of *Kibdelosporangium aridum* and *Streptoalloteichus hindustanus* in this clade is not supported by high bootstrap values.

2. Classification of alkalitolerant, thermophilic excellosporae

Almost complete 16S rRNA sequences were obtained for strains TA111 and TA113 (1,442 nucleotides). When these sequences were compared with those of representatives of the family *Thermomonosporaceae* and related taxa it was apparent that the two organisms fall within the range of the variation encompassed by genera classified

Table 2-17. 16S rRNA sequence similarities and G+C content of the 16S rRNA of *Excellospora* strains TA111 and TA113 and the type strains of related taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	G+C (mol%)
1. *Excellospora* strain TA111	**100**																		**59.5**
2. *Excellospora* strain TA113	**98.7**	100																	**59.5**
3. *Excellospora viridilutea* IFO 14480^T	**97.3**	97.7	100																60.0
4. *Actinomadura aurantiaca* JCM 8201^T	**94.5**	95.7	94.8	100															59.1
5. *Actinomadura atramentaria* IFO 14695^T	**96.2**	96.8	95.3	94.7	100														60.3
6. *Actinomadura coerulea* IFO 14679^T	**95.2**	96.1	95.1	95.1	96.4	100													59.8
7. *Actinomadura cremea* IFO 14183^T	**95.3**	95.6	95.3	95.1	95.5	97.2	100												59.5
8. *Actinomadura echinospora* IFO 14042^T	**95.7**	96.5	95.8	95.1	95.6	96.4	96.1	100											60.3
9. *Actinomadura fulvescens* IFO 14347^T	**96.2**	97.0	96.0	95.9	96.9	97.6	96.7	97.0	100										59.7
10. *Actinomadura kijaniata* IFO 14229^T	**95.7**	96.8	96.4	97.0	96.1	96.6	96.7	96.1	96.9	100									60.5
11. *Actinomadura libanotica* IFO 14095^T	**94.4**	95.7	94.6	99.1	95.0	95.3	95.3	95.3	96.1	96.6	100								60.2
12. *Actinomadura madurae* JCM 7436^T	**95.5**	96.2	95.4	95.3	96.4	98.5	96.7	96.5	97.4	96.5	95.1	100							59.7
13. *Actinomadura formosensis* JCM 7474^T	**96.1**	96.9	95.5	95.6	96.7	98.2	96.5	96.4	97.6	96.2	95.2	97.9	100						59.7
14. *Actinocorallia herbida* IFO 15485^T	**95.0**	96.4	94.8	98.4	95.7	94.9	94.8	95.5	96.3	96.1	98.0	95.1	95.8	100					59.9
15. *Spirilliplanes albida* IFO 12248^T	**95.8**	96.6	95.6	95.2	94.9	95.8	95.9	96.3	96.5	95.8	94.7	96.3	96.7	95.5	100				59.8
16. *Thermobifida alba* JCM 3077^T	**92.2**	92.6	92.2	92.1	91.3	91.5	91.2	93.0	92.0	91.7	92.1	91.4	91.9	91.9	91.8	100			59.6
17. *Thermobifida fusca* ATCC 27730^T	**92.6**	93.0	92.5	92.1	91.3	91.5	91.7	93.4	92.0	91.8	91.9	91.7	91.9	92.0	91.8	97.6	100		59.6
18. *Thermomonospora chromogena* JCM 6244^T	**93.1**	93.8	92.9	91.3	91.5	92.3	91.9	93.4	92.8	91.9	91.5	92.4	92.7	92.2	92.7	91.8	92.1	100	60.2
19. *Thermomonospora curvata* DSM 43183^T	**94.8**	95.1	96.0	93.5	94.4	94.9	94.2	96.2	94.4	94.4	93.5	95.2	94.9	94.0	94.2	92.1	92.7	92.7	60.2

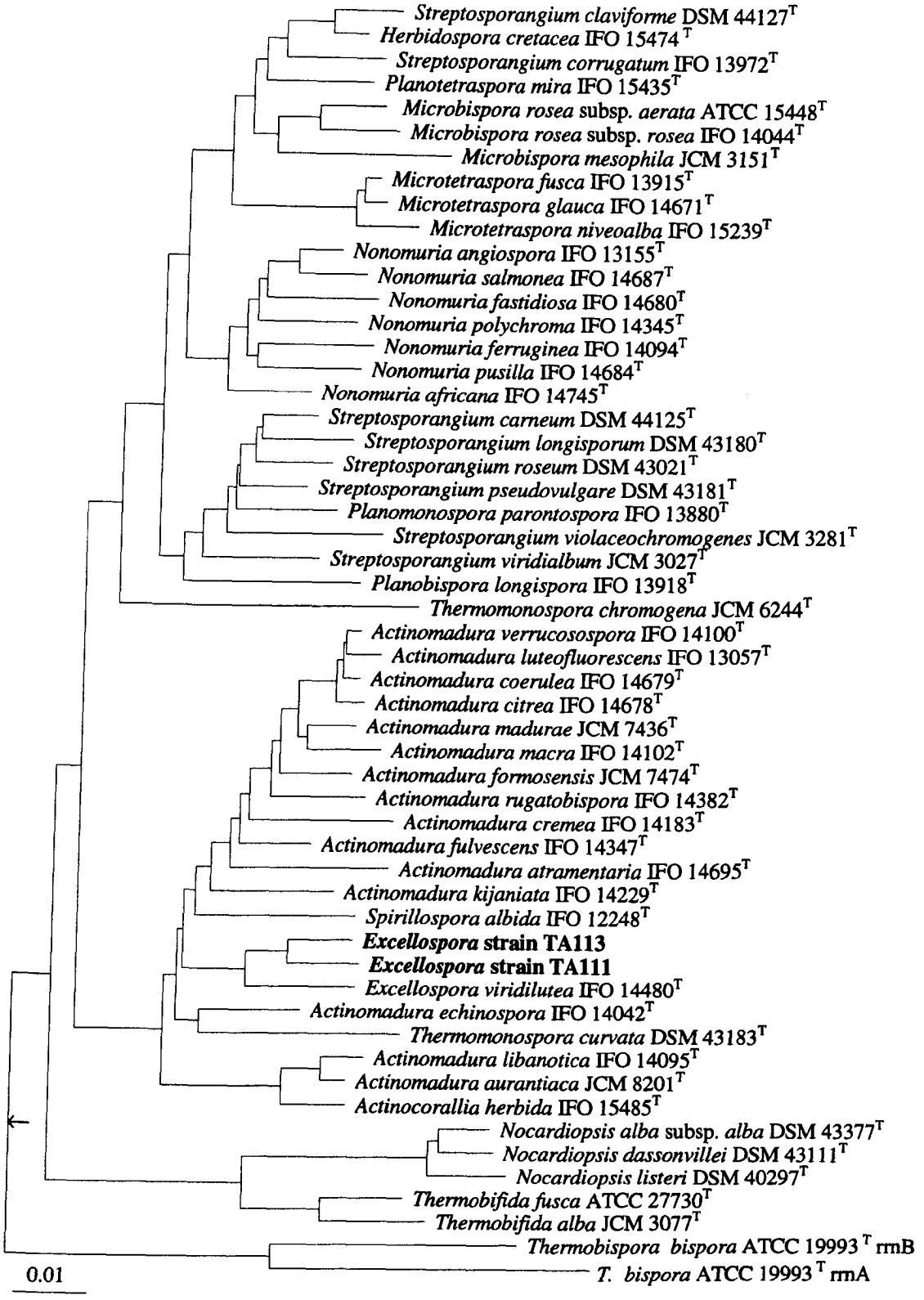


Figure 2-9. Least squares tree (Fitch & Margoliash, 1967) based on 16S rRNA sequences showing relationships between strains TA111 and TA113 and representatives of the family *Thermomonosporaceae* and some related taxa. The 16S rRNA sequences of *Arthrobacter globiformis* (M23411), *Bifidobacterium bifidum* (M38018) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The arrow points position of the root. The scale bar indicates 0.01 substitutions per nucleotide position.

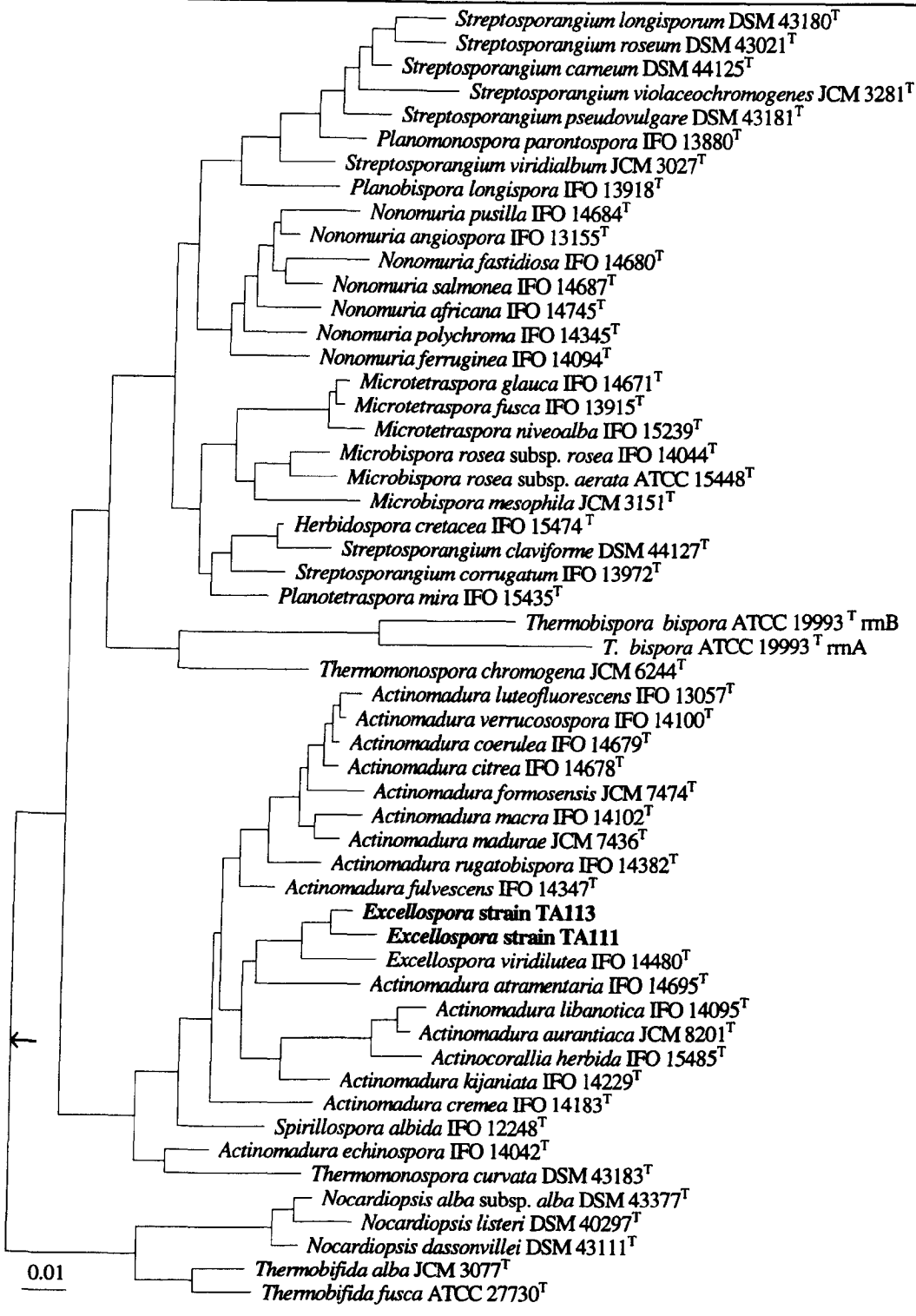


Figure 2-10. Maximum likelihood tree (Felsenstein, 1981) based on 16S rRNA sequences showing relationships between strains TA111 and TA113 and representatives of the family *Thermomonosporaceae* and some related taxa. The 16S rRNA sequences of *Arthrobacter globiformis* (M23411), *Bifidobacterium bifidum* (M38018) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The arrow points position of the root. The scale bar indicates 0.01 substitutions per nucleotide position.

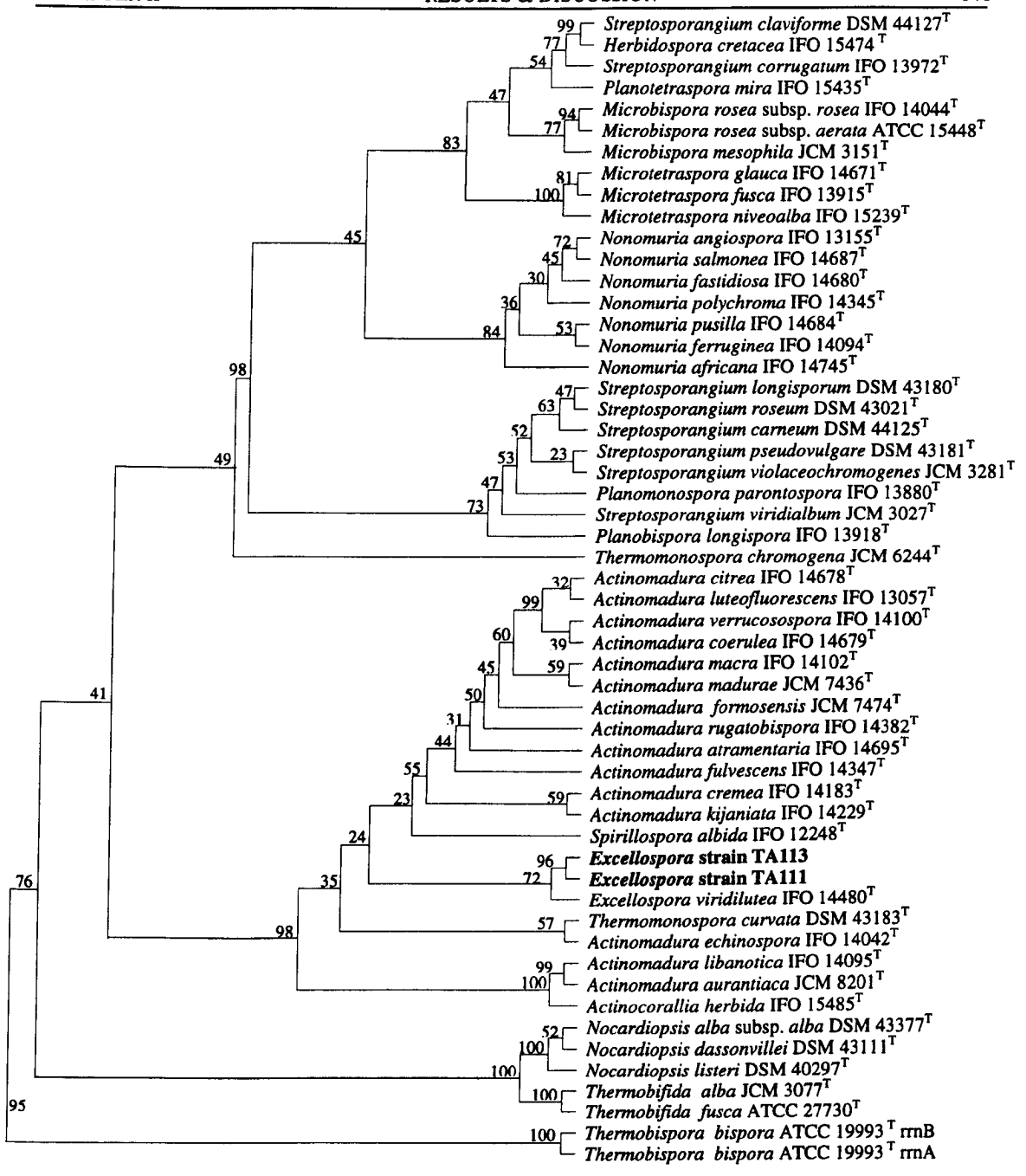


Figure 2-11. Consensus tree derived from bootstrap analysis (Felsenstein, 1985) of 1,000 re-sampled neighbour-joining trees (Saitou & Nei, 1987) based on 16S rRNA sequences showing relationships between strains TA111 and TA113 and representatives of the family *Thermomonosporaceae* and some related taxa. The 16S rRNA sequences of *Arthrobacter globiformis* (M23411), *Bifidobacterium bifidum* (M38018) and *Streptomyces violaceoruber* A3(2) (Y00411) were used as outgroups. The numbers (%) at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled data sets.

in the family *Thermomonosporaceae* (Table 2-17; Fig. 2-9, 2-10, 2-11). It is also apparent that strains TA111 and TA113 form a distinct clade together with *Excellospora viridilutea* IFO 14480^T within the range of variation encompassed by representatives of validly described *Actinomadura* species, *Actinocorallia herbida* IFO 15484^T, *Spirillospora albida* IFO 12248^T and *Thermomonospora curvata* JCM 3096^T. This relationship is supported by trees based on the least-squares (Fitch & Margoliash, 1967; Fig. 2-9) and maximum-likelihood treeing algorithms (Felsenstein, 1981; Fig. 2-10) and by the high bootstrap value (72 %) recorded using the neighbour-joining method (Saitou & Nei, 1987; Fig. 2-11). It is also clear from Table 2-17 that there is no clear difference between the 16S rDNA G+C contents of the thermophilic and mesophilic strains classified in the family *Thermomonosporaceae.*

Strains TA111 and TA113 share a 16S rDNA sequence similarity of 98.7 %, which corresponds to 19 differences out of 1,444 nucleotide positions. A comparable scale of difference exists between validly described species of genera classified in the family *Thermomonosporaceae* as exemplified by *Actinomadura citrea* IFO 14678^T and *Actinomadura madurae* JCM 7436^T (98.7 % similarity which corresponds to 19 nucleotide differences out of 1,406 nucleotides). The 16S rDNA similarities between strains TA111 and TA113 and *Excellospora viridilutea* IFO 14480^T were 97.4 % and 97.5 %, values which correspond to 38 and 36 differences out of 1,448 nucleotide positions, respectively.

It is also evident that the clade encompassing strains TA111, TA113 and *Excellospora viridilutea* IFO 14480^T is closely associated with representatives of the genus *Actinomadura* and with *Actinocorallia herbida* IFO 15484^T, *Spirillospora albida* IFO 12248^T and *Thermomonospora curvata* JCM 3096^T. Members of these taxa were also recovered within the phylogenetic radiation occupied by the members of the genus *Actinomadura* in an earlier 16S rRNA tree (Zhang *et al.*, 1998). Strains TA111 and TA113 together with *Excellospora viridilutea* IFO 14480^T share 16S rDNA nucleotide similarity

values of 94.4 to 97.0 % with the members of the genus *Actinomadura*, 95.6 to 96.6 % with *Spirillospora albida* IFO 12248^T and 94.8 to 96.0 % with *Thermomonospora curvata* JCM 3096^T. Further comparative studies, including an analysis of chemical and physiological properties, are needed to resolve the complex taxonomic structure shown by the members of family *Thermomonosporaceae*.

Strains TA86 and TA114 also belong to the *Excellospora* clade as they were found to share identical nucleotide sequences in a comparison of over 800 nucleotide positions (16S rDNA nucleotide positions from 59 to 933 according to the *Escherichia coli* numbering system; Brosius *et al.*, 1978) covering several variable regions with strains TA111 and TA113, repectively. It can, therefore, be concluded that strains TA86 and TA111 belong to a single species as do strains TA113 and TA114.

The test strains share a range of properties consistent with their assignment to the genus *Excellospora* Agre and Guzeva 1975. All four strains are aerobic, Gram-positive, non-acid fast, non-motile actinomycetes which grow between 37 and 55 °C and at pH 10.5. They produce a bluish-gray aerial spore mass and a pale yellow or non-distinctive substrate mycelium on oatmeal agar but do not form soluble pigment on glycerol asparagine agar, oatmeal agar, peptone yeast extract iron or tyrosine agars. Spiny spores are carried in straight spore chains (Fig. 2-12). Strains TA111 and TA113 contain *meso*-A_2pm as the major diamino acid of the peptidoglycan (Sahin, personal communication), major amounts of hexahydrogenated menaquinone with nine isoprene units (MK-9[H_6]) as the predominant isoprenologue, and DPG, PG, PI, PIDM with an unidentified polar lipids (Fig. 2-13; phospholipid type I *sensu* Lechevalier *et al.*, 1977). It can, therefore, concluded that the test strains should be classified in the same taxon genus as *Excellospora viridilutea*.

It is also interesting that *Actinomadura rubrobrunea* Kroppenstedt *et al.* 1990 DSM 43750^T and DSM 43751, which were previously classified as *Excellospora rubrobrunea* Agre and Guzeva 1975 and *Excellospora viridinigra* Agre and Guzeva 1975 share

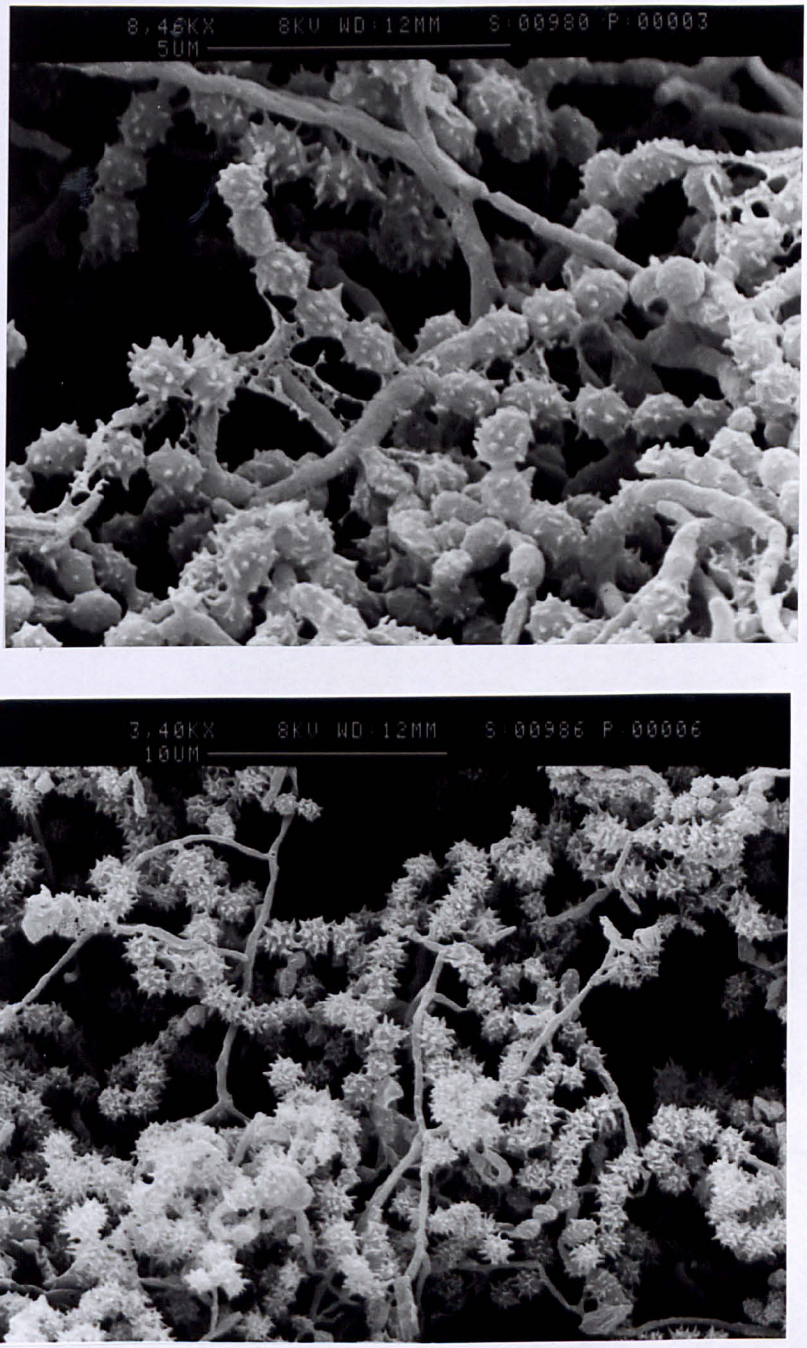


Figure 2-12. Scanning electron micrograph showing straight spore chains with spiny surfaces of (a) *Excellospora* strain TA86 and (b) *Excellospora* strain TA113. The organisms was grown on inorganic salt-starch agar (ISP medium 4) at 45 °C for 5 days. Bar = (a) 5 μm and (b) 10 μm.

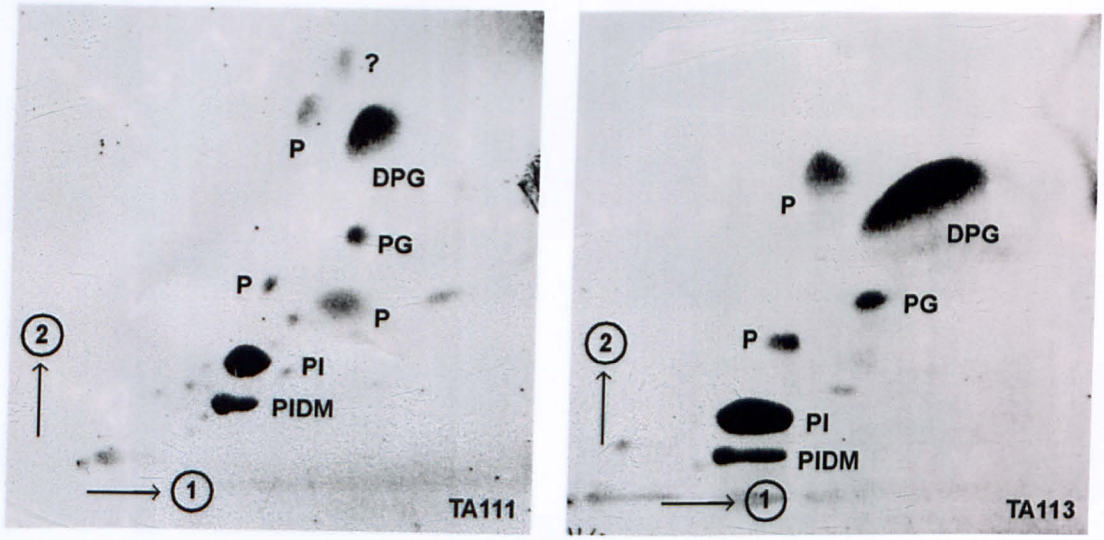


Figure 2-13. Two dimensional thin layer chromatography of polar lipids of *Excellospora* strain TA111 and TA113. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannoside; ?, unidentified phospholipid. Numbers indicate the order of chromatogrphic developments.

phenotypic properties with *Excellospora viridilutea* DSM 43934^T (Table 2-18). These data suggest that *Actinomadura rubrobrunea* strains should be returned to the genus *Excellospora* as *Excellospora rubrobrunea.* However, before any such recommendation can be made it will be necessary to determine whether the type strains of *Actinomadura rubrobrunea* falls within the same evolutionary clade as *Excellospora viridilutea* and the test strains.

The putative excellosporae share similar morphological and physiological characteristics but can be distinguished from representative strains of *Actinomadura rubrobrunea* and *Excellospora viridinigra* on the basis of the colour of their aerial and substrate mycelia (Table 2-18). Similarly, strains TA86 and TA111 can be distinguished from strains TA113 and TA114 by a number of phenotypic properties (Table 2-19). In addition, while TA 111 and TA113 share similar menaquinone profiles they can be distinguished as strain TA113 only contain a trace of octahydrogenated menaquinone with nine isoprene units (Table 2-20).

It can be concluded that strains TA113 and TA114, and strains TA86 and TA111 can be separated from one another and from *Excellospora viridilutea* IFO 14480^T using a combination of genotypic and phenotypic properties. It is, therefore, proposed that the validly desribed genus *Excellospora* merits continued recognition, and that strains TA86 and TA111, and strains TA113 and TA114 be recognised as new species within this taxon. The name *Excellospora alcalithermophila* is proposed for strains TA86 and TA111 and *Excellospora thermoalcalitolerans* for strains TA113 and TA114.

Emended description of the genus *Excellospora* Agre and Guzeva 1975^{AL}. Ex.cel'lo.spo.ra. M.L. fem. adj. from *excellens*, L. pr. part of *excello* prominent; Gr. n. *spora* seed (referring to the special structure of the spores).

The description is based on the data from this and earlier studies (Agre & Guzeva, 1975; Greiner-Mai *et al.*, 1987; Kroppenstedt *et al.*, 1990). Aerobic, Gram-positive, non-

Table 2-18. Phenotypic properties of the test strains and representative strains of *Actinomadura rubrobrunea* and *Excellospora viridilutea*

Properties	**Strains TA86, TA111, TA113 and TA114**	***Excellospora viridilutea* DSM 43934^{T} [a]**	***Actinomadura rubrobrunea* DSM 43750^{T} [a] and DSM 43751 [a]**
Morphological characteristics:			
Spores borne on aerial hyphae	+	+	+
Spore chain morphology	Straight	Spiral / hooked	Spiral
Number of spores	Below 20	1-20	1-20
Spore surface ornamentation	Spiny	Spiny	Spiny
Colour of aerial spore mass	Bluish-grey	Bluish-green	Grey to blue
Substrate mycelium colour	Not distinctive	Yellow to orange	Reddish brown
Diffusible pigments	Not distinctive	Not distinctive	Not distinctive
Degradation tests			
Adenine	-	ND	-
Arbutin	-	ND	-
Casein	+	ND	ND
Chitin	-	ND	+
DNA	+	ND	ND
Elastin	+	ND	ND
Esculin	-	ND	+
Gelatin	+	+	ND
Guanine	-	ND	ND
Hypoxanthine	+[b]	ND	-
Starch	+	+	-
Testosterone	+[b]	ND	ND
L-Tyrosine	+	ND	-
Xanthine	-	ND	-
Xylan	+	ND	ND
Nitrate reduction	ND	+	ND
Sole carbon sources at 1 %, w/v			
Arabinose	+	-	ND
Dulcitol	ND	-	ND
Galactose	+	+	ND
Glucose	ND	+	ND
Glycerol	ND	+	ND

Inositol	+	+	ND
Lactose	+	+	ND
Maltose	ND	+	ND
Mannitol	+	+	ND
Raffinose	-	+	ND
Rhamnose (0.1 %, w/v)	+	+	ND
Sodium acetate	+	+	ND
Sodium citrate	+	-	ND
Starch	+	+	ND
Xylose	+	-	ND

[a], Data taken from Agre & Guzeva (1975) and present study.

[b], Strain TA111 negative.

Table 2-19. Phenotypic characteristics of *Excellospora* strains TA86, TA111, TA113 and TA114

Characters:	Strains			
	TA86	**TA111**	**TA113**	**TA114**
DEGRADATION TESTS				
Adenine	-	-	-	-
Arbutin	-	-	-	-
Casein	+	+	+	+
Chitin	-	-	-	-
DNA	+	+	+	+
Elastin	+	+	+	+
Esculin	-	-	-	-
Gelatin	+	+	+	+
Guanine	-	-	-	-
Hypoxanthine	+	-	+	+
L-Tyrosine	+	+	+	+
Starch	+	+	+	+
Testosterone	+	-	+	+
Xanthine	-	-	-	-
Xylan	+	+	+	+
MORPHOLOGY AND PIGMENTATION				
Presence of aerial spores	+	+	+	+
Spore chain morphology:				
Rectiflexibiles	+	+	+	+
Colour of aerial spore mass:				
Bluish grey	+	+	+	+
Pigmentation of substrate mycelium:				
No distinctive substrate mycelium colour	+	+	+	+
Diffusible pigments:				
Production of diffusible pigments on ISP5	-	-	-	-
Melanin pigment production on ISP6	-	-	-	-
Melanin pigment production on ISP7	-	-	-	-
NUTRITIONAL TESTS:				
Sole carbon and energy sources *				
Sugar alcohols:				
Tetritols and pentitols:				
Adonitol	+	+	+	+
Arabitol	+	+	+	+
Xylitol	+	+	+	+
Hexitols:				
Meso-Inositol	+	+	+	+
Mannitol	+	+	+	+
Sorbitol	+	+	+	-
Carbohydrates				
Pentoses:				
L (+) Arabinose	+	+	+	+
D (+) Galactose	+	+	+	+
D-Lyxose	+	+	+	-
D (-) Ribose	+	+	+	+

Hexoses:				
D (-) Fructose	+	+	+	+
D (+) Mannose	+	+	+	-
D (+) Xylose	+	+	+	+
Deoxy-hexoses:				
α-L (+) Rhamnose	+	+	+	+
Turanose	+	+	+	+
Disaccharides:				
D (+) Cellobiose	+	+	+	+
D (+) Lactose	+	+	+	+
α-D (+) Melibiose	+	-	-	-
Sucrose	+	+	+	+
D (+) Trehalose	+	+	+	+
Trisaccharides:				
D (+) Melezitose	+	+	+	+
D (+) Raffinose	-	-	-	-
Polysaccharides				
Polyglucosides:				
Dextran	+	-	-	-
Inulin	+	-	-	-
Starch	+	+	+	+
Glycoside:				
Salicin	+	+	-	+
Synthetics:				
D-Gluconic acid (Na^+ salt)	+	+	+	+
D-Glucuronic acid (K^+ salt)	+	+	+	+
Carboxylic acids:				
Aliphatic acid:				
Quinic acid	+	+	-	-
Aromatic acids:				
Benzoic acid (Na^+ salt)	+	+	-	-
Urocanic acid	+	+	-	+
Monocarboxylic acid:				
Acetic acid (Na^+ salt)	+	+	+	+
Dicarboxylic acids:				
Malonic acid (Na^+ salt)	-	-	-	-
Pimelic acid	+	+	+	+
Sebacic acid	+	+	+	+
Hydroxy acids:				
Citric acid (Na^+ salt)	+	+	+	+
L(+) Tartaric acid	-	+	+	+
Keto acid:				
Pyruvic acid (Na+salt)	-	+	+	+
Aromatic hydroxy acids:				
Ferulic acid	-	-	-	-
D (+) Mandelic acid	-	-	-	-
Steroids and sterols:				
Androsterone	+	+	+	+
Cholesterol	+	+	+	+
Saponin	+	+	+	+
Miscellaneous carbon compounds:				
Amygdalin	+	+	+	+
Anthranilic acid	-	-	-	-
L-Ascorbic acid	-	+	+	+

Carboxymethylcellulose				
Cinnamic acid	-	+	+	+
Humic acid	+	+	-	-
1-Phenyldodecane	-	-	-	-
Sole nitrogen sources				
Amides:				
Acetamide	-	-	+	+
Glycinamide	+	+	+	+
Propionamide	+	+	+	+
Amine:				
DL-α-Amino-n-butyric acid	+	+	+	+
L-Arginine	+	+	+	+
L-Cysteine	+	+	+	+
L-Histidine	+	+	+	+
L-Hydroxyproline	+	+	+	+
L-*Iso*-Leucine	+	+	+	+
L-Methionine	+	-	-	-
L-β-Phenylalanine	+	+	+	+
L-Proline	+	+	+	+
L-Threonine	+	+	+	+
DL-Valine	+	+	-	-
L-Valine	+	+	+	+
Nitrogenous alkaloid:				
Papaverine	-	-	-	-
Miscellaneous compounds:				
Ammonium dihydrogen orthophosphate	+	+	+	+
Cadaverine	+	+	+	+
Creatine	+	-	-	-
Glycine anhydride	-	+	+	+
Glycine t-butyl ester	+	+	+	+
Hypoxanthine	-	+	+	+
Xanthine	-	+	+	+

PHYSIOLOGICAL TESTS

Growth in the presence of chemical inhibitors (%, w/v)				
Bismuth citrate (0.01)	-	-	-	-
Crystal violet (0.001)	+	+	+	+
Phenol (0.1%, v/v)	+	+	+	+
Phenyl ethanol (0.3%, v/v)	+	+	+	+
Potassium tellurite (0.001)	+	+	+	+
Sodium azide (0.005)	+	+	+	+
Sodium azide (0.01)	-	-	-	-
Sodium borohydride (0.1)	+	+	+	+
Sodium chloride (4.0)	+	+	+	+
Sodium chloride (7.0)	-	-	-	-
Sodium deoxycholate (0.005)	+	+	+	+
Sodium deoxycholate (0.01)	+	+	-	+
Sodium salicylate (0.1)	+	+	+	+
Sodium selenite (0.001)	+	+	+	+
Sodium selenite (0.005)	+	+	-	-
Tetrazolium salt (0.001)	+	+	+	+
Tetrazolium salt (0.01)	-	-	+	+
Tetrazolium salt (0.05)	-	-	-	-

Thallous acetate (0.001)	+	+	+	+
Thallous acetate (0.005)	+	+	-	-
Thallous acetate (0.01)	-	-	-	-
Growth in presence of heavy metal salts:				
Barium chloride (0.005)	+	+	+	+
Cobalt chloride (0.001)	+	+	+	+
Cobalt chloride (0.005)	+	+	+	+
Cobalt chloride(0.01)	-	-	-	-
Resistance to antibiotics and antibacterial compounds (μg/ml)				
Aminoglycosides:				
Amikacin (4)	+	-	+	+
Gentamycin sulphate (8)	+	+	+	+
Gentamycin sulphate (32)	+	-	+	+
Gentamycin sulphate (64)	+	-	+	+
Neomycin sulphate (8)	-	-	-	-
Neomycin sulphate (32)	-	-	-	-
Spiramycin (10)	+	+	+	+
Antitubercular drugs:				
Ethionamide(16)	+	+	+	+
Isoniazid (16)	+	+	+	+
Rifampicin (64)	+	+	+	+
Streptomycin sulphate (4)	+	-	+	+
Streptomycin sulphate (16)	+	-	+	+
Streptomycin sulphate (32)	+	-	+	+
Streptomycin sulphate (64)	+	-	+	+
Cephalosporins:				
Cefoxitin (32)	+	+	+	+
Cephaloridin (128)	+	+	+	+
Cephradin (16)	+	+	+	+
Cephradin (32)	+	-	+	+
Gramicidin (8)	+	+	+	+
Lincosamide:				
Lincomycin (64)	+	+	+	+
Lincomycin (128)	-	+	-	-
Macrolide:				
Oleandomycin phosphate (128)	+	+	+	+
Penicillins:				
Ampicillin (32)	+	+	+	+
Carbenicillin (12)	+	+	+	+
Lividomycin A (16)	-	-	-	-
Penicillin G (15 i.u)	+	+	+	+
Polypeptides:				
Bacitracin (32)	+	+	+	+
Polymyxin B sulphate (16)	+	+	+	+
Polymyxin B sulphate (32)	-	+	-	+
Viomycin (20)	+	+	+	+
Quinolone				
Nalidixic acid (32)	+	+	+	+
Tetracyclines				
Doxycyline (4)	+	+	+	+
Doxycyline (16)	-	-	-	-

Doxycyline (64)	-	-	-	-
Tetracyline (4)	+	+	+	+
Tetracyline (16)	-	-	-	-
Miscellaneous compounds				
Fusidic acid (4)	+	+	+	+
Fusidic acid (8)	+	-	+	+
Fusidic acid (16)	+	-	+	+
Novobiocin (4)	+	+	+	+
Novobiocin (16)	-	+	-	-
Novobiocin (32)	+	+	-	-
Tunicamycin (10)	+	+	+	+
Vancomycin (16)	-	-	-	-
Growth at				
pH 5	-	-	-	-
pH 6	+	+	+	+
pH 10	+	+	+	+
25 °C	-	-	-	-
30 °C	-	-	-	+
37 °C	+	+	+	+
50 °C	+	+	+	+
55 °C	+	+	+	+
60 °C	+	+	-	-

*, at 1 %, w/v apart from D-Glucuronic acid at 0.1 %, w/v.

Table 2-20. Menaquinone and polar lipid composition of the test strains and members of related taxa classified in the family *Thermomonosporaceae*[a]

Taxa[b]	Strains (DSM)	Menaquinone profiles: MK-9[c,d]			Polar lipids profiles[e]				
		H4	H6	H8	DPG	PE	PG	PI	PIDM
***Excellospora* strain TA111**	**44377**	+	+++	++	+	-	+	+	+
***Excellospora* strain TA113**	**44379**	+	+++	t	+	-	+	+	+
Actinomadura rubrobrunea	43750^T	+	+++	++	+	-	+	+	+
Actinomadura rubrobrunea	43751	+	+++	++	+	-	+	+	+
Actinomadura echinospora	43163^T	+	+++	t	+	-	+	+	-
Actinomadura aurantiaca	43924^T	+	+++	+	+	+	-	+	t
Actinomadura libanotica	43554^T	+++	++	+	+	-	-	+	+
Actinomadura atramentaria	43919^T	t	+++	+	+	-	+	+	-
Actinomadura citrea	43461^T	t	+++	+	+	-	+	+	t
Actinomadura coerulea	43675^T	t	+++	+	+	-	+	+	t
Actinomadura crema	43676^T	t	+++	t	+	-	+	+	-
Actinomadura flavescens	43923^T	+	+++	+	+	t	t	+	+
Actinomadura kijianata	43764^T	++	+++	t	+	+	+	+	-
Actinomadura livida	43677^T	t	+++	+	+	-	+	+	-
Actinomadura luteofluorescens	40398^T	+	+++	t	+	-	-	+	t

Actinomadura macra	43862 ^{T}	t	+++	+	+	t	-	+	t
Actinomadura madurae	43067 ^{T}	t	+++	+	+	-	+	+	t
Actinomadura malachitica	43462 ^{T}	t	+++	t	+	-	+	+	t
Actinomadura oligospora	43930 ^{T}	t	+++	+	+	-	-	+	t
Actinomadura pelletieri	43383 ^{T}	t	+++	+	+	-	+	+	+
Actinomadura spadix	43459 ^{T}	t	+++	++	+	-	+	+	t
Actinomadura umbrina	43927 ^{T}	t	+++	+	+	-	t	+	-
Actinomadura verrucosospora	43358 ^{T}	t	+++	+	+	t	+	+	-
Actinomadura vinacea	43765 ^{T}	t	+++	t	+	-	+	+	-
Actinomadura yumaensis	43931 ^{T}	t	+++	+	+	-	-	+	+

[a], All of the data except those for *Excellospora* strains TA111 and TA113 were taken from Kroppenstedt *et al.* (1990) who grew their test organisms in tryptic soy broth shake flasks at 28 °C (mesophilic strains) or 45 °C (thermophilic strains).

[b], The taxa are presented in the order they appeared in the least-squares tree (Fig. 2-9).

[c], The abbreviations of the menaquinones are exemplified by; MK-9(H_4) and MK-9(H_6), that is, tetra- and hexa-hydrogenated menaquinones with nine isoprene units, respectively.

[d], The main component in each series is denoted by '+ + +', components greater than 50% of the main peak by '+ +', components between 25 % and 50 % of the main peak and by '+', and 't' equals trace amount.

[e], Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannoside.

acid fast actinomycetes which form extensively branched, nonfragmenting substrate and aerial mycelia. Spores are borne in chains, singly or in pairs, on both aerial and substrate hyphae; the sporulating hyphae tend to undergo autolysis. The organisms contain *meso*-A_2pm, madurose, major amounts of branched-fatty acids with 14-methylpentadecanoic acid, 15-methylhexadecanoic acid and 16-methylheptadecanoic acid and minor proportions of 10-methyloctadecanoic acid; major amounts of hexahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue, and DPG, PG, PI and PIDM as major polar lipids. Excellosporae are euthermophilic actinomycetes which grow between 37 and 65 °C.

Description of *Excellospora alcalithermophila* sp. nov.

Excellospora alcalithermophila (al.ca.li.ther.mo.phil.a. N.L. n. *alcali* (from arabic *al.* end; *galiy* soda ash); Gr. n. *therme* heat; L. pres. part. *philos*, loving; M.L. part. adj. *alcalithermophila* thermophilic alkali tolerating).

The description is based on data taken from this and an earlier study (Sahin, unpublished). Aerobic, Gram-positive, non-acid-alcohol fast, non-motile actinomycetes which form extensively branched non-fragmenting aerial and substrate hyphae. Straight chains of spiny spores are borne on aerial hyphae. The colour of the aerial spore mass on inorganic salts-starch agar medium is bluish-grey. A pale yellow or non-distinctive substrate mycelium is formed on glycerol-asparagine and oatmeal agar media. Diffusible pigments, including melanin pigments, are not produced on glycerol-asparagine, peptone yeast extract iron or tyrosine agars. Growth occurs between 37 °C and 60 °C, and between pH 6.0 and 10.5.

Casein, DNA, elastin, gelatin, L-tyrosine, starch, and xylan are degraded but no activity is shown against adenine, arbutin, chitin, esculin, guanine or xanthine. The organisms grow on acetic acid, adonitol, amygdalin, androsterone, L-arabinose, arabitol, benzoic acid, D-cellobiose, cholesterol, citric acid, D-fructose, D-galactose, D-gluconic

acid, D-glucuronic acid, humic acid, *meso*-inositol, D-lactose, D-lyxose, D-mannitol, D-mannose, D-melezitose, pimelic acid, quinic acid, α-L-rhamnose, D-ribose, salicin, saponin, sebacic acid, D-sorbitol, starch, sucrose, D-trehalose, turanose, urocanic acid, xylitol and D-xylose as sole carbon and energy sources, but not on anthranilic acid, ferulic acid, malonic acid, D-mandelic acid, 1-phenyldodecane and D-raffinose.

The strains are sensitive to doxycyline hydrochloride (16 μg ml^{-1}), lividomycin A (4 μg ml^{-1}), neomycin sulphate (8 μg ml^{-1}), tetracyline hydrochloride (16 μg ml^{-1}) and vancomycin hydrochloride (16 μg ml^{-1}), but are resistant to ampicillin (32 μg ml^{-1}), bacitracin (32 μg ml^{-1}), carbenicillin (12 μg ml^{-1}), cefoxitin (32 μg ml^{-1}), cephaloridine (128 μg ml^{-1}), cephradine (16 μg ml^{-1}), doxycyline hydrochloride (4 μg ml^{-1}), ethionamide (16 μg ml^{-1}), fusidic acid (4 μg ml^{-1}), gentamycin sulphate (8 μg ml^{-1}), gramicidin (8 μg ml^{-1}), isoniazid (16 μg ml^{-1}), lincomycin hydrochloride (64 μg ml^{-1}), nalidixic acid (32 μg ml^{-1}), novobiocin (4 μg ml^{-1}), oleandomycin phosphate (128 μg ml^{-1}), penicillin G (15 i.u.), polymyxin B sulphate (16 μg ml^{-1}), rifampicin (64 μg ml^{-1}), spiramycin (10 μg ml^{-1}), tetracyline hydrochloride (4 μg ml^{-1}), tunicamycin (10 μg ml^{-1}) and viomycin sulphate (20 μg ml^{-1}).

The organisms were isolated from garden soil collected in Yogyakarta, Indonesia.

The type strain of *Excellospora alcalithermophilus* is TA111 (= DSM 44377).

Description of *Excellospora thermoalcalitolerans* sp. nov.

Excellospora thermoalcalitolerans (ther.mo.al.ca.li.to'le.rans. Gr. n. *therme* heat; N.L. n. *alcali* (from arabic *al.* end; *galiy* soda ash); L. pres. part. *tolerans*, tolerating, enduring; M.L. part. adj. *thermoalcalitolerans* thermophilic alkali tolerating).

The description is based on data taken from this and an earlier study (Sahin, unpublished). Aerobic, Gram-positive, non-acid-alcohol fast, non-motile actinomycetes which form extensively branched, non-fragmenting aerial and substrate hyphae. Straight

chains of spiny spores are borne on aerial hyphae. The aerial spore mass on inorganic salts-starch agar medium is bluish grey. A pale yellow or non-distinctive substrate mycelium on glycerol-asparagine and oatmeal agar media. Neither diffusible or melanin pigments are produced on glycerol-asparagine, peptone yeast extract iron or tyrosine agars. Growth occurs between 30 °C and 55 °C, and between pH 6.0 and 10.5.

Casein, DNA, elastin, gelatin, hypoxanthine, testosterone, L-tyrosine, starch, and xylan are degraded but no activity is shown against adenine, arbutin, chitin, esculin, guanine or xanthine. The organisms grow on acetic acid, adonitol, amygdalin, androsterone, L-arabinose, arabitol, L-ascobic acid, D-cellobiose, cholesterol, cinnamic acid, citric acid, D-fructose, D-galactose, D-gluconic acid, D-glucuronic acid, *meso*-inositol, D-lactose, D-mannitol, D-melezitose, pimelic acid, pyruvic acid, α-L-rhamnose, D-ribose, saponin, sebacic acid, starch, sucrose, L-tartaric acid, D-trehalose, turanose, xylitol and D-xylose as sole carbon and energy sources, but not on anthranilic acid, benzoic acid, dextran, ferulic acid, humic acid, inulin, malonic acid, D-mandelic acid, melibiose, 1-phenyldodecane quinic acid, and D-raffinose.

The strains are sensitive to doxycyline hydrochloride (16 μg ml^{-1}), lincomycin hydrochloride (128 μg ml^{-1}), lividomycin A (8 μg ml^{-1}), neomycin sulphate (8 μg ml^{-1}), novobiocin (16 μg ml^{-1}), tetracyline hydrochloride (16 μg ml^{-1}) and vancomycin (16 μg ml^{-1}), but are resistant to amikacin (4 μg ml^{-1}), ampicillin (32 μg ml^{-1}), bacitracin (32 μg ml^{-1}), carbenicillin (12 μg ml^{-1}), cefoxitin (32 μg ml^{-1}), cephaloridine (128 μg ml^{-1}), cephradine (32 μg ml^{-1}), doxycyline hydrochloride (4 μg ml^{-1}), ethionamide (16 μg ml^{-1}), fusidic acid (16 μg ml^{-1}), gentamycin sulphate (64 μg ml^{-1}), gramicidin (8 μg ml^{-1}), isoniazid (16 μg ml^{-1}), lincomycin hydrochloride (64 μg ml^{-1}), lividomycin A (4 μg ml^{-1}), nalidixic acid (32 μg ml^{-1}), novobiocin (4 μg ml^{-1}), oleandomycin phosphate (128 μg ml^{-1}), penicillin G (15 i.u.), polymyxin B sulphate (16 μg ml^{-1}), rifampicin (16, 32 and 64 μg ml^{-1}), spiramycin (10 μg

ml^{-1}), streptomycin sulphate (4, 16, 32 and 32 μg ml^{-1}), tetracyline hydrochloride (4 μg ml^{-1}), tunicamycin (10 μg ml^{-1}) and viomycin sulphate (20 μg ml^{-1}).

The organisms were isolated from garden soil collected in Yogyakarta, Indonesia.

The type strain of *Excellospora thermoalcalitolerans* is TA113 (= DSM 44379).

Separation of *Excellospora* from other genera classified in the family *Thermomonospora*. The revised genus *Excellospora* can be distinguished from the genera *Actinocorallia, Actinomadura, Spirillospora* and *Thermomonospora* using a combination of chemical and morphological characters (Table 2-8). The continued recognition of the genus *Excellospora* implies that the genus *Actinomadura* is still heterogeneous as members of the validly described species assigned to this taxon were assigned to several 16S rRNA clades (Fig. 2-9, 2-10 and 2-11). Additional comparative studies are needed to clarify the taxonomy of these and related taxa.

Chapter III:

Systematics of Thermophilic Streptomycetes

Introduction

1. Circumscription of the genus *Streptomyces*

The genus *Streptomyces* (Strep.to.my'ces. Gr. adj. *streptos* pliant, bent; Gr. n: *myces* fungus; M. L. masc. n.: *Streptomyces* pliant or bent fungus) is well circumscribed due to the extensive application of chemotaxonomic, molecular systematic and numerical phenetic methods (Williams *et al.*, 1983a, 1989; Witt & Stackebrandt, 1990; Kämpfer *et al.*, 1991; Goodfellow *et al.*, 1992; Embley & Stackebrandt, 1994; Stackebrandt *et al.*, 1997; Zhang *et al.*, 1997). The taxon encompasses aerobic, Gram-positive, non-acid-alcohol-fast actinomycetes which produce well developed vegetative hyphae (between 0.5-2.0 μm in diameter) that rarely fragment. Reproduction is by germination of non-motile spores carried on aerial hyphae. In members of most species, aerial hyphae differentiate into chains of three to many spores (more than 50 spores), which are borne in straight to flexuous, hooked, looped or spiral spore chains. The spore surfaces may be hairy, rugose, smooth, spiny or warty. In members of some species the aerial hyphae consist of long, straight filaments, bearing branches (3-6) at more or less regular intervals, arranged in whorls (verticils). Each branch of the verticil produces, at its apex, an umbel carrying from two to several chains of spherical to ellipsoidal smooth or rugose spores. Some streptomycetes bear short chains of spores on the substrate mycelium. Sclerotia, pycnidial-, sporangial-, and, synnemata-like structures may also be formed. In members of some species, such as *Streptomyces somaliensis*, spores have not been detected.

Streptomycetes form discrete and lichenoid, leathery or butyrous colonies. Initially colonies are relatively smooth but later develop a weft of aerial hyphae that may appear cottony, floccose, granular, powdery or velvety. A wide range of pigments is produced and these are responsible for the colour of the vegetative and aerial mycelia; coloured diffusible pigments may also be formed. Most strains show temperature optima between 25 to 35°C and grow well between pH 6.5 and 8.0. However, some strains grow at temperatures within the psychrophilic (Williams *et al.*, 1989) and thermophilic ranges (Goodfellow *et al.*, 1987; Kim, D. *et al.*, 1996, Kim, S. B. *et al.*, 1998) and others have acidic or alkaline pH requirements for growth (Mikami *et al.*, 1982, 1985; Goodfellow & Simpson, 1987; Korn-Wendisch & Kutzner, 1992; Sahin, 1995).

Streptomycetes are generally chemoorganotrophic, that is, they have an oxidative type of metabolism and are able to use a wide range of organic compounds as sole sources of carbon for energy and growth. Some thermophilic strains are obligate chemolithotrophs, oxidising carbon monoxide and hydrogen for growth (Gadkari *et al.*, 1990), or facultative carboxydotrophs, using single carbon compounds such as methanol or carbon monoxide (O'Donnell *et al.*, 1993b; Kim, S. B. *et al.*, 1998).

Streptomycetes have a cell wall peptidoglycan which contains major amounts of LL-diaminopimelic acid (LL-A_2pm; cell wall chemotype I *sensu* Lechevalier & Lechevalier, 1970a,b) and is of the A3γ type (glycine-LL-A_2pm; Schleifer & Kandler, 1972). They also contain major amounts of saturated, *iso/anteiso* branched and straight-chain fatty acids (e.g. *iso*-16 and *anteiso*-15/17; Kroppenstedt & Kutzner, 1978; Kroppenstedt, 1985), either hexa- or octahydrogenated menaquinones [(MK-9(H6) and MK-9(H8)] with nine isoprene units as the predominant isoprenologue (Collins *et al.*, 1984; Alderson *et al.*, 1985; Kroppenstedt, 1985) and complex polar lipid patterns that typically contain diphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides, but not glucosamine-containing phospholipids (phospholipid type 2 *sensu* Lechevalier *et al.*, 1977).

Streptomycetes are also characterised by the presence of teichoic acids (Naumova *et al.*, 1978; Vylegzhanina *et al.*, 1986), but lack mycolic acids (Goodfellow, 1989; Korn-Wendisch & Kutzner, 1992). The mol% G+C of the DNA is between 66 and 78 mol% (Williams *et al.*, 1989).

Streptomycetes are widely distributed in natural habitats and are especially common in soil, including composts. Members of a few species are pathogenic for animals, including humans and plants (Person & Martin, 1940; Mishra *et al.*, 1980; Healy & Lambert, 1991; Korn-Wendisch & Kutzner, 1992; McNeil & Brown, 1994).

The type species is *Streptomyces albus* (Rossi-Doria 1891) Waksman and Henrici 1943^{AL}.

It is apparent from 16S rRNA sequencing studies that streptomycetes form a distinct phyletic line within the order *Actinomycetales* (Fig. 1-1, page 21; Stackebrandt & Woese, 1981; Stackebrandt *et al.*, 1981, 1997; Witt & Stackebrandt, 1990; Embley & Stackebrandt, 1994). Members of the genus *Streptomyces* can be readily distinguished from other wall chemotype I genera using a combination of chemotaxonomic and morphological characters (Table 3-1).

The genus *Kitasatospora* (formerly *Kitasatosporia*) Omura *et al.* 1982 was proposed for actinomycetes that were phenotypically similar to *Streptomyces* but contained high proportions of *meso*-A_2pm. The aerial mycelia of *Kitasatospora* strains were subsequently found to contain LL- A_2pm as a major component of the peptidoglycan and whole-organism hydrolysates which were rich in galactose (Takahashi *et al.*, 1983). Wellington *et al.* (1992) noted that the 16S rRNA sequence of *Kitasatospora setae* NRRL B-16185^T showed 91.6 % similarity to that of *Streptomyces baldaccii* DPDU 0819^T and that a *Streptomyces*-specific oligonucleotide probe recognised representatives of four validly described species of *Kitasatospora.* These authors proposed that the name *Kitasatospora* should be reduced to a synonym of *Streptomyces* on the basis of these observations and on some phenotypic

Table 3-1. Characters that can be used to distinguish streptomycetes from other actinomycetes with a wall chemotype I.[a]

Characteristics	*Streptomyces* spp.	*Aeromicrobium* spp.	*Intrasporangium calvum*	*Friedmaniella antarctica*	*Kineosporia aurantiaca*	*Luteococcus japonicus*	*Nocardioides* spp.	*Propionibacterium propionicus*	*Propioniferax innocua*	*Sporichthya polymorpha*	*Terrabacter tumescens*
Cell morphology	branching hyphae	nonmotile rods and cocci	branching hyphae	cocci in packets	short hyphae	non-motile cocci	hyphae, rods and cocci	branched hyphae	rods (clusters and V-forms)	short hyphae	rods and motile cocci
Oxygen requirement	strict aerobe	aerobe	aerobe	aerobe	aerobe	facultative anaerobe	strict aerobe	Anaerobe	facultative anaerobe	facultative anaerobe	strict aerobe
Substrate mycelium:	+[b]	-	+	-	+	-	±	+	-	-	-
Spores	±	-	+	-	-	-	-	-	-	-	-
Spore vesicles	-	-	+	-	+	-	-	-	-	-	-
Motile spores	-	-	-	-	+	-	-	-	-	-	-
Fragmentation	-	-	+	-	-	-	+	+	-	-	-
Aerial mycelium:	+	-	-	-	-	-	±	-	-	+	-
Chains of arthrospores	+	-	-	-	-	-	±	-	-	+	-
Arthrospores in verticils	±	-	-	-	-	-	-	-	-	-	-
Motile spores	-	-	-	-	-	-	-	-	-	+	-
Sugars in whole-organism hydrolysates[c]	C	ND	ND	ND	gal, glu, man, rib, xyl	ara	ND	(gal, glu, rha)v, man	ara, man	C	C
Phospholipid type[d]	PII	PII	PIV	PI	PIII	PI	PI[e]	PI[f]	ND	PI	PII[g]
Predominant menaquinones[h]	MK-9 (H^6,H^8)	MK-9 (H^4)	MK-8	MK-9 (H^4)	MK-9 (H^4)	MK-9 (H^4)	MK-8 (H^4)	MK-9 (H^4)	MK-9 (H^4)	MK-9 (H^6)	MK-8 (H^4)
Fatty acids:											
Saturated straight chain	+	+	+	-	+	+	+	+	+	ND	+
Iso- and *anteiso-* branched	+	-	-	+	-	-	+	+	+	ND	+
Unsaturated	-	+	+	-	-	+	+	-	-	ND	+
10-methyl branched	-	+	-	-	+	-	+	-	-	ND	-
2-Hydroxy *iso*-branched	-	-	-	-	+	+	+	-	-	-	-
Mol% G + C of DNA	69 - 78	71 – 73	68	73	69	67	66 – 73	63 - 65	59 - 63	70	70 – 73

[a], Data taken from Charfreitag *et al.* (1988), Itoh *et al.* (1989), Locci and Schofield (1989), Williams *et al.* (1989), Miller *et al.* (1991), Korn-Wendisch and Kutzner (1992), Wellington *et al.* (1992), Rainey *et al.* (1993b), Collins *et al.* (1994), Tamura *et al.* (1994), Tamura and Yokota (1994), Yokota *et al.* (1994) and Schumann *et al.* (1997).

[b], Symbols: +, 90% or more of strains positive; -, 10% or less of strains positive;±, some strains positive; ND, not determined.

[c], Abbreviations: ara, arabinose; gal, galactose; glu, glucose; man, mannose; rha, rhamnose; rib, ribose; xyl, xylose; v, variable; C, no characteristic sugar.

[d], Categories of Lechevalier *et al.* (1977, 1981): PI, phosphatidylglycerol (variable); PII, only phosphatidylethanolamine; PIII, phosphatidylcholine (with phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidylglycerol variable, no phospholipids containing glucosamine); PIV, phospholipids containing glucosamine (with phosphatidylethanolamine and phosphatidylmethylethanolamine variable); all preparations contain phosphatidylinositol.

[e], Contains major amounts of acylphosphatidylglycerol and lacks diphosphatidylglycerol and nitrogenous phospholipids (Lechevalier *et al.*, 1977, 1981).

[f], Characteristic pattern consisting of diphosphatidylglycerol, phosphatidylglycerol and two incompletely characterised glycolipids (O'Donnell *et al.*, 1985).

[g], Also contains diphosphatidylglycerol (Collins *et al.*, 1989).

[h], MK-9(H_6,H_8), notation for a hexa- or octahydrogenated menaquinones with nine isoprene units.

properties. This proposal was supported by the results of an analysis of N-terminal sequences of ribosomal protein AT-L30 (Ochi & Hiranuma, 1994). In contrast, Kim, D. *et al.* (1996) found that three *Kitasatospora* strains formed a distinct clade outside the evolutionary line comprising *Streptomyces* species.

In an attempt to resolve the taxonomic status of the genus *Kitasatospora* Zhang *et al.* (1997) determined almost complete 16S rRNA sequences of 12 actinomycetes which had previously been classified either as *Kitasatospora* or as *Streptomyces* but were shown to contain major amounts of *meso*-A_2pm in whole-organism hydrolysates. The 16S-23S rRNA spacer regions of the test strains were also sequenced. These worker found that the *Kitasatospora* strains either formed a stable monophyletic clade within the genus *Streptomyces* or a sister taxon depending on the outgroup strains. However, the *Kitasatospora* and *Streptomyces* strains were consistently recovered as two distinct clades independent of the outgroup in phylogenetic trees based on the sequences of the 16S-23S rRNA spacer region. Zhang *et al.* (1997) proposed that the genus *Kitasatospora* Omura *et al.* 1982 should be revived on the basis of chemotaxonomic, phenotypic and phylogenetic evidence.

2. Thermophilic streptomycetes

Most of the emphasis in streptomycete systematics has been focused on mesophilic strains which grow between 15 and 37 °C with an optimum temperature around 25 °C. Thermophilic streptomycetes, that is, strains that grow well at 50 °C have received relatively little attention. Indeed, it was a matter of some controversy whether streptomycetes which grow at or above 45 °C should be assigned to distinct taxa or considered as thermotolerant variants of mesophilic species. Craveri and Pagani (1962) proposed the subgenus *Thermostreptomyces* for members of thermophilic taxa, but other workers regarded such organisms as thermotolerant rather than thermophilic (Corbaz *et al.*, 1963; Küster and Locci,

1963). *Thermostreptomyces* was listed as a *subgenus incertae sedis* in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Pridham & Tresner, 1974a).

Streptomyces glaucosporus (Krassilnikov *et al.* 1968a) Agre 1986, *Streptomyces macrosporus* Goodfellow *et al.* 1987, *Streptomyces megasporus* (Krassilnikov *et al.* 1968a) Agre 1983, *Streptomyces thermoautotrophicus* Gadkari *et al.* 1990, *Streptomyces thermocarboxydovorans* Kim, S. B. *et al.* 1998, *Streptomyces thermocarboxydus* Kim, S. B. *et al.* 1998, *Streptomyces thermodiastaticus* (Bergey *et al.* 1923) Waksman 1953, *Streptomyces thermogrieus* Xu *et al.* 1998, *Streptomyces thermolineatus* Goodfellow *et al.* 1987, *Streptomyces thermonitrificans* Desai and Dhala 1967, *Streptomyces thermoviolaceus* (Henssen 1957a) emended Goodfellow *et al.* 1987 and *Streptomyces thermovulgaris* (Henssen 1957a) emended Goodfellow *et al.* 1987 are validly described species which contain thermophilic streptomycetes. Additional thermophilic streptomycetes have been assigned to taxa which are not cited on the *Approved Lists of Bacterial Names*, notably "*Streptomyces thermoflavus*" (Kudrina and Maximova 1963) Pridham 1970, "*Streptomyces thermofuscus*" (Waksman *et al.* 1939) Waksman and Henrici 1948 and "*Streptomyces thermophilus*" (Gilbert 1904) Waksman and Henrici 1948 (syn. *Streptomyces rectus*; Henssen, 1957a).

Streptomyces albus (Rossi Doria 1891) Waksman and Henrici 1943^{AL} and *Streptomyces violaceoruber* (Waksman and Curtis 1916) Pridham 1970^{AL} have also been reported to grow at 55 °C (Lyons & Pridham, 1962; Fergus, 1964). *Streptomyces thermoautotrophicus* strain UBT1^{T} is unusual as it grows chemolithoautotrophically in a mineral medium using either CO or H_2 plus CO_2 as a sole carbon and energy sources (Gadkari *et al.*, 1990). This organism, which was isolated from a heated soil, has an optimal temperature of 65 °C but does not grow below 40 °C or over 65 °C.

3. Streptomycete systematics: the early years

Streptomycete systematics has had a long and tortuous history (Goodfellow *et al.*, 1992; Korn-Wendisch & Kutzner, 1992; Manfio *et al.*, 1995). Early descriptions of 'streptomycete' species by soil microbiologists were based on ecological requirements, pigmentation and spore chain morphology (Krainsky, 1914; Conn, 1916; Waksman & Curtis, 1916; Waksman, 1919; Jensen, 1930) and dichotomous keys for the identification of unknown strains rested on a few non-standardised tests, notably morphological and pigmentation characteristics (Krainsky, 1914; Waksman & Curtis, 1916; Waksman, 1919; Jensen, 1930; Krassilnikov, 1941). A turning point in streptomycete systematics came in 1943 when Waksman and Henrici proposed the genus *Streptomyces* for aerobic spore-forming actinomyces to avoid confusion with pathogenic microaerophilic organisms which retained the name *Actinomyces* Harz 1877.

It was only after the discovery that *Streptomyces antibioticus* produced actinomycin (Waksman & Woodruff, 1941) that streptomycetes were given serious attention. The realisation that these organisms were a rich source of commercially useful and hence highly profitable antibiotics prompted many workers to design new procedures for their isolation and growth. Lack of acceptable criteria for classification and identification led to new species being described usually on the basis of slight differences in morphological and cultural properties. This practice led to a proliferation of *Streptomyces* species (Waksman, 1957; Kurylowicz & Gyllenberg, 1988). Between 1940 and 1957 over a hundred *Streptomyces* species were described (Pridham *et al.*, 1958). This number increased to around 3,000 by 1970 though many of the new combinations were only cited in the patent literature (Trejo, 1970). Numerous artificial classifications were proposed to accommodate the ever increasing number of *Streptomyces* species. These classifications were mainly based on a few subjectively chosen characters, usually morphological and pigmentation properties, but, in some instances, biochemical, nutritional and physiological features were used. These

schemes enabled isolates to be 'identified' but the name given was dependent on the scheme used.

It had become clear by the early 1960s that streptomycete systematics was in a parlous state. The resultant practical problems were addressed in two co-operative investigations carried out between 1958 and 1962. One of the studies was performed under the auspices of the *Subcommittee on Actinomycetes* of the *Committee on Taxonomy* of the *American Society of Microbiology* (ASM; Gottlieb, 1961) and the other by the *Subcommittee on Taxonomy of Actinomycetes* of the *International Committee on Bacteriological Nomenclature* of the *International Association of Microbiological Societies* (AMS; Küster, 1959).

An attempt was made in each of these co-operative studies to evaluate the predictiveness of characters commonly used in streptomycete systematics. In the AMS study, 34 investigators examined 25 strains representing 21 streptomycete "series" using standard methods and growth conditions (Küster, 1959). The findings of the study were published (Küster, 1961; Szabó & Marton, 1964). Ten laboratories were involved in the corresponding ASM collaborative project which concluded that more work was required before reliable physiological tests could be recommended for the classification and identification of streptomycetes (Gottlieb, 1961).

It was clear from the results of the collaborative studies that developments in streptomycete systematics were being hampered by the use of variable and non-diagnostic characters that were often examined under non-standardised conditions. The reliance placed on subjectively weighted phenotypic features, the unavailability of extant type cultures of some species, and the difficulty in finding descriptions of species reported in the patent literature were all highlighted as serious problems. Although the co-operative projects raised as many problems as they answered they did pave the way for an extensive international collaborative study of the genus *Streptomyces*.

The *International Streptomyces Project* (ISP; Shirling & Gottlieb, 1966) was planned and executed by the *Subcommittee on Taxonomy of Actinomycetes* of the *International Committee on Bacteriological Nomenclature* and the *Subcommittee on Actinomycetes* of the *Committee on Taxonomy* of the *American Society of Microbiology* with the primary aim of providing reliable descriptions of extant and authentic type strains of *Streptomyces* and *Streptoverticillium* species. Existing type and neotype strains of species assigned to these genera were sent under code to at least three experts in different countries. The strains were examined using rigorously standardised procedures to determine their morphological, pigmentation, and carbon source utilisation properties. These characters were selected in light of the results derived from the earlier international co-operative studies (Küster, 1959; Gottlieb, 1961, 1963). The methods and new descriptions of the cultures were published (Shirling & Gottlieb, 1966, 1968a,b, 1969, 1972; Gottlieb & Shirling, 1967) and the type strains deposited in a number of internationally recognised service culture collections. The results of the International *Streptomyces* Project formed the basis of the classification of the genus *Streptomyces* in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Pridham & Tresner, 1974a,b). Seven thermophilic streptomycetes were included in the International *Streptomyces* Project, namely, *Streptomyces thermodiastaticus* ISP 5573^T, "*Streptomyces thermoflavus*" ISP 5574, *Streptomyces thermonitrificans* ISP 5579^T, "*Streptomyces thermophilus*" ISP 5365, "*Streptomyces thermotolerans*" ISP 5227, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* ISP 5443^T and *Streptomyces thermovulgaris* ISP 5444^T.

The participants in the International *Streptomyces* Project made a major contribution to streptomycete systematics as the practical problems outlined earlier were met. However, the very success of the project highlighted a number of serious weaknesses:

i) No attempt was made to detect synonyms or to devise a species concept for the genus;

ii) Few criteria were used to describe species and the ones that were applied were

essentially those which had been intuitively selected from a plethora of earlier classifications (Krainsky, 1914; Waksman & Curtis, 1916; Waksman, 1919, 1961; Jensen, 1930; Waksman & Henrici, 1948; Baldacci *et al.*, 1954; Hesseltine *et al.* 1954; Gauze *et al.*, 1957; Pridham *et al.*, 1958; Mayama, 1959; Nomi, 1960; Küster, 1961; Gottlieb, 1963; Hütter, 1967).

iii) An objective identification system was not produced although ISP data were used to generate dichotomous keys (Arai & Mikami, 1969; Küster, 1972; Nonomura, 1974; Szabó *et al.*, 1975). However, none of these schemes were widely used.

The reliance placed on the use of a limited number of intuitively chosen features, with heavy emphasis on morphology and pigmentation, represented a serious conceptual flaw in streptomycete systematics. The products of this approach to classification are intrinsically artificial and although some of them 'work', in the sense that a name is inevitably obtained for an unknown culture, they are essentially monothetic with rigid key characters and a limited information content. Indeed, it was only with the application of the numerical taxonomic procedure that attempts were made to construct polythetic classifications where organisms which share many features in common are grouped together with no single character being essential for group membership (Williams *et al.*, 1981; Goodfellow *et al.*, 1992; Manfio, 1995; Sahin, 1995).

4. Application of modern taxonomic methods

(a) Numerical taxonomy

The numerical taxonomy procedure was first applied to *Streptomyces* by Silvestri and his colleagues (Gilardi *et al.*, 1960; Hill *et al.*, 1961; Silvestri *et al.*, 1962) who examined nearly 200 mesophilic organisms for 100 unit characters. The test strains were assigned to 25 centres of taxonomic variation but some strains bearing the same specific name were recovered in different clusters. Several physiological and biochemical characters

highlighted in this study were used to construct identification keys (Hill & Silvestri, 1962). Results from factor analyses suggested that many characters used to described *Streptomyces* species were highly variable and prone to errors of interpretation (Gyllenberg, 1970). These early leads had little impact on developments in streptomycete systematics though a number of additional numerical phenetic studies were designed to clarify the taxonomy of specific groups of streptomycetes (Kurylowicz *et al.*, 1969, 1970, 1975; Paszkiewicz, 1972; Gyllenberg *et al.*, 1975; Szulga, 1978).

Sneath (1970) considered that a rigorous application of the numerical taxonomic procedure provided the only way of reclassifying the six hundred "species" of the genus *Streptomyces* since reliance on a few subjectively chosen tests could not be expected to reveal natural phenetic groups. The first comprehensive numerical taxonomic survey of the genus was carried out by Williams *et al.* (1983a) who examined 475 cultures, including 394 *Streptomyces* type strains from the International *Streptomyces* Project, for 139 unit characters. The data were analysed using the S_J and S_{SM} coefficients and clustering of similar strains achieved using the UPGMA algorithm. The resultant classification added to a wealth of evidence that eventually led to the genera *Actinopycnidium* Krassilnikov 1962, *Actinosporangium* Krassilnikov & Yuan 1961, *Chainia* Thirumalachar 1955, *Elytrosporangium* Falcão de Morais *et al.* 1966, *Kitasatoa* Matsumae *et al.* 1968, *Microellobosporia* Cross *et al.* 1963 and *Streptoverticillium* Baldacci 1958 becoming synonyms of the genus *Streptomyces* (Goodfellow *et al.*, 1986a-d; Witt & Stackebrandt, 1990). The type strains of the *Streptomyces* species were assigned to 19 major clusters (6 to 71 strains), which were provisionally considered as species-groups, and to 40 minor (2 to 5 strains) and 18 single-membered clusters that were equated with species. The seven thermophilic streptomycetes included in the International *Streptomyces* Project were also examined. "*Streptomyces thermoflavus*" ISP 5574, *Streptomyces thermonitrificans* ISP 5579^T and *Streptomyces thermovulgaris* ISP 5444^T formed a numerically defined cluster

whereas the other thermophilic strains were recovered at the periphery of clusters containing mesophilic streptomycetes. However, these results may be anomalous as the thermophilic strains were examined at 25 °C and hence may not have grown well on all of the test media. The results of this study provided the basis of the current taxonomy of the genus *Streptomyces* in *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989).

The classification of Williams *et al.* (1983a) was used to generate probabilistic schemes for the identification of unknown mesophilic streptomycetes to major and minor streptomycete clusters (Williams *et al.*, 1983b; Langham *et al.*, 1989). The computer-assisted approaches to the identification of streptomycetes rested on a balanced set of *a posteriori* weighted characters that accommodated some degree of strain variation. These approaches were in sharp contrast to previous streptomycete identification systems that were based on a few subjectively chosen features (Waksman, 1961; Pridham *et al.*, 1958; Hütter, 1967).

Goodfellow *et al.* (1992) re-examined most of the strains studied by Williams and his colleagues for all but two of the original 139 unit characters together with the results of rapid enzyme tests based on the fluorophores 7-amino-4-methylcoumarin and 4-methylumbelliferone. Excellent congruence was found with the earlier numerical classification though three taxa previously defined as subclusters, namely, *Streptomyces albidoflavus, Streptomyces anulatus* and *Streptomyces halstedii*, were recovered as separate, albeit related, clusters.

It is also encouraging that most of the major clusters defined by Williams *et al.* (1983a) were recognised by Kämpfer *et al.* (1991) who examined 821 *Streptomyces* (including *Streptoverticillium* spp.) for 329 physiological tests in a comprehensive numerical taxonomic study. Kämpfer and his colleagues concluded that the taxonomic status of many of their clusters, notably the minor and single membered clusters, were questionable. However, the results of their numerical taxonomic study were used to

construct a probability matrix for the numerical identification of streptomycetes (Kämpfer & Kroppenstedt, 1991).

Saddler (1988) isolated large numbers of alkalitolerant, mesophilic streptomycetes from a range of soils using isolation media adjusted to pH 10.0. An artificial classification of 731 alkalitolerant isolates based on pH requirements for growth, morphology and pigmentation properties revealed that 80 % of the taxonomically diverse strains were able to grow at pH 7.0 and pH 10.0. One hundred and seventy representatives of the 25 colour groups recognised by Saddler were compared with thirty-six marker neutrophilic strains of *Streptomyces* species for 136 unit characters and the resultant data examined using standard numerical taxonomic procedures. The test strains were assigned to eight multimembered and seven single-membered aggregate groups in the S_J, UPGMA analysis. The aggregate groups encompassed nine major (5 to 36 strains), eighteen minor (2 to 4 strains) and fifty-three single-membered clusters. The alkalitolerant isolates were largely distinct from the *Streptomyces* marker strains. There was considerable correlationship between cluster-group membership and the source, colour group and pH ranges of the strains.

Doering-Saad *et al.* (1992) examined eighty *Streptomyces* isolates, including 35 potato scab-inducing strains and 12 reference strains of *Streptomyces scabiei,* for 329 unit characters. The strains were assigned to three cluster-groups (A to C) defined at the 80 % similarity level in an S_{SM}, UPGMA analysis. Cluster-group A contained organisms that were related to either *Streptomyces exfoliatus* or *Streptomyces griseus*; cluster-group B encompassed strains which showed affinities to either *Streptomyces rochei* or *Streptomyces violaceus.* The majority of the pathogenic isolates and the reference strains assigned to cluster-group C were classified as either *Streptomyces griseus* or *Streptomyces violaceus.*

The first comprehensive numerical taxonomic study devoted to thermophilic streptomycetes was carried out by Goodfellow *et al.* (1987). These workers examined fifty thermophilic, neutrophilic streptomycetes from diverse habitats and compared the results

with corresponding data on representative mesophilic, neutrophilic marker strains that had been included in the extensive numerical taxonomic survey of Williams *et al.* (1983*a*). The thermophilic strains, which were grown at 45 °C, were examined for one hundred and thirty-five unit characters and the resultant data analysed using appropriate resemblance coefficients and clustering algorithms. Two aggregate clusters were detected, one contained the mesophilic streptomycetes and the other the thermophilic strains. The latter were assigned to two major (7 to 19 strains), four minor (2 to 3 strains) and two single-membered clusters. Three of these taxa were equated with validly described species, namely, *Streptomyces megasporus* (Krassilnikov *et al.* 1968a) Agre 1983, *Streptomyces thermoviolaceus* Henssen 1957b and *Streptomyces thermovulgaris* Henssen 1957b. The remaining cluster was raised to species status as *Streptomyces thermolineatus* Goodfellow *et al.* 1987.

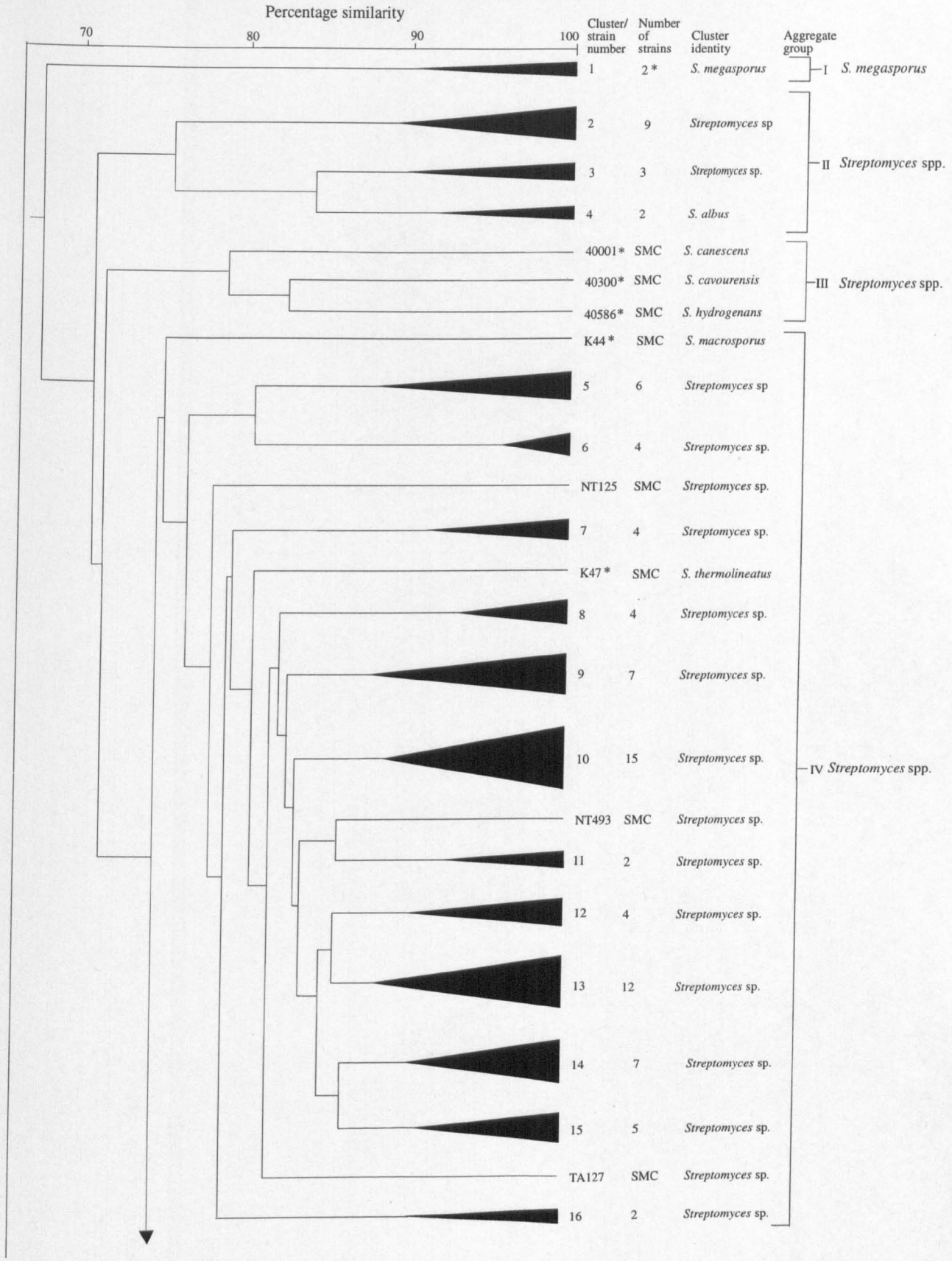
Fifty-four thermophilic, carboxydotrophic actinomycetes, isolated from soils and composts, were the subject of an extensive numerical phenetic survey together with representative mesophilic and thermophilic streptomycetes (O'Donnell *et al.*, 1993b). The test strains, which were grown at either 25 °C (mesophilic strains) or 45 °C (thermophilic strains), were examined for 119 unit characters and the data analysed using the D_P, S_J and S_{SM} coefficients and the UPGMA algorithm. The carboxydotrophic actinomycetes formed two major cluster-groups which were distinct from corresponding taxa equated with mesophilic and thermophilic streptomycetes. Most of the carboxydotrophic strains grew at 55 °C and all but two of them had a profile of chemical properties consistent with their assignment to the genus *Streptomyces*.

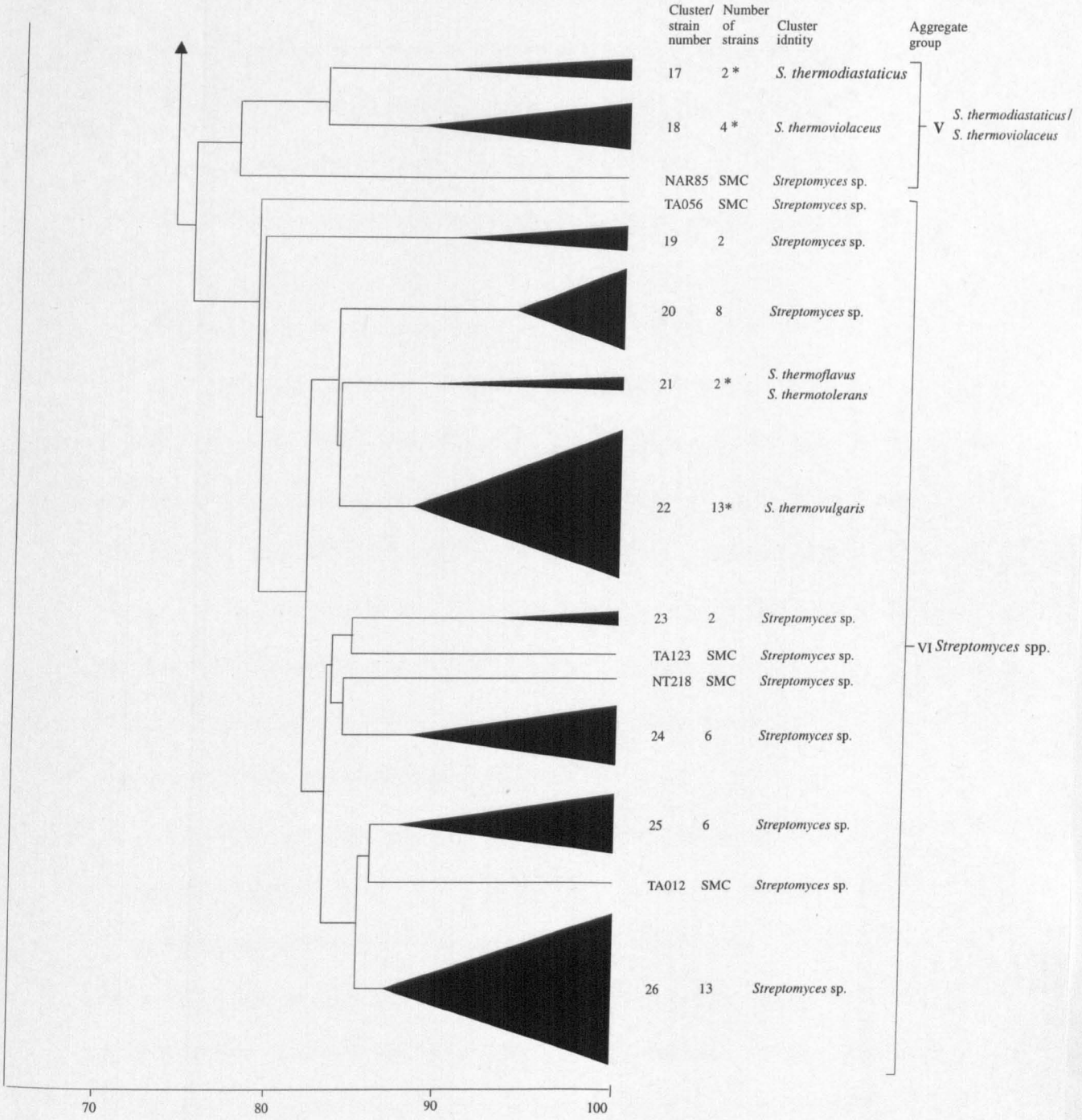
Sahin (1995) isolated large numbers of thermophilic streptomycetes from arid and tropical soil samples by incubating starch casein agar plates supplemented with cycloheximide and rifampicin, and adjusted to pH 7.0 or pH 10.5, at 55 °C for 5 days. Forty-five alkalitolerant, thermophilic streptomycetes, and eighty-five neutrophilic, thermophilic

streptomycetes were chosen to represent groups based on aerial spore mass colour, substrate mycelial pigmentation, diffusible pigment colour and on the production of melanin pigments. These organisms were examined with thirty-two marker neutrophilic, thermophilic streptomycetes, including *Streptomyces macrosporus* K44^T, *Streptomyces megasporus* K45^T, *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermolineatus* DSM41451^T, *Streptomyces thermoviolaceus* DSM 40443^T and *Streptomyces thermovulgaris* DSM 40444^T, for three hundred and thirty-nine unit characters together with three alkalitolerant, mesophilic organisms, namely, strain ISP 5001^T representing *Streptomyces canescens* Waksman 1957^{AL}, strain ISP 5300^T representing *Streptomyces cavourensis* subspecies *cavourensis* Skarbek and Brady 1978^{AL} and strain ISP 5586^T representing *Streptomyces hydrogenans* Lindner *et al.* 1958^{AL}. Eighteen randomly chosen duplicated cultures were studied under code to determine test error. A broad range of degradative, enzymatic, morphological, nutritional and physiological tests were performed to avoid undue emphasis on any particular character set. The enzymic tests were carried out using an automated system that involved the use of conjugated substrates based on the fluorophores 7-amino-4-methylcoumarin and 4-methylumbelliferone. Fifty-six unit characters were deleted from the raw database as they gave all positive or all negative results and a further twenty-three properties were removed because of high test error.

The final database contained information on one hundred fifty-nine test strains and two hundred and sixty unit characters. Good congruence was found between the classifications based on the standard resemblance coefficients (S_J, S_P and S_{SM}) and the single linkage and UPGMA clustering algorithms. The S_{SM}, UPGMA analysis was used as the baseline classification as it gave particularly good resolution of aggregate groups and clusters and a high cophenetic correlation value; six aggregate groups encompassed twelve major (5 to 15 strains), fourteen minor (2 to 4 strains) and thirteen single membered clusters (Fig. 3-1). Cluster composition was only marginally affected by the statistics used or by the

Figure 3-1. Abridged dendrogram showing aggregate groups and major, minor and single membered clusters defined by Sahin (1995) using the S_{SM} coefficient and the UPGMA algorithm (* type strain). The clusters were defined at the 87 % S-level.


Percentage similarity
70
80
90
100
Cluster/ strain number
Number of strains
Cluster identity
Aggregate group
1 2* S. megasporus
I S. megasporus
2 9 Streptomyces sp
3 3 Streptomyces sp.
4 2 S. albus
II Streptomyces spp.
40001* SMC S. canescens
40300* SMC S. cavourensis
40586* SMC S. hydrogenans
III Streptomyces spp.
K44* SMC S. macrosporus
5 6 Streptomyces sp
6 4 Streptomyces sp.
NT125 SMC Streptomyces sp.
7 4 Streptomyces sp.
K47* SMC S. thermolineatus
8 4 Streptomyces sp.
9 7 Streptomyces sp.
10 15 Streptomyces sp.
NT493 SMC Streptomyces sp.
11 2 Streptomyces sp.
12 4 Streptomyces sp.
13 12 Streptomyces sp.
14 7 Streptomyces sp.
15 5 Streptomyces sp.
TA127 SMC Streptomyces sp.
16 2 Streptomyces sp.
IV Streptomyces spp.


Cluster/ strain number
Number of strains
Cluster idntity
Aggregate group
17 2 * S. thermodiastaticus
18 4 * S. thermoviolaceus
V S. thermodiastaticus / S. thermoviolaceus
NAR85 SMC Streptomyces sp.
TA056 SMC Streptomyces sp.
19 2 Streptomyces sp.
20 8 Streptomyces sp.
21 2 * S. thermoflavus S. thermotolerans
22 13* S. thermovulgaris
23 2 Streptomyces sp.
TA123 SMC Streptomyces sp.
NT218 SMC Streptomyces sp.
24 6 Streptomyces sp.
25 6 Streptomyces sp.
TA012 SMC Streptomyces sp.
26 13 Streptomyces sp.
VI Streptomyces spp.
70
80
90
100

test error of 1.81%.

Thirty out of the forty-five alkalitolerant, thermophilic isolates were assigned to three major (6 to 13 strains), one minor and three single membered clusters in aggregate group VI. One of major clusters in aggregate group VI was identified as *Streptomyces thermovulgaris* as it contained the type strain of this species. The remaining fifteen alkalitolerant, thermophilic isolates were assigned to one major, one minor and one single-membered cluster in aggregate group IV and to one single-membered cluster in aggregate group V.

Sixty-one out of the eighty-five neutrophilic, thermophilic streptomycetes were recovered in aggregate group IV which encompassed six major (6 to 15 strains), six minor (2 to 4 strains) and three single membered clusters (Fig. 3-1). The two marker strains assigned to this aggregate group, *Streptomyces megasporus* K45^T and *Streptomyces thermolineatus* DSM 41451^T, also formed single membered clusters. Twelve neutrophilic, thermophilic isolates formed two putatively novel taxospecies that were assigned to aggregate cluster II together with two representatives of *Streptomyces albus*. The remaining twelve neutrophilic, thermophilic isolates were assigned to one major (10 strains), one minor and one single membered cluster in aggregate group VI.

The three remaining aggregate taxa were composed solely of marker strains. *Streptomyces canescens* DSM 40001^T, *Streptomyces cavourensis* subspecies *cavourensis* DSM 40300^T and *Streptomyces hydrogenans* DSM 40586^T were recovered as single membered clusters in aggregate group III and the two minor clusters which formed aggregate group V corresponded to the validly described species, *Streptomyces thermodiastaticus* and *Streptomyces thermoviolaceus*. Aggregate group I contained the two marker strains of *Streptomyces megasporus*.

The extensive numerical phenetic analyses considered above were partly designed to help determine the extent of streptomycete diversity and to provide a framework for

further developments in streptomycete systematics. It was, of course, recognised by the investigators that relationships depicted in numerical classifications can be influenced by test and strain selection, test error and the genetic instability of the test strains (Goodfellow & O'Donnell, 1993; Schrempf *et al.*, 1989, 1994) hence the need to evaluate numerical classifications in light of data derived from independent taxonomic methods.

(b) Molecular systematics

Relatively few attempts have been made to determine the taxonomic integrity of streptomycete clusters defined in the recent extensive numerical phenetic surveys using molecular systematic methods. This is surprising considering the ecological and commercial importance of streptomycetes but probably reflects the difficulty of examining a representative sample of strains from a genus which currently contains 465 validly described species, 44 subspecies (Bacterial Nomenclature, Deutsche Samlung von Mikroorganismen und Zellkulturen GmbH, 1998) and many putatively novel species. Most of the molecular taxonomic investigations that have been carried out have been focused on a few representatives of a relatively small number of the numerically defined taxa circumscribed by Williams *et al.* (1983a).

Nucleic acid sequencing studies. Nucleic acid cataloguing and sequencing studies have been used to determine taxonomic relationships between streptomycete species. 16S rRNA cataloguing experiments (Stackebrandt *et al.*, 1983) demonstrate a close relationship between representatives of the genera *Chainia* Thirumalachar 1955, *Elytrosporangium* Falcão de Morais *et al.* 1966, *Kitasatoa* Matsumae *et al.* 1968, *Microellobosporia* Cross *et al.* 1963, *Streptoverticillium* Baldacci 1958 and *Streptomyces* Waksman and Henrici 1943.

Almost complete 16S rRNA sequence data are available for 43 out of the 465 validly described species of *Streptomyces*. Even so, a number of phyletic lines can be detected in the emerging streptomycete tree as exemplified by sequences recorded for thermophilic streptomycetes (Fig. 3-2). Kim, D. *et al.* (1996) found that thermophilic streptomycetes

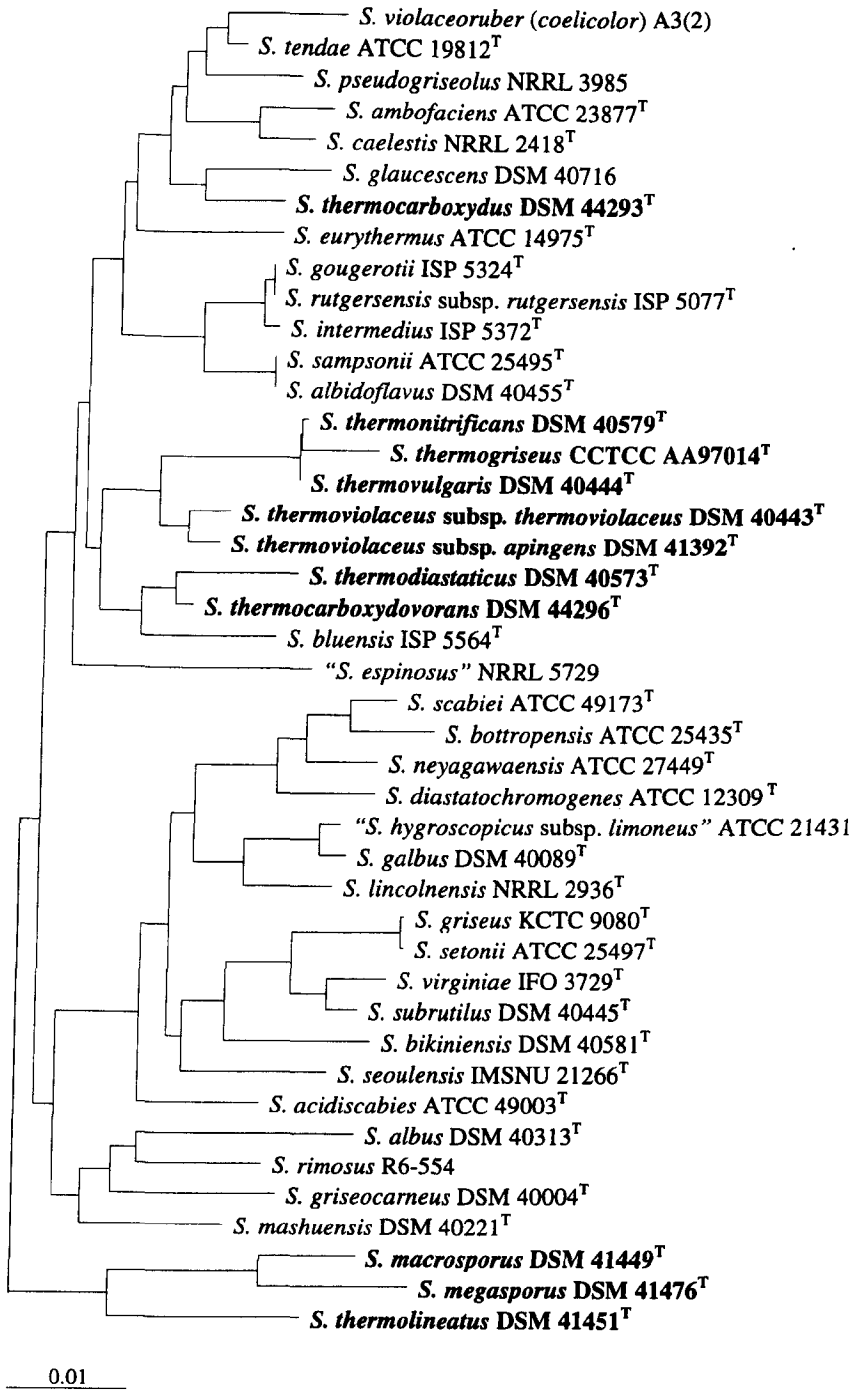


Figure 3-2. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16S rRNA sequences showing relationships between representatives of the genus *Streptomyces*. The 16S rRNA sequence of *Arthrobacter globiformis* DSM 20214^T (Accession number: M23411) was used as outgroup. Thermophilic streptomycetes are in bold. The scale bar indicates 0.01 substitutions per nucleotide position.

formed two distinct clades within the evolutionary radiation encompassed by the genus *Streptomyces*. One clade contained organisms classified as *Streptomyces macrosporus* Goodfellow *et al.* 1987, *Streptomyces megasporus* (Krassilnikov *et al.*, 1968a) Agre 1983 and *Streptomyces thermolineatus* Goodfellow *et al.* 1987 and the other representatives of *Streptomyces thermodiastaticus* (Bergey *et al.*, 1923) Waksman 1953^{AL}, *Streptomyces thermonitrificans* Desai and Dhala 1967, *Streptomyces thermoviolaceus* (Henssen, 1957b) emended Goodfellow *et al.* 1987 and *Streptomyces thermovulgaris* (Henssen, 1957b) emended Goodfellow *et al.* 1987. These results show that thermophilic streptomycetes form a diverse group and hence cannot be considered as a distinct subgroup within the genus *Streptomyces* as proposed by Craveri and Pagani (1962). Subsequently, *Streptomyces thermocarboxydovorans,* a thermophilic, carboxydotrophic streptomycete, was found to be closely related to *Streptomyces thermodiastaticus* on the basis of 16S rRNA sequence data (Kim, S. B. *et al.*, 1998). These authors also showed that another carboxydotrophic, thermophilic streptomycete, *Streptomyces thermocarboxydus,* formed a distinct phyletic line in the streptomycete tree. *Streptomyces thermogriseus* Xu *et al.* 1998, which has an upper temperature limit for growth of 65 to 68 °C, also belong to this groups.

Almost complete 16S rRNA sequence data have been used to help clarify the taxonomy of several streptomycete taxa, including streptomycetes which cause potato scab (Doering-Saad *et al.*, 1992; Goyer *et al.*, 1996; Healy & Lambert, 1991; Lambert & Loria, 1989a,b; Takeuchi *et al.*, 1996). The agents of potato scab formed a heterogeneous group thereby suggesting that the ability to cause this disease has arisen independently in several streptomycete lineages. However, *Streptomyces scabies* strains isolated from diverse geographical areas had either identical 16S rRNA sequences or ones which only differed by a single nucleotide.

Hain *et al.* (1997) found that *Streptomyces albidoflavus* strains DSM 40455^{T}, DSM 40792, DSM 40880 and DSM 46452, *Streptomyces canescens* DSM 40001^{T}, *Streptomyces*

coelicolor DSM 40233^T, *Streptomyces felleus* DSM 40130^T, *Streptomyces limosus* DSM 40131^T, *Streptomyces odorifer* DSM 40347^T, and *Streptomyces sampsonii* DSM 40394^T had identical 16S rRNA sequences when almost complete 16S rRNA sequences were determined. It was also clear that *Streptomyces gougerotii* DSM 40324^T, *Streptomyces intermedius* DSM 40372^T, and *Streptomyces rutgersensis* DSM 40077^T were closely related to *Streptomyces albidoflavus* though they showed 15, 14 and 16 nucleotide differences to representatives of this taxon. These results are interesting as Williams *et al.* (1989) considered that *Streptomyces canescens, Streptomyces coelicolor, Streptomyces felleus, Streptomyces limosus, Streptomyces odorifer* and *Streptomyces sampsonii* should be seen as subjective synonyms of *Streptomyces albidoflavus* on the basis of shared phenotypic properties.

It is becoming clear that 16S rRNA sequences should form part of the minimal descriptions of streptomycete species (Chun *et al.*, 1997; Kim, S. B. *et al.*, 1998). However, several recent descriptions of new species of *Streptomyces* have been based on phenotypic properties (Esnard *et al.*, 1995; Li, 1997) or on limited DNA:DNA relatedness data (Goyer *et al.*, 1996; Labeda *et al.*, 1997). The danger of this approach can be exemplified by the case of *Streptomyces spitsbergensis.* This organism, which was proposed by Wieczorek *et al.* (1993) based on DNA:DNA relatedness data obtained from a set of wrongly chosen reference strains, was subsequently found to belong to the same genomic species as *Streptomyces baldaccii* (Hatano *et al.*, 1997).

Several investigators have tried to clarify relationships within the genus *Streptomyces* by sequencing the more variable regions of 16S rRNA. Witt and Stackebrandt (1990) examined 520 nucleotides of the 16S rRNA of representatives of the genus *Streptomyces.* Sixteen out of the seventeen test strains fell into two phyletic lines. The first branch included *Streptomyces ambofaciens* ATCC 23877^T, *Streptomyces brasiliensis* DSM 43159^T, *Streptomyces diastaticus* DSM 40496^T, *Streptomyces indianensis* DSM 43803^T,

Streptomyces (coelicolor) violaceoruber A3(2) and *Streptomyces (lividans) violaceoruber* TK21, and the second *Streptomyces lavendulae* DSM 2014^T, *Streptomyces purpureus* DSM 43460^T and members of several *Streptoverticillium* species. *Streptomyces albus* DSM 40313^T formed a deep-rooted branch which was well separated from the two major branches. The 16S rRNA sequence data underpinned the close phenotypic relationship detected between *Streptomyces lavendulae* (cluster F61), *Streptomyces purpureus* (cluster F65) and *Streptoverticillium* strains (cluster group F) by Williams *et al.* (1983a). Witt and Stackebrandt went on to propose that the genus *Streptoverticillium* be reduced to a subjective synonym of the genus *Streptomyces* and emended the description of the genus *Streptomyces* accordingly.

Stackebrandt *et al.* (1991) analysed partial sequences of 16S and 23S rRNA preparations isolated from representatives of several *Streptomyces* species in a search for oligonucleotide signatures that could be used to assign unknown strains to the genus, species-groups and individual streptomycete species. 16S rRNA nucleotide 929 (*Streptomyces ambofaciens* numbering system [Pernodet *et al.*, 1989]; nucleotide 955 in the *Escherichia coli* numbering system [Brosius *et al.*, 1978]) was found to be unique to *Streptomyces* strains. A genus-specific probe (5'-GCGTCGAATTAAGCCACA-3') was generated incorporating this nucleotide position and its flanking regions.

Stackebrandt and his colleagues also found that regions α and β (nucleotides 982 through to 998 and 1102 through to 1122, respectively; *Streptomyces ambofaciens* numbering system) of 16S rRNA were relatively conserved though variations in these regions allowed the test strains to be classified into nineteen and twelve sequence groups, respectively. *Streptomyces* strains with identical nucleotide sequences for regions α and β were separated on the basis of variation in region γ of the 16S rRNA (nucleotides 158 through 203; *Streptomyces ambofaciens* numbering system). Partial sequencing of helix 54 of the 23S rRNA (between position 1518 and 1637; *Streptomyces ambofaciens* numbering

system [Pernodet *et al.*, 1989]) of six streptomycete strains, two other actinomycetes and two non-actinomycete reference strains revealed a high degree of variation within this region of the 23S rRNA molecule which was seen to have potential for designing species-specific probes.

The results of the studies outlined above suggested that stretches of 16S rRNA (regions α, β and γ) and helix 54 of the 23S rRNA showed sufficient variability to be used for the identification of members of some *Streptomyces* species. However, the significance of these findings for the classification of the genus *Streptomyces* has still to be established.

Stackebrandt and his colleagues (1992) considered that accurate phylogenetic trees could be generated by sequencing relatively small stretches of streptomycete 16S rRNA. The results of an analysis of the 226-nucleotide-stretch of 16S rRNA corresponding to the α and β variable regions from representatives of fifteen *Streptomyces* species were used to generate a phylogenetic tree which was found to correspond with one based on an analysis of 1137-nucleotide 16S rRNA sequences. Good congruence was also found between the phylogenetic classifications based on the α and β regions and the numerical phenetic classification of Williams *et al.* (1983a). However, an analysis based on a 204-nucleotide 16S rRNA sequence encompassing the highly variable γ region gave a phylogenetic tree which showed much less congruence with those of the α and β regions and the numerical phenetic data.

Kataoka *et al.* (1997) compared 120 nucleotides in the hypervariable region of 16S rRNA (nucleotide positions 158 to 277; *Streptomyces ambofaciens* numbering system [Pernodet *et al.*, 1989]) of 89 streptomycetes which represented eight cluster-groups assigned to category I in *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989). The test strains were assigned to fifty-seven 'identity groups' the members of which had common sequences. The identity groups fell into seven clusters each of which contained four or more strains. It was concluded that short hypervariable regions of the 16S rRNA

could be used to identify unknown strains to members of validly described *Streptomyces* species.

DNA:DNA relatedness studies. A widely accepted way of determining the taxonomic integrity of numerically defined taxospecies is to examine representative strains in DNA:DNA relatedness experiments. In general, good congruence has been found between bacterial classifications based on numerical phenetic and DNA relatedness data (Goodfellow *et al.*, 1997a). The evaluation of numerically defined clusters using DNA relatedness data rests on the premise that phenotypic diversity is a function of genotypic divergence. It has already been pointed out that genomic species can be defined as taxa that encompass strains which share 70% or more DNA:DNA relatedness with a difference in melting point (ΔTm) of 5 °C or less (Wayne *et al.*, 1987).

Early DNA:DNA relatedness studies on representatives of the genus *Streptomyces* were mainly based on a few strains. Monson *et al.* (1969) used a DNA hybridisation procedure to confirm the identity of strains assigned to *Streptomyces coelicolor* (Müller 1908) Waksman and Henrici 1948 and *Streptomyces violaceoruber* (Waksman and Curtis 1916) Pridham 1970; members of these species, notably *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* strains 66 and ISP 5434, are used as model organisms by streptomycete geneticists. *Streptomyces coelicolor* A3(2), a constituent of the species *Streptomyces violaceoruber,* is frequently mistaken for *Streptomyces coelicolor* Müller which is a synonym of *Streptomyces albidoflavus* (Williams *et al.*, 1983a; Goodfellow *et al.*, 1992). Hatano *et al.* (1994) clarified the tortuous nomenclatural history of these organisms when they confirmed that *Streptomyces coelicolor* A3(2), *Streptomyces lividans* strains 66 and ISP 5434 were *bona fide* members of the species *Streptomyces violaceoruber* (Waksman and Curtis 1916) Pridham 1970.

Farina and Bradley (1970) examined "sporangia-forming" actinomycetes and streptomycetes which had similar DNA base compositions (mol% G+C) using reference

DNA from strains CBS 107.58^{T}, a representative of *Actinoplanes philippinesis* Couch 1950^{AL}, and S13, a representative of *Streptomyces venezuelae* Ehrlich *et al.* 1948^{AL}. They concluded that while the members of the different genera shared a similar DNA base composition they had little DNA in common.

Okanishi *et al.* (1972) examined 57 *Streptomyces* strains, with an emphasis on the '*griseus* group', using reference DNA from strains ISP 5236^{T}, a representative of *Streptomyces griseus* (Krainsky 1914) Waksman & Henrici 1948, and ISP 5199^{T}, a representative of *Streptomyces globisporus* (Krassilnikov 1941) Waksman 1953. Six sub-groups were detected when the DNA relatedness data from taxa assigned to the genomic species corresponding to the '*griseus* group' were represented on an ordination diagram. Good correlation was found between the sub-groups and the discontinuous distribution of some biochemical and morphological characters. Additional studies demonstrated the heterogeneity of streptomycete groups which encompassed strains from industrial applications (Tewfik & Bradley, 1967; Tewfik *et al.*, 1968; Toyama *et al.*, 1974).

Stackebrandt *et al.* (1981) determined the genomic relatedness of representative strains classified in the families *Actinoplanaceae, Streptomycetaceae* and *Streptosporangiaceae.* The DNA-DNA reassociation data showed that strains from the same family were moderately related (*Actinoplanaceae* = 18-49 %; *Streptomycetaceae* = 23-41 %; *Streptosporangiaceae* = 31-38 %), a finding that was supported by the results of corresponding DNA-rRNA cistron similarity experiments. These workers also recovered representatives of the genera *Actinosporangium* Krassilnikov & Yuan 1961, *Chainia* Thirumalachar 1955, *Elytrosporangium* Falcão de Morais *et al.* 1966, *Kitasatoa* Matsumae *et al.* 1968, *Microellobosporia* Cross *et al.* 1963 and *Streptoverticillium* Baldacci 1958 in the same DNA and rRNA homology groups as *Streptomyces* strains.

Mordarski *et al.* (1986) examined representatives of the *Streptomyces albidoflavus* (cluster 1A), *Streptomyces anulatus* (cluster 1B), and *Streptomyces halstedii* (cluster 1C)

taxospecies defined by Williams *et al.* (1983a) together with marker strains of *Streptomyces albus* (cluster 16), *Streptomyces griseocarneum* (cluster 55) and *Streptomyces lavendulae* (cluster 61). The *Streptomyces albidoflavus* strains, including *Streptomyces coelicolor* ISP 5233^T, formed a distinct genomic species but much of the remaining data were difficult to interpret possibly due to poor strain selection.

Labeda and Lyons (1991a) demonstrated that *Streptomyces violaceusniger* (cluster 32; Williams *et al.*, 1983a) contained several genomic species when they examined five reference strains representing the cluster together with ten additional strains. Ten DNA relatedness groups were defined at similarity levels > 70%, seven of which consisted of single-membered strains. The multimembered clusters were equated with *Streptomyces hygroscopicus* (Jensen 1931) Labeda and Lyons 1991a and *Streptomyces violaceusniger* (Waksman and Curtis 1916) Labeda and Lyons 1991a. The latter two species were redescribed and a number of strains bearing different specific names reduced to synonyms of the newly described taxa. The fact that all of the cluster 32 strains had grey, smooth- or rough-surfaced spores borne in spiral chains (Williams *et al.*, 1983a) illustrates the danger of uncritically weighting morphological criteria when defining *Streptomyces* species.

In a further series of experiments, Labeda and Lyons (1991b) found that the *Streptomyces cyaneus* species-group (cluster 18; Williams *et al.*, 1983a) was markedly heterogeneous. Nine out of eighteen representatives of this cluster were assigned to two DNA:DNA relatedness groups defined at or above the 70% DNA relatedness level. The first group, which corresponded to *Streptomyces coeruleorubidus* (Preobrazhenskaya 1957) Labeda and Lyons 1991b, encompassed three organisms which formed blue spiny spores in spiral spore chains on a reddish substrate mycelium. The three strains included the type strains of *Streptomyces coeruleorubidus* Preobrazhenskaya 1957 (ISP 5145^T), *Streptomyces bellus* Margalith and Beretta 1960 (ISP 5185^T) and *Streptomyces curacoi* Cataldi 1963 (ISP 5107^T). It was proposed that the two latter species became subjective synonyms of

Streptomyces coeruleorubidus (Preobrazhenskaya 1957) Labeda and Lyons 1991b.

The second DNA:DNA relatedness group was equated with *Streptomyces purpurascens* (Lindenbein 1952) Labeda and Lyons 1991b and encompassed the type strains of *Streptomyces purpurascens* (ISP 5530^T), *Streptomyces afghaniensis* Shimo *et al.* 1959 (ISP 5228^T), *Streptomyces janthinus* (Artamonova and Krassilnikov 1960) Pridham 1970 (ISP 5206^T), *Streptomyces roseoviolaceus* (Sveshnikova 1957) Pridham *et al.* 1958 (ISP 5277^T) and *Streptomyces violatus* (Artamonova and Krassilnikov 1960) Pridham 1970 (ISP 5207^T). All five strains produced red spiny spores in spiral chains, formed reddish diffusible pigments and showed similar substrate mycelial pigmentation. It was proposed that *Streptomyces afghaniensis, Streptomyces janthinus, Streptomyces roseoviolaceus* and *Streptomyces violatus* be reduced to subjective synonyms of *Streptomyces purpurascens* (Lindenbein 1952) Labeda and Lyons 1991b according to the rule of priority.

The type strain of *Streptomyces cyaneus* (Krassilnikov 1941) Waksman 1953 (ISP 5081^T) formed a distinct single-membered cluster as it only shared a DNA:DNA relatedness value of around 20 % with the other test strains. An additional DNA homology group, which encompassed grey-spored strains, was highlighted when the DNA relatedness data were examined by principal components analysis though it was not possible to determine the taxonomic rank of this taxon. The remaining 16 test strains exhibited relatedness values between 0 and 57% when examined against reference DNA from *Streptomyces coeruleorubidus* ISP 5145^T and *Streptomyces purpurascens* ISP 5530^T.

The strains assigned to the *Streptomyces coeruleorubidus* and *Streptomyces purpurascens* genomic species were distinguished by a few biochemical and morphological properties. However, the remaining strains assigned to the *Streptomyces cyaneus* species-group were not clearly distinguished from the *Streptomyces coeruleorubidus* and *Streptomyces purpurascens* strains using the phenotypic properties used to describe these species. The *Streptomyces cyaneus* species-group has also been shown to be heterogeneous

on the basis of fatty acid (Saddler *et al.*, 1987) and whole-organism protein electrophoretic data (Manchester *et al.*, 1990).

In additional DNA:DNA relatedness studies (Labeda, 1992, 1993), strains assigned to cluster 61 (*Streptomyces lavendulae*: Williams *et al.*, 1983a) were examined together with representatives of *Streptomyces lavendulae* subspecies, five putative strains of *Streptomyces lavendulae* and *"Streptomyces majorciensis"* NRRL 15167. The cluster 61 strains were morphologically similar having rust-coloured to red, smooth, cylindrical to ovoid spores borne on sporophores that formed fairly large, open spirals. The numerical phenetic data, as reflected in *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989), indicated that all of the species assigned to cluster 61 should be considered as subjective synonyms of *Streptomyces lavendulae* (Waksman and Curtis 1916) Waksman and Henrici 1948. However, the 21 test strains were assigned to 14 DNA:DNA relatedness groups defined above the 70 % DNA relatedness level, including 10 single-membered clusters though *Streptomyces colombiensis* Pridham *et al.* 1958 was reduced to a synonym of *Streptomyces lavendulae* as it showed 83 % DNA relatedness to *Streptomyces lavendulae* NRRL B-1230^T. In contrast, the other cluster 61 strains showed less than 45 % DNA relatedness with strain NRRL B-1230^T and hence cannot be considered as synonyms of *Streptomyces lavendulae.*

Labeda (1998a), in a further examination of red-spored streptomycetes, determined the DNA relatedness between representatives of clusters 10 (*Streptomyces fulvissimus*; Williams *et al.*, 1983a) and 17 (*Streptomyces griseoviridis*; Williams *et al.*, 1983a). Members of these taxa were compared with one another and with twelve strains representing the DNA relatedness groups encompassed in the *Streptomyces lavendulae* phenotypic cluster, including *Streptomyces lavendulae* NRRL B-1230^T. The test strains were assigned to 15 DNA relatedness groups, including ten single membered clusters. The DNA relatedness data supported the separation of the members of the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* phenotypic clusters into six genomic species: *Streptomyces*

aureoverticillatus (Krassilnikov and Yuan 1960) Pridham 1970[AL], *Streptomyces fulvissimus* (Jensen 1930) Waksman and Henrici 1948[AL], *Streptomyces griseoviridis* Anderson *et al.* 1956[AL] (with *Streptomyces daghestanicus* (Sveshnikova 1957) Pridham *et al.* 1958[AL] as a subjective synonym), *Streptomyces longispororuber* Waksman 1953[AL] (with *Streptomyces chryseus* (Krassilnikov *et al.* 1965) Pridham 1970[AL] as a subjective synonym), *Streptomyces murinus* Frommer 1959[AL] and *Streptomyces spectabilis* Mason *et al.* 1961[AL]. None of the strains from the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* clusters shared high DNA relatedness values with *Streptomyces lavendulae* NRRL B-1230[T].

Labeda (1996) also determined the levels of DNA relatedness among 35 strains of *Streptomyces* species originally classified in the genus *Streptoverticillium.* These organisms represented eight out of the twenty-four phenotypic cluster groups described for the genus *Streptoverticillium* in *Bergey's Manual of Systematic Bacteriology* (Locci & Schofield, 1989). Average linkage clustering of the DNA:DNA relatedness data resulted in the delineation of twenty clusters, including thirteen single-membered clusters, at a DNA relatedness level of 70 %.

Comparable investigations (Labeda, 1992) with phytopathogenic strains of *Streptomyces ipomoeae* (Person and Martin 1940) Waksman and Henrici 1948, a pathogen of sweet potatoes, revealed a very close relationship (94-100% DNA:DNA relatedness) between strains isolated from several different locations in the USA and Japan thereby suggesting a common ancestry of the host root-stock from which the strains were isolated. *Streptomyces ipomoeae* strains were found to be unrelated to other plant pathogenic streptomycete species, namely, *Streptomyces acidiscabies* Lambert and Loria 1989b (17 % DNA relatedness) and *Streptomyces scabiei* (Thaxter 1891) Lambert and Loria 1989a (39 % DNA relatedness) or to other members of *Streptomyces* species that produced blue-spores. *Streptomyces acidiscabies* strains assigned to the same genomic species (Healy & Lambert, 1991) have been shown to be related on the basis of phenotypic properties (Doering-Saad *et*

al., 1992). However, members of the genomic species corresponding to *Streptomyces scabies* (Healy & Lambert, 1991) were assigned to different phenetic clusters by Doering-Saad *et al.* (1992).

The studies outlined above underline the need to evaluate the status of streptomycete clusters based on the numerical analysis of phenotypic data. The lack of congruence between much of the numerical phenetic and DNA relatedness data can be partly attributed to the difficulty of finding sufficient good quality phenotypic traits to distinguish between representatives of the many validly described species classified in the genus *Streptomyces*. However, DNA:DNA relatedness studies must also be interpreted with care as they can be hampered by technical difficulties and experimental test error (Sneath, 1983; Johnson, 1991; Goodfellow *et al.*, 1997a). In addition, information on taxonomic structure can be biased when small numbers of reference strains are examined in DNA relatedness experiments (Hartford & Sneath, 1988), as shown in the proposal for *Streptomyces spitsbergensis* (Wieczorek *et al.*, 1993; Hatano *et al.*, 1997). These workers showed that it is important to choose reference strains that are widely spread and representative of the species under study.

The interpretation of DNA:DNA relatedness studies can also be influenced by the subjective nature of cut-off points considered to correspond to species level relatedness. Johnson (1989) suggested that DNA relatedness values as low as 60 % might indicate species level relatedness. However, recent DNA relatedness studies on streptomycetes have implied that genomic relatedness greater than 80 % may actually equate to species level relatedness in this genus (Labeda, 1993, 1998a; Labeda & Lyons, 1992a,b).

It is important to recognise that the advantages of DNA:DNA relatedness studies outweigh their limitations. In the case of streptomycetes such studies are (i) giving a more uniform concept of what constitutes a streptomycete species, (ii) yielding valuable data for the evaluation of the status of phenotypically defined species, (iii) providing a means for

delineating taxospecies that accommodate substantial amounts of genetic diversity and (iv) highlighting groups that correspond to genomic species.

DNA fingerprinting studies. Representatives of the genus *Streptomyces* have been the subject of a number of DNA fingerprinting studies designed to separate closely related species and strains. Restriction endonuclease digestion of genomic DNA was used by Crameri *et al.* (1983) to determine relationships between *Streptomyces* strains belonging to different species. The use of frequently cutting restriction enzymes led to the generation of complex fingerprints which contained a large number of low molecular weight fragments. It was concluded that such complex patterns did not provide sufficient resolution for determining taxonomic relationships at the species level.

Beyazova and Lechevalier (1993) used the low-frequency restriction fragment analysis (LFRFA) technique to evaluate relationships between 59 strains belonging to six *Streptomyces* species, including organisms associated with human and plant infections, namely, *Streptomyces albus, Streptomyces ipomoeae* and *Streptomyces somaliensis*; eight representatives of *Streptomyces coeruleorubidus* Labeda and Lyons 1991b and *Streptomyces purpurascens* Labeda and Lyons 1991b were also examined. The *Streptomyces ipomoeae* strains, which represented a well defined genomic species (Labeda, 1992), were recovered as two distinct subclusters defined at the 75 % similarity level. In contrast, the remaining organisms, including the *Streptomyces purpurascens* and *Streptomyces coeruleorubidus* strains, were not recovered in taxonomically coherent groups.

Doering-Saad *et al.* (1992) examined 40 potato scab-inducing and nonpathogenic streptomycete isolates by ribotyping. The results revealed a high degree of diversity among the pathogenic strains with little correlation found with corresponding RFLP and numerical phenetic data. The authors concluded that genes coding for pathogenicity determinants were spread amongst members of different *Streptomyces* species by mobilizable elements and that RFLP data were of little importance in the classification of *Streptomyces* species.

Clarke *et al.* (1993) examined the RFLP profiles of the ribosomal RNA genes of representatives of fourteen *Streptomyces* species; all but one of the test strains had previously been shown to be closely related on the basis of numerical phenetic and DNA-DNA relatedness data (Williams *et al.*, 1983a; Mordarski *et al.*, 1986). Considerable variation was observed in the RFLP patterns of the representatives of the various species though several common fragments were also observed. However, when a method was designed to increase the number of fragments per strain on a single agarose gel good congruence was found with the results of the earlier studies, in particular *Streptomyces albidoflavus* and *Streptomyces anulatus* were shown to be good species. Clarke and his colleagues concluded that RFLP analysis of ribosomal RNA genes appeared was an accurate and rapid strain identification tool for establishing relationships between closely related *Streptomyces* species.

Fifteen clinically significant streptomycetes were included in the PCR-RFLP analysis of an amplified 439-bp segment (amplicon) of the 65-kDa heat shock protein gene together with *Streptomyces albus* ATCC 3004^T, *Streptomyces griseus* strains ATCC 10137 and ATCC 23345^T and *Streptomyces somaliensis* ATCC 33201^T (Steingrube *et al.*, 1997). The test strains were assigned to five groups. RFLP patterns that matched those exhibited by the type strains of *Streptomyces albus*, *Streptomyces griseus* and *Streptomyces somaliensis* were obtained from fourteen out of the nineteen *Streptomyces* isolates. In addition, all but one of these strains exhibited a unique *Hinf* I fragment of > 320 bp. It was concluded that PCR-RFLP analysis provides a rapid way of identifying clinically significant streptomycetes.

PCR-fingerprinting based on repetitive intergenic DNA sequences (rep-PCR) has been examined as a means of differentiating between closely related strains of plant pathogenic streptomycetes which in some cases were indistinguishable using other taxonomic criteria (Sadowsky *et al.*, 1996). It was shown that rep-PCR DNA fragments of *Streptomyces* strains with the BOXA1R primer were unique, stable and reproducible thereby

showing that the method might provide a useful and rapid way of determining strain identity and tracking strains for ecological and epidemiological investigations. In contrast, the method seemed to be of little value in discriminating between streptomycete species. The rep-PCR results were in good agreement with the DNA analyses of Doering-Saad *et al.* (1992) who also found that RFLP groupings of scab-forming streptomycetes did not correlate with numerical phenetic data.

Hain *et al.* (1997) examined the 16S-23S rRNA intergenic spacer region of twenty-one strains identified as *Streptomyces albidoflavus* by fatty acid analysis together with the type strains of nine proposed subjective synonyms of this taxon. The 16S-23S rRNA intergenic spacer region was found to vary in length and sequence composition among the strains; the variation in length was rapidly and accurately represented by high-resolution electrophoresis of dye-labelled PCR products. Dye-labelled amplication products of the 16S-23S rRNA intergenic spacer region were generated for the twenty-seven strains shown to have identical 16S rRNA sequences. Electrophoresis and fragment size analysis of these products revealed extensive variability in the number and size of the spacer regions and this led to the recognition of nineteen distinct banding patterns. The number of bands ranged from two to five with band sizes between 347 and 363 bp. This method was considered to be a useful for discriminating between streptomycetes at strain level. Strains with the same 16S-23S rRNA intergenic spacer banding pattern were distinguished by sequence composition of the spacer region.

(c) Chemotaxonomy

The detection of LL-A_2pm, hexa- and octahydrogenated menaquinones with nine isoprene units, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol dimannosides as major phospholipids and the absence of diagnostic sugars in whole-organism hydrolysates allows unknown sporoactinomycetes to be assigned to the genus *Streptomyces* (Table 3-2). Discrimination at the species level can be achieved

by analyses of fatty acids and cellular and ribosomal proteins. Some of these methods provide quantitative or semi-quantitative data, as in the case of cellular fatty acid and menaquinone analyses whereas others yield qualitative data including the procedures used to detect the isomers of A_2pm.

Actinophages. Phage host range studies have been found to be useful for the classification and identification of some streptomycetes species (Kutzner, 1961a,b; Korn *et al.*, 1978; Schneider *et al.*, 1990; Wellington & Williams, 1981; Williams *et al.*, 1993). Wellington and Williams (1981) isolated actinophages which were specific for *Streptoverticillium* species. Korn-Wendisch and Schneider (1992) found species-specific phages for *Streptomyces albus, Streptomyces coelicolor* Muller, *Streptomyces griseus, Streptomyces violaceuruber* and *Streptomyces viridochromogenes.* These authors also noted that the phage host range data were consistent with the recognition of *Streptoverticillium* as a synonym of the genus *Streptomyces.*

Curie-point pyrolysis mass spectrometry. Sanglier *et al.* (1992) carried out a number of experiments to determine the potential of Curie-point pyrolysis mass spectrometry in the classification, identification and typing of industrially important actinomycetes. They found the method to be of value in separating streptomycetes and for highlighting closely related strains. The analysis of environmental isolates of *Streptomyces violaceusniger* demonstrated that soil isolates could be distinguished from representative type strains assigned to the *Streptomyces violaceusniger* species-group (cluster 32; Williams *et al.*, 1983a) thereby indicating that PyMS might prove to be an effective way of detecting novel streptomycete taxa.

Ferguson *et al.* (1997) found good congruence between numerical phenetic and PyMS data in a study designed to evaluate the taxonomic integrity of *Streptomyces albidoflavus* (Williams *et al.*, 1983a). Principal component-canonical variates analysis of experimental data collected on thirty-two representative organisms showed that the

Streptomyces albidoflavus strains formed a distinct group. This result, when taken with earlier whole-organism protein (Manchester *et al.*, 1990), DNA:DNA relatedness (Mordarski *et al.*, 1986), 16S rRNA sequence (Hain *et al.*, 1997) and numerical taxonomic data (Williams *et al.*, 1983a), indicated that *Streptomyces albidoflavus* is a distinct and well defined species. In contrast, the species-groups equated with *Streptomyces anulatus* and *Streptomyces halstedii* were found to be heterogeneous and hence in need of further study. There is also evidence that analysis of PyMS data using artificial neural networks provides an effective way of identifying closely related species of *Streptomyces* (Chun *et al.*, 1993a,b, 1997).

Fatty acids. Saddler *et al.* (1987) examined fatty acid methyl esters prepared from members of the *Streptomyces cyaneus* species-group (cluster 18; Willimas *et al.*, 1983a), additional strains forming a blue aerial spore mass and blue-spored environmental isolates using soft independent modelling of class analogy (SIMCA) statistics. Disjoint principal component analysis and cross validation showed that most of the *Streptomyces cyaneus* strains and blue-spored soil isolates could be represented by statistically stable principal component models. The *Streptomyces cyaneus* strains fell into two major groups, the largest of which contained eighteen representatives of *Streptomyces cyaneus*. The second cluster was relatively heterogeneous as it contained a few of the *Streptomyces cyaneus* strains and all of the additional blue-spored organisms. A third group encompassed all of the blue-spored isolates from soil. However, apart from the blue-spored soil isolates, which formed a uniform group, the SIMCA groups included strains with diverse morphological properties (Williams *et al.*, 1983a). It was disappointing that there was no evidence of correlation between the *Streptomyces cyaneus* fatty acid groups and genomic species highlighted in DNA:DNA relatedness studies (Labeda & Lyons, 1991b); the *Streptomyces coeruleorubidus* and *Streptomyces purpurascens* strains, for instance, were scattered amongst the unrelated strains.

Multilocus enzyme electrophoresis. Oh *et al.* (1996) examined sixteen strains taken to represent the numerical phenetic classification of Williams *et al.* (1983a) together with eight additional *Streptomyces* strains by using multilocus enzyme electrophoresis (MEE) to determine the relative electrophoretic mobilities of eleven enzymes. Some enzymes, namely, hexokinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase, gave a limited number of constant and reproducible polymorphic patterns which were considered to be of potential value for the classification and identification of streptomycetes. It was particularly interesting that *Streptomyces griseolus* ISP 5067^T and *Streptomyces halstedii* ISP 5068^T (cluster 1C; Williams *et al.*, 1983a) shared identical patterns, as did *Streptomyces hygroscopicus* ISP 5578^T and *Streptomyces violaceusniger* ISP 5563^T (cluster 32; Williams *et al.*, 1983a).

Polyacrylamide gel electrophoresis of whole-organism proteins. Manchester *et al.* (1990) examined the whole-organism proteins of thirty-seven representative *Streptomyces albidoflavus* (cluster 1A; Williams *et al.*, 1983a), *Streptomyces anulatus* (cluster 1B), *Streptomyces cyaneus* (cluster 18), *Streptomyces rimosus* (cluster 42) and *Streptomyces (Streptoverticillium) griseocarneum* (cluster 55) strains by polyacrylamide gel electrophoresis. Most of the *Streptomyces albidoflavus* and *Streptomyces anulatus* strains were recovered in discrete clusters but the *Streptomyces cyaneus* and *Streptomyces (Streptoverticillium) griseocarneum* strains were scattered over several groups. These findings were in good agreement with DNA:DNA relatedness (Mordarski *et al.*, 1986), PyMS (Ferguson *et al.*, 1997) and 16S rRNA sequence data (Hain *et al.*, 1997) in showing that *Streptomyces albidoflavus* and *Streptomyces anulatus* were good species. The protein patterns also underpinned the DNA:DNA relatedness (Labeda & Lyons, 1991b) and fatty acid data (Saddler *et al.*, 1987) which highlight the heterogeneity of *Streptomyces cyaneus.*

Ribosomal proteins. Ochi (1989, 1992) demonstrated the usefulness of bi-dimensional polyacrylamide gel electrophoresis of ribosomal proteins in discriminating between

Streptomyces strains. He compared the ribosomal proteins of representatives of nine *Streptomyces* clusters defined by Williams *et al.* (1983a), including *Streptomyces antibioticus* ATCC 14888^T (cluster 31), *Streptomyces avidinii* ISP 5526^T (cluster 56), *Streptomyces griseoflavus* FERM 1805^T (cluster 37), *Streptomyces griseus* IFO 13189^T (cluster 1B), *Streptomyces hygroscopicus* ISP 5578^T (cluster 32), *Streptomyces lavendulae* subsp. *grasserius* ISP 5385^T, *Streptomyces lavendulae* subsp. *lavendulae* ISP 5069^T (cluster 61), *Streptomyces parvullus* ISP 5048^T (cluster 12), *Streptomyces venezuelae* ISP 5230^T (cluster 6), *Streptomyces violaceoruber (coelicolor)* A3(2) and *Streptomyces violaceus* ISP 5082^T (cluster 6). The ribosomal protein patterns were found to be species specific though *Streptomyces lavendulae* subsp. *grasserius* ISP 5385^T and *Streptomyces lavendulae* subsp. *lavendulae* ISP 5069^T had identical ribosomal protein patterns which were very similar to that of *Streptomyces avidinii* ISP 5526^T. This technique has not been used by other workers presumably because 2D-PAGE profiles of ribosomal proteins require elaborate sample preparation and are relatively difficult to standardise due to the fact that the protein patterns are highly complex and difficult to interpret.

Ochi and Hiranuma (1994) analysed the N-terminal sequences (20 amino acids) of ribosomal AT-L30 proteins extracted from forty-two strains representing thirty-five species classified in the genera *Streptomyces*, *Kitasatospora* and *Streptoverticillium*. They found that all of the representatives of the genera *Kitasatospora* and *Streptoverticillium* had identical or very similar sequences to *Streptomyces exfoliatus* JCM 4366^T. These strains also showed high levels of relatedness to *Streptomyces lavendulae* IFO 12789^T. It has been already pointed out that these results were used to support the view that the genera *Kitastaospora* and *Streptoverticillium* should become synonyms of the genus *Streptomyces* (Witt & Stackebrandt, 1990; Wellington *et al.*, 1992).

Ochi (1995) undertook an extensive analysis of the ribosomal AT-L30 proteins of eighty-one species taken to represent the genus *Streptomyces* (Williams *et al.*, 1983a). Forty-

nine strains were classified into four groups (I to IV) the members of which shared identical AT-L30 N-terminal amino acid sequences. A phylogenetic tree constructed on the basis of the levels of similarity of the AT-L30 N-terminal amino acid sequences revealed the existence of six major clusters. The first cluster contained the members of groups I and II together with representatives of three other species, namely, *Streptomyces chattanoogensis* JCM4571^T, *Streptomyces lydicus* JCM 4492^T and *Streptomyces misakiensis* JCM 4653^T; the second cluster, the members of groups III and IV and representatives of eight other species, namely, *Streptomyces albidoflavus* JCM 4446^T, *Streptomyces bambergiensis* JCM 4728^T, *Streptomyces cellulosae* JCM 4462^T, *Streptomyces diastaticus* JCM 4745^T, *Streptomyces griseoflavus* JCM 4479^T, *Streptomyces longisporaflavus* JCM 4396^T, *Streptomyces thermonitrificans* JCM 4841^T, *Streptomyces thermovulgaris* JCM 4520^T; the third cluster, *Streptomyces ramulosus* JCM 4604^T and *Streptomyces ochraceiscleroticus* JCM 4801^T; the fourth cluster, *Streptomyces rimosus* JCM 4667^T; the fifth cluster, *Streptomyces aurantiacus* JCM 4453^T and *Streptomyces tubercidicus* JCM 4558^T; and the sixth cluster *Streptomyces albus* JCM 4450^T and *Streptomyces sulphureus* JCM 4835^T. *Streptomyces thermonitrificans* JCM 4841^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* JCM 4843^T and *Streptomyces thermovulgaris* JCM 4520^T had identical AT-L30 N-terminal amino acid sequences and belonged to the first cluster; the same sequence was shown by *Streptomyces cellulosae* JCM 4462^T.

Good congruence was found between the AT-L30 N-terminal amino acid and the numerical phenetic data of Williams *et al.* (1983a) as exemplified by *Streptomyces griseus* JCM 4644^T and *Streptomyces anulatus* JCM 4721^T (the *Streptomyces anulatus* group [subcluster 1B]); *Streptomyces parvullus* ATCC 12434^T, *Streptomyces rochei* JCM 4074^T and *Streptomyces tendae* JCM 4610^T (the *Streptomyces rochei* group [cluster 12]); *Streptomyces lydicus* JCM 4492^T and *Streptomyces nigrescens* JCM 4401^T (the *Streptomyces lydicus* group [cluster 29]); *Streptomyces hygroscopicus* IFO 13472^T and

Streptomyces violaceusniger JCM 4850^T (the *Streptomyces violaceusniger* group [cluster 32]); *Streptomyces thermonitrificans* JCM 4841^T and *Streptomyces thermovulgaris* JCM 4520^T (the *Streptomyces thermovulgaris* group [cluster 36]); *Streptomyces cyanoalbus* JCM 4363^T, *Streptomyces griseoflavus* JCM 4479^T and *Streptomyces prasinopilosus* JCM 4404^T (the *Streptomyces griseoflavus* group [cluster 37]). Good congruence was also found between the AT-L30 sequence and corresponding 16S rRNA sequence data.

Serology. Cross and Spooner (1963) used an agar diffusion technique in an attempt to identify streptomycetes serologically but the results were difficult to interpret due to the difficulty of choosing representative strains. Ridell and Williams (1983) demonstrated that *Streptomyces* and *Streptoverticillium* strains which belonged to same numerical phenetic cluster (Williams *et al.* 1983a) shared a higher number of precipitinogens in common when compared with strains from other numerically defined clusters, as determined by using an immunodiffusion technique. Ridell *et al.* (1986) subsequently found that *Streptomyces* and *Streptoverticillium* strains belonging to the same phenetic clusters showed a high degree of immunogenic similarity.

5. Polyphasic taxonomy

It can be concluded from the studies outlined above that streptomycete systematics is still in a state of flux despite the results of extensive numerical phenetic surveys (Williams *et al.*, 1983a; Saddler *et al.*, 1988; Kämpfer et al., 1991; Sahin,1995). Nevertheless, it is clear that the description of *Streptomyces* species should be based on data derived from the application of chemotaxonomic, molecular systematic and phenotypic characterisation methods. Strategies similar to the one outlined in Table 3-2 are increasingly being used to assign unknown sporoactinomycetes to the genus *Streptomyces*, as exemplified by the descriptions of *Streptomyces caviscabies* Goyer *et al.* 1996, *Streptomyces seoulensis* Chun *et al.* 1997, *Streptomyces stramineus* Labeda *et al.* 1997, *Streptomyces*

Table 3-2. Strategies available for the reclassification of validly described *Streptomyces* species and for the recognition of novel species of streptomycetes

Pure cultures ↓	**Characterisation methods**		
	Chemical markers	**Genotypic features**	**Phenotypic markers**
1. Confirmation of genus identity	Detection of LL-A_2pm and no characteristic sugar* [a]; peptidoglycan type A3γ[b]; MK-9(H_6,H_8) as predominant menaquinones*; major amounts of saturated *iso/anteiso* branched and straight-chain fatty acids; DPG, PE, PI and PIDM as predominant polar lipids*[,c]; absence of mycolic acids	16S rRNA sequencing*, DNA rich in G+C (66 and 78 mol%)*	Characteristic colony morphology*; extensively branched aerial and substrate hyphae*; spore chain morphology and spore ornamentation*
↓			
2. Circumscription of species	Polyacrylamide gel electrophoresis of whole-organism proteins, pyrolysis mass spectrometry, phage host range studies, quantitative fatty acid profiles	16S rRNA sequencing*, DNA:DNA relatedness*, ribotyping* and sequencing of the 16S-23S rRNA spacer region	Morphological properties*: spore chain morphology, spore surface ornamentation, pigmentation properties; physiological properties*: degradation tests, sole carbon and energy sources, sole nitrogen sources, enzymatic profiles, resistance to antimicrobial and chemical compounds
↓			
3. Subspecific categories	Multilocus enzyme electrophoresis, serological comparisons	DNA fingerprinting: RAPyD's, ribotyping*, RFLP	Enzymatic profiles, resistance to antimicrobial and chemical compounds

* Indicates markers sought or techniques used in the present study.

Methods described by [a]Lechevalier & Lechevalier (1970a,b), [b]Schleifer & Kandler (1972) and [c]Lechevalier *et al.* (1977).

Abbreviations: RAPyD's, random amplified polymorphism of DNA; RFLP, restriction fragment length polymorphism.

thermocarboxydovorans Kim *et al.* 1998, *Streptomyces thermocarboxydus* Kim *et al.* 1998 and *Streptomyces turgidiscabies* Miyajima *et al.* 1998.

A polyphasic taxonomic approach has also been used to circumscribe three putatively novel species provisionally designated *Streptomyces* species-groups A, B and C (Manfio *et al.*, 1995; Atalan *et al.*, 1999). The provisional classification of these three taxa on the basis of morphology and pigmentation was supported by a combination of genotypic (DNA:DNA relatedness, ribotyping and 16S rRNA sequencing) and phenotypic data (fatty acids, numerical taxonomy, rapid fluorogenic enzyme tests, pyrolysis mass spectrometry and whole-organism protein electrophoresis).

The primary aim of the present study was to determine the taxonomic relationships of the putative type strains of *"Streptomyces thermoflavus"* (DSM 40574), *"Streptomyces thermophilus"* (DSM 40365) and *"Streptomyces thermotolerans"* (DSM 40227); three alkaltolerant streptomycetes, namely, *Streptomyces canescens* DSM 40001^T, *Streptomyces cavourensis* subsp. *cavourensis* DSM 40300^T and *Streptomyces hydrogenans* DSM 40586^T; *Streptomyces violaceoruber* DSM 40049^T, which grow at 55 °C; and representative alkalitolerant, thermophilic and neutrophilic, thermophilic streptomycete isolates included in the numerical phenetic study by Sahin (1995) using a polyphasic approach.

Materials and Methods

1. Strains and cultivation

Organisms and maintenance. The sources and histories of the reference strains and isolates are given in Table 3-3. All of the isolates representing clusters defined in the numerical phenetic study of Sahin (1995) are centrotype strains. The test strains were maintained on inorganic salt-starch agar (ISP medium 4, Difco; Shirling & Gottlieb, 1966) at room temperature and as glycerol suspensions (20 %, w/v) at –20 °C (Wellington & Williams, 1978). The glycerol suspensions were prepared in cryovials by scraping growth from sporulating strains incubated on inorganic salt-starch agar (ISP medium 4, Difco) plates at 45 °C for 5 days. As explained earlier, the frozen glycerol suspensions were used both for long-term preservation and as a ready source of inoculum. The test strains were examined using the taxonomic methods shown in Table 3-4.

Preparation of biomass. Single colonies from each of the test strains were used to inoculate 50 ml of Tryptic Soy broth (Difco Laboratories, Detroit, USA) held in 100 ml conical flasks. The inoculated flasks were shaken at 150 rpm for 3 to 5 days at 45 °C when growth was checked for purity by subculturing onto inorganic salt-starch agar (ISP medium 4, Difco; Shirling & Gottlieb, 1966) plates and then harvested by centrifugation at 6,000 rpm for 10 minutes. The cells used for the chemical studies were washed in distilled water and freeze-dried; those required for the molecular systematic investigations were washed in sterile TE buffer (Tris-HCL pH 8.0, 10mM; EDTA 1mM) and stored at –20 °C until needed.

2. Morphology and pigmentation

Test strains (Table 3-4) were examined for aerial spore mass colour following incubation on ISP 4 agar (Difco; Shirling & Gottlieb, 1966) for 5 days at 45 °C. Soluble

Table 3-3. Designations and histories of the test strains

Laboratory numbers	Taxa	Strain histories	Classification after Sahin (1995)	
			Cluster	Aggregate group
Thermophilic reference strains				
DSM 40573^T	*Streptomyces thermodiastaticus* (Bergey *et al.* 1923) Waksman 1953^{AL}	DSM 40573^T ← ISP 5573^T ← T. Cross, CUB 687^T ← J. R. Denison	17	V
DSM 40574	*"Streptomyces thermoflavus"* (Kudrina and Maximova 1963) Pridham 1970	DSM 40574← ISP 5574 ← T. Cross, CUB 75 ← NCIB 9670 ← N. Okafor, University of Nigeria.	Not included	
DSM 40579^T	*Streptomyces thermonitrificans* Desai and Dhala 1967^{AL}	DSM 40579^T ← ISP 5579^T ← NCIB 2007^T ← A. J. Desai & S. A. Dhala; soil, Bombay, India	Not included	
DSM 40365	*"Streptomyces thermophilus"* (Gilbert 1904) Waksman and Henrici 1948	DSM 40365 ← ISP 5365 ← CBS 275.66 ← A. Henssen, A 11; fresh horse manure	Not included	
DSM 40227	*"Streptomyces thermotolerans"* ex Pagano *et al.* 1959	DSM 40227 ← ISP 5227 ← F. Arnov, E.R. Squibb & Sons, M 4209.	Not included	
DSM 41392^T	*Streptomyces thermoviolaceus* subsp. *apingens* Henssen 1957^{AL}	DSM 41392^T ← ATCC 19994^T ← A. Henssen, $R_{89}{}^T$	Not included	
DSM 40443^T	*Streptomyces thermoviolaceus* subsp. *thermoviolaceus* Henssen 1957^{AL}	DSM 40443^T ← ISP 5443^T ← A. Henssen, R-77^T; mixed fresh horse and swine manure	18	V
DSM 40444^T	*Streptomyces thermovulgaris* Henssen 1957^{AL}	DSM 40444^T ← ISP 5444^T ← A. Henssen, MB $R_{10}{}^T$; fresh cow manure	22	VI
DSM 40049^T	*Streptomyces violaceoruber* (Waksman and Curtis 1916) Pridham 1970^{AL}	DSM 40049^T ← ISP 5049 ← IMRU 3030.	Not included	
Alkalitolerant reference strains				
DSM 40001^T	*Streptomyces canescens* Waksman 1957^{AL}	DSM 40001^T ← ISP 5001^T ← U. A. Phillips	SMC	III
DSM 40300^T	*Streptomyces cavourensis* subsp. *cavourensis* Skarbeck and Brady 1978^{AL}	DSM 40300^T ← ISP 5300^T ←D. Giolitti 829^T; soil, Italy	SMC	III

DSM 40586^T	*Streptomyces hydrogenans* Lindner *et al.* 1958^{AL}	DSM 40586^T ← ISP 5586^T ←ATCC 19631^T ←G. Nesemann, FHP 678^T; soil	SMC III
Alkalitolerant, thermophilic isolates			
NAR54	*Streptomyces* sp.	A.T Bull; sheep grazed soil, Canterbury, England, UK	6 IV
NAR84	*Streptomyces* sp.	A.T. Bull, lime soil, Canterbury, England, UK	5 IV
NAR85	*Streptomyces* sp.	A.T. Bull; lime soil,Canterbury, England, U.K.	SMC V
TA012	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	SMC VI
TA026	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	25 VI
TA034	Streptomyces sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	16 IV
TA056	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	SMC VI
TA061	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	24 VI
TA123	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	SMC VI
TA127	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	SMC IV
TA179	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	26 VI
TA265	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	23 VI
Neutrophilic, thermophilic isolates			
A1853	*Streptomyces* sp.	J. Lacey; A600, barley grain, Cambridge, England, UK	19 VI
A1956	*Streptomyces* sp.	J. Lacey; Nigeria	20 VI
B19	*Streptomyces* sp.	A. M. Al-Tai; poultry farm, Kuala Lumpur, Malaysia.	Not included
NT090	*Streptomyces* sp.	N. Sahin; garden soil, Bolu, Turkey	15 IV

NT123	*Streptomyces* sp	N. Sahin; arid soil, Van, Turkey	SMC	IV
NT218	*Streptomyces* sp.	N. Sahin; scrubland, Merida, Venezuela	SMC	VI
NT307	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	3	II
NT312	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	13	IV
NT322	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	8	IV
NT336	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	14	IV
NT358	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	7	IV
NT371	*Streptomyces* sp.	N. Sahin; arid soil, Merida, Venezuela	12	IV
NT381	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	11	IV
NT399	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	9	IV
NT493	*Streptomyces* sp.	N. Sahin; garden soil, Yogyakarta, Indonesia	SMC	IV
NT576	*Streptomyces* sp.	N. Sahin; garden soil, Bolu, Turkey	10	IV
Thermophilic, carboxydotrophilic isolates				
AT5	*Streptomyces* sp.	C. Falconer; garden soil, Newcastle upon Tyne	Falconer (1988)	
AT6	*Streptomyces* sp.	C. Falconer; garden soil, Newcastle upon Tyne	Falconer (1988)	
AT54	*Streptomyces* sp.	C. Falconer; garden soil, Newcastle upon Tyne	Falconer (1988)	

^{T}, Type strain; SMC, single-membered cluster.

A.T. Bull, Department of Bioscience, University of Kent, Canterbury, England, U.K.; **J. Lacey,** Department of Plant Pathology, Rothamsted Experimental Station, Harpenden, Hertfordshire, England, U.K.
ATCC, American Type Culture Collection, Rockville, MD., U.S.A.; **CUB**, Collection of the University of Bradford, Bradford, Yorkshire, England, UK; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124, Braunschweig, Germany; **IMRU**, Waksman Institute of Microbiology, Rutgers, the State University of New Jersey, Piscataway, NJ. U.S.A.; **ISP**, International *Streptomyces* Project, Ohio Wesleyan University, Delaware, Ohio, 43015, U.S.A.; **NCIB**, National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K.

Table 3-4. *Streptomyces* strains examined in the taxonomic studies

Strain	Name	Morphology	Physiological tests	Polar lipid and menaquinone analyses	16S rDNA sequencing	DNA:DNA pairing	Ribotyping	DNA base composirion
Reference strains								
DSM 40001^T	*Streptomyces canescens*				√			
DSM 40300^T	*Streptomyces cavourensis* subsp. *cavourensis*				√			
DSM 40586^T	*Streptomyces hydrogenans*				√			
DSM 40573^T	*Streptomyces thermodiastaticus*	√	√			√	√	√
DSM 40574	*"Streptomyces thermoflavus"*	√			√			
DSM 40579^T	*Streptomyces thermonitrificans*	√	√	√		√	√	√
DSM 40365	*"Streptomyces thermophilus"*	√			√			
DSM 40227	*"Streptomyces thermotolerans"*	√			√			
DSM 41392^T	*Streptomyces thermoviolaceus* subsp. *apingens*	√	√			√	√	
DSM 40443^T	*Streptomyces thermoviolaceus* subsp. *thermoviolaceus*	√	√			√	√	√
DSM 40444^T	*Streptomyces thermovulgaris*	√	√	√		√	√	√
DSM 40049^T	*Streptomyces vilaceoruber*				√			
Isolates								
A1853	*Streptomyces* sp.				√*	√	√	

Strain	Name	Morphology	Physiological tests	Polar lipid and menaquinone analyses	16S rDNA sequencing	DNA:DNA pairing	Ribotyping	DNA base composirion
A1956	*Streptomyces* sp.				√*	√	√	
AT5	*Streptomyces* sp.					√		
AT54	*Streptomyces* sp.					√		
AT6	*Streptomyces* sp.					√		
NAR54	*Streptomyces* sp.			√	√			
NAR84	*Streptomyces* sp.	√		√	√*			
NAR85	*Streptomyces* sp.	√	√		√	√	√	
NT090	*Streptomyces* sp.	√		√	√			
NT123	*Streptomyces* sp	√			√*			
NT218	*Streptomyces* sp.				√*	√	√	
NT307	*Streptomyces* sp.	√		√	√			
NT312	*Streptomyces* sp.	√			√*			
NT322	*Streptomyces* sp.	√		√	√			
NT336	*Streptomyces* sp.	√		√	√*			
NT358	*Streptomyces* sp.	√		√	√			
NT371	*Streptomyces* sp.			√	√*			

pigment production was detected on glucose asparagine agar (ISP 5, Difco; Shirling & Gottlieb, 1966), and the production of melanin pigments on peptone yeast extract iron (ISP 6, Difco; Shirling & Gottlieb, 1966) and tyrosine agars (ISP 7, Difco; Shirling & Gottlieb, 1966). Inoculated plates were incubated for 7 days at 45 °C. Spore chain morphology and spore surface ornamentation were observed after incubation for 5 days on ISP 4 agar (Shirling & Gottlieb, 1966) using light and scanning electron microscopy, as described earlier (Chapter II, pages 97 and 99).

3. Degradation and nutritional tests

The degradation and growth tests (Table 3-4) were carried out using the media and methods described earlier (Chapter II, pages 99 and 100).

4. Menaquinone and polar lipid analyses

The menaquinone and polar lipid composition of the test strains (Table 3-4) were determined following the procedures described earlier (Chapter II, pages 100 and 103).

5. DNA base composition

The base composition of genomic DNA preparations of the test strains (Table 3-4) were examined following the procedures outlined in Chapter II, page 103.

6. Sequencing and analysis of 16S rRNA

Extraction of genomic DNA and PCR amplification of the 16S rRNA from the test strains (Table 3-4) were carried out as described earlier (Chapter II, pages 103 and 121).

The almost complete and partial 16S rRNA sequences of the test strains were aligned manually with corresponding streptomycete nucleotide sequences (Table 3-5) retrieved from

Table 3-5. Test strains and their nucleotide sequence accession numbers

Species or subspecies	Strain[a]	Sources[b]	Accession no.	References
Almost complete nucleotide sequences (>1300)				
Isolates				
***Streptomyces* strain**	**B19**	**DSM 41700**	**-**	**This study**
***Streptomyces* strain**	**NT307**	**cluster 3[c]**	**-**	**This study**
***Streptomyces* strain**	**NAR54**	**cluster 6**	**-**	**This study**
***Streptomyces* strain**	**NT358**	**cluster 7**	**-**	**This study**
***Streptomyces* strain**	**NT322**	**cluster 8**	**-**	**This study**
***Streptomyces* strain**	**NT576**	**cluster 10**	**-**	**This study**
***Streptomyces* strain**	**NT381**	**cluster 11**	**-**	**This study**
***Streptomyces* strain**	**NT90**	**cluster 15**	**-**	**This study**
***Streptomyces* strain**	**NAR85**	**SMC, DSM 41740**	**AJ001434**	**This study**
***Streptomyces* strain**	**TA56**	**SMC, DSM 41741**	**AJ000284**	**This study**
Reference strains				
S. acidiscabies		ATCC 49003^T	D63865	Takeuchi *et al.* (1996)
S. albus	ISP 5313^T	DSM 40313^T	X53163	Stackebrandt *et al.* (1991)
S. albidoflavus	ISP 5455^T	DSM 40455^T	Z76676	Hain *et al.* (1997)
S. ambofaciens	ISP 5053^T	ATCC 23877^T	M27245	Pernodet *et al.* (1989)
S. bikiniensis	ISP 5581^T	DSM 40581^T	X79851	Mehling *et al.* (1995)
S. bluensis	ISP 5564^T	-	X79324	Mehling *et al.* (1995)
S. bottropensis	ISP 5262^T	ATCC 25435^T	D63868	Takeuchi *et al.* (1996)
S. caelestis	ISP 5084^T	NRRL 2418^T	X80824	Mehling *et al.* (1995)
S. canescens	**ISP 5001^T**	**DSM 40001^T**	**-**	**This study**
S. cavourensis subsp. cavourensis	**ISP 5300^T**	**DSM 40300^T**	**-**	**This study**
S. coeruleoprunus		DSM 41472^T	-	Stackebrandt, unpublished
S. diastatochromogenes	ISP 5449^T	ATCC 12309^T	D63867	Takeuchi *et al.* (1996)
"S. espinosus"		NRRL 5729	X80826	Mehling *et al.* (1995)
S. eurythermus	ISP 5014^T	ATCC 14975^T	D63870	Takeuchi *et al.* (1996)
S. fradiae		DSM 40063^T	-	Stackebrandt, unpublished
S. galbus	ISP 5089^T	DSM 40089^T	X79852	Mehling *et al.* (1995)
S. ghanaensis		DSM 40746^T	-	Stackebrandt, unpublished
S. glaucescens		DSM 40716	X79322	Mehling *et al.* (1995)
S. gougerotii	ISP 5324^T	DSM 40324^T	Z76687	Hain *et al.* (1997)
S. griseocarneus	ISP 5004^T	DSM 40004^T	X99943	Mehling *et al.* (1995)
S. griseus	ISP 5236^T	KCTC 9080^T	X61478	Kim *et al.* (1991)
S. hydrogenans	**ISP 5586^T**	**DSM 40586^T**	**-**	**This study**
"S. hygroscopicus subsp. *limoneus"*		ATCC 21431	X79853	Mehling *et al.* (1995)
S. lincolnensis	ISP 5355^T	NRRL 2936^T	X79854	Mehling *et al.* (1995)
S. macrosporus		DSM 41449^T	Z68099	Kim *et al.* (1996)
S. mashuense	ISP 5221^T	DSM 40221^T	X79323	Mehling *et al.* (1995)
S. megasporus		DSM 41476^T	Z68100	Kim *et al.* (1996)
S. neyagawaensis	ISP 5588^T	ATCC 27449^T	D63869	Takeuchi *et al.* (1996)
S. pseudogriseolus		NRRL 3985	X80827	Mehling *et al.* (1995)
S. rimosus	R6-554	-	X62884	Pujic *et al.*, unpublished
S. roseoflavus		DSM 40536^T	-	Stackebrandt, unpublished
S. scabiei		ATCC 49173^T	D63862	Takeuchi *et al.* (1996)

S. seoulensis		IMSNU 21266^T	Z71365	Chun *et al.*
S. subrutilus	ISP 5445^T	DSM 40445^T	X80825	Mehling *et al.* (1995)
S. thermocarboxydovorans	AT52^T	DSM 44296^T	U94489	Kim *et al.* (1998)
S. thermocarboxydus	AT37^T	DSM 44293^T	U94490	Kim *et al.* (1998)
S. thermodiastaticus	ISP 5573^T	DSM 40573^T	Z68101	Kim *et al.* (1996)
"S. thermoflavus"	**ISP 5574**	**DSM 40574**	-	**This study**
S. thermogriseus		CCTCC AA97014^T	AF056714	Xu *et al.*, 1998
S. thermolineatus		DSM 41451^T	Z68097	Kim *et al.* (1996)
S. thermonitrificans	ISP 5579^T	DSM 40579^T	Z68098	Kim *et al.* (1996)
"S. thermophilus"	**ISP 5365**	**DSM 40365**	-	**This study**
"S. thermotolerans"	**ISP 5227**	**DSM 40227**	-	**This study**
S. thermoviolaceus subsp. *apingens*		DSM 41392^T	Z68095	Kim *et al.* (1996)
S. thermoviolaceus subsp. *thermoviolaceus*	ISP 5443^T	DSM 40443^T	Z68096	Kim *et al.* (1996)
S. thermovulgaris	ISP 5444^T	DSM 40444^T	Z68094	Kim *et al.* (1996)
S. violaceoruber	**ISP 5049^T**	**DSM 40049^T**	-	**This study**
S. virginiae	ISP 5094^T	IFO 3729^T	D85119	Mehling *et al.* (1995)

Partial nucleotide sequences (<1300 nucleotides)

Isolates

***Streptomyces* strain**	**NAR84**	**cluster 5**	-	**This study**
***Streptomyces* strain**	**NT399**	**cluster 9**	-	**This study**
***Streptomyces* strain**	**NT371**	**cluster 12**	-	**This study**
***Streptomyces* strain**	**NT312**	**cluster 13**	-	**This study**
***Streptomyces* strain**	**NT336**	**cluster 14**	-	**This study**
***Streptomyces* strain**	**TA34**	**cluster 16**	-	**This study**
***Streptomyces* strain**	**A1853**	**cluster 19**	-	**This study**
***Streptomyces* strain**	**A1956**	**cluster 20**	-	**This study**
***Streptomyces* strain**	**TA61**	**cluster 24**	-	**This study**
***Streptomyces* strain**	**TA26**	**cluster 25**	-	**This study**
***Streptomyces* strain**	**TA179**	**cluster 26**	-	**This study**
***Streptomyces* strain**	**NT123**	**SMC**	-	**This study**
***Streptomyces* strain**	**NT218**	**SMC**	-	**This study**
***Streptomyces* strain**	**NT493**	**SMC**	-	**This study**
***Streptomyces* strain**	**TA12**	**SMC**	-	**This study**
***Streptomyces* strain**	**TA127**	**SMC**	-	**This study**

[T], Type strain.

[a], ISP, International *Streptomyces* Project codes (Gottlieb & Shirling, 1967; Shirling & Gottlieb, 1968a,b, 1969, 1972).

[b], ATCC, American Type Culture Collection, 10801 University Boulevard, Manassas, VA, U.S.A.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute of Fermentation, Osaka, Japan; IMSNU, Institute of Microbiology, Seoul National University, Seoul, Republic of Korea; KCTC, Korean Collection of Type Cultures, Korean Research Institute of Bioscience and Biotechnology, Taejeon, Republic of Korea; NRRL, Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

[c], Defined by Sahin (1995). SMC, single-membered cluster.

the RDP (Ribosomal Database Project; Maidak *et al.*, 1997) and EMBL/GenBank databases (Benson *et al.*, 1998) by using the AL16S program (Chun, 1995).

Evolutionary trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining algorithms (Saitou & Nei, 1987). Evolutionary distance matrices for the least-squares and neighbour-joining methods were generated as described by Jukes and Cantor (1969). The PHYLIP software package (Felsenstein, 1993) was used for generating all of the phylogenetic trees. The resultant unrooted tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method data based on 1000 re-samplings using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein, 1993). The root position of the unrooted tree based on the neighbour-joining method was estimated by using five outgroup organisms (*Arthrobacter globiformis* DSM 20214^T [accession number M23411], *Bacillus subtilis* [accession number K00637], *Escherichia coli* [accession number J01695], *Nocardia asteroides* ATCC 19247^T [accession number Z36934] and *Streptosporangium roseum* DSM 43021^T [accession number X70425], as described by Swofford and Olsen (1990).

The sequences of strains NT307 (centrotype strain of cluster 3; Sahin, 1995), NAR54 (centrotype strain of cluster 6; Sahin, 1995), NT358 (centrotype strain of cluster 7, Sahin, 1995), NT322 (centrotype strain of cluster 8, Sahin, 1995), NT576 (centrotype strain of cluster 10, Sahin, 1995) and NT381 (centrotype strain of cluster 11, Sahin, 1995) were compared with corresponding partial sequences of most of the validly described streptomycetes species held in DSMZ database (Stackebrandt *et al.*, unpublished); the partial 16S rRNA sequences were based on about 872 nucleotides between positions 33 and 474 and positions 804 and 1233 (*Streptomyces ambofaciens* numbering system; Pernodet *et al.* [1989]).

7. DNA:DNA relatedness studies

The DNA relatedness assays were performed on the nineteen strains (Table 3-4) by using the direct binding filter hybridisation method following a well-established procedure (Gillespie & Spiegelman, 1965; Denhardt, 1966; Meyer & Schleifer, 1978; Mordarski *et al.*, 1976), as described earlier (Chapter II, pages 121 and 129). The concentrations of SSC and the hybridization temperature were designed to achieve optimal hybridization conditions, that is, 25 °C below the melting temperature (*T*m) assuming that the mean DNA base composition of *Streptomyces* strains is 70 mol% G plus C.

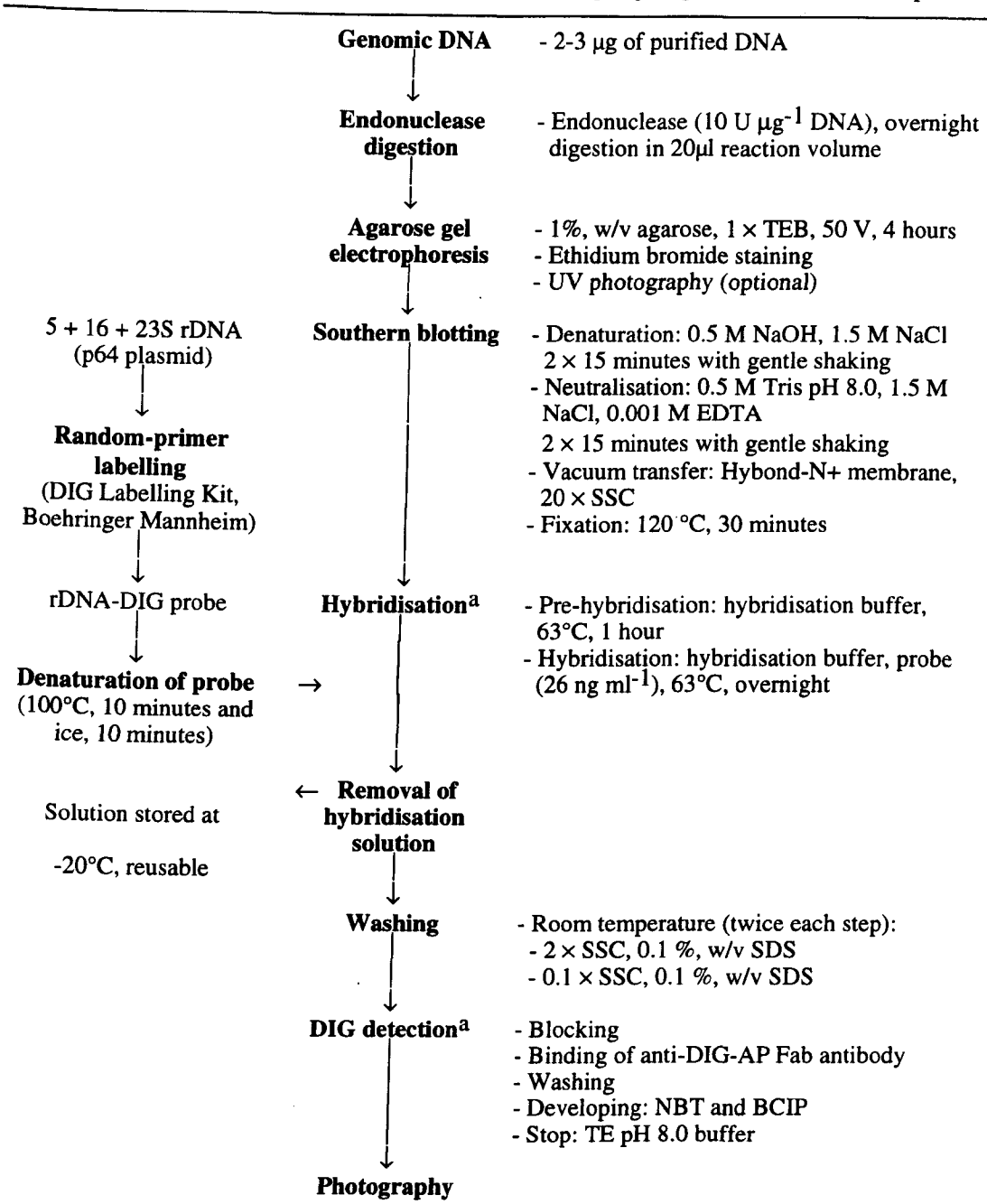
8. Molecular fingerprinting: Ribotyping

Ribotyping of the chromosomal DNA of sixteen test strains (Table 3-4) was performed as outlined in Figure 3-3.

Endonuclease digestion and agarose gel electrophoresis. It is known that *Bam* HI (Boeringer Mannheim, Germany) and *Sal* I (Boeringer Mannheim, Germany) produce relatively less strain-specific ribotyping patterns in streptomycetes (Zakrzewska-Czerwinska, personal communication). Restriction enzymes which tend to cut inside rRNA operons should be avoid.

Purified genomic DNA (*ca.* 2-3 µg), prepared as described earlier (Chapter II, small scale DNA extraction for 16S rRNA sequencing), was digested with *Bam* HI (recognition sequence G↓GATCC), *Sal* I (G↓TCGAC) and *Pvu* II (CAG↓CTG) restriction endonucleases using 10 units of enzyme per 1 µg of DNA in 25 µl volume reactions at 37°C overnight, as recommended by the manufacturer (Boehringer Mannheim Biochemica, 1996). The resultant DNA fragments were separated in 20 cm long agarose gels (1 %, w/v; 5 mm thickness) prepared using low electroendosmosis (EEO) agarose (Sigma). Electrophoresis was carried out at 50 V for 12 hours at room temperature in 1 × TEB buffer (Tris-borate-EDTA; Sambrook *et al.*, 1989). The gels were stained for 20 minutes with ethidium bromide

Figure. 3-3. Protocol for ribotyping experiments using digoxigenin labelled rDNA probes



[a], Hybridisation and detection were performed according to the recommendations of the DIG system manufacturer (Boehringer Mannheim Biochemica, 1993). Hybridisation buffer consisted of 5 × SSC, 0.1%, w/v N-laurylsarcosine, 0.02%, w/v SDS and 1%, w/v blocking reagent (Boehringer Mannheim Biochemica, 1993). AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium.

(0.5 μg ml-1 in water), destained in water and photographed under UV light.

Southern blotting of DNA. The DNA fragments were denatured *in situ* and transferred from gel to solid support, that is, to positively charged nylon membranes (Hybond-N^+; Amersham Life Science) by capillary action following a modified Southern blotting protocol (Southern, 1975). The relative position of the DNA fragments are preserved during their transfer to membranes. The DNA attached to the membrane was hybridised to a non-radioactive labelled DNA probe and the chemiluminescence reaction used to visualise the pattern of bands containing sequences complementary to the probe.

Procedure

1) After electrophoresis, trim away any unused areas of gel with a razor blade. Cut off the top left-hand corner of the gel; this serves to orientate the gel during subsequent operations.
2) Denature the DNA by soaking the gel twice for 15 minutes in 3 volumes of denaturation solution (0.5 N NaOH, 1.5 M NaCl) with gentle agitation. The depurination step was omitted. Transfer may be improved by nicking the DNA by brief and **controlled** depurination with acid or by UV exposure (5 minutes) prior to denaturation with alkaline.
3) Neutralise the gel by soaking twice for 15 minutes in 3 volumes of neutralisation solution (0.5 M Tris [pH 8.0], 1.5 M NaCl) at room temperature with gentle agitation.
4) Prepare the nylon membrane while the gel is in the neutralisation solution. Use gloves and blunt-ended forceps to handle the membrane. Using a clean cutter, cut a piece of membrane about 5mm wider than the gel and cut off a corner from the membrane to match the corner cut of the gel. Float the membrane on the surface of deionised water until it wets completely.

5) Transfer the denatured DNA from the gel to the membrane by capillary action using transfer solution (20 x SSC). (a) When the 3MM (Whatman) paper on top of the support is thoroughly wet, squeeze out all air bubbles with a glass rod; (b) place the gel and then the membrane on it making sure that there are no air bubbles between the gel and the membrane; (c) wet three pieces of 3MM paper (cut exactly to the size of the gel) in 20 x SSC and place them on top of the membrane, squeeze out any air bubbles with a glass rod; (d) pile paper towels (10 cm high) as big as or smaller than 3MM papers, put glass plates on top of the stack and weigh it down and wrap up this preparation, and (e) allow the transfer of DNA to proceed overnight.

6) Peel the membrane from the gel. Check under UV light to see if the DNA was transferred to membranes successfully. Wash the membrane in 6 x SSC to remove any agarose gel. Drain excess 6 x SSC on 3MM paper for 30 minutes.

7) The transferred DNA fragments are fixed on the membrane by UV-crosslinking (membrane should be wet). Next bake the membrane at 80 °C for 50 minutes. Alternatively the DNA can be fixed on the membrane by baking at 120 °C for 30 minutes. Store dried membrane at 4 °C.

Preparation of rRNA probe. The cloned rRNA operon containing 16S rRNA - 23S rRNA -5S rRNA genes from *Streptomyces (coelicolor) violaceoruber* DSM 41007 was used as probe (Zakrzewska-Czerwinska, 1989). The rRNA operon was cloned in the *Sal* I site of the pUC18 plasmid and the plasmid plus insert amplified following transformation into *Escherichia coli* JM109 (Promega). The 5S+16S+23S rRNA operon insert was removed by overnight digestion of 28 µg p64 plasmid DNA with approximately 40 U of *Sal* I in a 150 µl reaction volume for 3-4 hours, according to the recommendations of the manufacturer (Boehringer Mannheim Biochemica, 1996). The 7.2 kb rRNA fragment was separated from the linearised 2.8 kb plasmid by electrophoresis in a 1%, w/v preparative agarose gel

(Sambrook *et al.*, 1989). The band corresponding to the rRNA was cut from the gel, the DNA isolated and purified by using a standard method (Sambrook *et al.*, 1989) and resuspended in 20 µl TE buffer at pH 8.0. The DNA concentration was adjusted to approximately 1 µg μl^{-1} with sterile Milli-Q water.

Digoxigenin labelling of the rRNA fragment was performed by using a commercial kit, according to the recommendations of the manufacturer (Boehringer Mannheim Biochemica, 1996). Approximately 1 to 2 µg of purified DNA was labelled in a 50 µl volume labelling reaction. The DNA was heat denatured at 100 °C for 10 minutes followed by incubation in ice for 5 minutes prior to labelling. The labelling reaction was prepared from the reagents provided in the kit; the reagents were added to an ice cooled Eppendorf tube in the order listed below:

Labelling mix:

Template DNA.....1 µg

10 × Hexanucleotide mixture.....5 µl

dNTP labelling mixture.....5 µl

Milli-Q water.....up to 47.5 µl

Klenow DNA polymerase.....2.5 µl

The labelling reaction was incubated at 14 °C overnight then stopped by the addition of a 0.1 volume of 0.5 M EDTA (pH 8.0). The DNA was precipitated by the addition of a 0.1 volume of 4 M LiCl2 and 2.5 to 3.0 volumes of chilled ethanol. The preparation was mixed well then incubated at -20 °C overnight. DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes and washed once with 100 µl of chilled 70% ethanol (v/v) with centrifugation at 13,000 rpm for 5 minutes before discarding the 70% ethanol. The pellet was dried under vacuum, resuspended in approximately 25 µl of TE buffer and stored at -20 °C. The labelling efficiency was checked in a direct detection assay according to the protocol provided by Boehringer Mannheim Biochemica (1996).

Membrane hybridisation using DIG-labelled probe. Hybridisation of positively charged nylon membranes containing immobilised DNA was carried out in a hybridisation cassette following the protocol provided by Boehringer Mannheim Biochemica (1995). Membranes were inserted into the hybridisation cassette (Scotlab Ltd; Strathclyde, Scotland) and pre-hybridised with pre-hybridisation solution (20 ml per 100 cm^2 of membrane surface) without probe for at least 1 hour at 63 °C. The pre-hybridisation solution consisted of 5 × SSC, N-laurylsarcosine (0.1%, w/v), SDS (0.02%, w/v) and blocking reagent (1%, w/v). The blocking reagent consisted of an autoclaved stock solution of 10%, w/v hydrolysed casein (Boehringer Mannheim Biochemica, 1995), 100 mM maleic acid and 150 mM NaCl (pH 7.5).

The concentration of the probe in the hybridisation solution was approximately 26 ng per ml^{-1}. The rRNA DIG-labelled probe was denatured by boiling in a water bath for 10 minutes followed by incubation on ice for 5 minutes. At least 2.5 ml amounts of hybridisation solution were used per 100 cm^2 of membrane surface. The membranes were hybridised overnight at 63°C in a rotary oven (Biometra, Maidstone, UK) with gentle shaking. The filters were washed (50 ml of solution per 100 cm^2 of membrane surface) at room temperature, twice in 2 × SSC plus 0.1%, w/v SDS (5 minutes each wash) and twice at 63°C in 0.1 × SSC plus 0.1%, w/v SDS (15 minutes each wash). The membranes were not allowed to dry after washing.

Immunological detection of DIG-labelled probes. The immunological detection of the DIG-labelled DNA hybridised to DNA fragments containing the rRNA operon was carried out using an anti-digoxigenin Fab antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemica, 1995). The membranes were equilibrated in approximately 50 ml of buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5) for 1 minute at room temperature after hybridisation and post-hybridisation washes. They were then blocked by gentle agitation in sufficient volume of buffer 2 (1:10 dilution of 10%, w/v stock solution of the

blocking reagent diluted in buffer 1; Boehringer Mannheim Biochemica, 1995) to allow the membranes to flow freely in the container for at least 30 minutes.

After blocking, buffer 2 was discarded and the membranes incubated with gentle shaking for 30 minutes with the antibody solution. The anti-digoxigenin-AP Fab antibody was diluted 1:5000 in 20 ml of buffer 2 (per 100 cm^2 of membrane surface; Boehringer Mannheim Biochemica, 1995) to give a working concentration of 150 mU per ml. The solution was mixed gently by inversion and poured over the membrane in the container. After antibody binding, the membranes were transferred to a new tray and washed twice with 50 ml of buffer 1 (Boehringer Mannheim Biochemica, 1995), 15 minutes per wash, to remove unbound antibody prior to colorimetric detection.

Colorimetric detection was achieved using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). Ready-made stock solutions of BCIP (50 mg ml-1 BCIP, toluidinium salt in 100% dimethylformamide) and NBT (75 mg ml-1 NBT salt in 70% [v/v] dimethylformamide) were provided by the manufacturer (Boehringer Mannheim Biochemica, 1995) and stored at -20°C. The membranes were soaked in 20 ml (per 100 cm^2 of membrane surface) of alkaline phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2) at room temperature for 2 minutes.

The NBT/BCIP substrate solution was freshly prepared by mixing 45 µl of the NBT stock solution and 35 µl of the BCIP stock solution in 10 ml of the alkaline phosphatase buffer (per 100 cm^2 of surface membrane). A single membrane was placed on the bottom of a flat-surfaced plastic container with the side containing the immobilised DNA upwards and the NBT/BCIP solution carefully added then spread over the entire surface of the membrane by tilting the container. The latter was sealed and the reaction allowed to develop in the dark for several hours. Monitoring for the development of the colour reaction was carried out every 10 to 15 minutes taking great care not to shake the container while the colour was developing. Once the bands were detected the colorimetric reaction was stopped by washing

the membranes with 50 ml of TE pH 8.0 buffer. The membranes were kept wet until photographed.

Results

1. Phylogenetic analyses based on 16S rRNA sequences

(a) Reference strains

Almost complete 16S rRNA sequences (1400-1470 nucleotides between *Escherichia coli* positions 28 and 1524; Brosius *et al.*, 1978) were generated for *"Streptomyces thermoflavus"* DSM 40574, *"Streptomyces thermophilus"* DSM 40365, *"Streptomyces thermotolerans"* DSM 40227 and *Streptomyces violaceoruber* DSM 40049^T and for the three alkalitolerant marker strains, namely, *Streptomyces canescens* DSM 40001^T, *Streptomyces cavourensis* subsp. *cavourensis* DSM 40300^T and *Streptomyces hydrogenans* DSM 40586^T. The sequences of all seven test strains were compared with the corresponding sequences of representatives of the genus *Streptomyces,* including those of members of the validly described thermophilic *Streptomyces* species. As expected, all of the test strains fell within the range of variation encompassed by the genus *Streptomyces* (Fig. 3-4; Table 3-6). The 16S rRNA sequence similarity values found between the *Streptomyces* species were between 92.6 % and 100 % with an average value of 96.4 % (Table 3-6).

It is apparent from the streptomycete tree generated by using the least-squares algorithm (Fig. 3-4) that streptomycete strains can be assigned to four presumptive clades (A, B, C and D), three of which were also found in the phylogenetic trees generated by using the maximum-likelihood method. Clades B and C are supported by high bootstrap values based on the neighbour-joining algorithm.

Five of the test strains were recovered in clade A, namely, *Streptomyces canescens* DSM 40001^T, *"Streptomyces thermoflavus"* DSM 40574, *"Streptomyces thermophilus"* DSM 40365, *"Streptomyces thermotolerans"* DSM 40227 and *Streptomyces violaceoruber* DSM 40049^T. *Streptomyces canescens* DSM 40001^T had an identical 16S rRNA sequence to

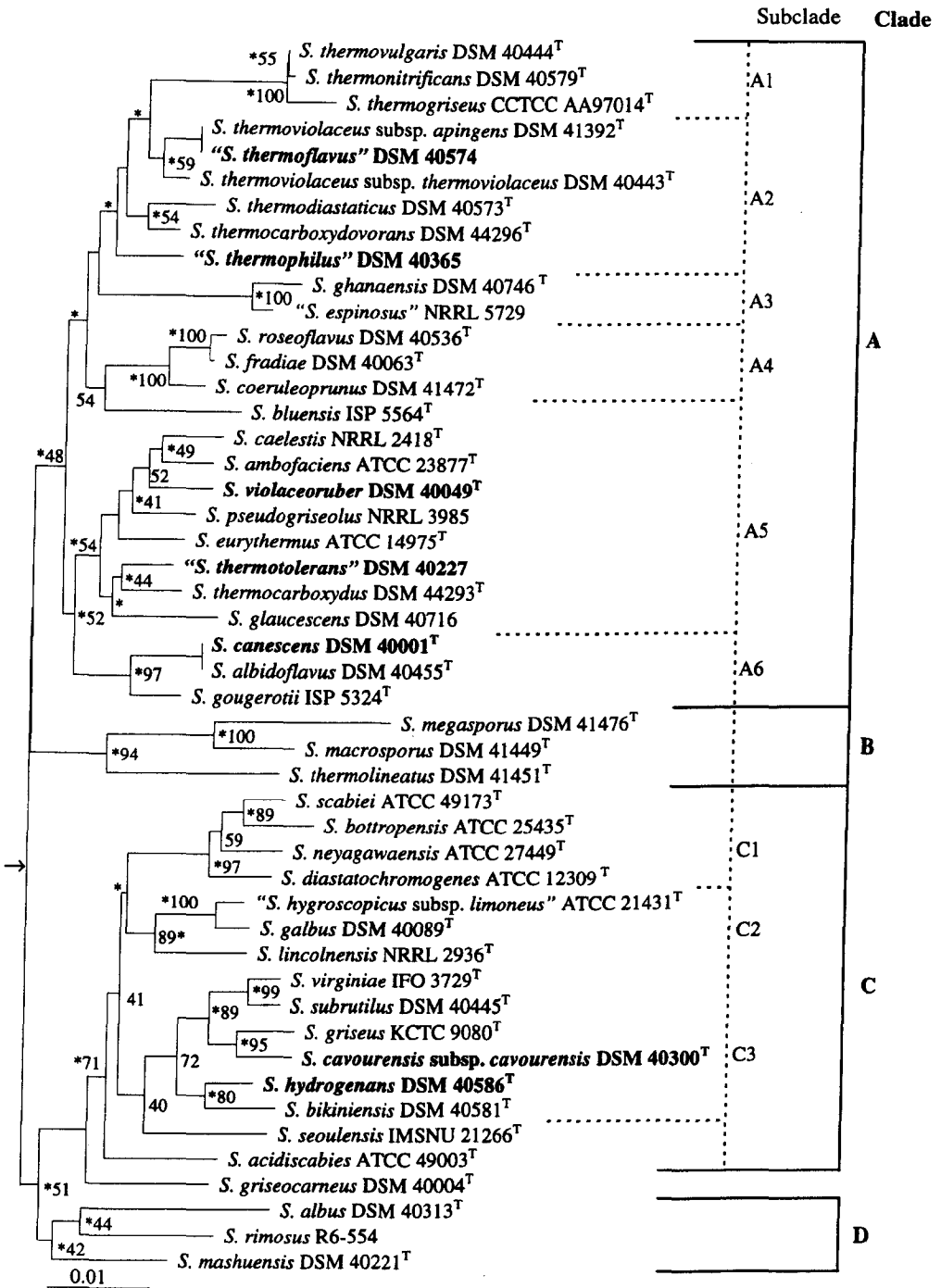


Figure 3-4. Least squares tree (Fitch & Margoliash, 1967) based on almost complete 16S rRNA sequences showing relationships between the test strains (in bold) and representatives of the genus *Streptomyces*. The corresponding 16S rRNA sequences of *Arthrobacter globiformis* (accession nuber M23411) was used as the outgroup. The asterisks indicate branches that were recovered using the maximum-likelihood algorithm (Felsenstein, 1981). The numbers at the nodes indicate the level (%) of bootstrap support based on a neighbour-joining analyses of 1000 resampled data sets; only values over 40 % are given. The arrow shows the root of the tree. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 3-6. The nucleotide sequence similarities (%) and differences found in the 16S rRNA of the test strains and representative *Streptomyces* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. *S. albidoflavus*	-	51	36	67	36	0	68	31	37	66	57	38	35	32	39	33	48	44	41	27	33	38	43	45
2. *S. albus*	96.0	-	50	50	51	51	55	50	60	52	48	55	52	51	60	44	60	56	43	57	44	48	55	55
3. *S. ambofaciens*	97.6	96.1	-	57	16	36	59	22	29	57	47	19	42	31	50	45	62	59	33	26	45	41	58	16
4. *S. bikiniensis*	95.5	96.1	96.1	-	65	67	32	48	65	31	16	54	54	58	58	51	70	66	44	62	51	50	65	50
5. *S. caelestis*	97.6	96.0	98.9	95.6	-	36	67	32	27	68	56	24	36	27	47	43	60	55	37	21	43	40	54	24
6. *S. canescens*	**100**	**96.0**	**97.5**	**95.4**	**97.5**	-	**68**	**31**	**37**	**66**	**57**	**38**	**35**	**32**	**39**	**33**	**48**	**44**	**42**	**27**	**33**	**38**	**43**	**45**
7. *S. cavourensis* subsp. *cavourensis*	**95.4**	**95.7**	**96.0**	**97.8**	**95.5**	**95.4**	-	**54**	**73**	**16**	**26**	**59**	**65**	**64**	**73**	**65**	**77**	**74**	**62**	**68**	**65**	**70**	**73**	**53**
8. *S. eurythermus*	97.9	96.1	98.5	96.8	97.8	97.9	96.3	-	34	57	42	20	32	35	41	37	52	49	28	32	37	36	48	20
9. *S. glaucescens*	97.5	95.3	98.0	95.6	98.2	97.5	95.1	97.7	-	74	59	27	35	24	40	35	51	47	41	24	35	33	46	37
10. *S. griseus*	95.5	96.0	96.1	97.9	95.4	95.5	98.9	96.1	95.0	-	22	59	66	62	71	63	79	75	59	70	63	67	74	56
11. *S. hydrogenans*	**96.0**	**96.2**	**96.7**	**98.9**	**96.0**	**95.9**	**98.2**	**97.0**	**95.8**	**98.4**	-	**49**	**53**	**53**	**59**	**58**	**70**	**67**	**46**	**60**	**58**	**54**	**66**	**46**
12. *S. pseudogriseolus*	97.4	95.7	98.7	96.3	98.4	97.4	96.0	98.6	98.2	96.0	96.5	-	36	21	46	41	51	47	27	22	41	37	46	20
13. *S. thermocarboxydovorans*	97.6	96.0	97.2	96.3	97.6	97.6	95.6	97.8	97.6	95.5	96.2	97.6	-	28	14	21	43	38	19	29	21	18	37	40
14. *S. thermocarboxydus*	97.8	96.0	97.9	96.1	98.2	97.8	95.7	97.6	98.4	95.8	96.2	98.6	98.1	-	35	31	48	43	31	16	31	29	42	35
15. *S. thermodiastaticus*	97.4	95.3	96.6	96.1	96.8	97.4	95.1	97.2	97.3	95.2	95.8	96.9	99.1	97.6	-	19	44	40	30	36	19	16	39	49
16. *"S. thermoflavus"*	**97.8**	**96.6**	**97.0**	**96.6**	**97.1**	**97.8**	**95.6**	**97.5**	**97.6**	**95.7**	**95.9**	**97.2**	**98.6**	**97.9**	**98.7**	-	**30**	**26**	**23**	**29**	**0**	**9**	**25**	**45**
17. *S. thermogriseus*	96.7	95.2	95.7	95.1	95.8	96.7	94.6	96.4	96.5	94.5	94.9	96.4	97.0	96.7	96.9	97.9	-	8	45	45	30	35	7	62
18. *S. thermonitrificans*	97.0	95.6	96.0	95.5	96.3	97.0	95.0	96.7	96.8	94.9	95.2	96.8	97.4	97.1	97.3	98.2	99.4	-	41	40	26	31	1	59
19. *"S. thermophilus"*	**97.2**	**96.7**	**97.8**	**97.0**	**97.5**	**97.1**	**95.8**	**98.1**	**97.2**	**96.0**	**96.7**	**98.2**	**98.7**	**97.9**	**98.0**	**98.4**	**96.9**	**97.2**	-	**30**	**23**	**14**	**40**	**32**
20. *"S. thermotolerans"*	**98.2**	**95.6**	**98.2**	**95.8**	**98.6**	**98.2**	**95.4**	**97.8**	**98.4**	**95.3**	**95.7**	**98.5**	**98.0**	**98.9**	**97.6**	**98.1**	**96.9**	**97.3**	**98.0**	-	**29**	**28**	**39**	**28**
21. *S. thermoviolaceus* subsp. *apingens*	97.8	96.6	97.0	96.6	97.1	97.8	95.6	97.5	97.6	95.7	95.9	97.2	98.6	97.9	98.7	100	97.9	98.2	98.4	98.0	-	9	25	45
22. *S. thermoviolaceus* subsp. *thermoviolaceus*	97.4	96.3	97.2	96.6	97.3	97.4	95.3	97.6	97.8	95.5	96.2	97.5	98.8	98.0	98.9	99.4	97.6	97.9	99.1	98.1	99.4	-	30	40
23. *S. thermovulgaris*	97.1	95.7	96.1	95.6	96.3	97.1	95.1	96.8	96.9	95.0	95.3	96.9	97.5	97.2	97.4	98.3	99.5	99.9	97.3	97.4	98.3	98.0	-	58
24. *S. violaceoruber*	**97.0**	**95.7**	**98.9**	**96.6**	**98.4**	**96.9**	**96.4**	**98.6**	**97.5**	**96.2**	**96.7**	**98.6**	**97.3**	**97.6**	**96.7**	**97.0**	**95.7**	**96.0**	**97.8**	**98.1**	**97.0**	**97.3**	**96.1**	-

1, *S. albidoflavus* DSM 40455^T; 2, *S. albus* DSM 40313^T; 3, *S. ambofaciens* ATCC 23877^T; 4, *S. bikiniensis* DSM 40581^T; 5, *S. caelestis* NRRL 2418^T; 6, *S. canescens* DSM 40001^T; 7, *S. cavourensis* subsp. *cavourensis* DSM 40300^T; 8, *S. eurythermus* ATCC 14975^T; 9, *S. glaucescens* DSM 40716; 10 *S. griseus* KCTC 9080^T; 11, *S. hydrogenans* DSM 40586^T; 12, *S. pseudogriseolus* NRRL 3985; 13, *S. thermocarboxydovorans* DSM 44296^T; 14, *S. thermocarboxydus* DSM 44293^T; 15, *S. thermodiastaticus* DSM 40573^T; 16, *"S. thermoflavus"* DSM 40574; 17, *S. thermogriseus* CCTCC AA97014^T; 18, *S. thermonitrificans* DSM 40579^T; 19, *"S. thermophilus"* DSM 40365; 20, *"S. thermotolerans"* DSM 40227; 21, *S. thermoviolaceus* subsp. *apingens* DSM 41392^T; 22, *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T; 23, *S. thermovulgaris* DSM 40444^T; 24, *S. violaceoruber* DSM 40049^T.

that of *Streptomyces albidoflavus* DSM 40455^T. An identical 16S rRNA sequence was also found between *"Streptomyces thermoflavus"* DSM 40574 and *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T. *"Streptomyces thermophilus"* DSM 40365 showed particularly high 16S rRNA sequence similarity values to *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (99.1 % nucleotide similarity) and *Streptomyces thermocarboxydovorans* DSM 44296^T (98.7 % nucleotide similarity). *"Streptomyces thermotolerans"* DSM 40227 and *Streptomyces violaceoruber* DSM 40049^T were found to be most closely related to *Streptomyces thermocarboxydus* DSM 44293^T (98.9 % nucleotide similarity) and *Streptomyces ambofaciens* ATCC 23877^T (98.9 % nucleotide similarity), repectively.

Clade A also contained eight thermophilic marker strains, namely, *Streptomyces thermocarboxydovorans* DSM 44296^T, *Streptomyces thermocarboxydus* DSM 44293^T, *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermogriseus* CCTCC AA97014^T, *Streptomyces thermonitrificans* DSM 40579^T, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T, and *Streptomyces thermovulgaris* DSM 40444^T together with thirteen mesophilic marker streptomycetes. In total, ten out of the thirteen thermophilic strains were recovered in clade A.

It is evident from Figure3-4 and Table 3-6 that twenty-five out of twenty-six strains assigned to clade A fall into six subclades. Subclade A1 encompasses three thermophilic streptomycetes, namely, *Streptomyces thermogriseus* CCTCC AA97014^T, *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T. Similarly, subclade A2 contains six thermophilic streptomycetes, that is, *Streptomyces thermocarboxydovorans* DSM 44296^T, *Streptomyces thermodiastaticus* DSM 40573^T, *"Streptomyces thermoflavus"* DSM 40574, *"Streptomyces thermophilus"* DSM 40365, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T and *Streptomyces*

thermoviolaceus subsp. *thermoviolaceus* DSM 40443^T. Subclade A3 encompasses the mesophiles, *"Streptomyces espinosus"* NRRL 5729 and *Streptomyces ghanaensis* DSM 40746^T. Similarly, subclade A4 contains *Streptomyces coeruleoprunus* DSM 41472^T, *Streptomyces fradiae* DSM 40063^T and *Streptomyces roseoflavus* DSM 40536^T. The largest group, subclade A5 contains eight strains including *Streptomyces ambofaciens* ATCC 23877^T, *Streptomyces thermocarboxydus* DSM 44293^T, *"Streptomyces thermotolerans"* DSM 40227 and *Streptomyces violaceoruber* DSM 40049^T. The final subclade, A6, encompasses three mesophilic strains, namely, *Streptomyces albidoflavus* DSM 40455^T, *Streptomyces canescens* DSM 40001^T and *Streptomyces gougerotii,* ISP 5324^T. The three remaining thermophilic streptomycetes, namely, *Streptomyces macrosporus* DSM 41449^T, *Streptomyces megasporus* DSM 41476^T and *Streptomyces thermolineatus* DSM 41451^T formed clade B.

Two of the test strains were recovered in clade C. *Streptomyces cavourensis* subsp. *cavourensis* DSM 40300^T showed its highest 16S rRNA sequence similarity with *Streptomyces griseus* KCTC 9080^T (98.9 % nucleotide similarity) whereas *Streptomyces hydrogenans* DSM 40586^T showed its highest similarity with *Streptomyces bikiniensis* DSM 40581^T (98.9 % nucleotide similarity).

Thirteen out of the fifteen strains in clade C can be assigned to three subclades (Fig. 3-4). Subclade C1 contains *Streptomyces bottropensis* ATCC 25435^T, *Streptomyces diastatochromogenes* ATCC 12309 ^{T}, *Streptomyces neyagawaensis* ATCC 27449^T and *Streptomyces scabiei* ATCC 49173^T, subclade C2 *Streptomyces galbus* DSM 40089^T, *"Streptomyces hygroscopicus* subsp. *limoneus"* ATCC 21431 and *Streptomyces lincolnensis* NRRL 2936^T, and subclade C3 *Streptomyces bikiniensis* DSM 40581^T, *Streptomyces cavourensis subsp. cavourensis* DSM 40300^T, *Streptomyces griseus* KCTC 9080^T, *Streptomyces hydrogenans* DSM 40586^T, *Streptomyces subrutilus* DSM 40445^T and *Streptomyces virginiae* IFO 3729^T.

Streptomyces albus DSM 40313^T, *Streptomyces mashuense* DSM 40221^T and *Streptomyces rimosus* R6-554 formed a loose grouping, clade D, at the periphery of clade C. However, this clade was not supported by high bootstrap values. *Streptomyces griseocarneus* DSM 40004^T could not be assigned to any of the caldes with confidence.

In general, the G+C content of the 16S rRNA of the thermophilic streptomycetes was higher than the corresponding data for the mesophilic streptomycetes (Table 3-7). The highest 16S rRNA G+C value, 61.6 mol%, was shown by *Streptomyces megasporus* DSM 41476^T. The other members of clade B, namely, *Streptomyces macrosporus* DSM 41449^T and *Streptomyces thermolineatus* DSM 41451^T showed values of 60.3 and 59.2 mol%, respectively. The G plus C values of the thermophilic reference streptomycetes assigned to clade A fell within the range 59.5 to 60.0 mol%. Thirteen out of the fifteen mesophilic streptomycetes in clade A contained 16S rRNA with G+C contents between 58.9 and 59.5 mol%, the exceptions, *"Streptomyces espinosus"* NRRL 5729 and *Streptomyces ghanaensis* DSM 40746^T gave values of 60.8 and 60.9 mol%, respectively. The fourteen mesophilic streptomycetes assigned to clade C contained 16S rRNA with G+C values within the range 58.1 and 59.1 mol%.

(b) Thermophilic isolates

Almost complete 16S rRNA sequences (1400-1470 nucleotides between *Escherichia coli* positions 28 and 1524; Brosius *et al.*, 1978) were generated for ten thermophilic isolates, namely, *Streptomyces* strain NT307 (centrotype strain of cluster 3, aggregate group II; Sahin,1995), *Streptomyces* strains NAR54, NT358, NT322, NT576, NT381 and NT90 (centrotype strains of clusters 6, 7, 8, 10, 11 and 15, respectively, aggregate group IV; Sahin,1995), *Streptomyces* strains NAR85 and TA56 (single-membered clusters, aggregate group V and VI, respectively; Sahin,1995) and *Streptomyces* strain B19, a strain isolated

Table 3-7. Guanine plus cytosine (mol% G+C) content of the 16S rRNA of the test strains*

Strain	G+C content	Strain	G+C content
Clade A		**Clade B**	
S. albidoflavus DSM 40455^T	59.4	***S. macrosporus* DSM 41449^T**	**60.3**
S. ambofaciens ATCC 23877^T	59.2	***S. megasporus* DSM 41476^T**	**61.6**
S. bluensis ISP 5564^T	59.1	***S. thermolineatus* DSM 41451^T**	**59.7**
S. caelestis NRRL 2418^T	59.3	**Clade C**	
S. canescens DSM 40001^T	59.4	*S. acidiscabies* ATCC 49003^T	59.1
S. coeruleoprunus DSM 41472^T	59.2	*S. bikiniensis* DSM 40581^T	59.1
"S. espinosus" NRRL 5729	60.8	*S. bottropensis* ATCC 25435^T	58.7
S. eurythermus ATCC 14975^T	58.9	*S. cavourensis subsp. cavourensis* DSM 40300^T	58.3
S. fradiae DSM 40063^T	59.4	*S. diastatochromogenes* ATCC 12309^T	58.7
S. ghanaensis DSM 40746^T	60.9	*S. galbus* DSM 40089^T	58.5
S. glaucescens DSM 40716	59.0	*S. griseus* KCTC 9080^T	58.4
S. gougerotii ISP 5324^T	59.3	*S. hydrogenans* DSM 40586^T	58.7
S. pseudogriseolus NRRL 3985	59.1	*S. lincolnensis* NRRL 2936^T	58.6
S. roseoflavus DSM 40536^T	59.3	*S. neyagawaensis* ATCC 27449^T	59.0
***S. thermocarboxydovorans* DSM 44296^T**	**59.8**	*S. scabiei* ATCC 49173^T	58.7
***S. thermocarboxydus* DSM 44293^T**	**59.7**	*S. seoulensis* IMSNU 21266^T	58.1
***S. thermodiastaticus* DSM 40573^T**	**59.5**	*S. subrutilus* DSM 40445^T	58.2
***"S. thermoflavus"* DSM 40574**	**59.7**	*S. virginiae* IFO 3729^T	58.2
***S. thermonitrificans* DSM 40579^T**	**60.0**	**Clade D**	
***"S. thermophilus"* DSM 40365**	**59.8**	*S. albus* DSM 40313^T	59.1
***"S. thermotolerans"* DSM 40227**	**59.7**	*S. mashuense* DSM 40221^T	58.9
***S. thermoviolaceus* subsp. *apingens* DSM 41392^T**	**59.7**	*S. rimosus* R6-554	59.3
***S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T**	**59.6**	**In search of clade**	
***S. thermovulgaris* DSM 40444^T**	**59.9**	*S. griseocarneus* DSM 40004^T	58.8
S. violaceoruber DSM 40049^T	59.1		

*, The G+C content of the 16S rRNA sequences was determined by using the AL16S program (Chun, 1995).

**, Clusters defined by Sahin (1995).

Thermophilic streptomycetes are given in bold.

from poultry faeces in Malaysia. The relationships found between the test and marker strains are presented in Figure 3-5 and Table 3-8.

It is evident from the phylogenetic tree based on almost complete 16S rRNA sequences (Fig. 3-5; Table 3-8) that nine out of the ten isolates fell into clade A. The remaining organism, *Streptomyces* strain NT307 was assigned to clade D. This organism shared its highest 16S rRNA similarity with *Streptomyces albus* DSM 40313^T (98.6 % nucleotide similarity).

Streptomyces strain TA56, which formed a single-membered cluster (Sahin, 1995), was recovered in subclade A1. This organism shared its highest 16S rRNA sequence similarity with *Streptomyces thermonitrificans* DSM 40579^T (98.9 % nucleotide similarity) and *Streptomyces thermovulgaris* DSM 40444^T (99.0 % nucleotide similarity). *Streptomyces* strain NAR85, the only isolate assigned to aggregate group V (Sahin, 1995), was recovered in subclade A2 and shared its highest 16S rRNA sequence similarities with *Streptomyces thermodiastaticus* DSM 40573^T (99.5 % nucleotide similarity), *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T (99.4 % nucleotide similarity) and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (99.4 % nucleotide similarity). Similarly, *Streptomyces* strain B19 shared its highest 16S rRNA similarity with *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T (98.6 % nucleotide similarity).

Five out of the six centrotype strains representing clusters in aggregate group IV (Sahin, 1995), namely, NAR54 (cluster 6), NT90 (cluster 15), NT358 (cluster 7), NT381 (cluster 11) and NT576 (cluster 10) were recovered in subclade A5. *Streptomyces* strain NAR54 showed the same highest 16S rRNA similarity with *Streptomyces* strain NT576 and *Streptomyces pseudogriseolus* NRRL 3985 (99.3 % nucleotide similarity). Similarly, *Streptomyces* strain NT90 was most closely related to *Streptomyces* strain NT381 and *Streptomyces caelestis* NRRL 2418^T (98.8 % nucleotide similarity). *Streptomyces* strain NT358 showed its highest 16S rRNA similarity with *Streptomyces eurythermus* ATCC

Figure 3-5. Least squares tree (Fitch & Margoliash, 1967) based on almost complete 16S rRNA sequences showing relationships between the isolates (in bold) and representatives of the genus *Streptomyces*. The corresponding 16S rRNA sequence of *Arthrobacter globiformis* (accession number M23411) was used as the outgroup. The numbers at the nodes indicate the level (%) of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets; only values over 40 % are given. The scale bar indicates 0.01 substitutions per nucleotide position. Alkalitolerant strains are marked with an asteriks. SMC=single-membered cluster.

The following strains had identical partial 16S rRNA sequences with organisms included in the tree: (a) *Streptomyces* strains **A1853** (Sahin, cluster 19, aggregate group VI), **A1956** (Sahin, cluster 20, aggregate group VI), **NT218** (Sahin, SMC, aggregate group VI), **TA12*** (Sahin, SMC, aggregate group VI), **TA26*** (Sahin, cluster 25, aggregate group VI), **TA34*** (Sahin, cluster 16, aggregate group IV), **TA54*** (Sahin, cluster 24, aggregate group VI), **TA123*** (Sahin, SMC, aggregate group VI), **TA179*** (Sahin, cluster 26, aggregate group VI) and **TA265*** (Sahin, cluster 23, aggregate group VI) with *Streptomyces thermovulgaris* DSM 40444^T (Sahin, cluster 22, aggregate group VI); (b) *Streptomyces* strain **NAR84*** (Sahin, cluster 5, aggregate group IV) with *Streptomyces* strain **NAR54*** (Sahin, cluster 6, aggregate group IV); (c) *Streptomyces* strains **NT312** (Sahin, cluster 13, aggregate group IV), **NT371**(Sahin, cluster 12, aggregate group IV), **NT399** (Sahin, cluster 9, aggregate group IV), **NT493** (Sahin, SMC, aggregate group IV), **TA127*** (Sahin, SMC, aggregate group IV) with *Streptomyces* strain **NT381** (Sahin, cluster 11, aggregate group IV), and (d) *Streptomyces* strain **NT336** (Sahin, cluster 14, aggregate group IV) with *Streptomyces* strain **NT90** (Sahin, cluster 15, aggregate group IV).

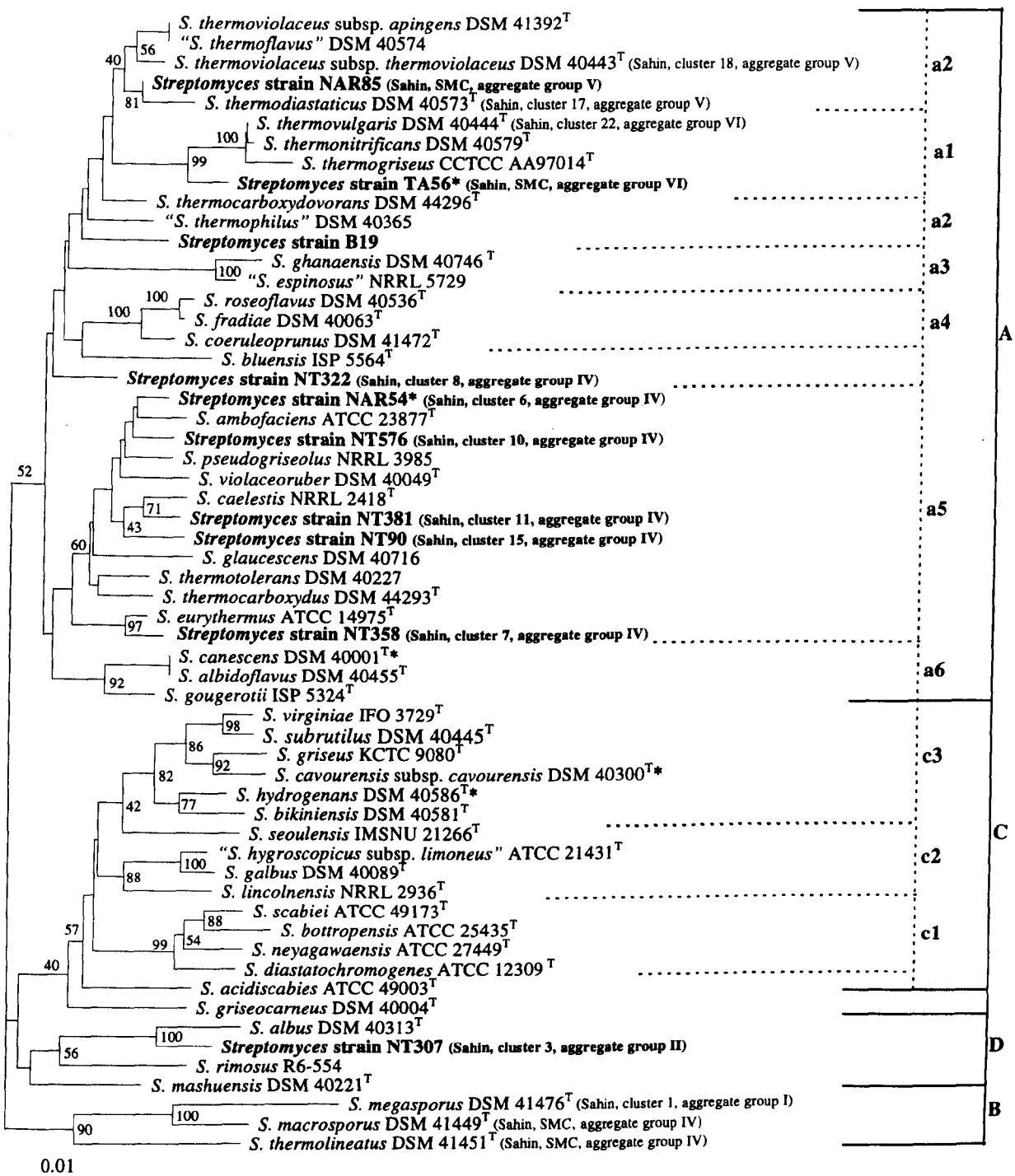

S. thermoviolaceus subsp. apingens DSM 41392T
"S. thermoflavus" DSM 40574
S. thermoviolaceus subsp. thermoviolaceus DSM 40443T (Sahin, cluster 18, aggregate group V)
Streptomyces strain NAR85 (Sahin, SMC, aggregate group V)
S. thermodiastaticus DSM 40573T (Sahin, cluster 17, aggregate group V)
S. thermovulgaris DSM 40444T (Sahin, cluster 22, aggregate group VI)
S. thermonitrificans DSM 40579T
S. thermogriseus CCTCC AA97014T
Streptomyces strain TA56* (Sahin, SMC, aggregate group VI)
S. thermocarboxydovorans DSM 44296T
"S. thermophilus" DSM 40365
Streptomyces strain B19
S. ghanaensis DSM 40746T
"S. espinosus" NRRL 5729
S. roseoflavus DSM 40536T
S. fradiae DSM 40063T
S. coeruleoprunus DSM 41472T
S. bluensis ISP 5564T
Streptomyces strain NT322 (Sahin, cluster 8, aggregate group IV)
Streptomyces strain NAR54* (Sahin, cluster 6, aggregate group IV)
S. ambofaciens ATCC 23877T
Streptomyces strain NT576 (Sahin, cluster 10, aggregate group IV)
S. pseudogriseolus NRRL 3985
S. violaceoruber DSM 40049T
S. caelestis NRRL 2418T
Streptomyces strain NT381 (Sahin, cluster 11, aggregate group IV)
Streptomyces strain NT90 (Sahin, cluster 15, aggregate group IV)
S. glaucescens DSM 40716
S. thermotolerans DSM 40227
S. thermocarboxydus DSM 44293T
S. eurythermus ATCC 14975T
Streptomyces strain NT358 (Sahin, cluster 7, aggregate group IV)
S. canescens DSM 40001T*
S. albidoflavus DSM 40455T
S. gougerotii ISP 5324T
S. virginiae IFO 3729T
S. subrutilus DSM 40445T
S. griseus KCTC 9080T
S. cavourensis subsp. cavourensis DSM 40300T*
S. hydrogenans DSM 40586T*
S. bikiniensis DSM 40581T
S. seoulensis IMSNU 21266T
"S. hygroscopicus subsp. limoneus" ATCC 21431T
S. galbus DSM 40089T
S. lincolnensis NRRL 2936T
S. scabiei ATCC 49173T
S. bottropensis ATCC 25435T
S. neyagawaensis ATCC 27449T
S. diastatochromogenes ATCC 12309T
S. acidiscabies ATCC 49003T
S. griseocarneus DSM 40004T
S. albus DSM 40313T
Streptomyces strain NT307 (Sahin, cluster 3, aggregate group II)
S. rimosus R6-554
S. mashuensis DSM 40221T
S. megasporus DSM 41476T (Sahin, cluster 1, aggregate group I)
S. macrosporus DSM 41449T (Sahin, SMC, aggregate group IV)
S. thermolineatus DSM 41451T (Sahin, SMC, aggregate group IV)
a2
a1
a2
a3
a4
a5
a6
c3
c2
c1
A
C
D
B
0.01

Table 3-8. The nucleotide sequence similarities (%) and differences found in the 16S rRNA of the test strains and representative *Streptomyces* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. *Streptomyces* strain B19	-	38	26	41	44	27	36	38	35	29	48	47	44	34	41	38	31	38	21	21	27	33	46
2. *Streptomyces* strain NAR54	97.4	-	36	23	60	25	29	20	11	44	52	12	20	37	23	11	22	44	40	40	37	49	19
3. *Streptomyces* strain NAR85	98.2	97.6	-	34	49	22	27	39	35	24	49	41	40	25	30	37	27	7	9	9	9	29	39
4. *Streptomyces* strain NT90	97.2	98.4	97.7	-	68	32	29	18	27	48	56	22	18	39	23	20	32	44	40	40	34	51	20
5. *Streptomyces* strain NT307	97.0	95.9	96.7	95.4	-	53	66	64	51	48	18	62	61	58	63	63	51	59	43	43	47	53	63
6. *Streptomyces* strain NT322	98.2	98.3	98.5	97.8	96.4	-	29	25	24	32	49	34	31	24	30	30	21	29	29	29	24	36	36
7. *Streptomyces* strain NT358	97.6	98.0	98.2	98.0	95.5	98.0	-	31	40	43	53	28	36	35	9	26	37	37	35	35	35	46	28
8. *Streptomyces* strain NT381	97.4	98.6	97.4	98.8	95.7	98.3	97.9	-	24	41	53	25	12	39	27	17	17	47	41	41	39	44	34
9. *Streptomyces* strain NT576	97.6	99.3	97.6	98.2	96.6	98.4	97.3	98.4	-	41	52	15	16	39	34	22	19	43	39	39	36	48	22
10. *Streptomyces* strain TA56	97.9	96.9	98.3	96.6	96.6	97.7	96.9	97.1	97.1	-	51	53	47	38	44	43	39	34	20	20	26	14	51
11. *S. albus*	96.3	96.0	96.2	95.6	98.6	96.2	95.9	95.9	96.0	96.0	-	50	51	55	50	55	51	60	44	44	48	55	55
12. *S. ambofaciens*	96.8	99.2	97.2	98.5	95.8	97.7	98.1	98.3	99.0	96.2	96.1	-	16	47	22	19	31	50	45	45	41	58	16
13. *S. caelestis*	97.0	98.6	97.3	98.8	95.9	97.9	97.6	99.2	98.9	96.7	96.0	98.9	-	45	32	24	27	47	43	43	40	54	24
14. *S. coeruleoprunus*	97.7	97.5	98.3	97.4	96.1	98.4	97.6	97.4	97.4	97.3	95.7	96.8	97.0	-	35	39	39	31	36	36	35	42	43
15. *S. eurythermus*	97.2	98.4	98.0	98.4	95.7	98.0	99.4	98.2	97.7	96.9	96.1	98.5	97.8	97.6	-	20	35	41	37	37	36	48	20
16. *S. pseudogriseolus*	97.4	99.3	97.5	98.6	95.7	98.0	98.2	98.8	98.5	96.9	95.7	98.7	98.4	97.4	98.6	-	21	46	41	41	37	46	20
17. *S. thermocarboxydus*	97.9	98.5	98.2	97.8	96.5	98.6	97.5	98.8	98.7	97.2	96.0	97.9	98.2	97.4	97.6	98.6	-	35	31	31	29	42	35
18. *S. thermodiastaticus*	97.4	97.0	99.5	97.0	96.0	98.0	97.5	96.8	97.1	97.6	95.3	96.6	96.8	97.9	97.2	96.9	97.6	-	19	19	16	39	49
19. *"S. thermoflavus"*	98.6	97.3	99.4	97.3	97.1	98.0	97.6	97.2	97.4	98.6	96.6	97.0	97.1	97.6	97.5	97.2	97.9	98.7	-	0	9	25	45
20. *S. thermoviolaceus* subsp. *apingens*	98.6	97.3	99.4	97.3	97.1	98.0	97.6	97.2	97.4	98.6	96.6	97.0	97.1	97.6	97.5	97.2	97.9	98.7	100	-	9	25	45
21. *S. thermoviolaceus* subsp. *thermoviolaceus*	98.2	97.5	99.4	97.7	96.8	98.4	97.6	97.4	97.6	98.2	96.3	97.2	97.3	97.6	97.6	97.5	98.0	98.9	99.4	99.4	-	30	40
22. *S. thermovulgaris*	97.8	96.7	98.0	96.5	96.4	97.6	96.9	97.0	96.8	99.0	95.7	96.1	96.3	97.2	96.8	96.9	97.2	97.4	98.3	98.3	98.0	-	58
23. *S. violaceoruber*	96.9	98.7	97.4	98.6	95.7	97.6	98.1	97.7	98.5	96.4	95.7	98.9	98.4	97.1	98.6	98.6	97.6	96.7	97.0	97.0	97.3	96.1	-

Reference strains: 11. *S. albus* DSM 40313^T; 12. *S. ambofaciens* ATCC 23877^T; 13. *S. caelestis* NRRL 2418^T; 14. *S. coeruleoprunus* DSM 41472^T; 15. *S. eurythermus* ATCC 14975^T; 16. *S. pseudogriseolus* NRRL 3985; 17. *S. thermocarboxydus* DSM 44293^T; 18. *S. thermodiastaticus* DSM 40573^T; 19. *"S. thermoflavus"* DSM 40574; 20. *S. thermoviolaceus* subsp. *apingens* DSM 41392^T; 21. *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T; 22. *S. thermovulgaris* DSM 40444^T and 23. *S. violaceoruber* DSM 40049^T.

14975^T (99.4 % nucleotide similarity). *Streptomyces* strain NT381 showed a correspondingly high similarity with *Streptomyces caelestis* NRRL 2418^T (99.2 % nucleotide similarity). The remaining isolate, *Streptomyces* strain NT576 showed its highest 16S rRNA similarity with strain NAR54 (99.3 % nucleotide similarity). Finally, strain NT322, which formed a distinct branch in clade A, is most closely related to *Streptomyces thermocarboxydus* DSM 44293^T (98.6 % nucleotide similarity).

Partial 16S rRNA sequences (700-1000 nucleotides between *Escherichia coli* positions 28 and 1150) were determined for fifteen thermophilic isolates, namely, *Streptomyces* strains NAR84, NT399, NT371, NT312 and NT336 (centrotype strains of clusters 5, 9, 12, 13 and 14, aggregate group IV; Sahin, 1995), *Streptomyces* strains A1853, A1956, TA265, TA61, TA26 and TA179 (centrotype strains of clusters 19, 20, 23, 24, 25 and 26, aggregate group VI; Sahin, 1995), *Streptomyces* strains NT493 and TA127 (single-membered clusters, aggregate group IV; Sahin, 1995), and *Streptomyces* strains TA123, NT218 and TA12 (single-membered clusters, aggregate group VI; Sahin, 1995). *Streptomyces* strains A1853, A1956, NT218, TA12, TA34, TA26, TA61, TA123 and TA179 had almost identical 16S rRNA sequences to those of to *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T differing from these organisms between 0 and 2 nucleotides. Similarly, *Streptomyces* strains NT312, NT371, NT399, NT493 and TA127 had identical partial 16S rRNA sequences to *Streptomyces* strain NT381, and *Streptomyces* strains NAR84 and NT336 identical partial 16S rRNA sequences to those of *Streptomyces* strains NAR54 and NT90, respectively.

Six out of the ten alkalitolerant, thermophilic isolates, that is, *Streptomyces* strains TA12 (single-membered cluster, aggregate group VI; Sahin, 1995), TA34 (cluster 16, aggregate group IV; Sahin, 1995), TA26 (cluster 25, aggregate group VI; Sahin, 1995), TA61 (cluster 24, aggregate group VI; Sahin, 1995), TA123 (single-membered cluster, aggregate group VI; Sahin, 1995) and TA179 (cluster 26, aggregate group VI; Sahin, 1995)

had almost identical 16S rRNA sequences to those of *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T (99.8 to 100 % nucleotide similarities). *Streptomyces* strain TA56 (single-membered cluster, aggregate group VI; Sahin, 1995) which shared its highest 16S rRNA similarity to *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T (98.9 % and 99.0 % nucleotide similarities, respectively) is also an alkalitolerant, thermophilic isolate. Two of the alkalitolerant, thermophilic isolates, namely, strain NAR54 (cluster 6, aggregate group IV; Sahin, 1995) and NAR84 (cluster 5, aggregate group IV; Sahin, 1995) had identical 16S rRNA sequences. The remaining alkalitolerant, thermophilic isolate, *Streptomyces* strain TA127, had an identical 16S rRNA sequence to *Streptomyces* strain NT90 (cluster 15, aggregate group IV; Sahin, 1995).

16S rRNA sequences of seven isolates, namely, *Streptomyces* strain NAR54 (centrotype strain of cluster 6, aggregate group IV; Sahin, 1995), NT90 (centrotype strain of cluster 15, aggregate group IV; Sahin, 1995), NT307 (centrotype strain of cluster 3, aggregate group II; Sahin, 1995), NT322 (centrotype strain of cluster 8, aggregate group IV; Sahin, 1995), NT358 (centrotype strain of cluster 7, aggregate group IV; Sahin, 1995), NT381 (centrotype strain of cluster 11, aggregate group IV; Sahin, 1995) and NT576 (centrotype strain of cluster 10, aggregate group IV; Sahin, 1995), were compared with the corresponding partial 16S rRNA sequences (16S rRNA positions 33 to 474 and 804 to 1233 [*Streptomyces ambofaciens* numbering system; Pernodet *et al.*, 1989]) held in the *Streptomyces* database at the DSMZ (Stackebrandt *et al.*, unpublished). *Streptomyces* strain NAR54 was found to be most closely related to *Streptomyces griseoincarnatus* DSM40274^T (99.0 % nucleotide similarity), *Streptomyces erythrogriseus* DSM 40116^T (99.0 % nucleotide similarity) and *Streptomyces griseoflavus* DSM 40456^T (99.0 % nucleotide similarity); *Streptomyces* strain NT90 to *Streptomyces plicatus* (99.1 % nucleotide similarity) and *Streptomyces cineoruber* (99.1 % nucleotide similarity); *Streptomyces* strain

NT307 to *Streptomyces flocculus* DSM 40327^T (98.6 % nucleotide similarity) and *Streptomyces albus* DSM 40313^T (98.0 % nucleotide similarity); *Streptomyces* strain NT358 to *Streptomyces lavenduligriseus* DSM 40487^T (98.9 % nucleotide similarity) and *Streptomyces eurythermus* DSM 40014^T (98.9 % nucleotide similarity); *Streptomyces* strain NT381 to *Streptomyces xantholiticus* DSM 40244^T (98.8 % nucleotide similarity) and *Streptomyces minutiscleroticus* DSM 40301^T (98.7 % nucleotide similarity); *Streptomyces* strain NT576 to *Streptomyces viridodiastaticus* DSM40249^T (99.2 % nucleotide similarity) and *Streptomyces albogriseolus* DSM 40003^T (99.0 % nucleotide similarity). The remaining organisms, *Streptomyces* strain NT322 showed the highest 16S rRNA sequence similarities with *Streptomyces coeruleoprunus* DSM 41472^T (97.6 % nucleotide similarity) and *Streptomyces fradiae* DSM 40063^T (97.6 % nucleotide similarity).

2. DNA:DNA relatedness study

The DNA relatedness data show that *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T belong to a single genomic species which is readily distinguished from a corresponding taxon which encompasses *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (Table 3-9). It is also clear from the DNA:DNA relatedness data that alkalitolerant, thermophilic isolates TA12, TA26, TA61, TA123, TA179 and TA265, and thermophilic, neutrophilic isolates A1853, A1956 and NT218 are *bona fide* members of *Streptomyces thermovulgaris* (Table 3-9). Similarly, *Streptomyces* strain AT5, a thermophilic, carboxydotrophic isolate, showed 95 % DNA:DNA relatedness with reference DNA prepared from *Streptomyces thermovulgaris* DSM 40444^T. The DNA:DNA relatedness data also showed that *Streptomyces* strain NAR85 should be classified as *Streptomyces thermodiastaticus* as it shares 97 % DNA relatedness with the type strain of

Table 3-9. Mean levels of DNA:DNA relatedness (%) found amongst representative thermophilic streptomycetes using the nitrocellulose filter method†

	Labelled strains							
Strains	DSM 40444^T	DSM 40579^T	AT5	TA56	DSM 40573^T	NAR85	DSM 41392^T	DSM 40443^T
S. thermovulgaris DSM 40444^T	100							
S. thermonitrificans DSM 40579^T	91	100						
Streptomyces strain AT5	95	ND	100					
Streptomyces strain TA56	62	50	ND	100				
S. thermodiastaticus DSM 40573^T	11	12	ND	17	100			
Streptomyces strain NAR85	8	ND	ND	ND	97	100		
S. thermoviolaceus subsp. *apingens* DSM 41392^T	12	ND	ND	ND	46	48	100	
S. thermoviolaceus subsp. *thermoviolaceus* DSM 40443^T	10	13	ND	17	58	55	95	100
S. griseus ISP 5236^T *	2	2	ND	1	8	9	6	6

^{T}, Type strain; ND, not determined; *, mesophilic control strain.

†, The pairwise DNA relatedness values in the table were obtained by averaging measurements of two sets of hybridisation. In each hybridisation, one of a pair of DNA preparations was labelled.

Streptomyces strains **A1853** (cluster 19, aggregate group VI), **A1956** (cluster 20, aggregate group VI), **NT218** (single-membered cluster, aggregate group VI), **TA12** (single-membered cluster, aggregate group VI), **TA26** (cluster 25, aggregate group VI), **TA61** (cluster 24, aggregate group VI), **TA123** (single-membered cluster, aggregate group VI), **TA179** (cluster 26, aggregate group VI) and **TA265** (cluster 23, aggregate group VI) shared significantly high DNA:DNA relatedness values (over 90 %) with labelled DNA preparations from *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T. Similarly, strain **AT6** and **AT54** shared DNA:DNA relatedness values over 70 % with labelled DNA preparations from strain AT5 and *Streptomyces thermovulgaris* DSM 40444^T.

this species. It is also evident from Table 3-9 that *Streptomyces* strain TA56 (single-membered cluster, aggregate group VI; Sahin, 1995) forms a distinct genomic species which shares a relatively close affinity with *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T.

Preliminary studies with labelled DNA from *Streptomyces* strain AT5 and *Streptomyces thermovulgaris* DSM 40444^T indicated that the DNA:DNA relatedness values between *Streptomyces thermovulgaris* DSM 40444^T and *Streptomyces* strains AT6 and AT54 were higher than 70 % and that the DNA relatedness values between strains AT5, AT6 and AT54 were greater than 87 %.

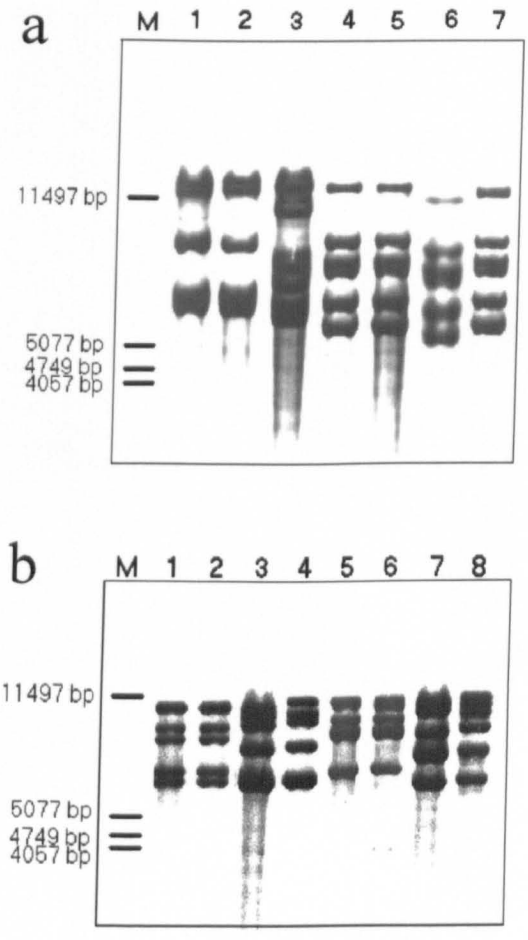
3. Ribotype patterns

It is evident from Figure 3-6a that the test strains showed three ribotype patterns when genomic DNA digests prepared using *Bam* HI restriction endonuclease were probed with the 7.2 kb DNA fragment from *Streptomyces violaceoruber* DSM 41007. *Streptomyces thermonitrificans* DSM 40579^T, *Streptomyces thermovulgaris* DSM 40444^T, *Streptomyces* strains A1853, A1956, NT218, TA26, TA61, TA123, TA179 and TA265 gave identical ribotype patterns, as did *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T and *Streptomyces* strain NAR85. The corresponding genomic DNA digest of *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T gave a very similar pattern to those shown by *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T and *Streptomyces* strain NAR85 though the ribotype bands were lower indicating that the molecular weight of the DNA fragments containing rRNA operons were lower (Fig. 3-6a). *Streptomyces* strain TA56 showed a distinct ribotype pattern (Fig. 3-6a).

Figure 3-6.

(a) Ribotyping patterns: 1, *S. thermovulgaris* DSM 40444^T; 2, *S. thermonitrificans* DSM 40579^T; 3, *Streptomyces* strain TA56; 4, *S. thermodiastaticus* DSM 40573^T; 5, *Streptomyces* strain NAR85; 6, *S. thermoviolaceus* subspecies *thermoviolaceus* DSM 40443^T; and 7, *S. thermoviolaceus* subspecies *apingens* DSM 41392^T generated from *Bam* HI genomic DNA digests hybridised with the digoxigenin-labelled rDNA probe. M, the size marker, is lambda DNA digested with *Pst* I.

(b) Ribotyping patterns: 1 and 5, *S. thermodiastaticus* DSM 40573^T; 2 and 6, *Streptomyces* strain NAR85; 3 and 7, *S. thermoviolaceus* subspecies *thermoviolaceus* DSM 40443^T; 4 and 8, *S. thermoviolaceus* subspecies *apingens* DSM 41392^T generated from *Sal* I (1-4) and *Pvu* II (5-8) genomic DNA digests hybridised with the digoxigenin labelled probe. M, the size marker, is lambda DNA digested with *Pst* I.


a
M 1 2 3 4 5 6 7
11497 bp
5077 bp
4749 bp
4057 bp
b
M 1 2 3 4 5 6 7 8
11497 bp
5077 bp
4749 bp
4057 bp

Streptomyces strain NAR85 gave the same banding pattern to that of *Streptomyces thermodiastaticus* DSM 40573^T with restriction endonucleases *Pvu* II and *Sal* I (Fig. 3-6b). This banding pattern served to distinguish these strains from the type strains of the two subspecies of *Streptomyces thermoviolaceus* which gave an identical banding pattern.

4. Chemotaxonomic markers

The results of the menaquinone analyses are shown in Table 3-10. Ten out of the eleven test strains were found to contain hexa- or octahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue. The exception, *Streptomyces* strain NT358, produced tetrahydrogenated menaquinones with nine isoprene units as the major component. The sole component found in *Streptomyces thermonitrificans* DSM 40579^T, *Streptomyces thermovulgaris* DSM 40444^T and *Streptomyces* strain TA56 was an octahydrogenated menaquinone with nine isoprene units. Identical menaquinone profiles were shown by *Streptomyces* strains NT90 and NT336.

The results of the polar lipid analyses are shown in Figure 3-7. All of the test strains contained PE, DPG, PI, PIDM and many unidentified phospholipids but the distribution of PG was variable. In addition, an unidentified glycolipid was detected in the polar lipid extract of *Streptomyces* strain TA56. *Streptomyces thermovulgaris* DSM 40444^T and *Streptomyces thermonitrificans* DSM 40579^T gave almost identical patterns, as did *Streptomyces* strains NT90 and NT336, and *Streptomyces* strains NAR54 and NAR84.

5. Phenotypic properties

The morphological properties of the test strains are summarised in Table 3-11. The spore chain morphology and spore surface ornamentation of individual strains are shown in Figure 3-8. It is evident that most of the test strains produced a grey aerial spore mass, spiral

Table 3-10. Menaquinone profiles of the test strains [a]

Strains	MK-9(H_4)	MK-9(H_6)	MK-9(H_8)
S. thermovulgaris DSM 40444^T (cluster 22, VI)			+++
S. thermonitrificans DSM 40579^T			+++
Streptomyces strain B19	++	+++	+
Streptomyces strain NT307 (cluster 3, II)[a]		++	+++
Streptomyces strain NAR84 (cluster 5, IV)	+	+++	++
Streptomyces strain NAR54 (cluster 6, IV)	+	+++	+
Streptomyces strain NT358 (cluster 7, IV)	+++	++(+)	+
Streptomyces strain NT322 (cluster 8, IV)		++	+++
Streptomyces strain NT576 (cluster 10, IV)	+	+++	++
Streptomyces strain NT336 (cluster 14, IV)		++(+)	+++
Streptomyces strain NT90 (cluster 15, IV)		++(+)	+++
Streptomyces strain TA56 (SMC, VI)			+++

[a], Clusters delineated in the numerical phenetic study of Sahin (1995); SMC, single-membered cluster. II; aggregate group II; IV, aggregate group IV; VI, aggregate group VI.

Abbreviations exemplified by MK-9 (H_4) and MK-9 (H_6), tetra- and hexahydrogenated menaquinones with nine isoprene units.

The main component in each series is denoted by '+ + +', any other component greater than 50% of the main peak by '+ +', and all other significant components by '+'. (+) indicates subtle difference in quantity.

Menaquinone components were eluted in the following order (retention time) under the conditions employed: dihydrogenated menaquinones with eight isoprene units, 8.4 minutes; dihydrogenated menaquinones with nine isoprene units, 9.7 minutes; tetrahydrogenated menaquinones with eight isopren units, 10.5 minutes; tetrahydrogenated menaquinones with nine isoprene units, 11.4 minutes; hexahydrogenated menaquinones with nine isoprene units, 13.5 minutes; tetrahydrogenated menaquinones with ten isoprene units, 14.6 minutes and octahydrogenated menaquinones with nine isoprene units, 16.1 minutes.

Figure. 3-7. Two dimensional thin-layer chromatography of the polar lipids of:

Streptomyces thermovulgaris DSM 40444^T (cluster 22, aggregate group VI),

Streptomyces thermonitrificans DSM 40579^T,

Streptomyces strain NT302 (cluster 3, aggregate group II),

Streptomyces strain NAR84 (cluster 5, aggregate group IV),

Streptomyces strain NAR54 (cluster 6, aggregate group IV),

Streptomyces strain NT322 (cluster 8, aggregate group IV),

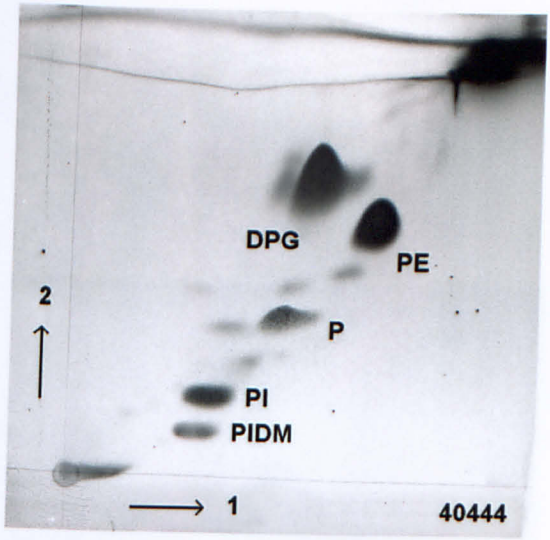
Streptomyces strain NT576 (cluster 10, aggregate group IV),

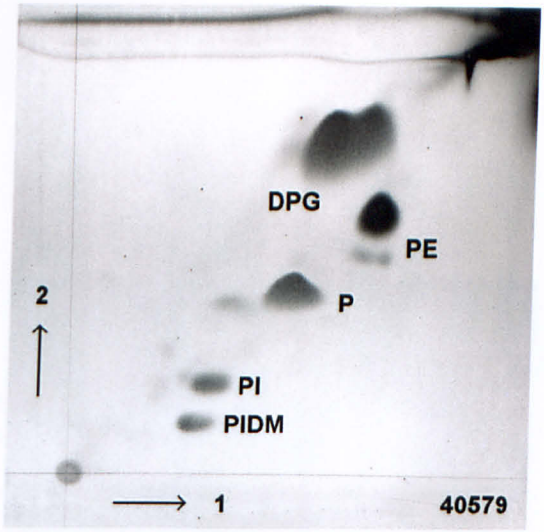
Streptomyces strain NT336 (cluster 14, aggregate group IV),

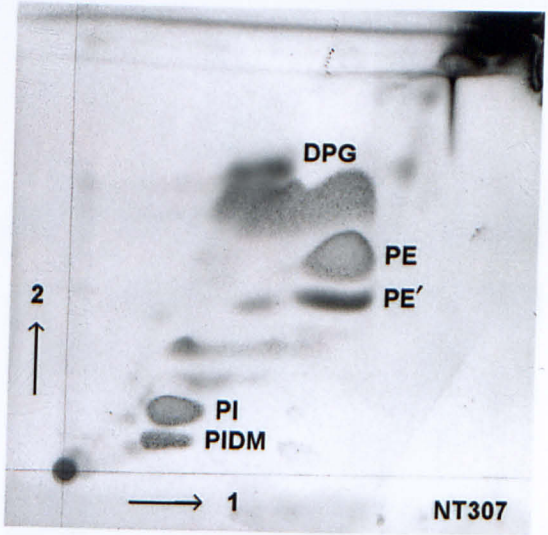
Streptomyces strain NT90 (cluster 15, aggregate group IV) and

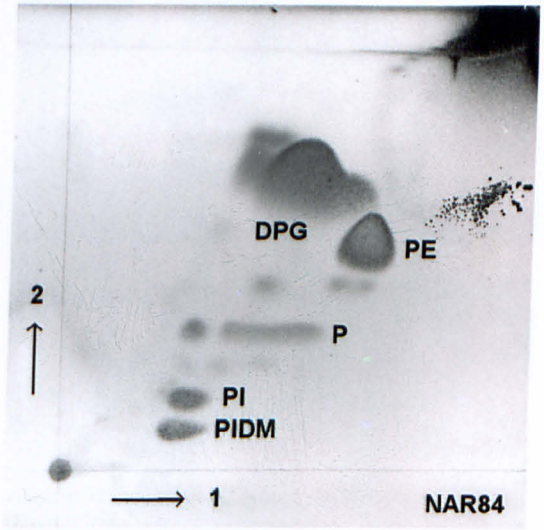
Streptomyces strain TA56 (single-membered cluster, aggregate group VI),

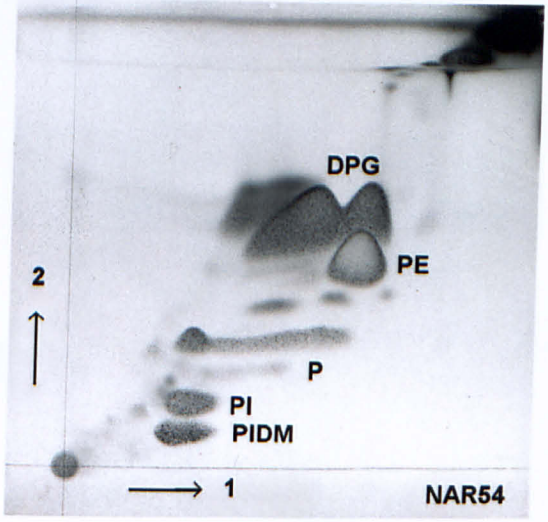
Chloroform-methanol-water (65:25:4, by volume) was used in the first direction followed by chloroform-acetic acid-methanol-water (80:18:12:5, by volume) in the second direction. Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PE', phosphatidylethanolamine derivative; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannoside; PIMs, phosphatidylinositol mannosides; G, glycolipids and P, unidentified phospholipids.

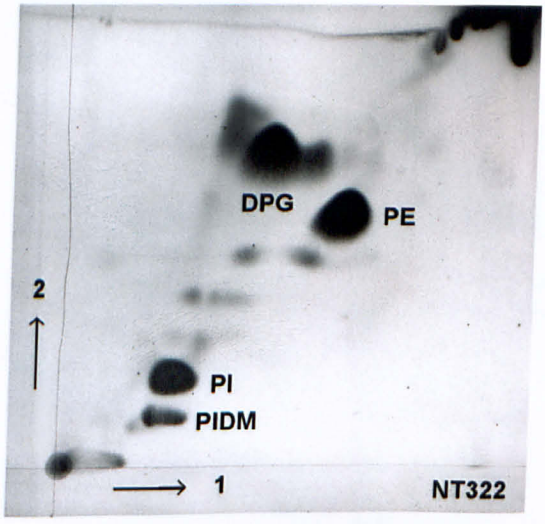

DPG
PE
P
PI
PIDM
2
1
40444


DPG
PE
P
PI
PIDM
2
1
40579


DPG
PE
PE′
PI
PIDM
2
1
NT307


DPG
PE
P
PI
PIDM
2
1
NAR84


DPG
PE
P
PI
PIDM
2
1
NAR54


DPG
PE
PI
PIDM
2
1
NT322

chains of spores, failed to produce melanin and did not form distinctive substrate mycelial pigments. *Streptomyces* strain NT576 was unusual amongst the test strains as it produced a green aerial spore mass. It is also interesting that *"Streptomyces thermoflavus"* DSM 40574, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T and *Streptomyces* strain NAR85 formed spores with a tuberculate surface ornamentation. *Streptomyces* strain TA56 was the only isolate to have warty spores.

A number of physiological tests were carried out to complement the results of Sahin (1995). *Streptomyces thermonitrificans* DSM 40579^T, *Streptomyces thermovulgaris* DSM 40444^T and *Streptomyces* strains NAR85 and TA56 reduced nitrate, and *Streptomyces thermocarboxydovorans* DSM 44296^T degraded adenine and xylan but not gelatin, and used L-arabinose, glucose and xylose as sole sources of carbon for energy and growth. Similarly, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T degraded adenine, casein and gelatin but not guanine, hypoxanthine, testosterone, xanthine or xylan, and used L-arabinose, glucose and xylose but not rhamnose as sole sources of carbon for energy and growth. *Streptomyces thermonitrificans* DSM 40579 ^{T} degraded adenine, casein and gelatin but not guanine, hypoxanthine, testosterone, xanthine or xylan, and used glucose and xylose but not L-arabinose or rhamnose as sole sources of carbon for energy and growth. *Streptomyces thermocarboxydovorans* DSM 44296^T, *Streptomyces thermonitrificans* DSM 40579 ^{T}, *Streptomyces thermovulgaris* DSM 40444^T and *Streptomyces* strain TA56 grew at pH 10 but *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T and *Streptomyces* strains NAR85 did not. All of the test strains grew at 25 °C but not at 15 °C.

Table 3-11. Morphological characteristics of the test strains

Strains	Colour of:		Production of:		Spore	Spore
	aerial spore mass	substrate mycelium	diffusible pigments	melanin pigments	chain morphology	surface ornamentation
Reference strains						
S. thermodiastaticus DSM 40573^T	Grey	Yellow-brown	-	-	Spiral	Tuberculate
"S. thermoflavus" DSM 40574	Light grey	Orange	-	-	Spiral	Tuberculate
S. thermonitrificans DSM 40579^T	Grey	Not distinctive	-	-	Hooked	Smooth
"S. thermophilus" DSM 40365	Grey	Not distinctive	-	+	Straight	Spiny
"S. thermotolerans" DSM 40227	Grey	Not distinctive	-	+	Hooked or spiral	Spiny
S. thermoviolaceus subsp. *apingens* DSM 41392^T	Grey	Not distinctive	-	-	Spiral	Tuberculate
S. thermoviolaceus subsp. *thermoviolaceus* DSM 40443^T	Grey	Violet	Violet	+	Spiral	Tuberculate
S. thermovulgaris DSM 40444^T	Grey	Not distinctive	-	-	Spiral	Smooth
Isolates*						
Aggregate group II						
NT307 (cluster 3)	White	Yellow-brown	-	-	Spiral	Smooth
Aggregate group IV						
NAR54 (cluster 6), NAR84 (cluster 5), NT322 (cluster 8), TA34 (cluster 16),	Grey	Not distinctive	-	-	Spiral	Smooth
NT90 (cluster 15), NT381 (cluster 11),	Grey	Not distinctive	-	-	Spiral	Not determined
NT125 (SMC)	Grey	Not distinctive	-	-	Straight-hooked	Not determined

NT312 (cluster 13), NT336 (cluster 14), NT399 (cluster 9)	Grey	Not distinctive	-	-	Spiral	Spiny
NT358 (cluster 7)	Brown-grey	Not distinctive	Not determined	Not determined	Straight	Smooth
NT371 (cluster 12)	Grey	Not distinctive	-	-	Spiral	Hairy-spiny
NT576 (cluster 10)	Green	Not distinctive	-	-	Spiral	Spiny
Aggregate group V						
NAR85 (SMC)	Grey	Not distinctive	-	-	Spiral	Tuberculate
Aggregate group VI						
A1853 (cluster 19), A1956 (cluster 20), NT218 (SMC), TA12 (SMC), TA26 (cluster 25), TA61 (cluster 24), TA123 (SMC), TA127 (SMC), TA179 (cluster 26), TA265 (cluster 23)	Grey	Not distinctive	-	-	Spiral	Not determined
TA56 (SMC)	Grey	Not distinctive	-	-	Spiral	Warty

SMC, single-membered cluster; -, absent; +, present. *, Aggregate groups and clusters after Sahin (1995).

Figure 3-8. Scanning electron micrograph showing spore chain morphology and spore surface ornamentation of:

(a) *Streptomyces thermodiastaticus* DSM 40573^T (cluster 17, aggregate group V), (b) *"Streptomyces thermoflavus"* DSM 40574,

(c) *Streptomyces thermonitrificans* DSM 40579^T, (d) *"Streptomyces thermophilus"* DSM 40365,

(e) *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T, (f) *Streptomyces thermovulgaris* DSM 40444^T,

(g) *Streptomyces* strain NAR84 (cluster 5, aggregate group IV), (h) *Streptomyces* strain NT358 (cluster 7, aggregate group IV),

(i) *Streptomyces* strain NT322 (cluster 8, aggregate group IV), (j) *Streptomyces* strain NT399 (cluster 9, aggregate group IV),

(k) *Streptomyces* strain NT576 (cluster 10, aggregate group IV), (l) *Streptomyces* strain NT371 (cluster 12, aggregate group IV),

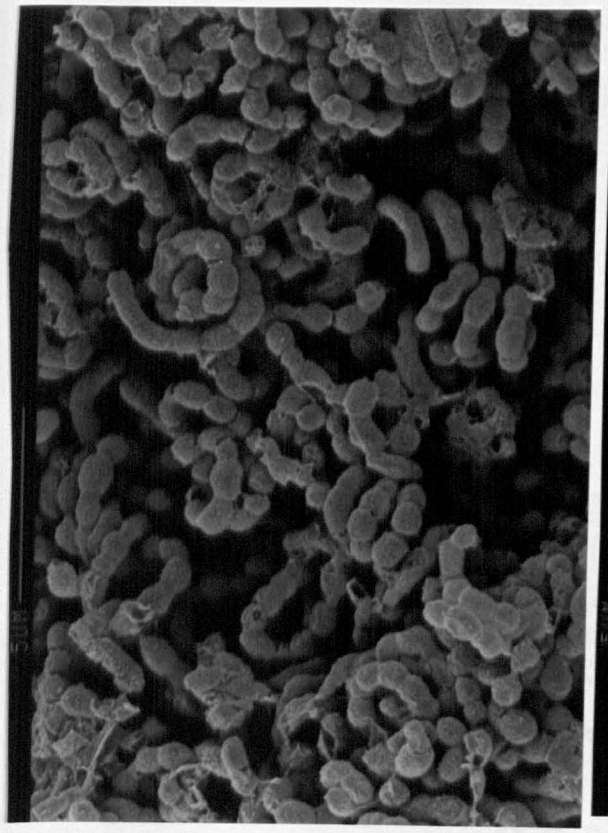
(m) *Streptomyces* strain NT312 (cluster 13, aggregate group IV), (n) *Streptomyces* strain NT336 (cluster 14, aggregate group IV),

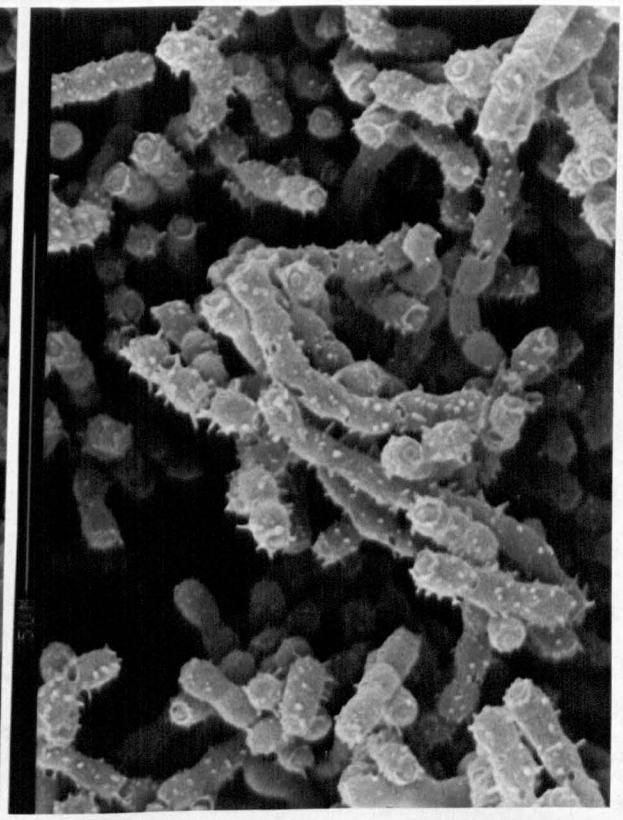
(o) *Streptomyces* strain TA56 (single-membered cluster, VI) and (p) *Streptomyces* strain B19.

The organisms were grown on inorganic salt-starch agar (ISP medium 4) at 45 °C for 5-7 days. Clusters and aggregate groups are taken from Sahin (1995).

DSM 40574

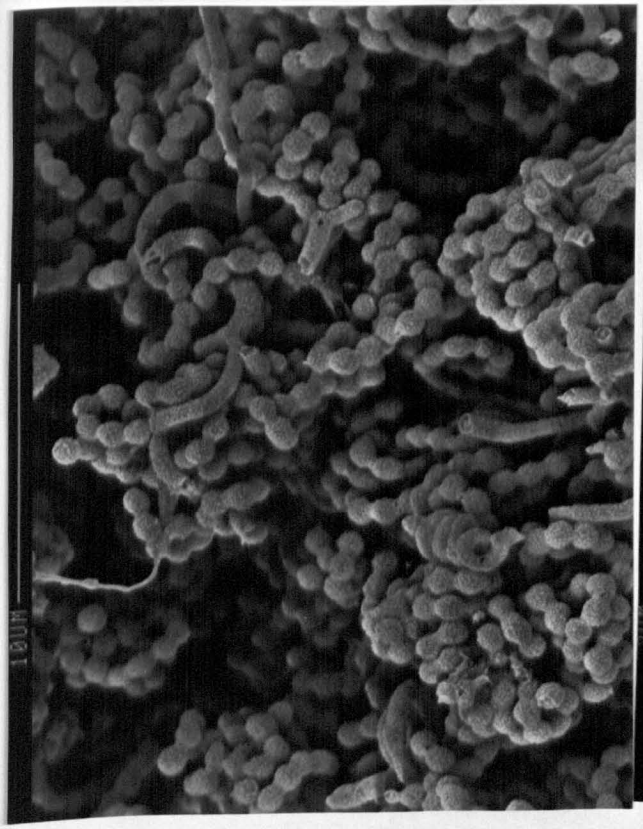
DSM 40365

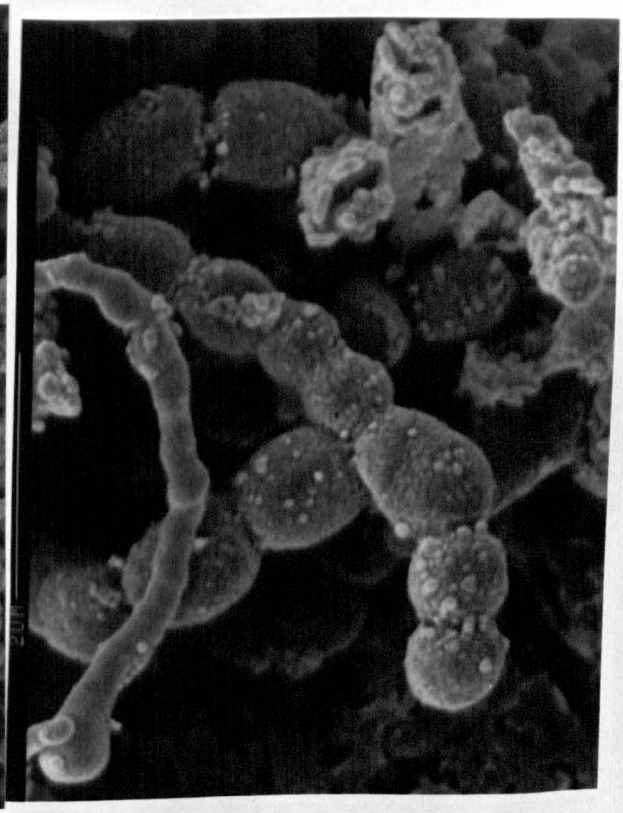




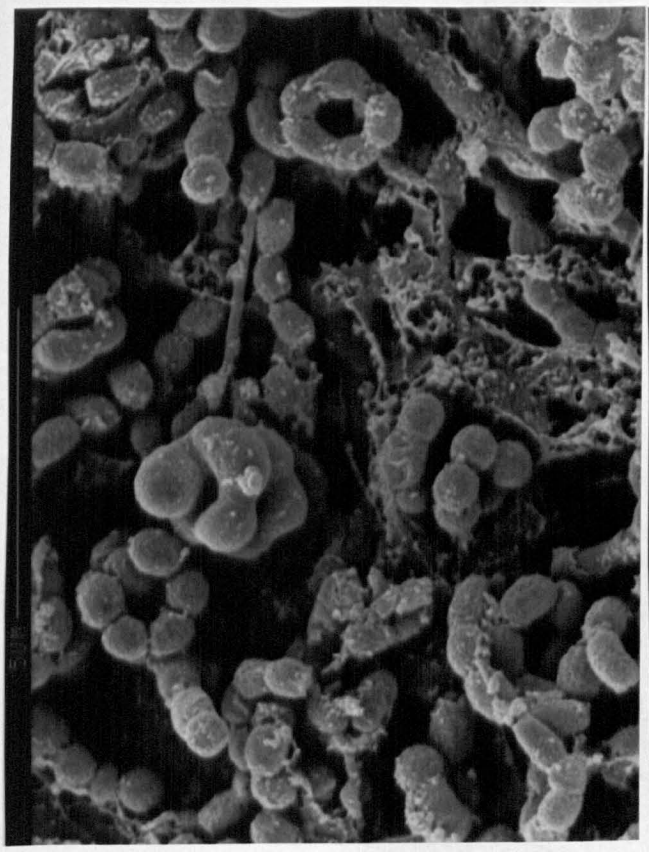
DSM 40573^{T}

DSM 40579^{T}

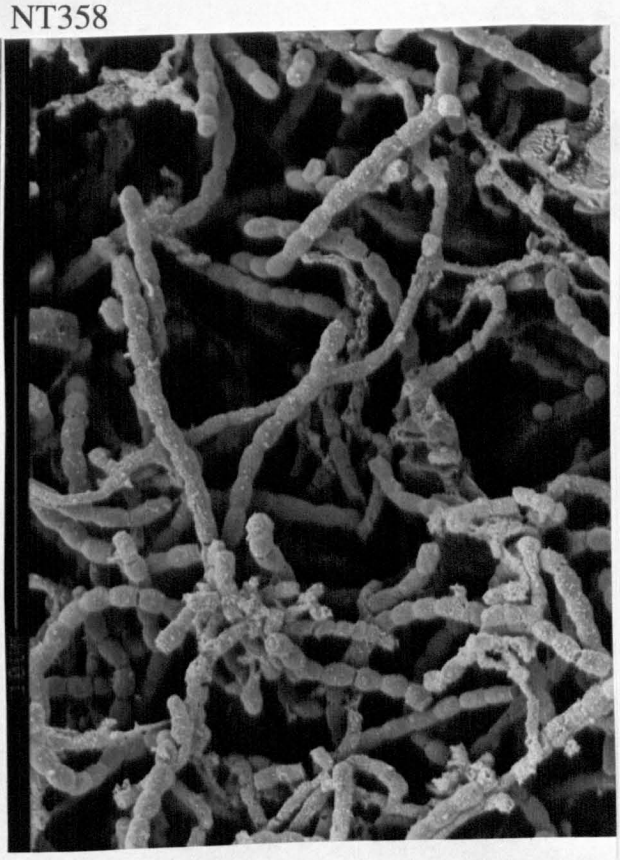




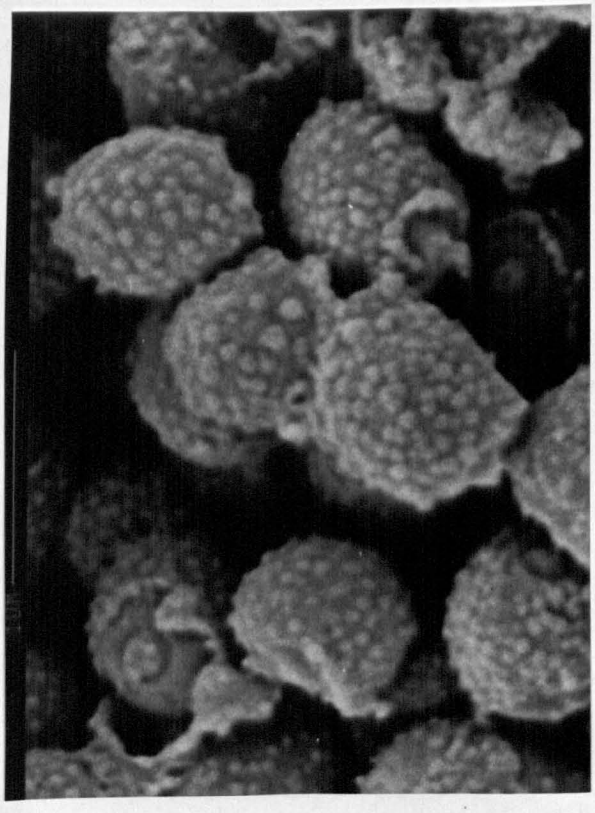
DSM 40444^T



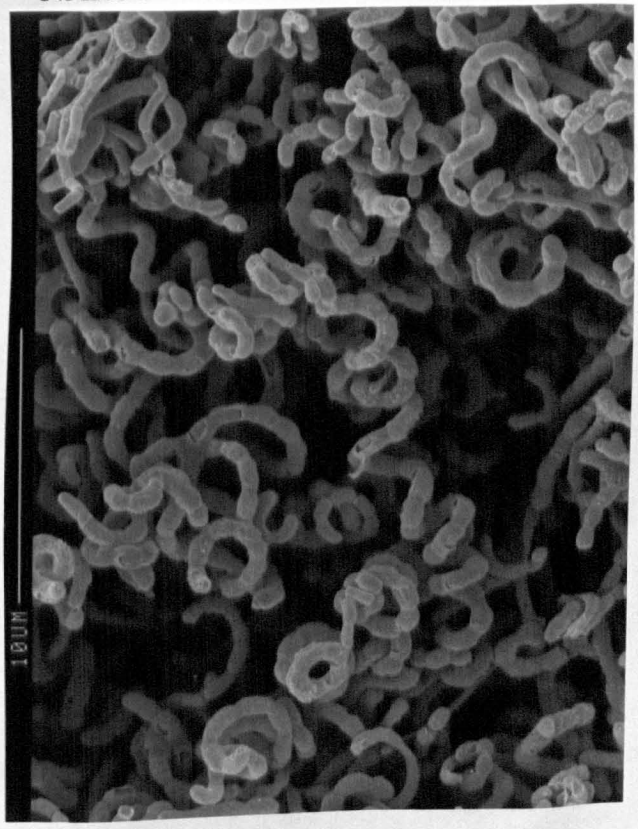
NT358



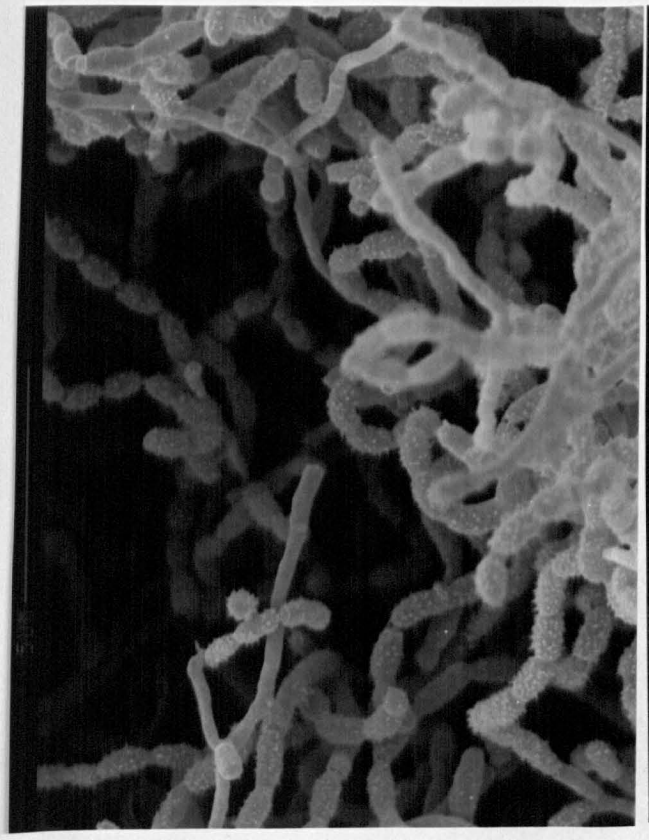
DSM 41392^T



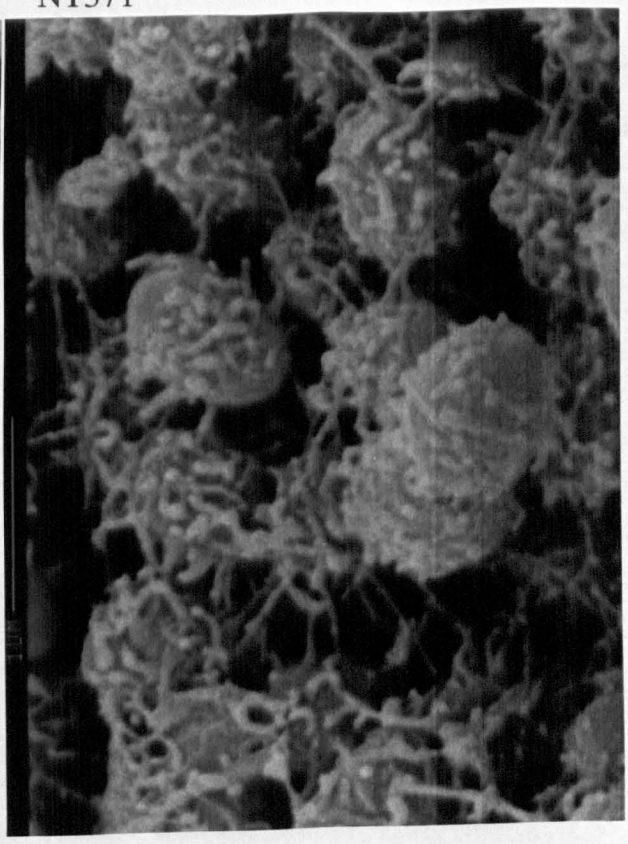
NAR84



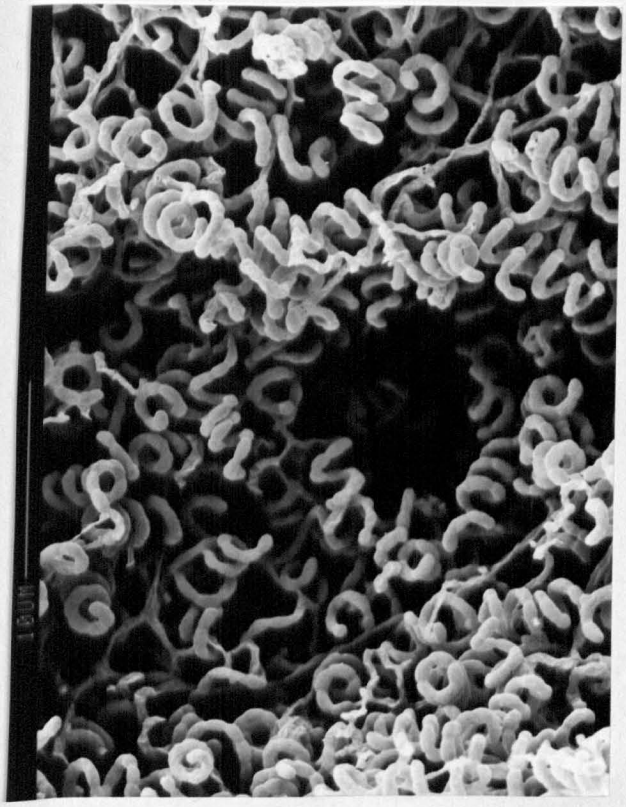
NT399



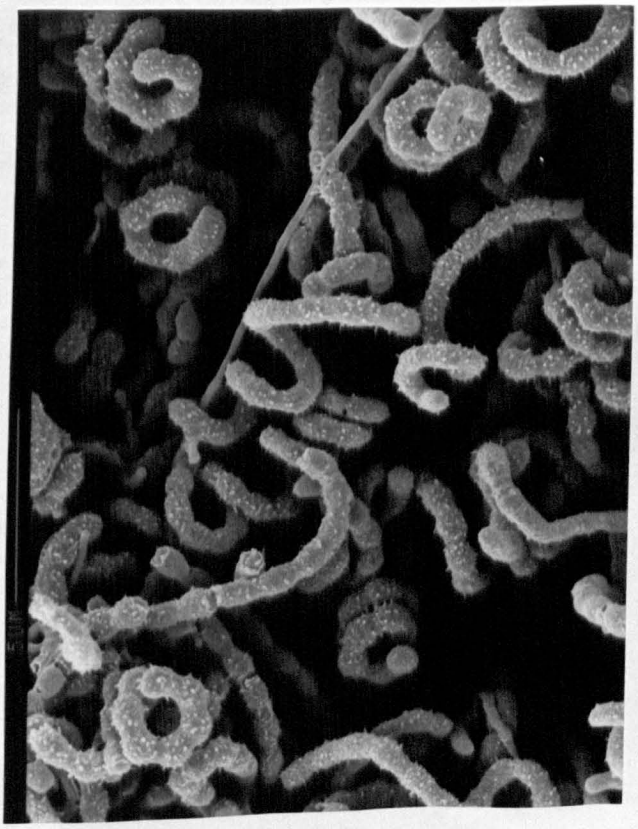
NT371

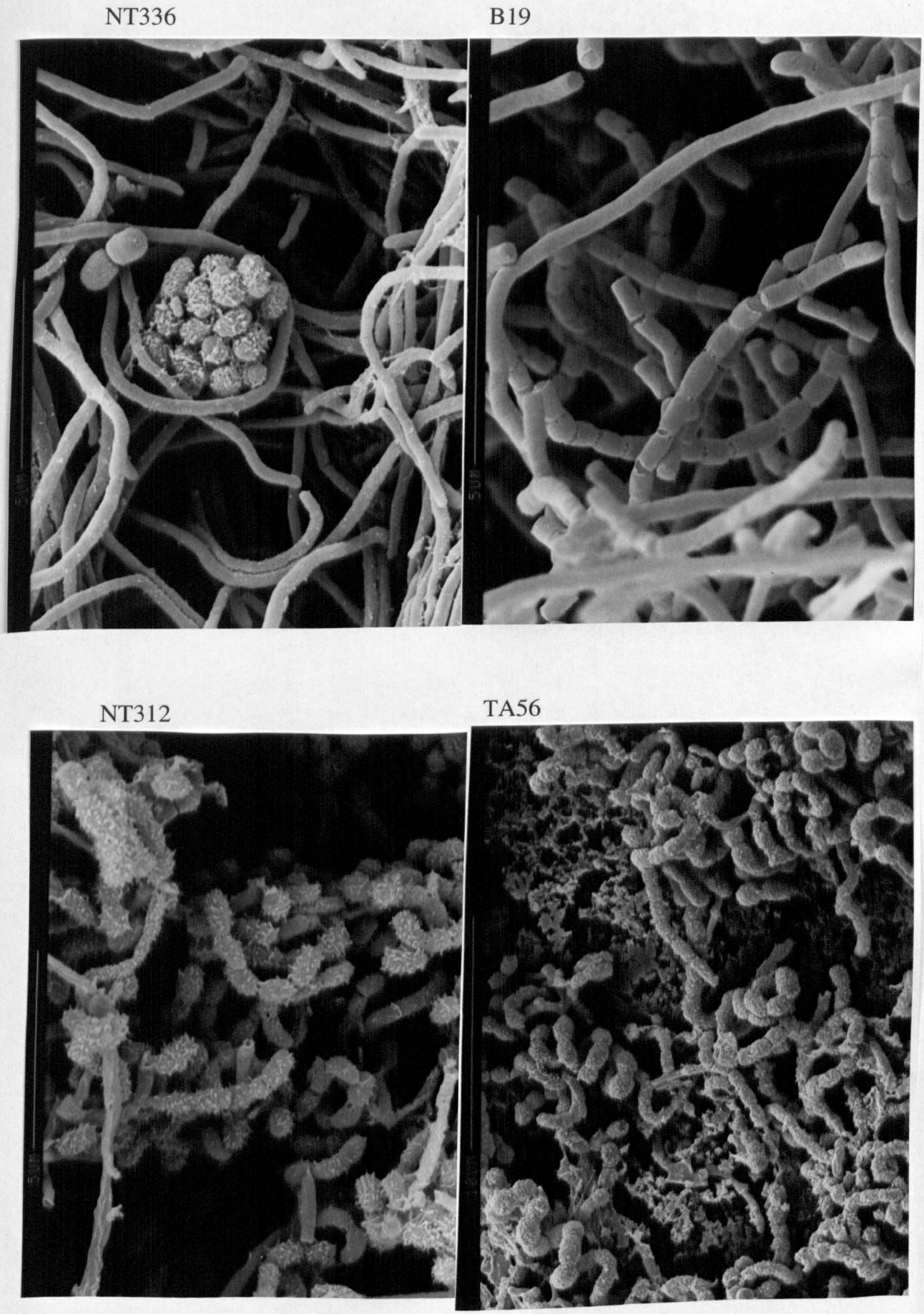


NT322



NT576




NT336
B19
NT312
TA56

Discussion

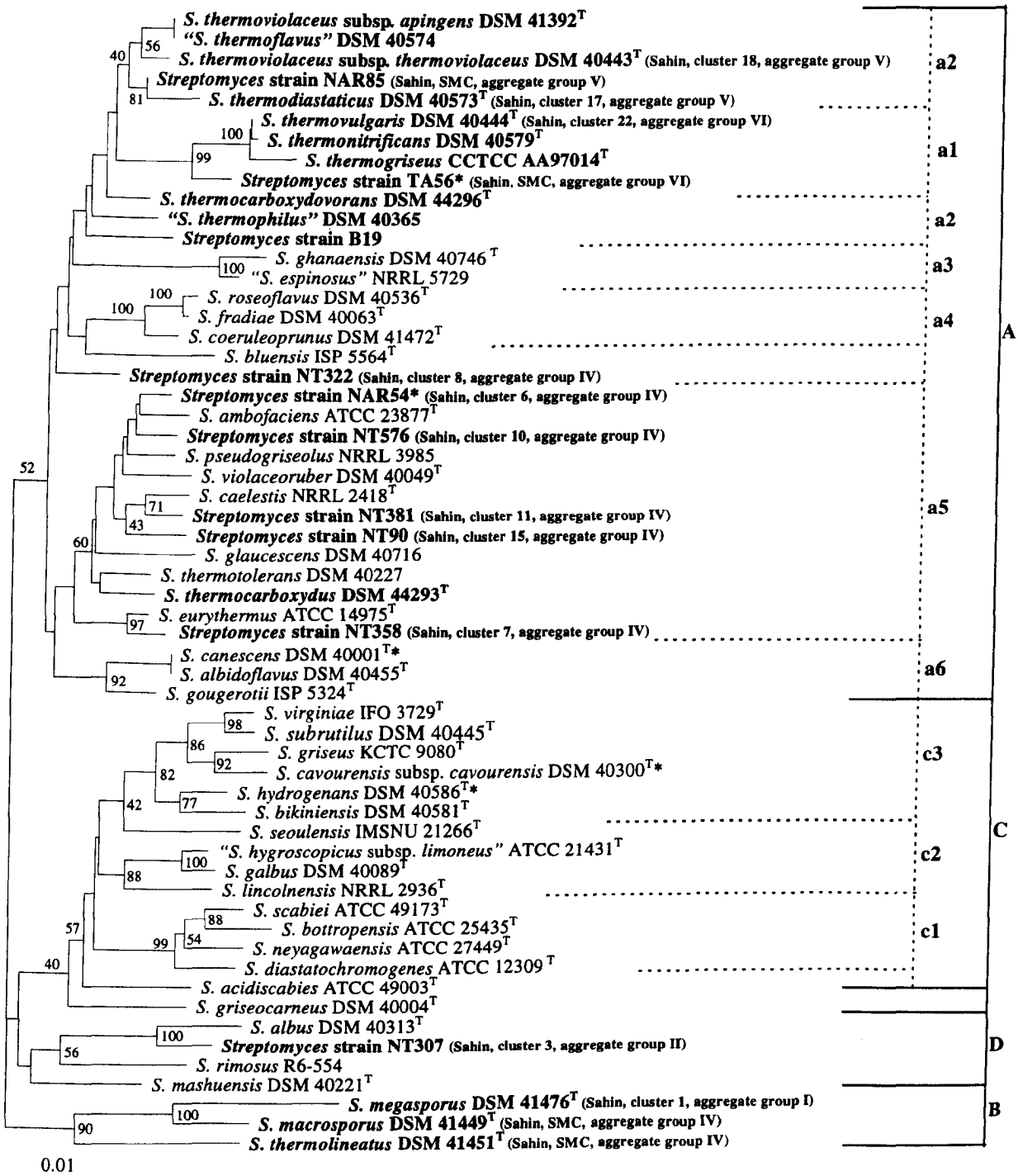
Phylogeny of thermophilic streptomycetes. Thermophilic streptomycetes fall into several distinct evolutionary lines based on 16S rRNA sequence data (Kim, D. *et al.*, 1996; Kim, S. B. *et al.*, 1998) and have been sharply distinguished from mesophilic streptomycetes in numerical phenetic surveys (Goodfellow *et al.*, 1987; O'Donnell *et al.*, 1993). It is, therefore, apparent from both genotypic and phenotypic data that these organisms do not form a single subgroup in the genus *Streptomyces* as proposed by Craveri and Pagani (1962). The present study confirms and extends the conclusions drawn from the earlier 16S rRNA sequence studies as thermophilic streptomycetes were assigned to two phyletic groups, provisionally labelled clades A and B (Fig. 3-9). The recovery of *Streptomyces macrosporus* DSM 41449^T, *Streptomyces megasporus* DSM 41476^T and *Streptomyces thermolineatus* DSM 41451^T as a distinct phyletic line, clade B, is in agreement with the results of the 16S rRNA sequencing studies mentioned above.

Thirty-six out of the forty thermophilic streptomycetes were recovered in clade A which also contained thirteen strains considered to be mesophilic streptomycetes. However, since many of the mesophilic strains have not the subject of temperature range studies it is quite possible that some of them may grow at high temperature. Fifty out of the fifty-two strains assigned to clade A were assigned to six subclades that were supported by the least-squares (Fitch & Margoliash, 1967) and maximum-likelihood algorithms (Felsenstein, 1981).

Subclade A1 encompasses three thermophilic reference strains, *Streptomyces thermogriseus* CCTCC AA97014^T, *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T and ten thermophilic isolates, namely, *Streptomyces* strains A1853 (centrotype strain of cluster 19, aggregate group VI; Sahin, 1995), A1956 (centrotype strain of cluster 20, aggregate group VI; Sahin, 1995), NT218

Figure 3-9. Least squares tree (Fitch & Margoliash, 1967) based on almost complete 16S rRNA sequences showing relationships between the thermophilic streptomycetes (in bold) and representatives of the genus *Streptomyces*. The corresponding 16S rRNA sequence of *Arthrobacter globiformis* (accession number M23411) was used as the outgroup. The numbers at the nodes indicate the level (%) of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets; only values over 40 % are given. The scale bar indicates 0.01 substitutions per nucleotide position. Alkalitolerant strains are marked with an asteriks. SMC=single-membered cluster.

The following strains had identical partial 16S rRNA sequences with organisms included in the tree: (a) *Streptomyces* strains **A1853** (Sahin, cluster 19, aggregate group VI), **A1956** (Sahin, cluster 20, aggregate group VI), **NT218** (Sahin, SMC, aggregate group VI), **TA12*** (Sahin, SMC, aggregate group VI), **TA26*** (Sahin, cluster 25, aggregate group VI), **TA34*** (Sahin, cluster 16, aggregate group IV), **TA54*** (Sahin, cluster 24, aggregate group VI), **TA123*** (Sahin, SMC, aggregate group VI), **TA179*** (Sahin, cluster 26, aggregate group VI) and **TA265*** (Sahin, cluster 23, aggregate group VI) with *Streptomyces thermovulgaris* DSM 40444^T (Sahin, cluster 22, aggregate group VI); (b) *Streptomyces* strain **NAR84*** (Sahin, cluster 5, aggregate group IV) with *Streptomyces* strain **NAR54*** (Sahin, cluster 6, aggregate group IV); (c) *Streptomyces* strains **NT312** (Sahin, cluster 13, aggregate group IV), **NT371**(Sahin, cluster 12, aggregate group IV), **NT399** (Sahin, cluster 9, aggregate group IV), **NT493** (Sahin, SMC, aggregate group IV), **TA127*** (Sahin, SMC, aggregate group IV) with *Streptomyces* strain **NT381** (Sahin, cluster 11, aggregate group IV), and (d) *Streptomyces* strain **NT336** (Sahin, cluster 14, aggregate group IV) with *Streptomyces* strain **NT90** (Sahin, cluster 15, aggregate group IV).


S. thermoviolaceus subsp. apingens DSM 41392T
"S. thermoflavus" DSM 40574
S. thermoviolaceus subsp. thermoviolaceus DSM 40443T (Sahin, cluster 18, aggregate group V)
Streptomyces strain NAR85 (Sahin, SMC, aggregate group V)
S. thermodiastaticus DSM 40573T (Sahin, cluster 17, aggregate group V)
S. thermovulgaris DSM 40444T (Sahin, cluster 22, aggregate group VI)
S. thermonitrificans DSM 40579T
S. thermogriseus CCTCC AA97014T
Streptomyces strain TA56* (Sahin, SMC, aggregate group VI)
S. thermocarboxydovorans DSM 44296T
"S. thermophilus" DSM 40365
Streptomyces strain B19
S. ghanaensis DSM 40746T
"S. espinosus" NRRL 5729
S. roseoflavus DSM 40536T
S. fradiae DSM 40063T
S. coeruleoprunus DSM 41472T
S. bluensis ISP 5564T
Streptomyces strain NT322 (Sahin, cluster 8, aggregate group IV)
Streptomyces strain NAR54* (Sahin, cluster 6, aggregate group IV)
S. ambofaciens ATCC 23877T
Streptomyces strain NT576 (Sahin, cluster 10, aggregate group IV)
S. pseudogriseolus NRRL 3985
S. violaceoruber DSM 40049T
S. caelestis NRRL 2418T
Streptomyces strain NT381 (Sahin, cluster 11, aggregate group IV)
Streptomyces strain NT90 (Sahin, cluster 15, aggregate group IV)
S. glaucescens DSM 40716
S. thermotolerans DSM 40227
S. thermocarboxydus DSM 44293T
S. eurythermus ATCC 14975T
Streptomyces strain NT358 (Sahin, cluster 7, aggregate group IV)
S. canescens DSM 40001T*
S. albidoflavus DSM 40455T
S. gougerotii ISP 5324T
S. virginiae IFO 3729T
S. subrutilus DSM 40445T
S. griseus KCTC 9080T
S. cavourensis subsp. cavourensis DSM 40300T*
S. hydrogenans DSM 40586T*
S. bikiniensis DSM 40581T
S. seoulensis IMSNU 21266T
"S. hygroscopicus subsp. limoneus" ATCC 21431T
S. galbus DSM 40089T
S. lincolnensis NRRL 2936T
S. scabiei ATCC 49173T
S. bottropensis ATCC 25435T
S. neyagawaensis ATCC 27449T
S. diastatochromogenes ATCC 12309T
S. acidiscabies ATCC 49003T
S. griseocarneus DSM 40004T
S. albus DSM 40313T
Streptomyces strain NT307 (Sahin, cluster 3, aggregate group II)
S. rimosus R6-554
S. mashuensis DSM 40221T
S. megasporus DSM 41476T (Sahin, cluster 1, aggregate group I)
S. macrosporus DSM 41449T (Sahin, SMC, aggregate group IV)
S. thermolineatus DSM 41451T (Sahin, SMC, aggregate group IV)
a2
a1
a2
a3
a4
a5
a6
c3
c2
c1
A
C
D
B
0.01

(single-membered cluster, aggregate group VI; Sahin, 1995), TA12 (single-membered cluster, aggregate group VI; Sahin, 1995), TA34 (centrotype strain of cluster 16, aggregate group IV; Sahin, 1995), TA26 (centrotype strain of cluster 25, aggregate group VI; Sahin, 1995), TA61 (centrotype strain of cluster 24, aggregate group VI; Sahin, 1995), TA56 (single-membered cluster, aggregate group VI; Sahin, 1995), TA123 (single-membered cluster, aggregate group VI; Sahin, 1995) and TA179 (centrotype strain of cluster 26, aggregate group VI; Sahin, 1995). Eleven out of thirteen strains assigned to subclade A1 shared almost identical 16S rRNA sequences (three nucleotide differences or less). The exceptions, *Streptomyces thermogriseus* CCTCC AA97014^T and *Streptomyces* strain TA56, showed their highest 16S rRNA sequence similarities with *Streptomyces thermovulgaris* DSM 40444^T (99.5 % which corresponds to seven nucleotide differences and 99.0 % which corresponds to fourteen nucleotide differences, respectively). The integrity of subclade A1 is supported by both the least-squares and maximum-likelihood trees and by high bootstrap values based on the neighbour-joining algorithm.

Subclade A2 contains *Streptomyces thermocarboxydovorans* DSM 44296^T, *Streptomyces thermodiastaticus* DSM 40573^T, *"Streptomyces thermoflavus"* DSM 40574, *"Streptomyces thermophilus"* DSM 40365, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T, *Streptomyces* strain NAR58 (single-membered cluster, aggregate group V; Sahin, 1995) and *Streptomyces* strain B19 isolated from poultry faeces. The strains in this subclade were closely related in terms of nucleotide similarity (average of nucleotide similarities, 98.8 % ± 1.2) but did not show monophyly. This apparently anomalous result can be attributed to the vagaries of the tree-algorithms which cannot reliably differentiate between the close relationships found between members of subclade A1 and A2; members of subclade A2

showed a 16S rRNA nucleotide similarity value of 97.8 % ± 0.5 to *Streptomyces thermovulgaris* DSM 40444^T.

Subclade A5 contains *Streptomyces eurythermus* ATCC 14975^T, *Streptomyces thermocarboxydus* DSM 44293^T, *"Streptomyces thermotolerans"* DSM 40227 and *Streptomyces violaceoruber* DSM 40049^T, and twelve thermophilic isolates, namely, *Streptomyces* strains NAR54 (centrotype strain of cluster 6, aggregate group IV; Sahin, 1995), NAR84 (centrotype strain of cluster 5, aggregate group IV; Sahin, 1995), NT90 (centrotype strain of cluster 15, aggregate group IV; Sahin, 1995), NT312 (centrotype strain of cluster 13, aggregate group IV; Sahin, 1995), NT336 (centrotype strain of cluster 14, aggregate group IV; Sahin, 1995), NT358 (centrotype strain of cluster 7, aggregate group IV; Sahin, 1995), NT371 (centrotype strain of cluster 12, aggregate group IV; Sahin, 1995), NT381 (centrotype strain of cluster 11, aggregate group IV; Sahin, 1995), NT399 (centrotype strain of cluster 9, aggregate group IV; Sahin, 1995), NT493 (single-membered cluster, aggregate group IV; Sahin, 1995), NT576 (centrotype strain of cluster 10, aggregate group IV; Sahin, 1995) and TA127 (single-membered cluster, aggregate group IV; Sahin, 1995). Subclade A5 also contained four strains, namely, *Streptomyces ambofaciens* ATCC 23877^T, *Streptomyces pseudogriseolus* NRRL 3985, *Streptomyces caelestis* NRRL 2418^T and *Streptomyces glaucescens* DSM 40716, considered as mesophilic streptomycetes. The integrity of this subclade is supported by the least-squares and maximum-likelihood trees and by high bootstrap values based on neighbour-joining method.

Subclades A3, A4 and A6 only contained organisms considered as mesophilic streptomycetes. Another mesophilic organism, *Streptomyces bluensis* ISP 5564^T, formed a distinct branch within clade A. The final thermophilic isolate, *Streptomyces* strain NT322, also formed a distinct branch within clade A.

Clade D contained *Streptomyces albus* DSM 40313^T, a thermotolerant streptomycete, two mesophilic reference streptomycetes, *Streptomyces mashuensis* DSM

Table 3-12. Comparison between the numerical phenetic and 16S rRNA sequence data

Numerical phenetic classification [a]	Strains	16S rRNA clade [b]
Aggregate group I		
Cluster 1	*S. megasporus* DSM 41476^T	B
Aggregate group II		
Cluster 3	*Streptomyces* strain NT307	D
Cluster 4	*S. albus* DSM 40313^T, K17 [c]	D
Aggregate group III		
SMC	*S. canescens* DSM 40001^T	A6
SMC	*S. cavourensis* DSM 40300^T	C
SMC	*S. hydrogenans* DSM 40586^T	C
Aggregate group IV		
Cluster 5	*Streptomyces* strain NAR84	A5
Cluster 6	*Streptomyces* strain NAR54	A5
Cluster 7	*Streptomyces* strain NT358	A5
Cluster 8	*Streptomyces* strain NT322	A[d]
Cluster 9	*Streptomyces* strain NT399	A5
Cluster 10	*Streptomyces* strain NT576	A5
Cluster 11	*Streptomyces* strain NT381	A5
Cluster 12	*Streptomyces* strain NT371	A5
Cluster 13	*Streptomyces* strain NT312	A5
Cluster 14	*Streptomyces* strain NT336	A5
Cluster 15	*Streptomyces* strain NT90	A5
Cluster 16	*Streptomyces* strain TA34	A1
SMC	*Streptomyces* strain NT125	A5
SMC	*Streptomyces* strain NT493	A5
SMC	*Streptomyces* strain TA127	A5
SMC	*S. thermolineatus* DSM 41451^T	B
SMC	*S. macrosporus* DSM 41449^T	B
Aggregate group V		
Cluster 17	*S. thermodiastaticus* DSM 40573^T	A2
Cluster 18	*S. themoviolaceus* subsp. *thermoviolaceus* DSM 40443^T	A2
Cluster	*Streptomyces* strain NAR85	A2
Aggregate group VI		
Cluster 19	*Streptomyces* strain A1853	A1

Cluster 20	*Streptomyces* strain A1956	A1
Cluster 22	*S. thermovulgaris* DSM 40444^T	A1
Cluster 23	*Streptomyces* strain TA265	A1
Cluster 24	*Streptomyces* strain TA 61	A1
Cluster 25	*Streptomyces* strain TA26	A1
Cluster 26	*Streptomyces* strain TA179	A1
SMC	*Streptomyces* strain TA56	A1
SMC	*Streptomyces* strain TA123	A1
SMC	*Streptomyces* strain TA218	A1
SMC	*Streptomyces* strain TA12	A1

[a], Classification by Sahin (1995)
[b], Based on the least-squares tree (Fig. 3-5).
[c], *S. albus* K17 was included in the numerical phenetic study whereas *S. albus* DSM 40313^T was included in the 16S rRNA sequence analysis.
[d], Phylogenetic position of strain NT322 within clade A is not clear.
SMC, single-membered cluster.

40221^T and *Streptomyces rimosus* R6-554, and a thermophilic isolate, strain NT307 (cluster 3, aggregate group II; Sahin, 1995). However, the integrity of this clade was not supported by the maximum-likelihood trees or by high bootstrap values based on the neighbour-joining method. Strain NT307 was most closely related to *Streptomyces albus* DSM 40313^T (98.6 % nucleotide similarity). It is also interesting that *Streptomyces albus* DSM 40313^T, the type species of the genus *Streptomyces*, was found to occupy a distinct position in earlier streptomycete trees based on 16S rRNA (Witt & Stackebrandt, 1990) and on N-terminal sequence of ribosomal protein AT-L30 (Ochi, 1995).

It is encouraging that good congruence was found in the present study between the numerical phenetic and 16S rRNA sequence data (Table 3-12). Aggregate group II was represented by *Streptomyces* strain NT307 (cluster 3) and *Streptomyces albus* strains K15 and K17. Strain NT307 and *Streptomyces albus* DSM 40313^T were recovered in clade D. Eleven out of the fifteen strains representing aggregate group IV were assigned to subclade A5. Similarly, the three strains representing clusters assigned to aggregate group V were assigned to subclade A2. All ten strains representing clusters assigned aggregate group VI by Sahin (1995) were assigned to subclade A1.

Reference strains. Numerical phenetic surveys designed to unravel the complicated taxonomic structure of the genus *Streptomyces* have yielded conflicting results with respect to members of some of the validly described taxa which contain thermophilic streptomycetes (Williams *et al.*, 1983a; Goodfellow *et al.*, 1987; Kämpfer *et al.*, 1991). In particular, the taxonomic standing of *Streptomyces thermonitrificans* Desai and Dhala 1967 is not clear as the type strain of this species has been reported to share genotypic (Ochi, 1995; Kim *et al.*, 1996) and phenotypic (Williams *et al.*, 1983a; Goodfellow *et al.*, 1987; Kämpfer *et al.*, 1991) properties in common with both *Streptomyces thermoviolaceus* (Henssen 1957) emend. Goodfellow *et al.* 1987 and *Streptomyces thermovulgaris* (Henssen 1957) emend. Goodfellow *et al.* 1987.

Streptomyces thermodiastaticus (Bergey *et al.* 1923) Waksman 1953 has many phenotypic characters in common with *Streptomyces thermoviolaceus* (Henssen 1957) emend. Goodfellow *et al.* 1987, including the ability to form spores with small hemispherical warts in spiral chains (Vobis & Henssen, 1983; Goodfellow *et al.*, 1987). The ultrastructure of the hemispherical warts of *Streptomyces thermoviolaceus* was examined by Vobis and Henssen (1983) who recommended that this type of spore ornamentation be designated "tuberculate". The close relationship between *Streptomyces thermodiastaticus* and *Streptomyces thermoviolaceus* is also evident from both the present and earlier 16S rRNA sequencing studies (Kim, D. *et al.*, 1996).

It has already been stressed that DNA:DNA relatedness studies can be used to resolve the finer taxonomic relationships between closely related organisms as it is generally agreed that genomic species should encompass strains which show approximately 70 % or more DNA:DNA relatedness under suitable experimental conditions (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Goodfellow *et al.*, 1997). In the present study, the DNA homology data show that *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T belong to a single genomic species which is readily distinguished from a corresponding taxon which encompasses *Streptomyces thermoviolaceus* subspecies *apingens* and *Streptomyces thermoviolaceus* subspecies *thermoviolaceus*. Relatively high DNA:DNA relatedness values also support the close relationship between representatives of *Streptomyces thermodiastaticus* and *Streptomyces thermoviolaceus*.

The type strains of *Streptomyces thermonitrificans* and *Streptomyces thermovulgaris* share almost identical 16S rRNA sequences (Kim, D. *et al.*, 1996) and similar ribosomal AT-L30 proteins (Ochi, 1995), have DPG, PE, PI, PIDM and unidentified phospholipids as major polar lipids, octahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue, and DNA rich in G plus C (70 and 72 mol%, respectively). The

identical ribotype patterns shown by these strains also serve to distinguish them from *Streptomyces thermodiastaticus* and *Streptomyces thermoviolaceus* and underpins the results of an earlier investigation where *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T were found to produce similar randomly amplified polymorphic DNA profiles albeit ones which were markedly different from those generated by *Streptomyces thermodiastaticus* DSM 40573^T and *Streptomyces thermoviolaceus* DSM 40443^T (Kim, S. B. *et al.*, 1998).

It is clear from both the present and previous investigations that *Streptomyces thermonitrificans* DSM 40579^T and *Streptomyces thermovulgaris* DSM 40444^T are members of the same species. This finding is also supported by some numerical phenetic data (Williams *et al.*, 1983a; Kämpfer *et al.*, 1991). It is, therefore, proposed that *Streptomyces thermonitrificans* Desai and Dhala 1967 be recognized as a subjective synonym of *Streptomyces thermovulgaris* (Henssen 1957) emend. Goodfellow *et al.* 1987. This proposal supersedes an earlier one where it was proposed that *Streptomyces thermonitrificans* be accepted as a subjective synonym of *Streptomyces thermoviolaceus* (Goodfellow *et al.*, 1987) on the basis of phenotypic data.

16S rRNA sequence data show that *Streptomyces thermodiastaticus* DSM 40573^T is most closely related to *Streptomyces thermocarboxydovorans* DSM 44296^T (99.0% nucleotide sequence similarity), *Streptomyces thermoviolaceus* subspecies *apingens* DSM 41392^T (99.1%), *Streptomyces thermoviolaceus* subspecies *thermoviolaceus* DSM 40443^T (99.4%) and *Streptomyces thermovulgaris* DSM 40444^T (97.7%) (Kim, D. *et al.*, 1996; Kim, S. B. *et al.*, 1998). However, it is clear from the DNA relatedness data that the type strains of *Streptomyces thermodiastaticus, Streptomyces thermoviolaceus* and *Streptomyces thermovulgaris* belong to different genomic species. Members of these taxa can also be distinguished from one another and from related taxa, including *Streptomyces thermocarboxydovorans,* using a combination of phenotypic properties (Kim, S. B. *et al.*,

1998). *Streptomyces thermodiastaticus* DSM 40573^T was also distinguished from the type strains of the two subspecies of *Streptomyces thermoviolaceus* when genomic digests prepared using *Pvu* II and *Sal* I restriction endonucleases were probed with the 7.2 kb DNA fragment from *Streptomyces violaceoruber* DSM 41007. However, all three of these strains gave similar banding patterns in the corresponding experiments with *Bam* HI genomic digests though the profile for the *Streptomyces thermoviolaceus* subspecies *thermoviolaceus* strain showed bands with slightly lower molecular weights than those of the *Streptomyces thermoviolaceus* subspecies *apingens* strain; these differences may be due to deletions in the DNA near the rRNA operons.

The inclusion of *Streptomyces thermodiastaticus* DSM 40573^T in the *Streptomyces halstedii* (Williams *et al.*, 1983) and *Streptomyces rochei* (Kämpfer *et al.*, 1991) numerical phenetic clusters can be attributed to the poor growth of this strain at the incubation temperatures used (25 °C and 28 °C, respectively), and to test and sampling error (Sneath & Johnson, 1972). Similar factors probably explain the assignment of *Streptomyces thermoviolaceus* DSM 40443^T to the *Streptomyces aurantiacus* (Williams *et al.*, 1983a) and *Streptomyces graminofaciens* clusters (Kämpfer *et al.*, 1991). It can be concluded from the present study that both *Streptomyces thermodiastaticus* (Bergey *et al.* 1923) Waksman 1953 and *Streptomyces thermoviolaceus* (Henssen 1957) emended Goodfellow *et al.* 1987 continue to merit recognition as validly described species.

"*Streptomyces thermoflavus*" (Kudrina and Maximova, 1963) Pridham 1970, "*Streptomyces thermophilus*" (Gilbert, 1904) Waksman and Henrici 1948 (syn. *Streptomyces rectus*; Henssen, 1957b) and "*Streptomyces thermotolerans*" ex Pagano *et al.* 1959 currently have no standing in nomenclature. "*Streptomyces thermoflavus*" DSM 40574, "*Streptomyces thermophilus*" DSM 40365 and "*Streptomyces thermotolerans*" DSM 40227, the putative type strains of these taxa, were included in the International *Streptomyces* Project (ISP; ISP 5574, ISP 5365 and ISP 5227, respectively) but were not

cited in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) and have not been validly published since 1 January 1980.

In the present study, "*Streptomyces thermoflavus*" DSM 40574 was found to have the same almost complete 16S rRNA sequence as *Streptomyces thermoviolaceus* subspecies *apingens* DSM 41392^T and was closely related to *Streptomyces thermoviolaceus* subspecies *thermoviolaceus* DSM 40443^T (99.4 % nucleotide similarity). "*Streptomyces thermoflavus*" DSM 40574, *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T all produce a grey aerial spore mass and spiral chains of spores with tuberculate surface ornamentation. However, in numerical phenetic surveys "*Streptomyces thermoflavus*" DSM 40574 has been assigned to the *Streptomyces thermovulgaris* (Williams *et al.*, 1883a) and *Streptomyces diastatochromogenes* clusters (Kämpfer *et al.*, 1991), and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T has been assigned to the *Streptomyces aurantiacus* (Williams *et al.*, 1983a) and *Streptomyces graminofaciens* clusters (Kämpfer *et al.*, 1991). "*Streptomyces thermoflavus*" DSM 40574 can be distinguished from strains of *Streptomyces thermoviolaceus* and other thermophilic streptomycetes by its characteristic orange-coloured substrate mycelium. Further comparative taxonomic studies are needed to establish the exact taxonomic position of "*Streptomyces thermoflavus*" DSM 40574.

"*Streptomyces thermophilus*" DSM 40365 showed its closest relationships to *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (99.1 % 16S rRNA nucleotide similarity) and *Streptomyces thermocarboxydovorans* DSM 44296^T (98.7 % 16S rRNA nucleotide similarity). Validly described species separated by such nucleotide differences have been shown to belong to different genomic species, for example, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T and *Streptomyces thermodiastaticus* DSM 40573^T share a 99.4 % 16S rRNA nucleotide similarity but showed 58 % DNA:DNA relatedness. "*Streptomyces thermophilus*" DSM 40365 and *Streptomyces*

thermoviolaceus subsp. *thermoviolaceus* DSM 40443^T have also been recovered in separate numerically circumscribed phenetic clusters. The former was assigned to the *Streptomyces chromofuscus* (Williams *et al.*, 1983a) and *Streptomyces rochei* clusters (Kämpfer *et al.*, 1991a) and as the latter to the *Streptomyces aurantiacus* (Williams *et al.*, 1983a) and *Streptomyces graminofaciens* clusters (Kämpfer *et al.*, 1991). "*Streptomyces thermophilus*" DSM 40365 can also be separated from related thermophilic streptomycetes, namely, *Streptomyces thermocarboxydovorans* DSM 44296^T, *Streptomyces thermodiastaticus* DSM 40573^T, *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T, "*Streptomyces thermoflavus*" DSM 40574 and *Streptomyces thermovulgaris* DSM 40444^T by its ability to produce straight chains of spiny spores and melanin pigments.

It is clear both from the present and earlier studies that "*Streptomyces thermophilus*" DSM 40574 is genotypically and phenotypically distinct from the related streptomycete taxa and should be recognised as valid species in the genus *Streptomyces*.

Description of *Streptomyces thermophilus* (Gilbert, 1904) Kim, B. and Goodfellow.

Streptomyces thermophilus (ther.mo.phi.lus; Gr. n. *therme* heat; Gr. adj. *philus* loving; M. L. part. adj. *thermophilus* heat-loving).

The description is based on data taken from this and earlier studies (Shirling & Gottlieb, 1972; Williams *et al.*, 1983a; Kämpfer *et al.*, 1991). Aerobic, Gram positive, thermophilic actinomycete which forms extensively branched substrate and aerial hyphae. Straight chains of spiny ornamented spores are borne on aerial hyphae. The organism forms a grey aerial spore mass but neither distinctive substrate mycelium colours nor diffusible pigments are formed. Melanin pigments are produced on peptone-yeast extract iron and tyrosine agars.

The organism degrades adenine, aesculin, arbutin, gelatin, casein and Tween 80. Acetate, L-arabinose, cellobiose, D-fructose, D-galactose, D-glucosamine, D-glucose, *meso*-inositol, D-lactose, D-mannitol, D-mannose, propionate, pyruvate, starch, sucrose, L-

tartrate, D-trehalose and D-xylose are used as sole sources of carbon for energy and growth but not *meso*-erythritol, inulin, α-D-raffinose, α-L-rhamnose or xylitol. Potassium nitrate, L-threonine and L-serine are used as sole sources of nitrogen but not L-hydroxyproline. The organism is sensitive to dimethylchlortetracycline (500 μg ml^{-1}), gentamicin sulphate (100 μg ml^{-1}), neomycin sulphate (50 μg ml^{-1}), oleandomycin (100 μg ml^{-1}), streptomycin sulphate (100 μg ml^{-1}), tobramycin (50 μg ml^{-1}), vancomycin hydrochloride (50 μg ml^{-1}), but not to cephaloridine (100 μg ml^{-1}). This strain does not grow at pH 4.3.

Isolated from fresh horse manure.

The type strain is ISP 5365^T (=DSM 40365^T).

The taxonomic position of "*Streptomyces thermotolerans*" DSM 40227 has still to be resolved. This organism was found to be most closely associated with *Streptomyces thermocarboxydus* DSM 44293^T. "*Streptomyces thermotolerans*" DSM 40227 should be considered as thermotolerant streptomycete as it does not grow well at 50 °C. Further studies are needed to determine whether it merits species status. It is encouraging that *Streptomyces violaceoruber* DSM 40049^T was found in the subclade A5 associated with thermophilic streptomycetes as members of this taxon were reported to grow at 55 °C (Fergus, 1964).

Mikami *et al.* (1982) found that out of all the ISP strains (Shirling & Gottlieb, 1966) only *Streptomyces canescens* DSM 40001^T (ISP 5001^T), *Streptomyces cavourensis* subsp. *cavourensis* DSM 40300^T (ISP 5001^T) and *Streptomyces hydrogenans* DSM 40586^T (ISP 5001^T) grew at pH 11.5. However, it is evident from the 16S rRNA sequencing data that these three alkalitolerant strains are not closely related. shared an identical 16S rRNA sequence with. The close relationship between *Streptomyces canescens* DSM 40001^T and *Streptomyces albidoflavus* DSM 40455^T is supported by the present 16S rRNA sequence data and the results of numerical phenetic (Williams *et al.*, 1983a; Kämpfer *et al.*, 1991),

pyrolysis mass spectrometric (Ferguson *et al.*, 1997) and 16S-23S rRNA spacer sequence studies (Hain *et al.*, 1997). It is clear from the genotypic and phenotypic data that *Streptomyces canescens* Waksman 1957^{AL} should be reduced to a synonym of *Streptomyces albidoflavus* (Rossi Doria 1891) Waksman and Henrici 1948^{AL}, as suggested by Williams *et al.* (1989). *Streptomyces cavourensis* subsp. *cavourensis* DSM 40300^{T} and *Streptomyces hydrogenans* DSM 40586^{T} showed their highest 16S rRNA similarities with *Streptomyces griseus* KCTC 9080T (98.9 % nucleotide similarity) and *Streptomyces bikinensis* DSM 40581T (98.9 % nucleotide similarity), respectively. However, further comparative studies are needed with other mesophilic streptomycetes to define their taxonomic status.

Isolates. Thermophilic, neutrophilic *Streptomyces* strains A1853 (centrotype strain of cluster 19, aggregate group VI; Sahin, 1995), A1956 (centrotype strain of cluster 20, aggregate group VI; Sahin, 1995) and NT218 (single-membered cluster, aggregate group VI; Sahin, 1995), and alkalitolerant, thermophilic *Streptomyces* isolates TA12 (single-membered cluster, aggregate group VI; Sahin, 1995), TA26 (centrotype strain of cluster 25, aggregate group VI; Sahin, 1995), TA61 (centrotype strain of cluster 24, aggregate group VI; Sahin, 1995), TA123 (single-membered cluster, aggregate group VI; Sahin, 1995) and TA179 (centrotype strain of cluster 26, aggregate group VI; Sahin, 1995) exhibited a number of phenotypic properties which are consistent with their classification in the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995). They all formed an extensively branched substrate mycelium, aerial hyphae which differentiated into long chains of smooth spores, and gave whole-organism hydrolysates that were rich in LL-diaminopimelic acid. It is evident from the DNA relatedness studies that all of these representatives of clusters assigned to aggregate group VI (Sahin, 1995) showed 90 % or more DNA relatedness with reference DNA prepared from *Streptomyces thermovulgaris* DSM 40444^{T}. The isolates also formed a grey aerial spore mass, lacked distinct substrate mycelium pigments, formed spores in hooked or spiral chains, and were melanin negative; all of these properties are typical of

bona fide members of the taxon *Streptomyces thermovulgaris* (Goodfellow *et al.*, 1987; Sahin, 1995). In addition, all of these strains gave the same ribotype pattern as *Streptomyces thermovulgaris* DSM 40444^T when *Bam* HI genomic digests were hybridised with the rRNA probe.

It is also evident from the DNA:DNA relatedness experiments and associated phenotypic data (O'Donnell *et al.*, 1993) that strains AT5, AT6 and AT54 have properties consistent with their assignment to *Streptomyces thermovulgaris.* This result was not unexpected as Kim, S. B. *et al.* (unpublished data) have shown that these carboxydotrophic, thermophilic streptomycetes have almost identical 16S rRNA sequences to that of *Streptomyces thermovulgaris* DSM 40444^T. This result raises the prospect that established members of *Streptomyces thermovulgaris* may be able to grow on CO as a sole source of energy and growth.

It is necessary to emend the description of *Streptomyces thermovulgaris* in light of the developments outlined above.

Description of *Streptomyces thermovulgaris* (Henssen 1957) Kim, B. *et al.* *Streptomyces thermovulgaris* (ther.mo.vul.ga'ris. Gr. n. *therme* heat; L. adj. *vulgaris* common; M. L. adj. *thermovulgaris* heat common, inferring common thermophile).

The description is based on data taken from this and earlier studies (Henssen, 1957; Goodfellow *et al.*, 1987; Falconer, 1988; Sahin, 1995). Aerobic, Gram positive, chemoorganotrophic, thermophilic actinomycete with extensively branched substrate and aerial hyphae. Some strains grow on carbon monoxide and methanol as sole carbon sources. Hooked or spiral chains of smooth surfaced spores are borne on aerial hyphae. The aerial spore mass colour is grey, but neither distinctive substrate mycelium colours nor diffusible pigments are formed. Melanin pigments are not produced on peptone-yeast extract iron or tyrosine agars.

More than 90 % of the strains examined in the present study degrade casein, gelatin, starch and L-tyrosine; utilise arabitol, D-cellobiose, cholesterol, D-fructose, D-galactose, gluconic acid (Na^+ salt), *meso*-inositol, D-lactose, mannitol, D-mannose, starch, D-trehalose, turanose and D-xylose as sole sole sources of carbon and energy; utilise DL-α-amino-n-butyric acid, ammonium dihydrogen orthophosphate, L-arginine, cadaverine, L-cysteine, glycinamide, glycine t-butyl ester, L-histidine, L-*iso*-leucine, L-methionine, L-β-phenylalanine, DL-β-phenylalanine, potassium nitrate, L-proline, L-threonine, DL-valine and L-valine as sole nitrogen sources. The strains grow in the presence of crystal violet (0.001 %, w/v), phenol (0.1 %, w/v), phenyl ethanol (0.3 %, w/v), sodium azide (0.005 %, w/v), sodium borohydride (0.1 %, w/v), sodium deoxycholate (0.005 %, w/v), sodium salicylate (0.01 %, w/v), sodium selenite (0.001 %, w/v), tetrazolium salt (0.001 %, w/v) and thallous acetate (0.001 %, w/v), and are not inhibited by ampicillin (8 µg/ml), carbenicillin (12 µg/ml), cefoxitin (32 µg/ml), cephradin (8 µg/ml), fusidic acid (8 µg/ml), isoniazid (16 µg/ml), nalidilic acid (32 µg/ml), oleandomycin phosphate (16 µg/ml), penicillin G (5 i.u), spiramycin (10 µg/ml), tetracyline (4 µg/ml) or tunicamycin (10 µg/ml), or by barium chloride (0.005 %, w/v), cobalt chloride (0.001 %, w/v) or zinc chloride (0.001 %, w/v).

More than 90 % of the strains studied are unable to degrade arbutin or xanthine; utilise adonitol, androsterone, anthranilic acid, L-arabinose, L-ascorbic acid, cinnamic acid, citric acid (Na^+ salt), ferulic acid, D-glucuronic acid, humic acid, D-lyxose, D-mandelic acid, pimelic acid, quinic acid, D-raffinose, sodium malonate (Na^+ salt), sorbitol, L-tartaric acid or xylitol as sole sources of carbon and energy. They are also unable to utilise papaverine, protamine sulfate, tetramethylammonium sulphate or xanthine as sole nitrogen sources. The strains does not grow in the presence of bacitracin (32 µg/ml), cephaloridin (64 µg/ml), doxycyline (16 µg/ml), gentamycin sulphate (8 µg/ml), lividomycin a (8 µg/ml),

neomycin sulphate (8 µg/ml), novobiocin (4 µg/ml), streptomycin sulphate (16 µg/ml), tetracyline (32 µg/ml) or vancomycin (16 µg/ml), or sodium chloride (7.0 %, w/v), tetrazolium salt (0.05 %, w/v), thallous acetate (0.005 %, w/v) or zinc chloride (0.005 %, w/v).

The organisms grow between 25 °C and 55 °C, from pH 6.0 to pH 10 (some strains grow at pH 11.5) and have DNA rich in G plus C (70-72 mol%).

The type strain is DSM 40444^T.

Streptomyces thermogrieus was proposed by Xu *et al.* (1998) to encompass four thermophilic streptomycetes isolated from habitats in Yunnan Province, China. However, in the present study, the type strain of this species shared a 99.5 % 16S rRNA nucleotide similarity (7 nucleotide differences) with *Streptomyces thermovulgaris* DSM 40444^T. Most of the seven nucleotide differences found between the sequences of these two strains were located in the conserved regions of 16S rRNA. It seems likely, therefore, that these apparent differences can be attributed to sequencing errors. This conclusion is also supported by the fact that *Streptomyces thermogrieus* and *Streptomyces thermovulgaris* both produce a grey aerial spore mass, and smooth surfaced spores in hooked or spiral chains. It seems likely that *Streptomyces thermogrieus* should be reduced to a synonym of *Streptomyces thermovulgaris* though further comparative taxonomic studies are needed to prove this legal doubt.

The remaining thermophilic isolate, *Streptomyces* strain TA56, formed a distinct single-membered cluster in aggregate group VI in the numerical phenetic study of Sahin (1995). This organism showed its closest 16S rRNA sequence similarity to *Streptomyces thermovulgaris* DSM 40444^T (98.9 %) but was distinguished from this and related strains on the basis of DNA:DNA relatedness data. A number of phenotypic features can also be weighted to differentiate *Streptomyces* strain TA56 from *Streptomyces thermovulgaris* strains, not least the ability of the former to form warty as opposed to smooth surfaced spores. The two strains were also distinguished by their polar lipid patterns as only

Streptomyces strain TA56 produced an unidentified glycolipid (α-naphthol and periodate-Schiff positive) which co-migrated with a derivative of phosphatidylethanolamine. *Streptomyces* strain TA56 and *Streptomyces thermovulgaris* DSM 40444^T also gave different ribotype patterns when high molecular weight DNA was digested with *Bam* HI and treated with the rRNA probe prepared from *Streptomyces violaceoruber* DSM 41007.

It is clear from both the genotypic and phenotypic data that *Streptomyces* strain TA56 is related to, but distinct from, *Streptomyces thermovulgaris.* It can also be differentiated from *Streptomyces thermoautotrophicus* for unlike the latter it is not an obligate chemolithoautotroph and does not grow at 65 °C (Gadkari *et al.*, 1990). Accordingly, the new species *Streptomyces thermoalcalitolerans* is proposed to accommodate *Streptomyces* strain TA56.

Description of *Streptomyces thermoalcalitolerans* sp. nov. *Streptomyces thermoalcalitolerans* (ther.mo.al.ca.li.to'le.rans. Gr. n. *therme* heat; N. L. n. *alcali* (from arabic *al.* end; *galiy* soda ash); L. pres. part. *tolerans* tolerating, enduring; M. L. part. adj. *thermoalcalitolerans* thermophilic alkali tolerating).

The description is based on data taken from this and an earlier study (Sahin, 1995). Aerobic, Gram positive, thermophilic actinomycete with extensively branched substrate and aerial hyphae. Spiral chains of warty surfaced spores are borne on aerial hyphae. The aerial spore mass colour is grey, neither distinctive substrate mycelium colours nor diffusible pigments are formed. Melanin pigments are not produced on peptone iron agar. Casein, DNA, gelatin, starch, testosterone, L-tyrosine and xylan are degraded but not adenine, arbutin, elastin, guanine, hypoxanthine or xanthine. Adonitol, L-arabinose, arabitol, D-cellobiose, D-fructose, D-galactose, D-glucose, *meso*-inositol, D-lactose, D-mannitol, D-mannose, D-melezitose hydrate, melibiose, α-L-rhamnose, D-ribose, D-sorbitol, sucrose, D-

trehalose, D-turanose, xylitol and D-xylose are used as sole carbon sources for energy and growth but D-raffinose is not.

Growth occurs between 25 °C and 55 °C, from pH 6.0 to pH 11.5, and in the presence of ampicillin (8 μg ml^{-1}), bacitracin (16 μg ml^{-1}), oleandomycin phosphate (16 μg ml^{-1}), penicillin G (15 international units), rifampicin (16 μg ml^{-1}), streptomycin sulphate (4 μg ml^{-1}), tetracycline hydrochloride (16 μg ml^{-1}) and tunicamycin (10 μg ml^{-1}). In contrast, growth was inhibited in the presence of gentamycin sulphate (8 μg ml^{-1}), lincomycin hydrochloride (32 μg ml^{-1}), neomycin sulphate (8 μg ml^{-1}), novobiocin (4 μg ml^{-1}), oleandomycin phosphate (32 μg ml^{-1}), polymyxin B phosphate (32 μg ml^{-1}), rifampicin (32 μg ml^{-1}), streptomycin sulphate (16 μg ml^{-1}), tetracycline hydrochloride (32 μg ml^{-1}), tobramycin sulphate (32 μg ml^{-1}) and vancomycin hydrochloride (16 μg ml^{-1}). The DNA composition of strain TA56 is 73 mol% G + C.

Isolated from tropical garden soil collected by M. Goodfellow from Yogyakarta, Central Java, Indonesia in 1991.

The putative type strain is TA56 (=DSM 41741).

Streptomyces thermoalcalitolerans TA56^T is closely related to *Streptomyces thermovulgaris* DSM 40444^T as the tow strains share a DNA relatedness value of 62 %, a value well below the 70 % cut-off point used to assign strains to single genomic species (Wayne *et al.*, 1987; Goodfellow *et al.*, 1997a). The two strains were also found to contain octahydrogenated menaquinone with nine isoprene units as the major isoprenologue.

Streptomyces strain NAR85 was the only neutrophilic, thermophilic isolate assigned to aggregate group V in the numerical phenetic study of Sahin (1995). in this study, the organism was most closely related to *Streptomyces thermodiastaticus* DSM 40573^T and *Streptomyces thermoviolaceus* DSM 40443^T. The close association with *Streptomyces thermodiastaticus* was underpinned by the results of the DNA:DNA relatedness studies as strain NAR85 showed 97 % DNA relatedness with labelled DNA prepared from

Streptomyces thermodiastaticus DSM 40573^T. This assignment was also supported by corresponding phenotypic, ribotyping and 16S rRNA sequence data. The nucleotide sequence data show that *Streptomyces* strain NAR85 is closely related to *Streptomyces thermodiastaticus* DSM 40573^T (99.5 % nucleotide sequence similarity) and *Streptomyces thermoviolaceus* DSM 40443^T (99.4 %). It is clear from both the genotypic and phenotypic data that *Streptomyces* strain NAR85 is a typical member of the species *Streptomyces thermodiastaticus. Streptomyces thermodiastaticus* strain NAR85 has been deposited in the DSMZ collection under the accession number DSM 41740.

It is evident from the 16S rRNA data that the neutrophilic, thermophilic isolate NT322 (centrotype strain of cluster 8, aggregate group IV; Sahin, 1995) is most closely related to *Streptomyces thermocarboxydus* DSM 44293^T (98.6 % nucleotide similarity), *Streptomyces coeruleoprunus* DSM 41472^T (98.4 % nucleotide similarity) and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (98.4 % nucleotide similarity). These similarity values are below the levels commonly found between validly described streptomycete species as exemplified by *Streptomyces thermocarboxydovorans* DSM 44296^T and *Streptomyces thermodiastaticus* DSM 40573^T (99.1 % nucleotide similarity). When the partial 16S rRNA sequence data of this strain was compared with the extensive DSMZ partial 16S rRNA database it was evident that strain NT322 was not closely related to any of the representatives of validly described *Streptomyces* species. In addition to *Streptomyces* strain NT322, cluster 8 contained three other strains, namely, *Streptomyces* strains NT342, NT346 and NT364. All four organisms were isolated from an arid soil sample collected from in Merida, Venezuela. The integrity of cluster 8 is also supported by pyrolysis mass spectrometric data (Sahin, 1995).

It can be concluded from the chemical fingerprinting, phenotypic and molecular systematic data that *Streptomyces* strains NT322, NT342, NT346 and NT364 merit species

status in the genus *Streptomyces*. It is, therefore, proposed that these strains be assigned to a new species for which the name *Streptomyces eurythermophilus* is given.

Description of *Streptomyces eurythermophilus* sp. nov. *Streptomyces eurythermophilus* (eur.y.ther'mo.phi.lus; Gr. pref. *eury* many; Gr. n. *therme* heat; Gr. adj. *philus* loving; M. L. part. adj. *eurythermophilus* thermophile with wide growth temperature range).

The description is based on data taken from this and an earlier study (Sahin, 1995). Aerobic, Gram positive, thermophilic actinomycete with extensively branched substrate and aerial hyphae. Spiral chains of smooth surfaced spores are borne on aerial hyphae. The aerial spore mass is brownish grey, but neither distinctive substrate mycelium colours nor diffusible pigments are formed on glycerol-asparagine, inorganic-salt starch or oatmeal agars. Melanin pigments are not formed peptone-yeast extract iron or tyrosine agars.

Adenine, casein, elastin, gelatin, hypoxanthine, testosterone, xanthine and xylan are degraded but not guanine or L-tyrosine. D-cellobiose, cholesterol, D-galactose, D-gluconic acid (Na^+ salt), humic acid, *meso*-inositol, D-lactose, D-mannitol, D-mannose, α-D-melibiose, pyruvic acid (Na^+ salt), quinic acid, α-L-rhamnose, salicin, saponin, sebacic acid, starch, sucrose, D-trehalose and D-xylose are used as sole carbon and energy sources but not adonitol, androsterone, anthranilic acid, L-arabinose, L-ascorbic acid, benzoic acid (Na^+ salt), carboxymethylcellulose, ferulic acid, D-glucuronic acid, inulin, D-lyxose, malonic acid (Na^+ salt), D-mandelic acid, D-melezitose, L-phenyldodecane, pimelic acid, D-raffinose, sorbitol, L-tartaric acid or xylitol. Ammonium dihydrogen orthophosphate, L-arginine, cadaverine, L-cysteine, glycinamide, glycine anhydride, glycine, t-butyl ester, L-histidine, hypoxanthine, L-*iso*-leucine, L-methionine, L-β-phenylalanine, potassium nitrate, L-proline, L-threonine, L-valine and xanthine are used as sole nitrogen source but not acetamide, DL-α-amino-n-butyric acid, creatine, propionamide, protamine sulphate, tetramethylammonium sulfate or DL-valine. Growth occurs between 20 °C and 55 °C, from pH 6.0 to pH 9.0.

Growth is inhibited by amikacin (4 μg ml^{-1}), cephaloridine (32 μg ml^{-1}), doxycyline (4 μg ml^{-1}), gentamycin sulphate (8 μg ml^{-1}), lividomycin A (4 μg ml^{-1}), novobiocin (4 μg ml^{-1}), streptomycin sulphate (4 μg ml^{-1}), vancomycin (16 μg ml^{-1}), streptomycin sulphate (4 μg ml^{-1}) and viomycin (20 μg ml^{-1}).

Strain NT322 contains DPG, PE, PI, PIDM and a few unidentified phospholipids as the major polar lipids, and hexa- and octahydrogenated menaquinones with nine isoprene units as the predominant isoprenologues.

Isolated from an arid soil sample collected from in Merida, Venezuela.

The type strain is *Streptomyces* strains NT322.

The remaining five thermophilic isolates, namely, NAR54 (centrotype strain of cluster 6; Sahin, 1995), NT358 (centrotype strain of cluster 7; Sahin, 1995), NT576 (centrotype strain of cluster 10; Sahin, 1995), NT381 (centrotype strain of cluster 11; Sahin, 1995) and NT90 (centrotype strain of cluster 15; Sahin, 1995), were recovered in subclade A5 in the streptomycete tree.

Identical partial 16S rRNA sequences were found between *Streptomyces* strains NAR54 and NAR84 (centrotype strain of cluster 5, aggregate group IV; Sahin, 1995), between *Streptomyces* strains NT90 and NT336 (centrotype strain of cluster 14, aggregate group IV; Sahin, 1995), and between *Streptomyces* strains NT381 and NT399 (centrotype strain of cluster 9, aggregate group IV; Sahin, 1995), NT312 (centrotype strain of cluster 13, aggregate group IV; Sahin, 1995), NT371 (centrotype strain of cluster 12, aggregate group IV; Sahin, 1995), TA127 (single-membered cluster, aggregate group IV; Sahin, 1995) and NT493 (single-membered cluster, aggregate group IV; Sahin, 1995). The close relationships between these groups of strains is supported by morphological data (Table 3-11).

The 16S rRNA sequences of seven isolates, namely, *Streptomyces* strain NAR54 (centrotype strain of cluster 6, aggregate group IV; Sahin, 1995), NT90 (centrotype strain of

cluster 15, aggregate group IV; Sahin, 1995), NT307 (centrotype strain of cluster 3, aggregate group II; Sahin, 1995), NT322 (centrotype strain of cluster 8, aggregate group IV; Sahin, 1995), NT358 (centrotype strain of cluster 7, aggregate group IV; Sahin, 1995), NT381 (centrotype strain of cluster 11, aggregate group IV; Sahin, 1995) and NT576 (centrotype strain of cluster 10, aggregate group IV; Sahin, 1995), were compared with the streptomycete partial 16S rRNA database held at DSMZ.

It is evident from Table 3-13 that some of these isolates have very similar morphological properties to those of their nearest neighbours. For instance, *Streptomyces* strain NT307 showed its highest nucleotide similarities to *Streptomyces flocculus* DSM 40327^T (98.6 % nucleotide similarity) and *Streptomyces albus* DSM 40313^T (98.0 % nucleotide similarity) and these strains shared white aerial spore mass colour and smooth spores in spiral chains. Similarly, *Streptomyces* strain NT381 showed its highest nucleotide similarities to *Streptomyces xantholiticus* DSM 40244^T (98.8 % nucleotide similarity) and *Streptomyces minutiscleroticus* DSM 40301^T (98.7 % nucleotide similarity) and these strains shared grey aerial spore mass colour and smooth spores in spiral chains. These findings suggest that these isolates may be the members of established streptomycete species though additional comparative taxonomic studies are needed to confirm this. These results also suggest that members of some established *Streptomyces* species may be able to grow at or above 50 °C. Further comparative studies are needed to determine the taxonomic relationships of these isolates and related strains.

The chemical and morphological properties of strain B19, which was isolated from a sample of poultry faeces collected from the poultry farm at the University of Malaya, are consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995). The organism forms an highly branched substrate mycelium, aerial hyphae which carry smooth surfaced spores in straight chains, contains LL-A_2pm in the peptidoglycan, lacks characteristic sugars and mycolic acids, has tetra-, hexa- and octahydrogenated

Table 3-13. Comparison of morphology between isolates and their nearest *Streptomyces* species

Isolates	Closest matches	16S rRNA similarity[a]	Spore surface ornamentation[b]	Spore chains[b]	Spore mass colour[b]
NT307 (cluster 3)			smooth	spiral	white
	S. flocculus DSM 40327^T	98.6 %	smooth	spiral	white
	S. albus DSM 40313^T	98.0 %	smooth	spiral	white
NAR54 (cluster 6)			smooth	spiral	grey
	S. erythrogriseus DSM 40116^T	99.0 %	spiny	hooked or spiral	grey-red
	S. griseoflavus DSM 40456^T	99.0 %	spiny	spiral	grey
	S. griseoincarnatus DSM40274^T	99.0 %	spiny	hooked of spiral	grey-red
NT358 (cluster 7)			smooth	straight	grey
	S. eurythermus DSM 40014^T	98.9 %	smooth	hooked	grey
	S. lavenduligriseus DSM 40487^T	98.9 %	smooth	hooked or straight	grey
NT576 (cluster 10)			spiny	spiral	grey
	S. viridodiastaticus DSM40249^T	99.2 %	small- spiny	hooked or spiral	grey
	S. albogriseolus DSM 40003^T	99.0 %	warty	hooked or spiral	grey
NT381 (cluster 11)			smooth	spiral	grey
	S. xantholiticus DSM 40244^T	98.8 %	smooth	spiral	grey
	S. minutiscleroticus DSM 40301^T	98.7 %	smooth	spiral	grey-yellow
NT90 (cluster 15)			spiny	spiral	grey
	S. cineoruber	99.1 %	smooth	straight	grey
	S. plicatus	99.1 %	smooth	hooked or long-spiral	grey

[a], Partial 16S rRNA sequences positions between 33 and 474 and between 804-1233 (*Streptomyces ambofaciens* numbering system; Pernodet *et al.*, [1989]) of most of the validly described streptomycete species held in DSMZ database were compared.

[b], Descriptions of established taxa were taken from this and earlier studies (Shirling & Gottlieb, 1968a,b, 1969, 1972 and Gottlieb & Shirling, 1967).

menaquinones with nine isoprene units, contains DPG, PE, PI and PIDM as major polar lipids, and has a DNA base composition of 68.6 mol % G+C. The assignment of the strain to the genus *Streptomyces* is also supported by 16S rRNA sequence data.

Comparison of the almost complete 16S rRNA sequence (1492 nucleotides) of *Streptomyces* strain B19 with corresponding streptomycete sequences showed that this organism lies at the periphery of the subclade A2 which is occupied by *Streptomyces thermodiastaticus* and allied taxa. The mean 16S rRNA similarity value found between the test strain and members of the *Streptomyces thermodiastaticus* clade was 97.9 %. *Streptomyces* strain B19 shares particularly high percentage nucleotide sequence similarities with *Streptomyces thermoviolaceus* subsp. *apingens* (98.6 %) and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* (98.2 %); these values correspond to 21 and 27 nucleotide differences out of 1475 nucleotide positions. Nucleotide similarities within this range have been reported for several validly described species belonging to the *Streptomyces thermodiastaticus* clade, for instance, between *Streptomyces thermocarboxydovorans* and *Streptomyces thermoviolaceus* (98.8 %; Kim, S. B. *et al.*, 1998). Members of these taxa form distinct genomic species and can be separated using a set of phenotypic properties (Goodfellow *et al.*, 1987; Kim, S. B. *et al.*, 1998).

Streptomyces strain B19 forms a grey aerial spore mass and produces smooth surfaced spores in straight chains. It also produces a brown substrate mycelium and brown diffusable pigments on Czapek Dox and yeast extract-malt extract agars, but not on glycerol-asparagine, inorganic salts-starch or oatmeal agars. Melanin pigments are formed on peptone iron and tyrosine agars. The organism can be distinguished from members of the *Streptomyces thermodiastaticus* clade using a combination of phenotypic properties (Table 3-14, pages 289 and 290). This latter observation is in line with recent studies which show that members of streptomycete species found to be closely related on the basis of genotypic data can be also be distinguished using phenotypic properties, notably morphological and

pigmentation features (Labeda & Lyons, 1991; Chun *et al.*, 1997b; Labeda *et al.*, 1997; Kim, S. B. *et al.*, 1998).

It is apparent from the genotypic and phenotypic data that strain B19 forms a distinct centre of taxonomic variation within the genus *Streptomyces*. It is, therefore, proposed that this organism be recognised as a new species, for which the name *Streptomyces thermocoprophilus* is given.

Description of *Streptomyces thermocoprophilus* sp. nov. *Streptomyces thermocoprophilus* (ther. mo. co. pro. phi. lus. Gr. n. *therme* heat; Gr. n. *copro* dung; Gr. adj. *philus* loving; M. L. adj. *thermocoprophilus* dung loving thermophile).

The description is based on the present and earlier studies by Al-Tai *et al.* (unpublished data). Aerobic, Gram positive, moderately thermophilic actinomycete which forms highly branched substrate and aerial hyphae. The latter differentiate into long straight spore chains. The spores are cylindrical shaped and have smooth surfaces. The aerial spore mass colour is grey. Diffusable pigments are formed on some standard media such as inorganic salts starch agar. Melanin pigments are produced on peptone iron and tyrosine agars. Casein, starch, xanthine and xylan are degrade but not adenine is not.

L-arabinose, D-fructose, D-galactose, D-glucose, *meso*-inositol, maltose, D-mannitol, D-mannose and D-xylose are used as sole carbon sources for energy and growth but not carboxymethylcellulose, D-raffinose, starch or sucrose.

Growth occurs between 20 °C and 50 °C (optimum temperatures for growth are 37 to 50 °C) and in the presence of ampicillin (10 $\mu g\ ml^{-1}$), erythromycin (15 $\mu g\ ml^{-1}$) and sodium chloride (7 %, w/v) but not at 10 °C or 55 °C. Growth is inhibited by chloramphenicol (30 $\mu g\ ml^{-1}$), gentamycin sulphate (15 $\mu g\ ml^{-1}$), kanamycin sulphate (30 $\mu g\ ml^{-1}$), neomycin sulphate (30 $\mu g\ ml^{-1}$), streptomycin sulphate (10$\mu g\ ml^{-1}$) and tetracycline hydrocholoride (30 $\mu g\ ml^{-1}$). Antimicrobial activity is shown against *Bacillus subtilis* NCIB

3610 but not against *Escherichia coli* NCIB 9132 or *Staphylococcus aureus* ATCC 12600, or against representative strains of *Candida albicans, Curvularia lunata, Pestalotiopsis gnepini, Pyricularia oryzae* and *Trichoderma viride.*

The G+C ratio of the genomic DNA is 68.6 mol%.

The organism was isolated from a sample of poultry faeces collected from the poultry farm at the University of Malaya.

The type strain is B19 (=DSM 41700).

Some phenotypic properties differentiating thermophilic streptomycete species are shown in Table 3-14.

It is evident from these studies that a phylogenetic approach is needed to clarify the taxonomy of existing validly described species of *Streptomyces.* The resultant framework is needed to help describe the many strains of streptomycetes which remain to be isolated from natural habitats and given formal designations. It seems likely from the results of the present study that many more species of the streptomycetes remain to be described.

Table 3-14. Phenotypic properties of the thermophilic *Streptomyces* species

	S. eurythermophilus NT322^T	*S. thermoalcaliitolerans* DSM 41741^T	*S.thermocarboxydovorans*	*S. thermocarboxydus* DSM 44293^T	*S. thermocoprophilus* B19^T	*S. thermodiastaticus* DSM 40573^T	*S. thermophilus* DSM 40365^T	*S. thermoviolaceus* subsp.*thermoviolaceus* DSM 40443^T	*S. thermoviolaceus* subsp.*apingens* DSM 41392^T	*S. thermovulgaris*
Aerial spore mass color	Brown-grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey	Grey
Pigmentation of substrate mycelium	Not distinctive	Not distinctive	Not distinctive	Not distinctive	Not distinctive	Yellowish brown	Not distinctive	Yellow/ Violet [a]	Yellow	Not distinctive
Spore chain	SP	SP	RF	RA	RF	SP	RF	SP	SP	SP
Spore surface	Smooth	Warty	Smooth	Warty	Smooth	Tuberculate	Spiny	Tuberculate	Tuberculate	Smooth
Melanin production	-	-	-	-	+	-	+	-	-	-
Nitrate reduction	ND	+	+	+	ND	-	ND	-	-	+
Degradation of:										
Adenine	+	-	+	ND	-	+	+	+	+	v
Casein	-	+	ND	ND	+	+	+	+	ND	+
Elastin	+	-	+	+	-	+	ND	+	+	+
Gelatin	+	+	-	+	+	+	+	+	+	+
Hypoxanthine	+	-	+	+	+	-	ND	-	+	-
Starch	ND	+	+	+	+	+	ND	+	+	+
Testosterone	+	+	+	+	-	+	ND	-	-	v
L-Tyrosine	-	+	+	-	+	+	ND	+	+	+
Xanthine	+	-	+	+	+	-	ND	-	-	-
Xylan	+	+	+	-	+	-	ND	-	-	-

Growth on sole carbon sources:										
L-Arabinose	-	+	-	ND	+	-	+	-	+	-
meso-Inositol	+	+	-	+	+	+	+	+	+	+
Mannitol	+	+	-	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	+	-	-	-	-
Sucrose	+	+	-	-	-	-	+	-	+	+
Growth at:										
10 °C	-	-	-	-	-	-	-	-	-	-
15 °C	-	-	-	-	-	-	ND	-	-	-
20 °C	ND	-	+	+	+	-	ND	+	+	-
25 °C	+	+	+	+	+	+	+	+	+	+
50 °C	+	+	+	+	+	+	+	+	+	+
55 °C	+	+	+	+	-	+	+	+	+	+
60 °C	-	-	-	-	-	-	ND	-	-	-

Symbols: +, positive or more than 90% of strains positive in the case of the *S. thermocarboxydovorans* and *S. thermovulgaris* strains;
-, negative or more 90% of strains negative in the case of the *S. thermocarboxydovorans* and *S. thermovulgaris strains.*
ND, not determined; v, variable.

Data taken from this and previous studies (Shirling & Gottlieb, 1969, 1972; Williams *et al*., 1983a; Goodfellow *et al*., 1987; Kampfer *et al.*, 1991; Sahin, 1995; Kim, S. B. *et al.*, 1998).
Abbreviations: RF, Rectiflexibiles; SP, Spirals.

[a], Substrate mycelium is initially yellow but becomes purple after 5 days due to the formation of a diffusible purple pigment.

References

Abed, Y., Davin-Regli, A., Bollet, C. & De Micco, P. (1995). Efficient discrimination of *Mycobacterium tuberculosis* strains by 16S-23S spacer region-based random amplified polymorphic DNA analysis. *Journal of Clinical Microbiology* **33**, 14184-1420. [1]

Agre, N. S. (1983). Genera *Streptomyces*, *Streptoverticillium* and *Chainia*. In *A guide for the determination of actinomycetes*, pp. 52-71. Edited by T. F Gause, T. P. Preobrazhenskaya, M. A. Sveshnikova, L. P. Terekhova & T. S. Maksimova. Moscow: Nauka. (in Russian) [3]

Agre, N. S. & Guzeva L. N. (1975). New actinomycetes genus *Excellospora* gen. nov. *Microbiology* (English Translation of *Mikrobiologiya*) **44**, 518-523. [2]

Akimov, V. N., Evtushenko, L. I. & Dobritsa, S. V. (1989). *Pseudoamycolata halophobica* gen. nov., sp. nov. *International Journal of Systematic Bacteriology* **39**, 457-461. [2]

Al-Tai, A. M. & Ruan, J. S. (1994). *Nocardiopsis halophila* sp. nov., a new halophilic actinomycete isolated from soil. *International Journal of Systematic Bacteriology* **44**, 474-478. [2]

Alderson, G. & Goodfellow, M. (1979). Classification and identification of actinomycetes causing mycetoma. *Postepy Higieni i Medycyny Doswiadezalnej* **33**, 109-124. [2]

Alderson, G., Goodfellow, M. & Minnikin, D. E. (1985). Menaquinone composition in the classification of *Streptomyces* and other sporoactinomycetes. *Journal of General Microbiology* **131**, 1671-1679. [3]

Alm, R. A. & Manning, P. A. (1990). Biotype-specific probe for *Vibrio cholerae* seogroup O1. *Journal of Clinical Microbiology* **28**, 823-824. [1]

Al-Saif, N. M., O'Neill, G. L., Magee, J. T., Brazier, J. S. & Duerden, B. I. (1998). PCR-ribotyping and pyrolysis mass spectrometry fingerprinting of environmental and hospital isolates of *Clostridium difficile*. *Journal of Medical Microbiology* **47**, 117-121. [1]

Amann, R. I., Ludwig, W. & Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143-169. [1]

Amikam, D., Glaser, G. & Razin, S. (1984). Mycoplasmas (Mollicutes) have a low number of rRNA genes. *Journal of Bacteriology* **158**, 376-378. [1]

Anzai, Y., Okuda, T. & Watanabe, J. (1994). Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. II. Actinomycetes. *Journal of Antibiotics* **47**, 183-193. [1]

Applied Biosystems. (1993). *Taq DyeDeoxyTM terminator cycle sequencing kit.* Rev. E ed., vol. Part number 901497. Applied Biosystems Inc, Foster City (USA). [2]

Arai, T. & Mikami, Y. (1969). Identification keys for antibiotics producing *Streptomyces*. I. Antifungal antibiotics producers. *Annual Report of the Institute of Food Microbiology, Chiba University* **22**, 59-79. [3]

Arbeit, R., Slutsky, A., Barber, T. W., Maslow, J. N., Niemczyk, S., Falkinham III, J. O., O'Connor, G. T. & von Reyn, F. (1993). Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. *The Journal of Infectious Disease* **167**, 1384-1390. [1]

Asano, K., Sano, H., Masunaga, I. & Kawamoto, I. (1989). 3-*O*-methylrhamnose: identification and distribution in *Catellatospora* species and related actinomycetes. *International Journal of Systematic Bacteriology* **39**, 56-60. [1]

Assulieau, J. (1966). *The bacterial lipids*. Hermann: Paris. [1]

Atalan, E.; Manfio, G. P.; Ward, A.C. Kroppenstedt, R. M. & Goodfellow, M. (1999). Biosystematic studies on novel streptomyces from soil. *Microbiology* (in press). [3]

Athalye, M., Goodfellow, M. & Minnikin, D. E. (1984). Menaquinone composition in the classification of Actinomadura and related taxa. *Journal of General Microbiology* **130**, 817-823. [2]

Athalye, M., Goodfellow, M., Lacey, J. & White, R. P. (1985). Numerical classification of *Actinomadura* and *Nocardiopsis*. *International Journal of Systematic Bacteriology* **35**, 86-98. [2]

Auerbach, S. B., McNeil, M. M., Brown, J. M., Lasker, B. A. & Jarvis, W. R. (1992). Outbreak of pseudoinfection with *Tsukamurella paurometabolum* traced to laboratory contamination: Efficacy of joint epidermiologica and laboratory investigation. *Clinical Infectious Diseases* **14**, 1015-1022. [1]

Baldacci, E. (1958). Development in the classification of actinomycetes. *Giornale di Microbiologia* **6**, 10-27. [3]

Baldacci, E., Farina, G. & Locci, R. (1966). Emendation of the genus *Streptoverticillium* Baldacci (1958) and revision of some species. *Giornale di Microbiologia* **14**, 153-171. [3]

Baldacci, E., Spalla, C. & Grein, A. (1954). The classification of the *Actinomyces* species (=*Streptomyces*). *Archiv für Mikrobiologie* **20**, 347-357. [3]

Barrett, S. J. & Sneath, P. H. A. (1994). A numerical phenotypic taxonomic study of the genus *Neisseria*. *Microbiology* **140**, 2867-2891. [1]

Barsotti, O., Decoret, D., Benay, G., Carlotti, A., Freney, J., Guerin-Faublee, V. & Morrier, J.-J. (1994). rRNA gene restriction patterns as possible taxonomic tools for the genus *Actinomyces*. *Zentralblatt für Bakteriologie* **281**, 433-441. [1]

Bassam, B. J., Caetano-Anollés, G. & Gresshoff, P. M. (1992). DNA amplification fingerprinting of bacteria. *Applied Microbiology & Biotechnology* **38**, 70-76. [1]

Becker, B., Lechevalier, M. P. & Lechevalier, H. A. (1965). Chemical composition of cell wall preparations from strains of various form-genera of aerobic actinomycetes. *Applied Microbiology* **13**, 236-243. [2]

Beiss, U. (1964). Zur Papierchromatographischen aufrennung von Pflanzenlipiden. *J. Chromatogr.* **13**, 104-110. [2]

Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J. & Ouellette, B. F. (1998). GenBank. *Nucleic Acids Research* **26**, 1-7. [1,2,3]

Bercovier, H., Kafri, O. & Sela, S. (1986). Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. *Biochemical Research Communications* **136**, 1136-1141. [1]

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. & Huntoon, F. M. (1923). *Bergey's Manual of Determinative Bacteriology*, 1st Edn. Baltimore: The Williams and Wilkins Co. [3]

Beyazova, M. & Lechevalier, M. P. (1993). Taxonomic utility of restriction endonuclease fingerprinting of large DNA fragments from *Streptomyces* strains. *International Journal of Systematic Bacteriology* **43**, 674-682. [3]

Birtles, R. J. & Raoult, D. (1996). Comparison of partial citrate synthase gene (gltA) sequences for phylogenetic analysis of *Bartonella* species. IJSB **46**, 891-897. [1]

Blanchard, R. (1896). Parasites végetaux à l'exclusion des bactéries, PP. 811-932. In *Traité de pathologie générale*, vol. 2. Edited by R. Blanchard. Paris: G. Masson. [2]

Boeringer Mannheim GmbH. (1995). *The DIG System User's Guide for Filter Hybridization.* Mannheim, Germany. [2,3]

Boiron, P., Provost, F. & Duport, B. (1993). *Laboratory Methods for the diagnosis of Nocardiosis.* Institut Pasteur: Paris. [2,3]

Böttger, E. C. (1989). Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiology Letters* **65**, 171-176. [1]

Bowen, T., Stackebrandt, E., Dorsch, M. & Embley, T. M. (1989). The phylogeny of *Amycolata autotrophica, Kibdelosporangium aridum* and *Saccharothrix australiensis. Journal of General Microbiology* **135**, 2529-2536. [1,2]

Brenner, D. J. (1984). Family *Enterobacteriaceae*. In *Bergey's manual of systematic bacteriology*, vol. 1. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins. [1]

Brenner, D. J., Fanning, G. R., Rake, A. V. & Johnson, K. E. (1969) Batch procedure for thermal elution of DNA from hydroxyapatite. *Anal Biochem* **28**, 447-459. [1]

Brenner, D. J., Fanning, G. R., Skerman, F. J. & Falkow, S. (1972). Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *Journal of Bacteriology* **109**, 953-965. [1]

Brenner, D. J., Fanning, G. R., Miklos, G. V. & Steigerwalt, A. G. (1973). Polynucleotide sequence relatedness among *Shigella* species. *International Journal of Systematic Bacteriology* **23**, 1-7. [1]

Brock, T. D. (1978). *Thermophilic Microorganisms and Life at High Temperatures.* New York: Springer-Verlag. [2]

Brock, T. D. (1986). Introduction: An overview of the thermophiles. In *Thermophiles: General, Molecular, and Applied Microbiology*, pp 1-17. Edited by T. D. Brock. New York, John Wiley & Sons. [2]

Brock, T. D. & Freeze, H. (1969). *Thermus aquaticus* gen. n., a nonsporulating extreme thermophile. *Journal of Bacteriology* **98**, 289-297. [2]

Brocq-Rousseau, D. (1904). Sur un *Streptothrix* cause de l'alteration des aviones moisies. *Rev. Bot.* **16**, 219-230. [2]

Brosius, J., Palmer, J. L., Kennedy, J. P. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 4801-4805. [2,3]

Brousseau, R., Saint-Onge, A., Prefontaine, G., Masson, L. & Cabana, J. (1993). Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Applied & Environmental Microbiology* **59**, 114-119. [1]

Buchanan, R. E. (1917). Studies on the nomenclature and classification of the bacteria. II. The primary subdivision of the schizomycetes. *Journal of Bacteriology* **2**, 155-164. [1]

Buchanan, R. E. (1918). Studies in the classification and nomenclature of the bacteria. VIII. Subgroups and genera of the *Actinomycetales*. *Journal of Bacteriology* **3**, 403-406. [1]

Bull, A. T., Goodfellow, M. & Slater, J. H. (1992) Biodiversity as a source of innovation in biotechnology. Annual Review of Microbiology **46**, 219-252. [1]

Burggraf, S., Olsen, G. J., Stetter, K. O. & Woese, C. R. (1992) A phylogenetic analysis of *Aquifex pyrophilus*. *Systematic and Applied Microbiology* **15**,352-356. [2]

Butler, W. R., Kilburn, J. O. & Kubica, G. P. (1987). High-performance liquid chromatography analysis of mycolic acids as an aid in laboratory identification of *Rhodococcus* and *Nocardia* species. *Journal of Clinical Microbiology* **25**, 2126-2131. [1]

Caetano-Anollés, G., Bassam, B. J. & Gresshoff, P. M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* **9,** 553-557 [1]

Canhos, V. P., Manfio, G. P. & Blaine, L. D. (1993). Software tools and databases for bacterial systematics and their disseminaztion *via* global networks. *Antonie van Leeuwenhoek* **64**, 205-229. [1]

Charfreitag, O., Collins, M. D. & Stackebrandt, E. (1988). Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. *International Journal of Systematic Bacteriology* **38**, 354-357. [3]

Chester, F. D. (1897). Report of the mycologist, bacteriological work. *Delaware Agricultural Experimental Station, Bulletin* **9,** 38-145. [1]

Chiodini, R. J. (1990) Characterization of *Mycobacterium paratuberculosis* and organisms of the *Mycobacterium avium* complex by restriction polymorphism of the rRNA gene region. *Journal of Clinical Microbiology* **28**, 489-494. [1]

Christova, K., Sholeva, Z., Chipeva, V., Najdenova, M. & Todorova, D. (1997) Differentiation of Streptomyces strains by multilocus enzyme electrophoresis. *Biotechnology & Biotechnological Equipment* 11, A47-A52. [3]

Chun, J. (1995). *Computer-assisted classification and identification of actinomycetes.* Ph.D. Thesis, University of Newcastle upon Tyne, UK. [1,2,3]

Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* **45,** 240-245. [1,2,3]

Chun, J., Atalan, E., Kim, S.-B., Kim, H.-J., Hamid, M. E., Trujillo, M. E., Magee, J. G., Manfio, G. P., Ward, A. C. & Goodfellow, M. (1993a). Rapid identification of streptomycetes by artificial neural network analysis of pyrolysis mass spectra. *FEMS Microbiology Letters* **114**, 115-120. [1]

Chun, J., Atalan, E., Ward, A. C. & Goodfellow, M. (1993b). Artificial neural network analysis of pyrolysis mass spectrometric data in the identification of *Streptomyces* strains. *FEMS Microbiology Letters* **107**, 321-326. [1]

Chun, J., Kang, S. O., Hah, Y. C., Goodfellow, M. (1996). Phylogeny of mycolic acid-containing actinomycetes. *Journal of Industrial Microbiology* 1996 **17**, 205-213. [1]

Chun, J., Blackall, L. L., Kang, S.-O., Hah, Y. C. & Goodfellow, M. (1997a). A proposal to reclassify *Nocardia pinensis* Blackall et al. as *Skermania piniformis* gen. nov., com. nov. *International Journal of Systematic Bacteriology* **47**, 127-131. [1]

Chun, J., Youn, H. -D., Yim, Y. -I., Lee, H., Kim, M. Y., Hah, Y. C. & Kang, S. -O. (1997b). *Streptomyces seoulensis* sp. nov. *International Journal of Systematic Bacteriology* **47**, 492-498. [3]

Chun, J., Kim, S. B., Oh, Y. K., Seong, C-. N., Lee, D-. H., Bae, K. S., Kim, M. Y., Hah, Y. C. & Goodfellow, M. (1998a). *Amycolatopsis thermoflava* sp. nov. *International Journal of Systematic Bacteriology* (submitted). [2]

Chun, J., Seong, C.-N., Bae, K. S., Lee, K.-J., Kang, S.-O., Goodfellow, M. & Hah, Y. C. (1998b). *Nocardia flavorosea* sp. nov. *International Journal of Systematic Bacteriology* (submitted). [1]

Cilia, V., Lafay, B. & Christen, R. (1996). Sequence heterogeneities among 16S ribosomal sequences, and their effect on phylogenetic analyses at the species level. *Molecular Biology and Evolution* **13**, 451-461. [1]

Claridge, M. F., Dawah, H. A. & Wilson, M. R. (1997) Practical approaches to species concepts for living organisms. In *Species: The Units of Biodiversity*, pp. 1-16. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall. [1

Clarke, S. D., Ritchie, D. A. & Williams, S. T. (1993) Ribosomal DNA restriction fragment analysis of some closely-related Streptomyces species. *Systematic and Applied Microbiology* **16**, 256-260. [3]

Collins, D. M. (1984). DNA restriction endonuclease analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. *Journal of Clinical Microbiology* **130**, 1019-1021. [1]

Collins, D. M. (1985). DNA restriction endonuclease analysis of *Mycobacterium bovis* and other members of the tuberculosis complex. *Journal of Clinical Microbiology* **21**, 562-564. [1]

Collins, M. D. (1994). Isoprenoid quinones. In *Chemical methods in Prokaryotic Systematics*, pp. 265-309. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: John Wiley and Sons . [1]

Collins, M. D. & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiological Reviews* **45**, 316-354. [1]

Collins, M. D. & Jones, D. (1982). Lipid composition of *Corynebacterium paurometabolum* Steinhaus. *FEMS Microbiology Letters* **13**, 13-16. [2]

Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *Journal of General Microbiology* **100**, 221-230. [2]

Collins, M. D., Faulkner, M. & Keddie, R. M. (1984). Menaquinone composition of some sporeforming actinomycetes. *Systematic and Applied Microbiology* **5**, 20-29. [2,3]

Collins, M. D., Kroppenstedt, R. M., Tamaoka, J., Komagata, K. & Kinoshita, T. (1988). Structures of the tetrahydrogenated menaquinones from *Actinomadura angiospora, Faenia rectivirgula* and *Saccharothrix australiensis*. *Current Microbiology* **17**, 275-279. [1,2]

Collins, M. D., Dorsch, M. & Stackebrandt, E. (1989). Transfer of *Pimelobacter tumescens* to *Terrabacter* gen. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to

Nocardioides as *Nocardioides jensenii* comb. nov. *International Journal of Systematic Bacteriology* **39**, 1-6. [3]

Collins, M. D., Cockcroft, S. & Wallbanks, S. (1994). Phylogenetic analysis of a new LL-diaminopimelic acid-containing coryneform bacterium from herbage, *Nocardioides plantarum* sp. nov. *International Journal of Systematics Bacteriology* **44**, 523-526. [3]

Colwell, R. R. (1970). Polyphasic taxonomy of bacteria. In *Culture collections of microorganisms*, pp. 421-436. Edited by H. Iizuka & T. Hasegawa. Baltimore: University Park Press. [1,3]

Colwell, R. R. & Mandel, M. (1964). Adansonian analysis and deoxyribonucleic acid base composition of some Gram-negative bacteria. *Journal of Bacteriology* **87**, 1412-1422. [1]

Conn, H. J. (1916). A possible function of actinomycetes in soil. *Journal of Bacteriology* **1**, 197-207. [3]

Consden, R. & Gordon, A. H. (1948). Effect of salt on partition chromatograms. *Nature (London)* **162**, 180-181. [1,2]

Corbaz, R., Gregory, P. H. & Lacey, M. E. (1963). Thermophilic and mesophilic actinomycetes in mouldy hay. *Journal of General Microbiology* **32**, 449-455. [3]

Corkill, J. E., Sisson, P. R., Smyth, A., Deveney, J., Freeman, R., Shears, P., Heaf, D. & Hart, C. A. (1994). Application of pyrolysis mass spectrometry and SDS-PAGE in the study of the epimediology of *Pseudomonas cepacia* in cystic fibrosis. *Journal of Medical Microbiology* **41**, 106-111. [1]

Couch, J. N. (1950). *Actinoplanes* a new genus of the *Actinomycetales*. *Journal of the Elisha Mitchell Scientific Society* **66**, 87-92. [3]

Couch, J. N. (1955). A new genus and family of the *Actinomycetales*, with a revision of the genus *Actinoplanes*. *Journal of Elisha Mitchell Scientific Society* **71**, 148-155. [2]

Couch, J. N. (1963). Some new genera and species of the *Actinoplanaceae*. *Journal of Elisha Mitchell Scientific Society* **79**, 53-70. [2]

Crameri, R. G., Hintermann, G. & Hütter, R. (1983). Deoxyribonucleic acid restriction endonuclease fingerprint characterization of actinomycete strains. *FEMS Microbiology Letters* **23**, 652-655. [3]

Craveri, R. & Pagani, H. (1962). Thermophilic micro-organisms among actinomycetes in the soil. *Ann Microbiol* **12**, 115-130. [3]

Crawford, D. L. (1975). Cultural, morphological, and physiological characteristics of *Thermomonospora fusca* (strain 190Th). *Canadian Journal of Microbiology* **21**, 1841-1848. [2]

Crawford, D. L. & M. A. Gonda (1977). The sporulation process in *Thermomonospora fusca* as revealed by scanning electronic microscopy and transmission electronic microscopy. *Canadian Journal of Microbiology* **23**, 1088-1095. [2]

Cross. T. (1968) Thermophilic actinomycetes. *Journal of Applied Bacteriology* **31**, 36-53. [2]

Cross, T. (1981). Aquatic actinomycetes: a critical survey of the occurrence, growth and role of actinomycetes in aquatic habitats. *Journal of Applied Bacteriology* **50,** 397-423. [2]

Cross, T. (1982). Actinomycetes: a continuing source of new metabolites. *Developments in Industrial Microbiology* **23**, 1-18. [2]

Cross, T. & Goodfellow, M. (1973). Taxonomy and classification of the actinomycetes. In *Actinomycetales : Characteristics and Practical Importance*, pp. 11-112. Edited by G. Sykes & F. A. Skinner. London: Academic Press. [2]

Cross, T. & Spooner, D. F. (1963). The serological identification of streptomycetes by agar gel diffusion techniques. *Journal of General Microbiology* **33**, 275-282. [3]

Cross, T., Lechevalier, M. P. & Lechevalier, H. (1963). A new genus of the *Actinomycetales*: *Microellobosporia* gen. nov. *Journal of General Microbiology* **31,** 421-429. [3]

Daffe, M., McNeil, M. & Brennan, P. J. (1993). Major structural features of the cell wall arabinogalactans of *Mycobacterium*, *Rhodococcus*, and *Nocardia*. **249**, 383-398. [1]

Dams, E., Hendriks, L., Van De Peer, Y., Neefs, J.-M., Smiths, G., Vandenkempt, J. & De Wachter, R. (1988). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* **16**, 87-175. [1]

De Boer, L., Dijkhuizen, L., Grobben, G., Goodfellow, M., Stackebrandt, E., Parlett, J. H., Whitehead, D. & Witt, D. (1990). *Amycolatopsis methanolica* sp. nov., a facultatively methylotrophic actinomycete. *International Journal of Systematic Bacteriology* **40,** 194-204. [2]

De Ley, J. (1970). Molecular techniques and applications in bacterial taxonomy. In *The* Actinomycetales, pp. 317-327. Edited by H. Prauser. Jena: Veb Gustav Fischer Verlag. [1]

De Rijk, P., Caers, A., Van de Peer, Y. & De Wachter, R. (1998). Database on the structure of large ribosomal subunit RNA. *Nucleic Acids Research* **26**, 183-186. [1]

De Smedt, J. & De Ley, J. (1977). Intra- and intergeneric similarities of *Agrobacterium* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* **27**, 222-240. [1]

De Zoysa, A., Efstratiou, A., George, R. C., Jahkola, M., Vuopio-Varkila, J., Deshevoi, S., Tseneva, G. & Rikushin, Y. (1995). Molecular epidemiology of *Corynebacterium diphtheriae* from northwestern Russia and surrounding countries studied by using ribotyping and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **33**, 1080-1083. [1]

Denhardt, D. T. (1966). A membrane-filter technique for the detection of complementary DNA. *Biochemical and Biophysical Research Communications* **23**, 641-646. [2,3]

Desai, A. J. & Dhala, S. A. (1967). *Streptomyces thermonitrificans* sp. n., a thermophilic streptomycete. *Antonie van Leeuwenhoek* **33**, 137-144. [3]

Dijkhuizen, L. (1996). Evolution of metabolic pathways. In *Evolution of Microbial Life*, pp. 243-266. Edited by D. McL. Roberts, P. Sharp, G. Alderson, & M. A. Collins. Cambridge: Cambridge University Press. [2]

Dittmer, J. C. F. & R. L. Lester (1964) A simple, specific spray for the detection of phospholipids on thin-layer chromatography. *Journal of Lipid Research* **5**, 126-127. [2]

Doering-Saad, C., Kämpfer, P., Manulis, S., Kritzman, G., Schneider, J., Zakerzewska-Czerwinska, J., Schrempf, H. & Barash, I. (1992). Diversity among *Streptomyces* strains causing potato scab. *Applied & Environmental Microbiology* **58**, 3932-3940. [1,3]

Deutsche Samlung von Mikroorganismen und Zellkulturen GmbH. (1998). *Bacterial Nomenclature up-to-date October 1998.* DSMZ-Deutsche Samlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany. [1,3,Appendix]

Dittmer, J. C. & Lester, R. L. (1964). A simple specific spray for the detection of phospholipids on thin-layer chromatography. *Journal of Lipid Research* **5**, 126-127. [1,2]

Domenech, P., Menendez, M. C. & Garcia, M. J. (1994). Restriction fragment length polymorphisms of 16S rRNA genes in the differentiation of fast-growing mycobacterial species. *FEMS Microbiology Letters* **116**, 19-24. [1]

Dryden, S. & Kaplan, S. (1990). Localization and structural analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides*. *Nucleic Acids Research* **18**, 7267-7277. [1]

Drucker, D. B. (1981). Analysis of structural components of microorganisms by gas chromatography. In *Microbiological applications of gas chromatography*, pp. 166-296. Edited by D. B. Drucker. Cambridge: Cambridge University Press. [1]

Duerden, B. I., Eley, A., Goodwin, L., Magee, J. T., Hindmarch, J. M. & Bennett, K. W. (1989). A comparison of *Bacteroides ureolyticus* isolates from different clinical sources. *Journal of Medical Microbiology* **29**, 63-73. [2]

Eardly, B. D. Wang, F.-S. & Berkum, P. van. (1996). Corresponding 16S rRNA gene segments in *Rhizobiaceae* and *Aeromonas* yield discordant phylogenies. *Plant and Soil* **186**, 69-74. [1]

East, A. K., Allaway, D. & Collins, M. D. (1992). Analysis of DNA encoding 23S rRNA and 16S-23S rRNA intergenic spacer regions from *Plesiomonas shigelloides*. *FEMS Microbiology Letters* **95**, 57-62. []

Edwards, C. (1990) Thermopiles. In *Microbiology of Extreme Environments*, pp. 1-32. Edited by C. Edwards. Milton Keynes: Open University Press. [2]

Efron, B. & Gong, G. (1983). A leisurely look at the bootstrap, the jacknife, and cross-validation. *American Statistician* **37**, 36-48. [1]

Embley, T. M. (1991). The linear PCR reaction: a simple and robust method for sequencing amplified rDNA genes. *Letters in Applied Microbiology* **13**, 171-174. [1]

Embley, T. M. (1992). The family *Pseudonocardiaceae*. In *The Prokaryotes*, 2nd Edition, pp. 996-1027. Edited by A. Balows, H.G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Berlin: Springer-Verlag. [2]

Embley, T. M. & Stackebrandt, E. (1994). The molecular phylogeny and systematics of the actinomycetes. *Annual Reviews of Microbiology* **48**, 257-289. [1, 2, 3]

Embley, T. M. & Stackebrandt, E. (1997) Species in practice: exploring unclutured prokaryote diversity in natural samples. In *Species: The Units of Biodiversity*, pp. 61-82. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall. [1]

Embley, T. M. & Wait, R. (1994). Structural lipids of eubacteria. In *Chemical methods in Prokaryotic Systematics*, pp. 121-161. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: John Wiley and Sons. [1]

Embley, T. M., Goodfellow, M., Minnikin, D. E. & Austin, B. (1983). Fatty acids, isoprenoid quinone and polar lipid composition of *Renibacterium salmoninarum*. *Journal of Applied Bacteriology* **55**, 37. [1]

Embley, T.M., O'donnell, A. G., Rostron, J & Goodfellow, M. (1988a). Chemotaxonomy of wall type IV actinomycetes which lack mycolic acids. *Journal of General Microbiology* **134**, 953-960. [2]

Embley, T. M., Smida, J. & Stackebrandt, E. (1988b). The phylogeny of mycolate-less wall chemotype IV actinomycetes and description of *Pseudonocardiaceae* fam. nov. *Systematic and Applied Microbiology* **11**, 44-52. [2]

Ettlinger, L., Corbaz, R. & Hütter, R. (1958). Zur Systematik der Actinomyceten. 4. Eine Arteinteilung der Gattung *Streptomyces* Waksman et Henrici. *Archiv für Mikrobiologie* **31**, 326-358. [3]

Exmelin, L., Malbruny, B.,Vergnaud, M., Prosvost, F., Boiron, P & Morel, C. (1996). Molecular study of nosocomial nocardiosis outbreak inovlving heart transplant recipients. *Journal of Clinical Microbiology* **34**, 1014-1016. [1]

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane- filter

hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *International Journal of Systematic Bacteriology* **39**, 224-229. [1]

Falcäo de Morais, J. O., Batista, A. C. & Masa, D. M. G. (1966). *Elytrosporangium*: a new genus of the *Actinomycetales*. *Mycopathologia et Mycologia Applicata* **30**, 161-171. [3]

Farina, G. & Bradley, S. G. (1970). Reassociation of deoxyribonucleic acids from *Actinoplanes* and other actinomycetes. *Journal of Bacteriology* **102**, 30-35. [3]

Felsenstein, J. (1978). Numerical methods for inferring evolutionary trees. *Quarterly Review Biology* **57**, 379-404. [1]

Felsenstein, J. (1981). Evolutionary tree from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368-376. [1,2,3]

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791. [1,2,3]

Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* **22**, 521-565. [1,2,3]

Felsenstein, J. (1993). *PHYLIP (phylogenetic inference package),* version 3.5c. Seattle: Department of Genetics, University of Washington. [1,2,3]

Fergus, C. L. (1964). Thermophilic and thermotolerant moulds and actinomycetes of mushroom compost during peak heating. *Mycologia* **56**, 267-284. [3]

Ferguson, E. V., Sanglier, J. -J., Ward, A. C. & Goodfellew, M. (1997). Evalaution of *Streptomyces* species-groups by pyrolysis mass spectrometry. *Zentralblatt für Bakteriologie* **285**, 169-181. [3]

Fischer, A., Kroppenstedt, R. M. & Stackebrandt. E. (1983). Molecular-genetic and chemotaxonomic studies on *Actinomadura* and *Nocardiopsis*. *Journal of General Microbiology* **129**, 3433-3446. [2]

Fitch, W. M. (1971). Towards defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* **20**, 406-416. [1,2,3]

Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome c sequences is of general applicability. *Science* **155**, 279-284. [1,2,3]

Flaig, W. & Kutzner, H. J. (1960). Beitrag zur Systematik der Gattung *Streptomyces* Waksman et Henrici. *Archiv für Mikrobiologie* **35**, 105-138. [3]

Fournier, P. E., Roux, V. & Raoult, D. (1998). Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. IJSB **48**, 839-849. [1]

Fowler, V. J., Ludwig, W. & Stackebrandt, E. (1985). Ribosomal ribonucleic acid cataloguing in bacterial systematics: the phylogeny of *Actinomadura*. In *Chemical methods in bacterial systematics*, 1st edition, pp. 17-40. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press. [2]

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic Bacteriology* **42**, 166-170. [1]

Freeman, R., Sisson, P. R., Lightfoot, N. F. & McLauchlin, J. (1991a). Analysis of epidemic and sporadic strains of *Listeria monocytogenes* by pyrolysis mass spectrometry. *Letters in Applied Microbiology* **12**, 133-136. [1]

Freeman, R., Gould, F. K., Sisson, P. R. & Lightfoot, N. F. (1991b). Strain differentiation of capsule type 23 penicillin-resistant *Streptococcus pneumoniae* from nosocomial infections by pyrolysis mass spectrometry. *Letters in Applied Microbiology* **13**, 28-31. [1]

Freeman, R., Sisson, P. R., Noble, W. C. & Lightfoot, N. F. (1997). An apparent outbreak of infection with *Acinetobacter calcoaceticus* reconsidered after investigation by pyrolysis mass spectrometry. *Zentralblatt für Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **285**, 234-244. [1]

Frothingham, R. & Wilson, K. H. (1993). Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *Journal of Bacteriology* **175**, 2818-2825. [1]

Fry, N. K., Warwick, S., Saunders, N. A. & Embley, T. M. (1991). The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family *Legionellaceae*. *Journal of General Microbiology* **137**, 1215-1222. [1]

Funke, G., Osorio, C. R., Frei, R., Riegel, P. & Collins, M. D. (1998). *Corynebacterium confusum* sp. nov., isolated from human clinical specimens. *International Journal of Systematic Bacteriology* **48**, 1291-1296. [1]

Gadkari, D., Schricker, K., Acker, G., Kroppenstedt, R. M. & Meyer, O. (1990). *Streptomyces thermoautotrophicus* sp. nov., a thermophilic CO- and H_2-oxidizing obligate chemolithotroph. *Applied Environmental Microbiology* **56**, 3727-3734. [2,3]

Garnier, T., Canard, B. & Cole, S. T. (1991). Cloning, mapping, and molecular characterization of the rRNA operons of *Clostridium perfringens*. *Journal of Bacteriology* **173**, 5431-5438. [1]

Gauze, G. F., Preobrazhenskaya, T. P., Kudrina, E. S., Blinov, N. O., Ryabova, I. D. & Sveshnikova, M. A. (1957). *[Problems of classification of actinomycetes-antagonists (in Russian).* Government Publishing House of Medical Literature, Medzig, Moscow. [3]

Gauze, G. F., Maksimova, T. S., Olkhovatova, O. L., Sveshnikova, M. A., Kochetkova, G. V. & Ilchenko, G. B. (1974). Production of madumycin, an antibacterial antibiotic, by *Actinomadura flava* sp. nov. *Antibiotiki* **19,** 771-775. [2]

Gauze, G. F., Preobrazhenskaya, T. P., Sveshnikova, M. A., Terekhova, L. P. & Maximova, T. S. (1983). *A guide for the determination of actinomycetes. Genera* Streptomyces, Streptoverticillium, *and* Chainia. Moscow: Nauka. [3]

Gerke, C. W., McCune, R. A., Gama-Sosa, M. A., Ehrlich, M. & Kuo, K. C. (1984). Quantitative reversed-phase high-performance liquid chromatography of major and modified nucleosides in DNA. *Journal of Chromatography* **301,** 199-219. [2,3]

Gilardi, E., Hill, L. R., Turri, M. & Silvestri, L. G. (1960). Quantitative methods in the systematics of *Actinomycetales*. I. *Giornale di Microbiologia* **8,** 203-218. [3]

Gilbert, R. (1904). Über *Actinomyces thermophilus* und andere Actinomyceten. *Z Hyg Infektionskr* **47,** 383-406. [3]

Gillespie, D. & Spiegelman, S. (1965). A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *Journal of Molecular Biology* **12,** 829-842. [2]

Gochnauer, M. B., Leppard, G. G., Komaratat, P., Kates, M., Novitsky, T. & Kushner, D. J. (1975). Isolation and characterisation of *Actinopolyspora halophila* gen. et sp. nov., an extremly halophilic actinomycete. *Canadian Journal of Microbiology* **21,** 1500-1511. [2]

Goodacre, R. & Kell, D. B. (1996). Pyrolysis mass-spectrometry and its applications in biotechnology. *Current Opinion in Biotechnology* **7,** 20-28. [3]

Goodacre, R., Timmins, E. M., Burton, R., Kaderbhai, N., Woodward, A. M., Kell, D. B. & Rooney, P. J. (1998). Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiology* **144,** 1157-1170 [1]

Goodfellow, M. (1989). The actinomycetes I. Suprageneric classification of actinomycetes. In *Bergey's manual of systematic bacteriology*, Volume 4, pp. 2333-2339. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins. [3]

Goodfellow, M. (1992). The family *Nocardiaceae.* In *The Prokaryotes*, 2nd edition, pp. 1188-1213. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag. [1]

Goodfellow, M (1995) Inter-strain comparison of pathogenic microorganisms by pyrolysis mass-spectrometry. *Binary-Computing in Microbiology* **7,** 54-60. [1,3]

Goodfellow, M. (1998). Numerical taxonomy. In *Biology of the Prokaryotes.* Edited by H. G. Schlegel & J. Lengeler, W. George. Stuttgart: Thieme Verley (in press). [2]

Goodfellow, M. & Cross, T. (1974). Actinomycetes. In *Biology of Plant Litter Decomposition.* pp. 269-302. Edited by C. H. Dickinson & G. J. F. Pugh. London: Academic Press. [1]

Goodfellow, M. & Cross, T. (1984). Classification. In *The Biology of the Actinomycetes,* pp. 7-164. Edited by M. Goodfellow, M. Mordarski & S.T. Williams. London: Academic Press. [1,2]

Goodfellow, M. & Dickinson, C. H. (1985) Delineation and description of microbial populations using numerical methods. In *Computer-Assisted Bacterial Systematics*, pp. 165-225. Edited by M. Goodfellow, D. Jones & F. G. Priest. London: Academic Press. [1]

Goodfellow, M. & Minnikin, D. E. (1981). Introduction to the coryneform bacteria. In: *The Prokaryotes*, Volume II, pp. 1811-1826. Edited by M. P. Starr, H. Stolp, H. G. Truper, A. Balows & H. G. Schlegel. Berlin: Springer Verlag. [1]

Goodfellow, M. & Minnikin, D. E. (eds) (1985) *Chemical Methods in Bacterial Systematics.* London: Academic Press. [1]

Goodfellow, M. & O'Donnell, A. G. (1989). Search and discovery of industrially-significant actinomycetes. In *Microbial products: new approaches*, pp. 343-383. Edited by S. Baumberg, I. S. Hunter & P. M. Rhodes. Cambridge: Cambridge University Press. [1]

Goodfellow, M. & O'Donnell, A. G. (1993). Roots of bacterial systematics. In *Handbook of New Bacterial Systematics,* pp. 3-54, Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1,2,3]

Goodfellow, M. & O'Donnell, A. G. (1994). Chemosystematics: current state and future prospects. In *Chemical methods in prokaryotic systematics*, pp. 1-20. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: John Wiley & Sons. [1]

Goodfellow, M. & Pirouz, T. (1982). Numerical classification of sporoactinomycetes containing *meso*-diaminopimelic acid in the cell wall. *Journal of General Microbiology* **128**, 503-527. [2]

Goodfellow, M. & Simpson, K. E. (1987). Ecology of streptomycetes. *Frontiers in Applied Microbiology* **2**, 97-125. [3]

Goodfellow, M. & Wayne, L. G. (1982). Taxonomy and nomenclature. In *The biology of the Mycobacteria. Physiology, Identification and Classification*, vol. I. pp. 471-521. Edited by C. Ratledge & J. S. Standford. London: Academic Press. [1]

Goodfellow, M., Alderson, G. & Lacey, J. (1979). Numerical taxonomy of *Actinomadura* and related actinomycetes. *Journal of General Microbiology* **112**, 95-111. [2]

Goodfellow, M., Jones, D. & Priest, F. G. (1985) *Computer-Assisted Bacterial Systematics.* London: Academic Press. [1]

Goodfellow, M., Williams, S. T. & Alderson, G. (1986a). Transfer of *Actinosporangium violaceum* Krasil'nikov and Yuan, *Actinosporangium vitaminophilum* Shomura *et al.* and *Actinopycnidium caeruleum* Krasil'nikov to the genus *Streptomyces*, with amended descriptions of the species. *Systematic and Applied Microbiology* **8**, 61-64. [3]

Goodfellow, M., Williams, S. T. & Alderson, G. (1986b). Transfer of *Chainia* species to the genus *Streptomyces* with emended description of species. *Systematic and Applied Microbiology* **8**, 55-60. [3]

Goodfellow, M., Williams, S. T. & Alderson, G. (1986c). Transfer of *Elytrosporangium brasiliense* Falcão de Morais *et al.*, *Elytrosporangium carpinense* Falcão de Morais *et al.*, *Elytrosporangium spirale* Falcão de Morais, *Microellobosporia cinerea* Cross *et al.*, *Microellobosporia flavea* Cross *et al.*, *Microellobosporia grisea* (Konev *et al.*) Pridham and *Microellobosporia violacea* (Tsyganov *et al.*) Pridham to the genus *Streptomyces*, with emended descriptions of the species. *Systematic and Applied Microbiology* **8**, 48-54. [3]

Goodfellow, M., Williams, S. T. & Alderson, G. (1986d). Transfer of *Kitasatoa purpurea* Matsumae and Hata to the genus *Streptomyces* as *Streptomyces purpureus comb. nov. Systematic and Applied Microbiology* **8**, 65-66. [3]

Goodfellow, M., Lacey J. & Todd, C. (1987). Numerical classification of thermophilic streptomycetes. *Journal of General Microbiology* **133**, 3135-3149. [1,2,3]

Goodfellow, M., Stackebrandt, E. & Kroppenstedt, R. M. (1988). Chemotaxonomy and actinomycete systematics, pp. 11-18. In *Biology of actinomycetes '88.* Edited by Y. Okami, T. Beppu & H. Ogawara. Tokyo: Japan Scientific Societies Press. [2]

Goodfellow, M., Stanton, L. J., Simpson, K. E. & Minnikin, D. E. (1990). Numerical and chemical classification of *Actinoplanes* and some related actinomycetes. *Journal of General Microbiology* **136**, 19-36. [2]

Goodfellow, M., Ferguson, E. V. & Sanglier, J. J. (1992). Numerical classification and identification of *Streptomyces* species. *Gene* **115**, 225-233. [3]

Goodfellow, M., Manfio, G. P. & Chun, J. (1997a). Towards a practical species concept for cultivatable bacteria. In *Species: The units of biodiversity,* pp. 25-59. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall. [1,3]

Goodfellow, M, Freeman, R, Sisson, P. R. (1997b). Curie-point pyrolysis mass spectrometry as a tool in clinical microbiology *Zentralblatt fur Bakteriologie - International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **285**, 133-156. [1,3]

Goodfellow, M., Brown, A., Cia, J., Chun, J. & Collins, M. D. (1997c). *Amycolatopsis japonicum* sp. nov., an actinomycete producing (S,S)-N,N'-ethylenediaminedisuccinic acid. *Systematic and Applied Microbiology* **20**, 78-84. [2]

Ghuysen, J. M. (1968). Use of bacteriolytic enzymes in determination of of wall structure and their role in cell metabolism. *Bacteriological Reviews* **32**, 425-464. [1]

Gordon, R. E. & Mihm, J. M. (1962). Identification of *Nocardia caviae* nov. comb. *Annals of the New York Academy of Sciences* **98**, 628-636. [2,3]

Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H. N. (1974). *Nocardia coeliaca, Nocardia autotrophica*, and the Nocardin strain. *International Journal of Systematic Bacteriology* **24**, 54-63. [2]

Gottlieb, D. & Shirling, E. B. (1967). Cooperative description of type cultures of *Streptomyces*. I. The International *Streptomyces* Project. *International Journal of Systematic Bacteriology* **17**, 315-322. [3]

Gottlieb, D. (1961). An evaluation of criteria and procedures used in the description and characterization of the streptomycetes. A co-operative study. *Applied Microbiology* **9**, 55-65. [3]

Gottlieb, D. (1963). Recommendations for description of some *Actinomycetales* appearing in patent applications. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **13**, 159-160. [3]

Gottlieb, D. (1973). General consideration and implications of the *Actinomycetales*. In *Actinomycetales: Characteristics and Practical Importance*, pp. 1-10. Edited by G. Skyes & F. A. Skinner. London: Academic Press. [1]

Gottlieb, D. (1974). *Actinomycetales*. In *Bergey's Manual of Determinative Bacteriology*, pp. 657-659. Edited by R. E. Buchnan & N. E. Gibbons. Baltimore: The Williams and Wilkins. [1]

Gould, F. K., Freeman, R., Sisson, P. R., Cookson, B. D. & Lightfoot, N. F. (1991). Inter-strain comparison by pyrolysis mass spectrometry in the investigation of *Staphylococcus aureus* nosocomial infection. *Journal of Hospital Infection* **19**, 41-48. [1]

Goyer, C., Otrysko, B. & Beaulieu, C. (1996). Taxonomic studies on streptomycetes causing potato common scab. - A review *Canadian Journal of Plant Pathology* **18**, 107-113. [3]

Gray, M. W. & Spencer, D. F. (1996). Organellar evolution. In *Evolution of Microbial Life*, pp. 109-126. Edited by D. McL. Roberts, P. Sharp, G. Alderson, & M. A. Collins. Cambridge: Cambridge University Press. [2]

Greiner-Mai, E., Kroppenstedt, R. M., Korn-Wendisch, F. & Kutzner, H. J. (1987). Morphological and biochemical characterization and emended descriptions of thermophilic actinomycetes species. *Systematics and Applied Microbiology* **9**, 97-109. [2]

Grimont, F. & Grimont, P. (1986). Ribosomal nucleic acids gene restriction patterns as possible taxonomic tools. *Ann Inst Pasteur / Microbiol* (Paris) **137B**, 165-175. [1]

Grimont, F., Verger, J. M., Cornelis, P., Limet, J., Lefevre, M., Grayon, M., Regnault, B., Van Broeck, J. & Grimont, P. A. (1992). Molecular typing of *Brucella* with cloned DNA probes. *Research in Microbiology* **143**, 55-65. [1]

Grund, E. & Kroppenstedt, R. M. (1989). Tansfer of five nocardiopsis species to the genus Saccharothrix Labeda et al. 1984. *Journal of Systematic Applied Microbiology* **12**, 267-274. [2]

Grund, E. & Kroppenstedt, R. M. (1990). Chemotaxonomy and numerical taxonomy of the genus *Nocardiopsis* Meyer 1976. *International Journal of Systematic Bacteriology* **40**, 5-11. [2]

Guerrero, C., Bernasconi, C., Burki, D., Bodmer, T. & Telenti, A. (1995). IS*1245*: a novel insertion element from *Mycobacterium avium* is a specific marker for analysis of strain relatedness. *Journal of Clinical Microbiology* **33**, 304-307. [1]

Gunstone, F. D. & Jacobsberg, F. R. (1972). Fatty acids, part 35: The preparation and properties of a complete series of methyl epoxyoctadecanoates. Chemestry and Physics of Lipids **9**, 26-64. [2]

Gürtler, V. & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **42**, 3-16. [1,3]

Gutteridge, C. S. (1987). Characterisation of microorganisms by pyrolysis mass spectrometry. *Methods in Microbiology* **19**, 227-272. [1]

Gyllenberg, H. G. (1970). Factor analytical evaluation of patterns of correlated characteristics in streptomycetes. In *The Actinomycetales*, pp. 101-105. Edited by H. Prauser. Jena: Veb Gustav Fischer Verlag. [3]

Gyllenberg, H. G., Niemelä, T. K. & Niemi, J. S. (1975). A model for automatic identification of streptomycetes. *Postepy Higieni i Medycyny Doswiadezalnej* **29**, 357-383. [3]

Hackel, C., Houard, S., Portaels, F., van Elsen, A., Herzog, A. & Bollen, A. (1990). Specific identification of *Mycobacterium leprae* by the polymerase chain reaction. *Molecular & Cellular Probes* **4**, 205-210. [1]

Hain, T., Ward-Rainey, N., Kroppenstedt, R. M., Stackebrandt, E. & Rainey, F. A. (1997) Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S-23S ribosomal DNA intergenic spacers. *International Journal of Systematic Bacteriology* **47**, 202-206. [1,3]

Hamid, M. E. (1994). *Classification and Identification of Actinomycetes Associated with Bovine Farcy*. Ph.D. Thesis, University of Newcastle upon Tyne. [1]

Hancock, I. C. (1994). Analysis of cell wall constituents of Gram-positive bacteria. In *Chemical methods in prokaryotic systematics*, pp. 63-84. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: John Wiley & Sons. [1]

Hartford, T. & Sneath, P. H. A. (1988). Distortion of taxonomic structure from DNA relationships due to different choice of reference strains. *Systematic and Applied Microbiology* **10**, 241-250. [3]

Hartford, T. & Sneath, P. H. A. (1990). Experimental error in DNA-DNA pairing: a survey of the literature. *Journal of Applied Bacteriology* **68**, 527-542. [1,3]

Harz, C. O. (1877) *Actinomyces bovis*, ein neuer Schimmel in den Geweben des Rindes. *Jahresbericht der Königlichen Centralen Thierarzneischule Müchen für 1877/1878* **5**, 125-140. [3]

Hasegawa, T. (1988). *Actinokineospora* a new species of the *Actinomycetales*. *Actinomycetologica* **2**, 31-45. [2]

Hasegawa, T., Tanida, S. & Ono, H. (1986). *Thermomonospora formosensis* sp. nov. *International Journal of Systematic Bacteriology* **36**, 20-23. [2]

Hatano, K., Nishii, T. & Mordarska, H. (1997). *Streptomyces spitzbergensis* Wieczorek *et al.* 1993 is a later subjective synonym of *Streptomyces baldacii* (Farina and Locci 1966) Witt and Stackebrandt 1991. *International Journal of Systematic Bacteriology* **47**, 573-574. [3]

Hatano, K., Tamura, T. & Nishii, T. (1994). Taxonomic status of *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66. *Actinomycetologica* **8**, 47-50. [3]

Healy, F. G. & Lambert, D. H. (1991). Relationships among *Streptomyces* spp. causing potato scab. *International Journal of Systematic Bacteriology* **41**, 479-482. [3]

Heinonen, T. Y., Schnare, M. N. & Gray, M. W. (1990). Sequence heterogeneity in the duplicate large subunit ribosomal RNA genes of *Tetrahymena pyriformis* mitochondrial DNA. *Journal of Biological Chemistry* **265**, 22336-22341. [1]

Henssen, A. (1957a). Beitrage zur Morphologie und Systematik der thermophilen Actinomyceten. (Contibutions to the morphology and systematics of the thermophilic actinomycetes.) *Archiv für Mikrobiologie* **26**, 373-414. [2,3]

Henssen, A. (1957b). Über die Bedeutung der thermophilen Mikroorganismen für der Zersetzung des Stallmistes. *Archiv für Mikrobiologie* **27**, 63-81. [3]

Henssen, A. (1970). Spore formation in thermophilic actinomyceetes, pp. 205-210. In *The Actinomycetales*. Edited by H. Prauser. Jena: Gustav Fischer Verlag. [2]

Henssen, A. & Schäfer, D. (1971). Amended description of the genus *Pseudonocardia* Henssen and description of a new species *Pseudonocardia spinosa* Schäfer. *International Journal of Systematic Bacteriology* **21**, 29-34. [2]

Henssen, A & Schnepf, E. (1967). Zur kenntnis thermophiler Actinomyceten. *Archiv für Mikrobiologie* 57, 214-231. [2]

Henssen, A., Kothe, H. W. & Kroppenstedt, R. M. (1987). Transfer of *Pseudonocardia azurea* and "*Pseudonocardia fastidiosa*" to the genus *Amycolatopsis*, with emended species description. *International Journal of Systematic Bacteriology* **37**, 292-295. [2]

Hesseltine, C. W., Benedict, R. G. & Pridham, T. G. (1954). Useful criteria for species differentiation in the genus *Streptomyces*. *Annals of the New York Academy of Sciences* **60**, 136-151. [3]

Hill, L. R. & Silvestri, L. G. (1962). Quantitative methods in the systematics of *Actinomycetales*. III. The taxonomic significance of physiological-biochemical characters and the construction of a diagnostic key. *Giornale di Microbiologia* **10**, 1-28. [3]

Hill, L. R., Turri, M., Gilardi, E. & Silvestri, L. G. (1961). Quantitative methods in the systematics of *Actinomycetales*. - II. *Giornale di Microbiologia* **9**, 56-72. [3]

Hillis, D. M., Allard, W. & Miyamoto, M. M. (1993). Analysis of DNA sequence data: phylogenetic inferrence. *Methods in Enzymology* **224**, 456-487. [1]

Hirayama, H., Tamaoka, J. & Horikoshi, K. (1996). Improved immobilization of DNA to microwell plates for DNA-DNA hybridization. *Nucleic Acids Research* 24, 4098-4099. [1]

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology, Ninth Edition.* Baltimore: Williams & Wilkins. [2]

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology.* 9th ed. Williams & Wilkins, Co., Baltimore. [3]

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985). *Genetic manipulation of Streptomyces. A laboratory manual.* Norwich: The John Innes Foundation. [2,3]

Huber, R., Langworthy, T. A., Köning, H., Thomm, M., Woese, C. R., Sleytr, U. B. & Stetter, K. O. (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C. *Archives of Microbiology*. **144**, 324-333. [2]

Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., König, H., Rachel, R., Rockinger, I., Fricke, H. & Stetter, K. O. (1992). *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacterium. *Systematic and Applied Microbiology* **15**, 340-351. [2]

Hull, D. L. (1997). The ideal species concept - and why we can't get it. In *Species: The Units of Biodiversity*, pp. 357-380. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall. [1]

Hütter, R. (1967). Systematik der Streptomyceten unter besonderer Berücksichtigung der von ihnen gebildeten Antibiotica. S. Karger. Basel, Switzerland. [3]

Iinuma, S., Yokota, A., Hasegawa, T. & Kanamaru, T. (1994) *Actinocorallia* gen. nov., a new genus of the order *Actinomycetales*. *International Journal of Systematic Bacteriology* 44, 230-234. [2]

Imhoff, J. F. & Suling, J. (1996). The phylogenetic relationship among *Ectothiorhodospiraceae* - a reevaluation of their taxonomy on the basis of 16S rDNA analyses. *Archives of Microbiology* **165,** 106-113. [1]

Ingledew, W. J. & Poole, R. K. (1984). The respiratory chains of *Escherichia coli*. *Microbiological Reviews* **48,** 222-271. [1]

Irwin, W. J. (1982). *Analytical Pyrolysis: A Comprehensive Guide*. New York: Marcel Dekker. [1]

Itoh, T., Kudo, T., Parenti, F. & Seino, A. (1989). Amended description of the genus *Kineosporia*, based on chemotaxonomic and morphological studies. *International Journal of Systematic Bacteriology* **39**, 168-173. [3]

Jacin, H. & Mishkin, A. R. (1965). Separation of carbohydrates on borate impregnated silica gel G plates. *Journal of Chromatography* **18,** 170-173. [1,2]

Jahnke, K. D. (1994). A modified method of quantitative colorimetric DNA-DNA hybridization on membrane filters for bacterial identification. *Journal of Microbiological Methods* **20**, 273-288. [1]

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. & Kersters, k. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiolgy* **142**, 1881-1893. [1]

Jensen, H. L. (1930). Actinomycetes in Danish soils. *Soil Science* **30**, 59-77. [2]

Jensen, H. L. (1931a). Contribution to our knowledge of *Actinomycetales*. II. The definition and subdivision of the genus *Actinomyces*, with a preliminary account of Australian soil actinomycetes. *Proceedings of the Linnean Society of New South Wales* **56**, 345-370. [3]

Jensen, H. L. (1931b). The microbiology of farmyard manure decomposition in soil. I. Changes in the microflora and their relation to nitrification. *Journal of Agricultural Science* **21**, 38. [3]

Jensen, K. A. (1932). Reinzuch und Typen Bestimmung von Tuberkelbazillenstämmen. *Zentralblatt für Bakteriologie.* **125**, 222-239. [2]

Ji, Y. E., Colston, M. J. & Cox, R. A. (1994). The ribosomal RNA (rrn) operons of fast-growing mycobacteria: Primary and secondary structures and their relation to rrn operons of pathogenic slow-growers. *Microbiology* **140**, 2829-2840. [1]

Jiang, C., Xu, L., Yang, Y.-R., Guo, G.-Y., Ma, J. & Liu, Y. (1991). *Actinobispora*, a new genus of the order *Actinomycetales*. *International Journal of Systematic Bacteriology* **41**, 526-528. [2]

Jin, L. & Nei, M. (1990). Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molecular Biology and Evolution* **7**, 82-102. [1]

Johnson, J. L. (1989). Bacterial classification III. Nucleic acids in bacterial classification. In *Bergey's manual of systematic bacteriology*, vol. 4, pp. 2306-2309. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins. [1]

Johnson, J. L. (1991). DNA reassociation experiments. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 21-44. Edited by E. Stackebrandt & M. Goodfellow. Chichester: John Wiley and Sons. [1]

Johnson, J. L. & Ault, D. A. (1978). Taxonomy of the *Bacteroides*. II. Correlation of phenotypic characterizations with deoxyribonucleic acid homology groupings for *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. *International Journal of Systematic Bacteriology* **28**, 257-265. [1]

Jones, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *Journal of Bacteriology* **57**, 141-145. [2,3]

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian protein metabolism*, vol. 3, pp. 21-132. Edited by H. N. Munro. New York: Academic Press. [1,2,3]

Kämpfer, P. & Kroppenstedt, R. M. (1991). Probabilistic identification of streptomycetes using miniaturized physiological tests. *Journal of General Microbiology* **137**, 1893-1902. [3]

Kämpfer, P., Kroppenstedt, R. M. & Dott, W. (1991). A numerical classification of the genera *Streptomyces* and *Streptoverticillium* using miniaturized physiological tests. *Journal of General Microbiology* **137**, 1831-1891. [3]

Kanaujia, G. V., Katoch, V. M., Shivannavar. C. T., Sharma, V. D. & Patil, M. A. (1991). Rapid characterization of *Mycobacterium fortuitum-chelonae* complex by restriction fragment length polymorphism of ribosomal RNA genes. *FEMS Microbiology Letters* **77**, 205-208. [1]

Kataoka, M., Ueda, K., Kudo, T., Seki, T. & Yoshida, T. (1997). Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus *Streptomyces*. *FEMS Microbiology Letters* 151, 249-255. [3]

Kates, M. (1964). Bacterial lipids. *Advances in Lipid Research* **2**, 17-90. [1]

Kawaguchi, A., Seyama, Y., Sasaki, K., Okuda, A. & Yamakawa, T. (1979). Thermal regulation of fatty acid synthetase from *Brevibacterium ammoniagenes*. *Journal of Biochemistry* **85**, 865-869. [1]

Kay, H. E., Coutinho, H. L. C., Fattori, M., Manfio, G. P., Goodacre, R., Nuti, M. P., Basaglia, M. & Beringer, J. E. (1994). The identification of *Bradyrhizobium japonicum* strains isolated from italian soils. *Microbiology* **140**, 2333-2339. [1]

Kilpper-Bälz, R. (1991). DNA-rRNA hybridization. In *Nucleic acid techniques in bacterial systematics*, pp. 45-68. Edited by E. Stackebrandt & M. Goodfellow. New York: John Wiley and Sons. [1,2,3]

Kim, D., Chun, J., Sahin, N., Hah, Y.-C. & Goodfellow, M. (1996). Analysis of thermophilic clades within the genus *Streptomyces* by 16S ribosomal DNA sequence comparisons. *International Journal of Systematic Bacteriology* **46**, 581-587. [1,2,3]

Kim, S. B. & Goodfellow, M. (1998). Reclassification of *Amycolatopsis rugosa* Lechvalier *et al.* 1986 as *Prauserella rugosa* gen. nov., sp. com. nov. *International Journal of Systematic Bacteriology* (in press) [1,2]

Kim, S. B., Yoon, J. H., Kim, H., Lee, S. T., Park, Y. H. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Saccharomonospora* conducted with 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* **45**, 351-356. [1,2]

Kim. S. B., Falconer, C., Williams, E. & Goodfellow, M. (1998). *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., Two moderately thermophilic carboxydotrophic strains from soil. *International Journal of Systematic Bacteriology* **48**, 59-68. [2,3]

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111-120. [1,2,3]

Klijn, N., Weerkamp, A. H. & de Vos W. M. (1991). Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. *Applied & Environmental Microbiology* **57**, 3390-3393. [1]

Korn, F., Weingärtner, B. & Kutzner, H. J. (1978). A study of twenty actinophages: morphology, serological relationships and host range. In *Genetics of the* Actinomycetales, pp. 251-270. Edited by E. Freerksen, I. Tarnok & J. Thumin. Stuttgart: Gustav Fischer Verlag. [3]

Korn-Wendisch, F. & Kutzner, H. J. (1992). The family *Streptomycetaceae*. In *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*, 2nd ed., vol. 1., pp. 921-995. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. -H. Schleifer. New York: Springer-Verlag. [2, 3]

Korn-Wendish, F. & Schneider, J. (1992). Phage typing - a useful tool in actinomycete systematics. *Gene* **115**, 243-247. [3]

Korn-Wendish, F., Kempf, A., Grund, E., Kroppenstedt, R. M. & Kutzner, H. J. (1989). Transfer of *Faenia rectivirgula* Kurup and Agre 1983 to the genus *Saccharopolyspora* Lacey and Goodfellow 1975, elevation of *Saccharopolypsora hirsuta* subsp. *taberi* Labeda 1987 to species level, and

emended description of the genus *Saccharopolyspora*. *International Journal of Systematic Bacteriology* **39**, 430-441. [2]

Korn-Wendisch, F., Rainey, F., Kroppenstedt, R. M., Kempf, A., Majazza, A., Kutzner, H. J. & Stackebrandt, E. (1995). *Thermocrispum* gen. nov., a new genus of the order *Actinomycetales*, and description of *Thermocrispum municipale* sp. nov. and *Thermocrispum agreste* sp. nov. *International Journal of Systematic Bacteriology* **45**, 67-77. [2]

Krainsky, A. (1914). Die Aktinomyceten und ihren Bedeutung in der Natur. Zentralblatt für Bakteriologie Parasitendkunde Infektionskrankheiten und Hygiene, Abteilung II **41**, 649-688. [3]

Krassilnikov, N. A. (1941). *Keys to Actinomycetales*. Moscow: Akademii Nauk SSSR. (In Russian). [2,3]

Krassilnikov, N. A. (1949). *Opredelitelv Bakterii i Actinomicetov*. Moscow: Akademii Nauk SSSR. [1,3]

Krassilnikov, N. A. (1962). A new actinomyces genus *Actinopycnidium* n. gen. of the *Actinomycetaceae* family (in Russian). *Mikrobiologiya (Moscow)* **31**, 250-253. [3]

Krassilnikov, N. A. & Agre, N. S. (1964a). On two new species of *Thermopolyspora*. *Hind. Antibiot. Bull.* **6**, 97-107. [2]

Krassilnikov, N. A. & Agre, N. S. (1964b). A new genus of the actinomycetes - *Actinobifida*. the yellow group *Actinobifida dichotomica Mikrobiologiya* **33**, 935-943. [2]

Krassilnikov, N. A. & Agre, N. S. (1965). Two brown group of *Actinobifida chromogena* n. sp. *Mikrobiologiya* **34**, 284-291. [2]

Krassilnikov, N. A. & Yuan, T. (1961). *Actinosporangium* - a new genus in the family *Actinoplanaceae*. *Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya* **8**, 113-116. [3]

Krassilnikov, N. A., Agre, N. S., Dorokhova, L. A. & Sokolov, A. A. (1968a). Three new species of thermophilic actinomycetes. *Mikrobiologiya* **37**, 75-83 (in Russian). [3]

Krassil'nikov, N. A., Agre, N. S. & El-Regestan, G. I. (1968b). New thermophilic species of the genus *Micropolyspora*. *Mikrobiologiya* **37**, 1065-1072. [2]

Krieg, N. R. (1988). Bacterial classification: an overview. *Canadian Journal of Microbiology* **34**, 536-540. [1]

Kristjansson, J. K. & Stetter, K. O. (1992) Thermophilic bacteria. In *Thermophilic Bacteria*, pp. 1-18. Edited by J. K. Kristjansson. Boca Raton: CRC Press. [2]

Kristjansson, J. K., Hjorleifsdottir, S., Marteinsson, V. T. & Alfredsson, G. A. (1994) *Thermus scotoductus* sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. x-1. *Systematic and Applied Microbiology* **17**, 44- 50. [2]

Kristjansson. J. K., Hreggvidsson, G. O. & Grant, W. D. (1998). Taxonomy of extremophiles. In *Applied Microbial Systematics*. Edited by F. G. Priest & M. Goodfellow (in press). [2]

Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical methods in bacterial systematics*, 1st ed., pp. 173-199. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press. [1,2,3]

Kroppenstedt, R. M. & Goodfellow, M. (1992) The family Thermomonoapora. In *The Prokaryotes*, 2nd edition, pp. 1085-1114. Edited by A. Balows, H. G. Truper, M. Dworkin, W. Harder & K. -H. Schleifer. New York: Springer. [2]

Kroppenstedt, R. M. & Kutzner, H. J. (1978). Biochemical taxonomy of some problem actinomycetes. In *Nocardia and Streptomyces*, pp. 125-133. Edited by M. Mordarski, W. Kurylowicz, & J. Jeljaszewicz. Stuttgart: Gustav Fischer Verlag. [2,3]

Kroppenstedt, R. M., Stackebrandt, E. & Goodfellow, M. (1990). Taxonomic revision of the actinomycete genera *Actinomadura* and *Microtetraspora*. *Systematic and Applied Microbiology* **13,** 148-160. [1,2]

Kroppenstedt, R. M., Stackebrandt, E., Goodfellow, M. (1991). *In* Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List no. 36. *International Journal Systematic Bacteriology* **41**,178-179. [2]

Kudo, T. (1997). Family *Themomonospora*. In *Atlas of Actinomycetes*, pp. 82-83. Edited by S. Miyadoh. Tokyo: Asakura Publishing. [2]

Kudrina, E. S. & Maximova, T. S. (1963). Some species of thermophilic *Actinomyces* from soils of China and their antibiotic properties. *Mikrobiologiya* **32**, 623-631 (in Russian). [3]

Kurtzman, C. P. (1992). rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *International Journal of Systematic Bacteriology* **42,** 1-6. [1]

Kurup, V. P. & Agre, N. S. (1983). Transfer of *Micropolyspora rectivirgula* (Krassilnikov and Agre 1964) Lechevalier, Lechevalier, and Becker 1966 to *Faenia* gen. nov. *International Journal of Systematic Bacteriology* **33**, 663-665. [2]

Kurylowicz, W. & Gyllenberg, H. G. (1988). Problems in taxonomy of streptomycetes. In *Biology of actinomycetes '88*, pp. 11-18. Edited by Y. Okami, T. Beppu & H. Ogawara. Tokyo: Japan Scientific Societies Press. [3]

Kurylowicz, W., Paszkiewicz, A., Szulga, T., Woznicka, W. & Kurzatkowski, W. (1975). Numerical classification and identification methods in industrial microbiology. In *Proceedings of the Symposium on Automation in Microbiology, Copenhagen, 1975*, pp. 8-22. [3]

Kurylowicz, W., Woznicka, W. & Gyllenberg, H. G. (1969). On the taxonomy of *Streptomyces* (in French). *Review of Immunology (Paris)* **33,** 27-38. [3]

Kurylowicz, W., Woznicka, W., Paskiewicz, A. & Malinowski, K. (1970). Application of numeric taxonomy in streptomycetes. In *The Actinomycetales*, pp. 107-122. Edited by H. Prauser Jena: Veb Gustav Fischer Verlag. [3]

Küster, E. (1959). Outline of a comparative study of criteria used in characterization of the actinomycetes. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **9,** 97-104. [2,3]

Küster, E. (1961). Results of a comparative study of criteria used in the classification of the actinomycetes. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **11,** 91-98. [3]

Küster, E. (1972). Simple working key for the classification of and identification of named taxa included in the International *Streptomyces* Project. *International Journal of Systematic Bacteriology* **22,** 139-148. [3]

Küster, E. & Locci, R. (1963). Studies on peat and peat microorganisms. I. Taxonomic studies on thermophilic actinomycetes isolated from peat. *Archives of Microbiology* **45,** 188-197. [2,3]

Kutzner, H. J. (1961a). Effect of various factors on the efficiency of plating and plaque morphology of some *Streptomyces* phages. *Pathol. Microbiol.* **24,** 30-51. [3]

Kutzner, H. J. (1961b). Specificity of actinophages within a selected group of *Streptomyces. Pathol. Microbiol.* **24,** 170-191. [3]

Labeda, D. P. (1986). Transfer of "*Nocardia aerocolonigenes*" (Shinobu and Kawato 1960) Pridham 1970 into the genus *Saccharothrix* Labeda, Testa, Lechevalier and Lechevalier 1984 as *Saccharothrix aerocolonigenes* sp. nov. *International Journal of Systematic Bacteriology* **36,** 109-110. [2]

Labeda, D. P. (1992). DNA-DNA hybridization in the systematics of *Streptomyces. Gene* **115,** 249-253. [3]

Labeda, D. P. (1993). DNA relatedness among strains of the *Streptomyces lavendulae* phenotypic cluster group. *International Journal of Systematic Bacteriology* **43,** 822-825. [3]

Labeda, D. P. (1995). *Amycolatopsis coloradensis* sp. nov., the avoparin (LL-AV290)-producing strain. *International Journal of Systematic Bacteriology* **45,** 124-127. [2]

Labeda, D. P. (1996). DNA relatedness among verticil-forming *Streptomyces* species (formerly *Streptoverticillium* species). *International Journal of Systematic Bacteriology* **46,** 699-703. [3]

Labeda, D. P. (1998a) DNA relatedness among the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* phenotypic cluster groups. *International Journal of Systematic Bacteriology* **48,** 829-832. [3]

Labeda, D. P. (1998b). Phylogenetic analysis of the genus *Saccharothrix* and related taxa. In *Abstracts of the 98th General Meeting of the American Society for Microbiology,* p. 435. Atlanta, Georgia: American Society for Microbiology. [2]

Labeda, D. P. & Lechevalier, M. P. (1989). Amendment of the genus *Saccharothrix espanaensis* sp. nov., *Saccharothrix cryophilis* sp. nov., and *Saccharothrix mutabilis* comb. nov. *International Journal of Systematic Bacteriology* **39**, 420-423. [2]

Labeda, D. P. & Lyons, A. J. (1991a). The *Streptomyces violaceusniger* cluster is heterogeneous in DNA relatedness among strains: emendation of the descriptions of *S. violaceusniger* and *Streptomyces hygroscopicus*. *International Journal of Systematic Bacteriology* **41**, 398-401. [3]

Labeda, D. P. & Lyons, A. J. (1991b). Deoxyribonucleic acid relatedness among species of the *Streptomyces cyaneus* cluster. *Systematic and Applied Microbiology* **14**, 158-164. [3]

Labeda, D. P., Testa, R. T., Lechevalier, M. P. & Lechevalier, H. A. (1984). *Saccharothrix*: a new genus of the *Actinomycetales* related to *Nocardiopsis*. *International Journal of Systematic Bacteriology* **34**, 426-431. [1,2]

Labeda, D. P., Lechevalier, M. P. & Testa, R. T. (1997). *Streptomyces stramineus* sp. nov. a new species of the verticillate streptomycetes. *International Journal of Systematic Bacteriology* **47**, 747-753. [3]

Lacey, J. & Goodfellow, M. (1975). A novel actinomycete from sugar-cane bagasse: *Saccharopolyspora hirsuta* gen. et sp. nov. *Journal of General Microbiology* **88**, 75-85. [2]

Lacey, J., Goodfellow, M. & Alderson, G. (1978). The genus *Actinomadura* Lechevalier and Lechevalier, pp 107-117. In *Nocardia and Streptomyces, Proceedings of the International Symposium on Nocardia and Streptomyces*. Edtied by M. Mordarski, W. Kurylowicz & J. Jeljaszewicz. Stuttgart: Gustav Fischer Verlag. [2]

Lambert, D. H. & Loria, R. (1989a). *Streptomyces acidiscabies* sp. nov. *International Journal of Systematic Bacteriology* **39**, 393-396. [3]

Lambert, D. H. & Loria, R. (1989b). *Streptomyces scabies* sp. nov., nom. rev. *International Journal of Systematic Bacteriology* **39**, 387-392. [3]

Lan, R. T. & Reeves, P. R. (1996). Gene transfer is a major factor in bacterial evolution. *Molecular Biology and Evolution* **13**, 47-55. [1]

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics*, pp. 115-175. Edited by E. Stackebrandt & M. Goodfellow. New York: John Wiley & Sons. [2,3]

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955-6959. [1]

Langham, C. D., Williams, S. T., Sneath, P. H. A. & Mortimer , A. M. (1989a). New probability matrices for identification of *Streptomyces*. *Journal of General Microbiology* **135**, 121-133. [3]

Larsson, L., Sonesson, A., Jantzen, E. & Bryn, K. (1989). Detection and identification of bacteria using gas-chromatographic analysis of cellular fatty acids. In *Rapid methods and automation in microbiology and immunology*, pp. 389-395. Edited by A. Balows. Brixia: Academic Press. [1]

Lasker, B.A., Brown, J. M. & McNeil, M. M. (1992). Identification and epidemiological typing of chemical and enviornmental isolates of the genus *Rhodococcus* with use of a digoxigenin-labeled rDNA probe. *Clinical Infectious Diseases* **15**, 223-233. [1]

Laurent, F., Carlotti, A., Boiron, P., Villard, J. & Freney, J. (1996). Ribotyping: a tool for taxonomy and identification of the *Nocardia asteroides* complex species, *Journal of Clinical Microbiology* **34**, 1079-82. [1]

Laveran, M. (1906). Tumeur provoquee par un microcoque rose en zooglées. *C. R. Hebd. Soc. Biol.* **2**, 340-341. [2]

Lechevalier, M. P. & Gerber, N. N. (1970). The identity of 3-O-methyl-D-galactose with madurose. *Carb. Res.* **13**, 451-454. [2]

Lechevalier, M. P. & Lechevalier, H. A. (1970a). Composition of wholecell hydroysates as a criterion in the classification of aerobic actinomycetes. In *The Actinomycetales*, pp 311-316. Edited by H. Prauser. Jena, Germany: Gustav Fisher Verlag. [1,2,3]

Lechevalier, M. P. & Lechevalier, H. A. (1970b). Chemical composition as a criterion in the classification of aerobic actinomycetes. *International Journal of Systematic Bacteriology* **20**, 435-443. [1,2,3]

Lechevalier, H. A. & Lechevalier, M. P. (1970c). A critical evaluation of the genera of aerobic actinomycetes. In *The Actinomycetales*, pp. 393-405. Edited by H. Prauser. Jena: Gustav Fischer Verlag. [2]

Lechevalier, M. P. & Lechevalier, H. A. (1980). The chemotaxonomy of actinomycetes, Actinomycete Taxonomy, Special Publication 6, pp 227-291, Edited by A. Dietz, D. & D. Thayer. Arlington, USA: Society for Industrial Microbiology. [1]

Lechevalier, M. P., De Bièvre, C. & Lechevalier, H. A. (1977). Chemotaxonomy of aerobic actinomycetes: phospholipid composition. *Biochemical Systematics and Ecology* **5**, 249-260. [1,2,3]

Lechevalier, M. P., Stern, A. E. & Lechevalier, H. A. (1981). Phospholipids in the taxonomy of actinomycetes, pp. 111-116. In *Actinomycetes*. Edited by K. P. Schaal & G. Pulverer. Stuttgart: Gustav Fischer Verlag. [1,3]

Lechevalier, M. P., Prauser, H., Labeda, D. P. & Ruan, J. S. (1986). Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. *International Journal of Systematic Bacteriology* **36**, 29-37. [2]

Lévy-Frébault, F., Grimont, F., Grimont, P. A. D. & David, H. L. (1986). Deoxyribonucleic acid

relatedness study of the *Mycobacterium fortuitum - Mycobacterium chelonae* complex. *International Journal of Systematic Bacteriology* **36**, 458-460. [1]

Lévy-Frébault, V. V. & Portaels, F. (1992). Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *International Journal of Systematic Bacteriology* **42**, 315-323. [1]

Liesack, W., Weyland, H. & Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbial Ecology* **21**, 191-198. [1]

Lind, A. & Ridell, M. (1976). Serological relationships between *Nocardia*, *Mycobacterium*, *Corynebacterium* and the 'rhodochrous' taxon. In *The Biology of Nocardiae*, pp. 220-235. M. Goodfellow, G. H. Brownell & J. A. Serrano, eds. London: Academic Press. [1]

Locci, R. & Schofield, G. M. (1989). Genus *Streptoverticillium* Baldacci 1958, 15, emend. mut. char. Baldacci, Farina and Locci 1966, 168AL. In *Bergey's manual of systematic bacteriology*, vol. 4., pp. 2492-2504. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins. [3]

Locci, R., Baldacci, E. & Petrolini, B. (1967). Contribution to the study of oligosporic actinomycetes. I. Description of new species of *Actinobifida*: *Actinobifida alba* sp. nov. and revision of the genus. *G. Microbiol.* **15**, 79-91. [2]

Loughney, K., Lund, E. & Dahlberg, J. E. (1982). tRNA genes are found between 16S and 23S rRNA genes in *Bacillus subtilis*. *Nucleic Acids Research* **10**, 1607-1624. [1]

Loutit, J. S. &Tompkins, L. S. (1991). Restriction enzyme and Southern hybridization analyses of *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. *Journal of Clinical Microbiology* **29**, 2897-900. [1]

Low, J. C., Chalmers, R. M., Donachie, W., Freeman, R., McLauchlin, J. & Sisson, P. R. (1992). Pyrolysis mass spectrometry of *Listeria monocytogenes* isolates from sheep. *Research in Vertebrate Science* 53, 64-67. [1]

Ludwig, W. (1991). DNA sequencing in bacterial systematics In *Nucleic acid techniques in bacterial systematics*, pp. 69-94. E. Stackebrandt & M. Goodfellow, eds. John Wiley and Sons: New York. [1]

Ludwig, W. & Schleifer, K. H. (1994). Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbioloy Reviews* **15**, 155-173. [1]

Ludwig, W., Kirchhof, G., Klugbauer, N., Weizenegger, M., Betzl, D., Ehrmann, M., Hertel, C., Jilg, S., Tatzel, R., Zitzelsberger, H., Liebl, S., Hochberger, M., Shah, J., Lane, D., Wallnofer, P. R. & Schleifer, K. H. (1992). Complete 23s ribosomal RNA sequences of Gram-positive bacteria with a low DNA G+C content. *Systematic and Applied Microbiology* **15**, 487-501. [1]

Ludwig, W., Neumaier, J., Klugbauer, N., Brockmann, E., Roller, C., Jilg, S., Reetz, K., Schachtner, I., Ludvigsen, A., Bachleitner, M., Fischer, U. & Schleifer, K. H. (1993). Phylogenetic relationships of *Bacteria* based on comparative sequence analysis of elongation factor Tu and ATP-synthase *B*-subunit genes. *Antonie van Leeuwenhoek* **64**, 285-305. [1]

Lungu, O., Latta, P. D., Weitzman, I. & Silverstein, S. (1994). Differntiation of *Nocardia* from rapidly growing *Mycobacterium* species by PCR-RFLP analysis. *Diagnostic Microbiology & Infectious Disease*. **18**,13-18. [1]

Lyon, A. J. & Pridham, T. G. (1962). Proposal to designate strain ATCC 3004 (IMRU 3004) as the neotype strain of *Streptomyces albus* (Rossi-Doria) Waksman and Henrici. *Journal of Bacteriology* **83**, 370-380. [3]

Maden, B. E., Dent, C. L., Farrell, T. E., Garde, J., McCallum, F. S. & Wakeman, J. A. (1987). Clones of human ribosomal DNA containing the complete 18 S rRNA and 28S rRNA genes. Characterization, a detailed map of the human ribosomal transcription unit and diversity among clones. *Biochemical Journal* **246**, 519-27. [1]

Magee, J. G. (1993). Whole-organism fingerprinting. In: *Handbook of New Bacterial Systematics*, pp. 383-427. Edited by M. Goodfellow & A.G. O'Donnell. London: Academic Press. [1]

Magee, J. G. (1994). Analytical fingerprinting methods. In: *Chemical Methods in Prokaryotic Systematics*, pp. 523-553. Edited by M. Goodfellow & A.G. O'Donnell. London: John Wiley and Sons. [1]

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997). The Ribosomal Database Project (RDP). *Nucleic Acids Res* **5**, 109-111. [1,2,3]

Maltsev, I. I., Kalinovsky, A. I., Zgurskaya, H. I. & Evtsushenko, L. I. (1992). Tyvelose in *Agromyces* cell walls. *Systematic and Applied Microbiology* **15**, 187-190. [1]

Manafi, M., Kneifel, W. & Bascomb, S. (1991). Fluorogenic and chromogenic substrates used in bacterial diagnostics. *Microbiological Reviews* **55**, 335-348. [1]

Manchester, L., Pot, B., Kersters, K. & Goodfellow, M. (1990). Classification of *Streptomyces* and *Streptoverticillium* species by numerical-analysis of electrophoretic protein-patterns. *Systematic and Applied Microbiology* **13**, 333-337. [3]

Manchester, L. N., Toole, A. & Goodacre, R. (1995). Characterisation of *Carnobacterium* species by pyrolysis mass spectrometry. *Journal of Applied Bacteriology* **78**, 88-96. [1]

Mandel, M. (1966). Deoxyribonucleic acid base composition in the genus *Pseudomonas*. *Journal of General Microbiology* **43**, 273-292. [1]

Manfio, G. P. (1995). *Towards Minimal Standards for The Description of Streptomyces Species*. Ph. D. Thesis, University of Newcastle upon Tyne, U.K. [3]

Manfio, G. P., Zakrezewska-Czerwinska, J., Atalan, E. & Goodfellow, M. (1995). Towards minimal standards for the description of *Streptomyces* species. *Biotechnologia* **7-8**, 242-253. [1,3

Margalith, P. & Beretta, G. (1960). A new antibiotic producing *Streptomyces: Str. bellus* nov. sp. *Mycopathologia et Mycologia Applicata* **12**, 189-195. [2]

Marmur, J. & Doty, P. (1961). Thermal renaturation of deoxyribonucleic acids. *Journal of Molecular Biology* **3**, 585-594. [1]

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* **5**, 109-118. [1]

Martinez-Murcia, A. J., Benlloch, S. & Collins, M. D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *International Journal of Systematic Bacteriology* **42**, 412-421. [1]

Matsiota-Bernard, P., Waser, S., Tassios, P. T., Kyriakopoulos, A. & Legakis, N. J. (1997). Rapid discrimination of *Mycobacterium avium* strains from AIDS patients by randomly amplified polymorphic DNA analysis. *Journal of Clinical Microbiology* **35**, 1585-1588. [1]

Matsumae, A. M., Ohtani, M., Takeshima, H. & Hata, T. (1968). A new genus of the *Actinomycetales*: *Kitasatoa* gen. nov. *Journal of Antibiotics (Tokyo)* **21**, 616-625. [3]

Mayama, M. (1959). A consideration on the classification of streptomycetes. *Annual Report of Shionogi Research Laboratories (Japan)* **9**, 1185-1212. [3]

McAdam, R. A., Guilhot, C. & Gicquel, B. (1994). Transposition in mycobacteria. In: *Tuberculosis: pathogenesis, protection, and control*, pp 199-216. Edited by B. R. Bloom. Washingon, D.C.: American Society for Microbiology. [1]

McCarthy, A. J. (1989). *Saccharomonospora.* In *Bergey's Manual of Systematic Bacteriology*, volume 4 , pp. 2402-2404. Baltimore: Williams & Wilkins . [2]

McCarthy, A. J. & CROSS, T. (1981). A note on selective isolation medium for the thermophilic actinomycete *Thermomonospora chromogena. Journal of Applied Bacteriology* 51, 299-302. [2]

McCarthy, A. J. & CROSS, T. (1984). A taxonomic study of *Thermomonospora* and other monosporic actinomycetes. *Journal of General Microbiology* **130**, 5-25. [2]

McClung, N. M. (1974). Genus *Nocardia* Trevisan 1989, 9. In *Bergey's Manual of Determinative Bacteriology, Volume 8.* pp. 726-746. Edited by R. E. Buchnan & N. E. Gibbons. Baltimore: Williams and Wilkins. [2]

McNabb, A., Shuttleworth, R., Behme, R. & Colby, W. D. (1997). Fatty acid characterization of rapidly growing pathogenic aerobic actinomycetes as a means of identification. *Journal of Clinical Microbiology* **35**, 1361- 1368. [1]

McNeil, M. M. & Brown, J. M. (1994). The medically important aerobic actinomycetes: epidemiology and microbiology. *Clinical Microbiological Reviews* **7**, 357-417. [3]

McVeigh, H., Munro, J. & Embley, T. M. (1994). The phylogenetic position of *Pseudoamycolata halophobica* (Akimov *et al.*) and a proposal to reclassify it as *Pseudonocardia halophobica*. *International Journal of Systematic Bacteriology* **44**, 300-302. [2]

Mertz, F. P. & Yao, R. C. (1993). *Amycolatopsis alba* sp. nov., isolated from soil. *International Journal of Systematic Bacteriology* **43**, 715-720. [2]

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid-chromatography. *International Journal of Systematic Bacteriology* **39**, 159-167. [1,2,3]

Meyer, J. (1976). *Nocardiopsis*, a new genus of the order *Actinomycetales*. *International Journal of Systematic Bacteriology* **26**, 487-93. [2]

Meyer, J. (1979). New species of the genus *Actinomadura*. *Z. Allg. Mikrobiol.* **19**, 37-44. [2]

Meyer, J. (1989). Genus *Actinomadura* Lechvalier and Lechvalier 1970. In *Bergey's Manual of Systematic Bacteriology*, Vol. 4, pp. 2511-2526. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams and Wilkins. [2]

Meyer, S. A. & Schleifer, K. H. (1978). Deoxyribonucleic acid reassociation in the classification of coagulase-positive staphylococci. *Archives of Microbiology* **117**, 183-188. [2]

Mikami, Y., Miyashita, K. & Arai, T. (1982). Diaminopimelic acid profiles of alkalophilic and alkali-resistant strains of actinomycetes. *Journal of General Microbiology* **128**, 1709-1712. [3]

Mikami, Y., Miyashita, K. & Arai, T. (1985). Alkalophilic actinomycetes. In *The actinomycetes*, vol. 19, no. 3., pp. 176-191. Edited by H. A. Lechevalier. New Jersey: Rutgers University Publications Department. [3]

Miller, L. T. & Berger, T. (1985). Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett-Packard Application Note no. 228-241. Hewlett-Packard, USA. [1]

Miller, E. S., Woese, C. R. & Brenner, S. (1991). Description of the erythromycin-producing bacterium *Arthrobacter* sp. strain NRRL B-3381 as *Aeromicrobium erythreum* gen. nov., sp. nov. *International Journal of Systematic Bacteriology* **41**, 363-368. [3]

Minnikin, D. E (1988). Isolation and purification of mycobacterial wall lipids. In: *Bacterial Cell Surface Techniques*, pp. 125-135. Edited by I. C. Hancock & I. C. Poxton. Chichester: John Wiley & Sons. [1]

Minnikin, D. E. (1993). Mycolic acids. In *CRC Handbook of Chromatography*, pp. 339-347. Edited by K. D. Mukherjee & N. Weber. Boca Raton: CRC Press. [1]

Minnikin, D. E. & Goodfellow, M. (1980). Lipid composition in the classification and identification of acid fast bacteria. In: *Microbial Classification and Identification*, pp. 189-256. Edited by M. Goodfellow & R. G. Board. London: Academic Press. [1]

Minnikin, D. E. & Goodfellow, M. (1981). Lipids in the classification of actinomycetes. In *Actinomycetes*, pp. 99-109. Edited by K. P. Schaal & G. Pulverer. Stuttgart: Gustav Fischer Verlag. [1]

Minnikin, D. E., Patel, P. V., Alshamaony, L. & Goodfellow, M. (1977). Polar lipid composition in the classification of *Nocardia* and related bacteria. *International Journal of Systematic Bacteriology* **27**, 104-117. [2]

Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980). Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *Journal of Chromatography* **188**, 221-233. [2

Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233-241. [1,2,3]

Mishra, S. K., Gordon, R. E. & Barnett, D. A. (1980). Identification of nocardiae and streptomycetes of medical importance. *Journal of Clinical Microbiology* **11**, 728-736. [3]

Miyadoh, S., Anzai, H., Amano, S. & Shomura, T. (1989). *Actinomadura malachitica* and *Microtetraspora viridis* are synonyms and should be transferred as *Actinomadura viridis* comb. nov. *International Journal of Systematic Bacteriology* **39**, 152-158. [2]

Miyajima, K., Tanaka, F., Takeuchi, T. & Kuninaga, S. (1998). *Streptomyces turgidiscabies* sp. nov. *International Journal of Systematic Bacteriology* **48**, 495-502. [3]

Monson, A. M., Bradley, S. G., Enquist, L. W. & Cruces, G. (1969). Genetic homologies among *Streptomyces violaceoruber* strains. *Journal of Bacteriology* **99**: 702-706. [3]

Moore, W. E. C., Cato, E. P. & Moore, L. V. H. (1985). Index of the bacterial and yeast nomenclatural changes published in the *International Journal of Systematic Bacteriology* since the *1980 Approved Lists of Bacterial Names* (1 January 1980 to 1 January 1985). *International Journal of Systematic Bacteriology* **35**, 382-407. [2]

Mordarski, M., Szyba, K., Pulverer, G. & Goodfellow, M. (1976). Deoxyribonucleic acid

reassociation in the classification of the *"rhodochrous"* complex and allied taxa. *Journal of General Microbiology* **94**, 235-245. [2,3]

Mordarski, M., Goodfellow, M., Tkacz, A., Pulverer, G. & Schaal, K. P. (1980). Ribosomal ribonucleic acid similarities in the classification of *Rhodococcus* and related taxa. *Journal of General Microbiology* **118**, 313-319. [1]

Mordarski, M., Tkacz, A., Goodfellow, M., Schaal, K. P. & Pulverer, G. (1981). Ribosomal ribonucleic acid similarities in the classification of actinomycetes. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. I. Abteilung, Supplement* **11**, 79-85.

Mordarski, M., Goodfellow, M., Williams, S. T. & Sneath, P. H. A. (1986). Evaluation of species groups in the genus *Streptomyces*. In *Biological, biochemical and biomedical aspects of actinomycetes*, vol. B, pp. 517-525. Edited by G. Szabó, S. Biró & M. Goodfellow. Budapest: Akadémiai Kiadó. [2,3]

Moss, M. T., Malik, Z. P., Tizard, M. L., Green, E. P., Sanderson, J. D., Hermon-Taylor, J. (1992). IS902, an insertion element of the chronic-enteritis-causing *Mycobacterium avium* subsp. *silvaticum*. *Journal of General Microbiology* **138**, 139-45. [1]

Müller, R. (1908). Eine Diphtheridee und eine *Streptothrix* mit gleichen blauen Farbstoff sowie Untersuchungen"uber Streptothrixarten im allegemeinen. *Zentralblatt für Bakteriologie Parasitendkunde Infektionskrankheiten und Hygiene, Abteilung I, Originale* **46**, 195-222. [3]

Muller, K. D., Schmid, E. N. & Kroppenstedt, R. M. (1998). Improved identification of mycobacteria by using the Microbial Identification System in combination with additional trimethylsulfonium hydroxide pyrolysis. *Journal of Clinical Microbiology* **36**, 2477- 2480. [1]

Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E. & Zavarzin, G. A. (1990). Report of the *ad hoc* committee on approaches to taxonomy with proteobacteria. *International Journal of Systematic Bacteriology* **40**, 213-215. [1]

Mylvaganam, S. & Dennis, P. P. (1992). Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* **130**, 399-410. [1]

Naumann, D., Helm, D. & Schultz, C. (1994). Characterization and identification of micro-organisms by FT-IR spectroscopy and FT-IR microscopy. In *Bacterial Diversity and Systematics (FEMS Symposium No. 75).* New York: Plenum Press. [3]

Naumova, I. B., Zaretskaya, M. S., Dmitrieva, N. F. & Streshinskaya, G. M. (1978). Structural features of teichoic acids of certain *Streptomyces* species. In *Nocardia and Streptomyces*, pp. 75-83. Edited by M. Mordarski, W. Kurilowicz & J. Jeljaszewicz. Stuttgart: Gustav Fischer Verlag. [3]

Neefs, J.-M., van de Peer, Y., de Rijk, P., Chapelle, S. & de Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Research* **21**, 3025-3049. [2,3]

Nei, M. (1987). *Molecular Evolutionary Genetics*. Columbia University Press, New York. [1]

Nelson, W. H., Manoharan, R. & Sperry, J. F. (1992). UV resonance Raman studies of bacteria. *Applied Spectroscopy Reviews* **27**, 67-124. [3]

Nijhuis, A., deJong, S. & Vandeginste, B. G. M. (1997). Multivariate statistical process control in chromatography. *Chemometrics and Intelligent Laboratory Systems* **38**, 51-62. [1]

Ninet, B., Traitler, H., Aeschlimann, J.-M., Horman, I., Hartmann, D. & Bille, J. (1992). Quantitative analysis of cellular fatty acids (CFA) composition of seven species of *Listeria*. *Systematic and Applied Microbiology* **15**, 423-427. [1]

Ninet, B., Monod, M., Emler, S., Pawlowski, J., Metral, C. Rohner, P., Auckenthaler, R. & Hirschel, B. (1996). Two different 16S rRNA genes in a mycobacterial strain. *Journal of Clinical Microbiology* **34**, 2531-2536. [1]

Nomi, R. (1960). Studies on the classification of streptomycetes, part XIII. The microscopic morphology and other characteristics of whorl forming strains. *Journal of General and Applied Microbiology* **5**, 191-199. [3]

Nonomura, H. (1974). Key for classification and identification of 458 species of the streptomycetes included in the ISP. *Journal of Fermentation Technology* **52**, 78-92. [3]

Nonomura, H. & Y. Ohara. (1957). Distribution of actinomycetes in soil. II. *Microbispora*, a new genus of the *Streptosporangiaceae*. *Journal of Fermentation Technology* **35**, 307-311. [2]

Nonomura, H. & Y. Ohara. (1971). Distribution of actinomycetes in soil. (X) New genus and species of monosporic actinomycetes. *Journal of Fermentation Technology* **49**, 895-903. [2]

Nonomura, H. & Ohara, Y. (1974). Distribution of actinomycetes in soil. XII. A new species of actinomycetes, *Thermononospora mesouviformis*, sp. nov. *Journal of Fermentation Technology* **52**, 10-13. [2]

O'Donnell, A. G. (1985). Numerical analysis of chemotaxonomic data. In *Computer-assisted bacterial systematics*, pp. 403-414. Edited by M. Goodfellow, D. Jones & F. G. Priest. London: Academic Press. [1]

O'Donnell, A. G., Minnikin, D. E., Goodfellow, M. & Parlett, J. H. (1982). The analysis of actinomycete wall amino acids by gas chromatography. *FEMS Microbiology Letters* **15**, 75-78. [1]

O'Donnell, A. G., Minnikin, D. E., Goodfellow, M., Parlett, J. H., Schofield, G. M. & Schaal, K. P. (1985). Lipid and wall amino acid composition in the classification and identification of *Arachnia propionica*. *Zentralblatt für Mikrobiologie und Hygiene Series* **A260**, 300-310. [1]

O'Donnell, A. G., Embley, T. M. & Goodfellow, M. (1993a). Future of bacterial systematics. In: *Handbook of New Bacterial Systematics*, pp. 513-524. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1]

O'Donnell, A. G., Falconer, C., Goodfellow, M., Ward, A. C. & Williams, E. (1993b). Biosystematics and diversity amongst novel carboxydotrophic actinomycetes. *Antonie van Leeuwenhoek* **64**, 325-340. [2,3]

Ochi, K. (1989). Heterogeneity of ribosomal proteins among *Streptomyces* species and its application to identification. *Journal of General Microbiology* **135**, 2635-2642. [1,3]

Ochi, K. (1992). Polyacrylamide gel electrophoresis analysis of ribosomal protein: a new approach for actinomycete taxonomy. *Gene* **115**, 261-265. [1,3]

Ochi, K. (1995). A taxonomic study of the genus *Streptomyces* by analysis of ribosomal protein AT-L30. *International Journal of Systematic Bacteriology* **45**, 507-514. [1,3]

Ochi, K. & Hiranuma, H. (1994). A taxonomic review of the genera *Kitasatosporia* and *Streptoverticillium* by analysis of ribosomal protein AT-L30. *International Journal of Systematic Bacteriology* **44**, 285-292. [1,3]

Ochi, K. & Miyadoh, S. (1992). Polyacrylamide gel electrophoresis analysis of ribosomal protein AT-L30 from an actinomycete genus, *Streptosporangium*. *International Journal of Systematic Bacteriology* **42**, 151-155. [1]

Ochi, K. & Yoshida, M. (1991). Polyacrylamide gel electrophoresis analysis of mycolateless wall chemotype IV actinomycetes. *International Journal of Systematic Bacteriology* **41**, 402-405. [1]

Ochi, K., Miyadoh, S. & Tamura, T. (1991). Polyacrylamide gel electrophoresis analysis of ribosomal protein AT-L30 as a novel approach to actinomycete taxonomy: application to the genera *Actinomadura* and *Microtetraspora*. *International Journal of Systematic Bacteriology* **41**, 234-239. [2]

Oh, C. H., Ahn, M. H. & Kim, J. H. (1996). Use of electrophoretic enzyme patterns for streptomycete systematics. *FEMS Microbiology Letters* **140**, 9-13. [3]

Okanishi, M., Akagawa, H. & Umezawa, H. (1972). An evaluation of taxonomic criteria in streptomycetes on the basis of deoxyribonucleic acid homology. *Journal of General Microbiology* **72**, 49-58. [3]

Olsen, G. J. & Woese, C. R. (1993). Ribosomal RNA: a key to phylogeny. *FASEB Journal* **7**, 113-123. [1, 2]

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applications in Biological Sciences* **10**, 41-48. [1]

Omura, S., Takahashi, Y., Iwai, Y. & Tanaka, H. (1982). *Kitasatosporia*, a new genus of the order *Actinomycetales*. *Journal of Antibiotics* **35**, 1013-1019. [3]

Orr, K., Gould, F. K., Sisson, P. R., Lightfoot, N. F., Freeman, R. & Burdess, D. (1991). Rapid interstrain comparison by pyrolysis mass spectrometry in nosocomial infection with *Xanthomonas maltophilia*. *Journal of Hospital Infection* **17**, 187-195. [1]

Orr, K. E., Lightfoot, N. F., Sisson, P. R., Harkis, B. A., Tweddle, J. L., Boyd, P., Carroll, A., Jackson, C. J., Wareing, D. R. A. & Freeman, R. (1995). Direct milk excretion of *Campylobacter jejuni* in a dairy cow causing cases of human enteritis. *Epidemiology and Infection* **114**, 15-24. [1]

Oyarzabal, O. A., Wesley, I. V., Harmon, K. M., Schroeder-Tucker, L., Barbaree, J. M., Lauerman, L. H., Backert, S. & Conner, D. E. (1997). Specific identification of *Campylobacter fetus* by PCR targeting variable regions of the 16S rDNA. *Veterinary Microbiology* **58**, 61-71. [1]

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* **276**, 734-740. [1]

Pace, N. R., Olsen, G. J. & Woese, C. R. (1986a). Ribosomal RNA phylogeny and the primary lines of evolutionary descent. *Cell* **45**, 325-326. [1]

Pace, N. R., Stahl, D. A., Lane, D. J. & Olsen, G. J. (1986b). The analysis of natural microbial populations by ribosomal RNA sequences. *Advances in Microbial Ecology* **9**, 1-55. [1]

Pagano, Donovick, Dutcher & Heuser. (1959). U.S. Patent 2,902,412 September 1, 1959. [3]

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357-358. [2,3]

Palittapongarnipm, P., Chomic, S., Fanning, A. & Kunimoto, D. (1993). DNA fragment length polymorphism analysis of *M. tuberculosis* isolates by arbitrarily primed polymerase chaim reaction. *Journal of Infectious Diseases* **167**, 975-978. [1]

Park, Y.-H., Yim, D.-G., Kim, E., Kho, Y.-H., Mheen, T.-I., Lonsdale, J. & Goodfellow, M. (1991). Classification of acidophilic, neutrotolerant and neutrophilic streptomycetes by nucleotide sequencing of 5S ribosomal RNA. *Journal of General Microbiology* **137**, 2265-2269.[3]

Park, Y.-H., Suzuki, K.-I., Yim, D.-G., Lee, K.-C., Kim, E., Yoon, J., Kim, S., Kho, Y.-H., Goodfellow, M. & Komagata, K. (1993). Suprageneric classification of peptidoglycan group B actinomycetes by nucleotide sequencing of 5S ribosomal RNA. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **64**, 307-313. [1]

Pascual, C., Lawson, P. A., Farrow, J. A. E., Gimenez, M. N. & Collins, M. D. (1995). Phylogenetic analysis of the genus *Corynebacterium* based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* **45,** 724-728. [1]

Paszkiewicz, A. (1972). Application of numerical methods to the taxonomy of the genus *Streptomyces. Archivum Immunologiae et Therapiae Experimentalis (Warszawa)* **20,** 307-332. [3]

Pernodet, J.L., Boccard, F., Alegre, M. T., Gagnat, J. & Guerineau, M. (1989). Organization and nucleotide sequence analysis of a ribosomal RNA gene-cluster from *Streptomyces ambofaciens. Gene* .**79,** 33-46. [3]

Person, L. H. & Martin, W. H. (1940). Soil rot of sweet potato in Louisiana. *Phytopathology* **30,** 913-926. [3]

Perttula, M, Konradsdottir, M, Pere, J, Kristjansson, Jk, Viikari, L. (1991). Removal of acetate from NSSC sulfite pulp-mill condensates using thermophilic bacteria. *Water Research* **25,** 599-604. [2]

Pinoy, E. (1912). Isolement et culture d'une vouvelle oospora pathogene. Mycetoma a grains rouges de la paroi thoracique. *Bull. Soc. Path. Exot.* **5,** 585-589. [2]

Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology* **8,** 151-156. [2,3]

Pittenger, R. C. & Brigham, R. B. (1956). *Streptomyces orientalis* n. sp., the source of vancomycin. *Antibiotics and Chemotheraphy* **6,** 642-647. [2]

Pitulle, C., Dorsch, M., Kazda, J., Wolters, J. & Stackebrandt, E. (1992). Phylogeny of rapidly growing members of the genus *Mycobacterium. International Journal of Systematic Bacteriology* **42,** 337-343. [1]

Plikaytis, B. B., Plikaytis, B. D., Yakrus, M. A., Butler, W. R., Woodley, C. L., Silcox, V. A. & Shinnick, T. M. (1992). Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology* **30,** 1815-1822. [1]

Portaels, F., Fonteyne, P. -A., De Beenhouwer, H., de Rijik, P. Guédénon, A., Hayman, J. & Meyers, W. M. (1996). Variability in 3' end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. *Journal of Clinical Microbiology* **34,** 962-965. [1]

Postic, D., Assous, M. V., Grimont, P. A. D. & Baranton, G. (1994). Diversity of *Borellia burdorferi sensu* Lato evidenced by restriction fragment length polymorphism of *rrf*(5S)-*rrl*(23S) intergenic spacer amplicons. *International Journal of Systematic Bacteriology* **44,** 743-752. [1]

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical methods in prokaryotic systematics*, pp. 493-521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: John Wiley & Sons. [1]

Poulet, S. & Cole, S. T. (1995). Repeated DNA sequences in mycobacteria. *Archives of Microbiology* **163**, 79-86. [1]

Prauser, H. (1970). Characters and genera arrangement in the *Actinomycetales*. In *The Actinomycetales*. pp. 407-418. H. Prauser, ed. Gustav Fischer Verlag: Jena. [1]

Prauser, H. (1978). Considerations on taxonomic relations among Gram-positive, branching bacteria. *Zentralblatt für Bakteriologie, Parasitenfunde, Infektionskrankheiten und Hygiene. I. Abteilung Supplement* **6**, 3-12. [1]

Prauser, H. (1981). Nocardioform orgainsms: general charcaterization and taxonomic relationships. *Zentralblatt für Bakteriologie, Parasitenfunde, Infektionskrankheiten und Hygiene. I. Abteilung Supplement* **11**, 17-24. [1]

Preobrazhenskaya, T. P. & Sveshnikova, M. A. (1974). Newsspecies of the genus Actinomadura. *Mikrobiologiya* **43**, 864-868. [2]

Pridham, T. G. (1970). New names and new combinations in the order *Actinomycetales* Buchanan 1917. *Technical Bulletin Agricultural Research Service, United States Department of Agriculture* no. 1424. [3]

Pridham, T. G. & Tresner, H. D. (1974a). Family VII. *Streptomycetaceae* Waksman and Henrici 1943, 339. In *Bergey's manual of determinative bacteriology*, 8th ed., pp. 747-748. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins. [3]

Pridham, T. G. & Tresner, H. D. (1974b). Genus I. *Streptomyces* Waksman and Henrici. In *Bergey's manual of determinative bacteriology*, pp. 748-829. Edited by R. E. Buchanan & N. E. Gibbons. 8th ed. Baltimore: Williams & Wilkins. [3]

Pridham, T. G., Hesseltine, C. W. & Benedict, R. G. (1958). A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. *Applied Microbiology* **6**, 52-79. [2,3]

Priest, F. G. & Williams, S. T. (1993). Computer-assisted identification. In: *Handbook of New Bacterial Systematics*, pp. 361-381. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1]

Priest, F.G. & Austin, B. (1993). *Modern Bacterial Taxonomy.* 2nd ed. London: Chapman & Hall. [1]

Promega Corporation. (1993). *Promega 1993/94 catalog: biological research products.* Promega Corporation, Madison. [3]

Qu, L. H., Michot, B. & Bachellerie, J. P. (1983). Improved methods for structure probing in large RNA's: a rapid "heterogenous" sequencing approach is coupled to the direct mapping of nuclease accessible sites: application to the 5' terminal domain of eukaryotic 28S rRNA. *Nucleic Acids Research* **11**, 5903-5919. [1]

Rainey, F. A., Janssen, P. H., Morgan, H. W. & Stackebrandt, E. (1993a). A biphasic approach to the determination of the phenotypic and genotypic diversity of some anaerobic, cellulotytic, thermophilic, rod-shaped bacteria. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **64**, 341-355. [1]

Rainey, F. A., Ward, N. L., Morgan, H. W. & Stackebrandt, E. (1993b). A phylogenetic analysis of anaerobic thermophilic bacteria: an aid for their reclassification. *Journal of Bacteriology* **175**, 4774-4779. [1, 3]

Rainey, F. A., Weiss, N. & Stackebrandt, E. (1994). *Coriobacterium* and *Atopobium* are phylogenetic neighbors within the actinomycetes line of descent. *Systematic and Applied Microbiology* **17**, 202-205. [1]

Rainey, F. A., Burghardt, J., Kroppenstedt, R. M., Klatte, S. & Stackebrandt, E. (1995a). Phylogenetic analysis of the genera *Rhodococcus* and *Nocardia* and evidence for the evolutionary origin of the genus *Nocardia* from within the radiation of *Rhodococcus* species. *Microbiology* **141**, 523-528. [1]

Rainey, F. A., Klatte, S., Kroppenstedt, R. M. & Stackebrandt, E. (1995b). *Dietzia*, a new genus including *Dietzia maris* comb. nov., formerly *Rhodococcus maris*. *International Journal of Systematic Bacteriology* **45**, 32-36. [1]

Rainey, F. A., Wardrainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage - proposal of *Nocardiopsaceae* fam. nov. *International Journal of Systematic Bacteriology* **46**, 1088-1092. [1,2]

Ramsey, E. D., Collins, M. D. & Mellon, F. A. (1988). Tandem mass spectrometric studies of menaquinones. Potential for rapid location of points of saturation. *Rapid Communications in Mass Spectrometry* **2**, 273-274. [1]

Ratledge, C. & Wilkinson, S. G. (1988). An overview of microbial lipids. In *Microbial lipids*, vol. 1, pp. 3-22. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press. [1]

Ravin, A. W. (1961). The genetics of transformation. *Advances in Genetics* **10**, 61-163. [1]

Reichert, K., Lipski, A., Pradella, S., Stackebrandt, E. & Altendorf, K. (1998). *Pseudonocardia asaccharolytica* sp. nov. and *Pseudonocardia sulfidoxydans* sp. nov., two new dimethyl disulfide-

degrading actinomycetes and emended description of the genus *Pseudonocardia*. IJSB 48, 441-449. [1]

Reischl, U., Emler, S., Horak, Z., Kaustova, J., Kroppenstedt, R. M., Lehn, N. & Naumann, L. (1998). *Mycobacterium bohemicum* sp. nov., a new slow-growing scotochromogenic mycobacterium. *International Journal of Systematic Bacteriology* **48,** 1349-1355. [1]

Rheims, H., Schumann, P., Rohde, M. & Stackebrandt, E. (1998). *Verrucosispora gifhornensis* gen, nov., sp. nov., a new member of the actinobacterial family *Micromonosporaceae. International Journal of Systematic Bacteriology* **48,** 1119-1127. [1]

Ridell, M. & Williams, S. T. (1983). Serotaxonomical analysis of some streptomycetes and related organisms. *Journal of General Microbiology* **129,** 2857-2861. [3]

Ridell, M., Wallerström, G. & Williams, S. T. (1986). Immunodiffusion analyses of phenetically defined strains of *Streptomyces, Streptoverticillium* and *Nocardiopsis. Systematic and Applied Microbiology* **8,** 24-27. [3]

Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113,** 237-251. [3]

Roberts, P. D., Hodge, N. C., Bouzar, H., Jones, J. B., Stall, R. E., Berger, R. D. & Chase, A. R. (1998). Relatedness of strains of *Xanthomonas fragariae* by restriction fragment length polymorphism, DNA-DNA reassociation, and fatty acid analyses. *Applied and Environmental Microbiology* **64,** 3961-3965. [1]

Rogall, T., Wolters, J., Flohr, t. & Böttger, E. C. (1990). Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium. International Journal of Systematic Bacteriology* **40,** 323-330. [1]

Roiz, M. P., Palenque, E., Guerrero, C. & Garcia, M. J. (1995). Use of restriction fragment length polymorphism as a genetic marker for typing *Mycobacterium avium* strains. *Journal of Clinical Microbiology* **33,** 1389-1391. [1]

Ross, B. C., Raios, K., Jackson, K., Sievers, A. & Dwyer, B. (1991). Differentiation of *Mycobacterium tuberculosis* strains by use of a nonradioactive southern blot hybridization method. *The Journal of Infectious Diseases* **163,** 904-907. [1]

Rossi-Doria, T. (1891). Su di alcune specie di *'Streptothrix'* trovate nell'aria studate in rapporto a quelle giá note a specialmente all' *'Actinomyces. Annali dell' Instituto d'Igiene Sperimentale della Rendiconti Universita di Roma* **1,** 399-438. [3]

Ruan, J.-S., Lang, Y., Shi, Y., Qu, L. & Yu, X. (1994). Chemical and molecular classification of *Saccharomonospora* strains. *International Journal of Systematic Bacteriology* **44,** 704-707. [1]

Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M. & Christen, R. (1994). Phylogenetic analysis and assessment of the genera *Vibrio, Photobacterium, Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *International Journal of Systematic Bacteriology* **44**, 416-426. [1]

Runmao, H., Lin, C. & Guizhen, W., (1988). *Saccharomonospora cyanea* sp. nov. *International Journal of Systematic Bacteriology* **4**, 444-446. [2]

Rychlik, W. & Rhoads, R. E. (1989). A computer program for choosing optimal oligonucleotides for filter hybridisation, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Research* **17**, 8543-8551. [2]

Sackin, M. J. & Jones, D. (1993). Computer-assisted classification. In: *Handbook of New Bacterial Systematics*, pp. 281-313. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1]

Saddler, G. S. (1988). *Selective isolation and rapid identification of streptomycetes.* Ph.D. Thesis, University of Newcastle upon Tyne. [3]

Saddler, G. S., Goodfellow, M., Minnikin, D. E. & O'Donnell, A. G. (1986). Influence of the growth cycle on the fatty acid and menaquinone composition of *Streptomyces cyaneus* NCIB 9616. *Journal of Applied Bacteriology* **60**, 51-56. [1]

Saddler, G. S., O'Donnell, A. G., Goodfellow, M. & Minnikin, D. E. (1987). SIMCA pattern recognition in the analysis of streptomycete fatty acids. *Journal of General Microbiology* **133**, 1137-1147. [1,3]

Saddler, G. S., Falconer, C. & Sanglier, J. J. (1988). Preliminary experiments for the selection and identification of actinomycetes by pyrolysis mass spectrometry. *Actinomycetologica* **2**, S3-S4. [1]

Saddler, G. S., Tavecchia, P., Lociuro, S., Zanol, M., Colombo, L. & Selva, E. (1991). Analysis of madurose and other actinomycete whole cell sugars by gas chromatography. *J Microbiol Methods* **14**, 185-191. [1]

Sadowsky, M. J., Kinkel, L. L., Bowers, J. H. & Schottel, J. L. (1996). Use of repetitive intergenic DNA-sequences to classify pathogenic and disease-suppressive Streptomyces strains. *Applied and Environmental Microbiology* **62**, 3489-3493. [3]

Sahin, N. (1995). *Selective isolation, characterisation and classification of novel alkaliphic streptomycetes.* Ph.D. Thesis, Department of Microbiology, University of Newcastle upon Tyne (UK). [1,2,3]

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuch, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplication of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491. [1]

Saito, H. & Miura, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochimica et Biophysica Acta* **72**, 619-629. [2,3]

Saitou, N. & Nei, M. (1987). The neighbor joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425. [1,2,3]

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual,* 2nd edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. [2,3]

Sanglier, J.-J., Whitehead, D., Saddler, G. S., Ferguson, E. V. & Goodfellow, M. (1992). Pyrolysis mass spectrometry as a method for the classification, identification and selection of actinomycetes. *Gene* **115**, 235-242. [1]

Schaal, K. P. (1985). Identification of clinically significant actinomycetes and related bacteria using chemical techniques. In *Chemical Methods in Bacterial Systematics*, pp. 359-381. M. Goodfellow & D. E. Minnikin, eds. London: Academic Press. [1]

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Review* **36**, 407-477. [1,2]

Schleifer, K. H. & Stackebrandt, E. (1983). Molecular systematics of prokaryotes. *Annual Review of Microbiology* **37**, 143-187. [3]

Schleifer, K. H., Ludwig, W. & Amman, R. (1993). Nucleic acid probes. In *Handbook of New Bacterial Systematics*, pp. 463-510. M. Goodfellow & A. G. O'Donnell eds. London: Academic Press. [1]

Schneider, J., Korn-Wendisch, F. & Kutzner, H. J. (1990). øSC623, a temperate actinophage of *Streptomyces coelicolor* Müller, and its relatives øSC347 and øSC681. *Journal of General Microbiology* **136**, 767-772. [3]

Schrempf, H., Kessler, A., Brönneke, V., Dittrich, W. & Betzler, M. (1989). Genetic instability in streptomycetes. In *Microbiology '89*, pp. 133-140. Edited by C. L. Hershberger, St. W. Queener & G. Hegeman. Washington, D.C.: American Society for Microbiology. [3]

Schrempf, H., Müller, D. & Kämpfer, P. (1994). Spontaneous variants of streptomycetes lack important phenotypic characteristics relevant for numerical identification. In Abstracts *of the 9th International Symposium on the Biology of Actinomycetes (ISBA'94)*, p. 246. Moscow, July 10-15, 1994. [3]

Shaw, N. (1968). The detection of lipids on thin-layer chromatograms with the periodate-Schiff reagent. *Biochimica et Biophysica Acta* **164**, 435-436. [1,2]

Shearer, M. C., Colman, P. M. & Nash III, C. H. (1983). Nocardiopsis mutabilis, a new species of nocardioform bacteria isolated from soil. *International Journal of Systematic Bacteriology* **33**,369-374. [2]

Shearer, M. C., Colman, P. M., Ferrin, R. M., Nisbet, L. J. & Nash, C. H. Iii. (1986). A new genus of the *Actinomycetales*: *Kibdelosporangium aridum* gen. nov., sp. nov. *International Journal of Systematic Bacteriology* **36**, 47-54. [2]

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology* **16**, 313-340. [2,3]

Shirling, E. B. & Gottlieb, D. (1968a). Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. *International Journal of Systematic Bacteriology* **18**, 69-189. [3]

Shirling, E. B. & Gottlieb, D. (1968b). Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. *International Journal of Systematic Bacteriology* **18**, 279-392. [3]

Shirling, E. B. & Gottlieb, D. (1969). Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. *International Journal of Systematic Bacteriology* **19**, 391-512. [3]

Shirling, E. B. & Gottlieb, D. (1972). Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. *International Journal of Systematic Bacteriology* **22**, 265-394. [3]

Silvestri, L. & Hill, L. R. (1965). Agreement between deoxyribonucleic acid base composition and taxonomic classification of Gram-positive cocci. *Journal of Bacteriology* **90**, 136-140. [1]

Silvestri, L. G., Turri, M., Hill, L. R. & Gilardi, E. (1962). A quantitative approach to the systematics of actinomycetes based on overall similarity. In *Microbial Classification*, pp. 333-360. G. C. Ainsworth & P. H. A. Sneath, eds. Cambridge University Press: Cambridge. [3]

Sisson, P. R., Freeman, R., Lightfoot, N. F. & Richardson, I. R. (1991). Incrimination of an environmental source of a case of Legionnaires' disease by pyrolysis mass spectrometry. *Epidermiology and Infection* **107**, 127-132. [1]

Skerman, V. B. D., McGowan, V. & Sneath, P. H. A. (1980). Approved lists of bacterial names. *International Journal of Systematic Bacteriology* **30**, 225-420. [1, 2,3]

Sneath, P. H. A. (1970). Application of numerical taxonomy to *Actinomycetales*: problems and prospects. In *The Actinomycetales*. pp. 371-377. H. Prauser, ed. Gustav Fischer Verlag: Jena. [1,3]

Sneath, P. H. A. (1983). Distortions of taxonomic structure from incomplete data on a restricted set of reference strains. *Journal of General Microbiology* **129**, 1045-1073. [3]

Sneath, P. H. A. (1989a). Bacterial nomenclature. In *Bergey's manual of systematic bacteriology*, pp. 2317-2321. Edited by Williams, S. T.; Sharpe, M. E. & Holt, J. G., vol. 4. Baltimore: Williams & Wilkins . [1]

Sneath, P. H. A. (1989b). Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. *Systematic and Applied Microbiology* **12**, 15-31. [1]

Sneath, P. H. A. (1992). *International Code of Nomenclature of Bacteria: 1990 Revision.* Washington, D. C.: American Society for Microbiology. [2]

Sneath, P. H. A. (1993). Evidence from *Aeromonas* for genetic crossing-over in ribosomal sequences. *International Journal of Systematic Bacteriology* **43**, 626-629. [1]

Sneath, P. H. A. & Johnson, R. (1972). The influence on numerical taxonomic similarities of error in microbiological tests. *Journal of General Microbiology* **72**, 377-392. [3]

Sneath, P. H. A. & Sokal, R. R. (1973) *Numerical Taxonomy: The Principle and Practice of Numerical Classification.* San Francisco: Freeman. [1]

Soina, V. S., Sokolov, A. A. & Agre, N. S. (1975). Ultrastructure of mycelium and spores of *Actinomadura fastidiosa* sp. nov. *Mikrobiologiya* **44**, 883-887. [2]

Soini, H. & Viljanen, M. K. (1997). Diversity of the 32-kilodalton protein gene may form a basis for species determination of potentially pathogenic mycobacterial species. *Journal of Clinical Microbiology* **31**, 769-773. [1]

Sokal, R. R. & Sneath, P. H. A. (1963). *The Principle of Numerical Taxonomy.* San Francisco: W. H. Freeman. [1]

Soto, A., Pitcher, D. G. & Soriano, F. (1991). A numerical analysis of ribosomal RNA gene patterns for typing clinical isolates of *Corynebacterium* group D2. *Epidemiology and Infection* **107**, 263-272. [1]

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517. [3]

Srivastava, A. K. & Schlessinger, D. (1990). Mechanism and regulation of bacterial ribosomal RNA processing. *Annual Review of Microbiolog* **44**,105-29. [1]

St-Laurent, L., Bousquet, J., Simon, L. & Lalonde, M. (1987). Seperation of various *Frankia* strains in the *Alnus* and *Elaeagnus* host specificity groups using sugar analysis. *Canadian Journal of Microbiology* **33**, 764-772. [1]

Stackebrandt, E. (1992). Unifying phylogeny and phenotypic diversity. In *The Prokaryotes*, 2nd edition, pp. 19-47. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag. [1]

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* **44**, 846-849. [1,2,3]

Stackebrandt, E. & Goodfellow, M. (eds) (1991). *Nucleic Acid Techniques in Bacterial Systematics.* Chichester: John Wiley and Sons. [1]

Stackebrandt, E. & Liesack, W. (1993). Nucleic acds and classification. In *Handbook of New Bacterial Systematics*, pp. 151-194. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1]

Stackebrandt, E. & Ludwig, W. (1994). The importance of using outgroup reference organisms in phylogenetic studies: the *Atopobium* case. *Systematic and Applied Microbiology* **17**, 39-43. [1]

Stackebrandt, E. & Rainey, F. A. (1995). Partial and complete 16S rDNA sequences, their use in genertation of 16S rDNA phylogenetic trees and their implications in molecular ecological studies. pp, 1-17, In: *Molecular microbial ecology manual.* Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Netherlands: Kluwer Academic Publishers. [1]

Stackebrandt, E. & Scheifer, K. H. (1984). Molecular systematics of actinomycetes and related organisms. In *Biological, Biochemical and Biomedical Aspectes of Actinomycetes,* pp. 485-504. L. Ortiz-Oritiz, L. F. Bajalil & V. Yakoleff, eds. Academic Press: Orlando. [1]

Stackebrandt, E. & Woese, C. R. (1981). Towards a phylogeny of the actinomycetes and related organisms. *Current Microbiology* **5**, 131-136. [1,2,3]

Stackebrandt, E., Lewis, B. J. & Woese, C. R. (1980). The phylogenetic structure of the coryneform group of bacteria. *Zentralblatt für Bakteriologie, Parasitenfunde, Infektionskrankheiten und Hygiene. I. Abteilung, Originale Reihe* **C1**, 137-149. [1]

Stackebrandt, E., Wunner-Füssl, B., Fowler, V. J. & Schleifer, K. -H. (1981). Deoxyribonucleic acid homologies and ribosomal ribonucleic acid similarities among sporeforming members of the order *Actinomycetales. International Journal of Systematic Bacteriology* **31**, 420-431. [1,2,3]

Stackebrandt, E., Ludwig, W., Seewaldt, E. & Schleifer, K. H. (1983). Phylogeny of sporeforming members of the order *Actinomycetales. International Journal of Systematic Bacteriology* **33**, 173-180. [1]

Stackebrandt, E., Witt, D., Kemmerling, C., Kroppenstedt, R. & Liesack, W. (1991). Designation of streptomycete 16S and 23S rRNA-based target regions for oligonucleotide probes. *Applied and Environmental Microbiology* **57**, 1468-1477. [3]

Stackebrandt, E., W. Liesack & Witt, D. (1992). Ribosomal RNA and rRNA sequence analysis. *Gene* **115**, 255-260.[1,3]

Stackebrandt, E., Kroppenstedt, R. M., Jahnke, K.-D., Kemmerling, C. & Gurtler, H. (1994). Transfer of *Streptosporangium viridogriseum* (Okuda *et al.* 1966), *Streptosporangium viridogriseum* subsp. *kofuense* (Nonomura and Ohara 1969), and *Streptosporangium albidum* (Furumai *et al.* 1968) to *Kutzneria* gen. nov. as *Kutzneria viridogrisea* comb. nov., *Kutzneria kofuensis* comb. nov., and

Kutzneria albida comb. nov., respectively, and emendation of the genus *Streptosporangium*. *International Journal of Systematic Bacteriology* **44**, 265-269. [2]

Stackebrandt, E., Rainey, R. A. & Ward-Rainey, N. L. (1997) Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *International Journal of Systematic Bacteriology* **47**, 479-491. [1, 2, 3]

Stahl, D. A. & Urbance, J. W. (1990). The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *Journal of Bacteriology* **172**, 116-124. [1]

Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226-231. [1,2,3]

Stapleton, R. D., Ripp, S., Jimenez, L., Cheol-Koh, S., Fleming, J. T., Gregory, I. R. & Sayler, G. S. (1998). Nucleic acid analytical approaches in bioremediation: site assessment and characterization. *Journal of Microbiological Methods* **32**, 165-178. [1]

Stead, D. E., Sellwood, J. E., Wilson, J. & Viney, I. (1992). Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *Journal of Applied Bacteriology* **72**, 315-321. [1]

Steingrube, V. A., Wilson, R. W., Brown, B. A., Jost, K. C., Blacklock, Z., Gibson, J. L. & Wallace, R. J., Jr. (1997). Rapid identification of clinically significant species and taxa of Aerobic actinomycetes, including *Actinomadura*, *Gordona*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* isolates, by DNA amplification and restriction endonuclease analysis. *Journal of Clinical Microbiology* **35**, 817-822. [1,3]

Steingrube, V. A., Gibson, J. L., Brown, B. A., Zhang, Y., Wilson, R. W., Rajagopalan, M. & Wallace, R. J. Jr. (1995). PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *Journal of Clinical Microbiology* **33**,149-153. [1,3]

Stephan, R., Schraft, H. & Untermann, F. (1994). Characterization of *Bacillus licheniformis* with RAPD technique (randomly amplified polymorphic DNA). *Letters in Applied Microbiology* **18**,260-263. [1]

Strässle, A., Putnik, J., Weber, R., Fehr-Merhof, A., Wust, J. & Pfyffer, G. E. (1997). Molecular epidemiology of *Mycobacterium tuberculosis* strains isolated from patients in a human immunodeficiency virus cohort in Switzerland. *Journal of Clinical Microbiology* **35**, 374-378. [1]

Stull, T. L., LiPuma, J. J. & Edlind, T. D. (1988). A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *Journal of Infectious Diseases* **157**, 280-286. [1]

Sultana, S., Magee, J. T. & Duerden, B. I. (1995). Analysis of *Bacteroides* species by pyrolysis mass-spectrometry. *Clinical Infectious Diseases* **20**, S122-S127. [1]

Suzuki, K.-I. & Komagata, K. (1983). Taxonomic significance of cellular fatty acids composition in some coryneform bacteria. IJSB **33**, 188-200. [1]

Swaminathan, B. & Matar, G. M. (1993). Molecular typing methods. In: *Diagnostic Molecular Microbiology, Principles and Applications,* pp. 26-51. Edited by D. H. Persing, T. F. Smith, F. C. Tenover & T. J. White. Washington, D.C.: American Society for Microbiology. [1]

Swanson, D. S., Pan, XI. & Musser, J. M. (1996). Identification and subspecific differentiation of *Mycobacterium scrofulaceum* by automated sequencing of a region of the gene (*hsp65*) encoding a 65-kilodalton heat shock protein. *Journal of Clinical Microbiology* **34**, 3151-3159. [1]

Swanson, D. S., Kapur, V., Stockbauer, K., Pan, XI., Frothingham, R. & Musser, J. M. (1997). Subspecific differentiation of *Mycobacterium avium* complex strains by automated sequencing of a region of the gene (*hsp65*) encoding a 65-kilodalton heat shock protein. *International Journal of Systematic Bacteriology* **47**, 414-419. [1]

Swofford, D. L. & Olsen, G. J. (1990). Phylogenetic reconstruction. In *Molecular systematics,* pp. 411-501. Edited by D, Hillis & C. Moritz. Sunderland,U.S.A.: Sinauer Associates. [1,3]

Szabó, I. & Marton, M. (1964). Comments on the first results of the international cooperative work on criteria used in characterization of streptomycetes. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **14**, 17-38. [3]

Szabó, I., Marton, M., Buti, I. & Fernandez, C. (1975). A diagnostic key for the identification of "species" of *Streptomyces* and *Streptoverticillium* included in the International *Streptomyces* Project. *Acta Botanica Academiae Scientiarum Hungaricae* **21**, 387-418. [3]

Szulga, T. (1978). A critical evaluation of taxometric procedures applied to *Streptomyces*. In *Nocardia and Streptomyces,* pp. 31-42. Edited by M. Mordarski, W. Kurylowicz & J. Jeljaszewicz. Stuttgart: Gustav Fischer Verlag. [3]

Takeuchi, T., Sawada, H., Tanaka, F. & Matsuda, I. (1996). Phylogenetic analysis of *Streptomyces* spp. causing potato scab based on 16S ribosomal-RNA sequences. *International Journal of Systematic Bacteriology* **46,** 476-479. [3]

Tamaoka, J. (1994). Determination of DNA base composition. In *Chemical methods in prokaryotic systematics,* pp.463-470. Edited by M. Goodfellow and A. G. O'Donnell. Chichester: John Wiley and Sons. [1,2,3]

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiology Letters* **25,** 125-128. [1]

Tamura, T. & Yokota, A. (1994). Transfer of *Nocardioides fastidiosa* Collins and Stackebrandt

1989 to the genus *Aeromicrobium* as *Aeromicrobium fastidiosum* comb. nov. *International Journal of Systematic Bacteriology* **44**, 608-611. [1,3]

Tamura, T., Takeuchi, M. & Yokota, A. (1994). *Luteococcus japonicus* gen. nov., sp. nov., a new Gram-positive coccus with LL-diaminopimelic acid in the cell wall. *International Journal of Systematic Bacteriology* **44**, 348-356. [3]

Tamura, T, Hayakawa, M & Hatano, K. (1997) A new genus of the order *Actinomycetales*, *Spirilliplanes* gen. nov., with description of Spirilliplanes yamanashiensis sp. nov. *International Journal of Systematic Bacteriology* **47**, 97-102. [2]

Tateno, Y., Fukami-Kobayashi, K., Miyazaki, S., Sugawara, H. & Gojobori, T. (1998). DNA Data Bank of Japan at work on genome sequence data. *Nucleic Acids Research* **26**, 16-20. [1]

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C. & Bodmer, T. (1993). Rapid identification of mycobacteria to the species level by polymearase chain reaction and restriction enzyme analysis. *Journal of Clinical Microbiology* **35**,149-153. [1]

Tewfik, E. M. & Bradley, S. G. (1967). Characterization of deoxyribonucleic acids from streptomycetes and nocardiae. *Journal of Bacteriology* **94**, 1994-2000. [3]

Tewfik, E., Bradley, S. G., Kuroda, S. & Wu, R. Y. (1968). Studies on deoxyribonucleic acids from streptomycetes and nocardiae. *Developments in Industrial Microbiology* **9**, 242-249. [3]

Thaxter, R. (1891). The potato scab. *Conn. Agricultural Experiment Station Report* **1890,** 81-95. [3]

Thiemann, J. E. & Beretta, G. (1968). A new genus of the *Actinoplanaceae*: *Planobispora* gen. nov. *Archive of Microbiology* **62**, 157-166. [2]

Thiemann, J. E., Pagani, H. & Beretta, G. (1967). A new genus of the *Actinoplanaceae*: *Planomonospora* gen. nov. *Giorn. Microbiol.* **15**, 27-38. [2]

Thiemann, J. E., Pagani, H. & Beretta, G. (1968). A new genus of the *Actinoplanaceae*: *Microtetraspora* gen. nov. *Journal of General Microbiology* **50**, 295-303. [2]

Thirumalachar, M. J. (1955). *Chainia*, a new genus of the *Actinomycetales*. *Nature* **176**, 934-935. [3]

Timmins, E. M., Howell, S. A., Alsberg, B. K., Noble, W. C. & Goodacre, R. (1998) Rapid differentiation of closely related *Candida* species and strains by pyrolysis mass spectrometry and Fourier transform-infrared spectroscopy. *Journal of Clinical Microbiology* 36, 367-374. [3]

Tomita, K., Nakakita, Y., Hoshino, Y., Numata, K. & Kawaguchi, H. (1987). New genus of the *Actinomycetales: Streptoalloteichus hidustanus* gen. nov., nom. rev.; sp. nov., nom. rev. *International Journal of Systematic Bacteriology* **37**, 211-213. [2]

Towner, K. J. & Cockayne, A. (1993). *Molecular Methods for Microbial Identification and Typing*, UK: Chapman & Hall. [1]

Toyama, H., Okanishi, M. & Umezawa, H. (1974). Heterogeneity among whorl-forming streptomycetes determined by DNA reassociation. *Journal of General Microbiology* **80**, 507-514. [3]

Tram, C. Simonet, M., Nicolas, M. H., Offredo, C., Grimont, F., Lefevre, M.,Ageron, E., Debure, A. & Grimont, P. A. (1990). Molecular typing of nosocomial isolates of *Legionella pneumophila* serogroup 3. *Journal of Clinical Microbiology* **28**, 242-245. [1]

Trejo, W. H. (1970). An evaluation of some concepts and criteria used in the speciation of streptomycetes. *Transactions of the New York Academy of Sciences, Series II* **32**, 989-997. [3]

Tresner, H. D., Davies, M. C. & Backus, E. J. (1961). Electron microscopy of Streptomyces spore morphology and its role in species differentiation. *Journal of Bacteriology* **81**, 70-80. [2,3]

Trujillo, M. E. & Goodfellow, M. (1997). Polyphasic taxonomic study of clinically significant actinomadurae including the description of *Actinomadura latina* sp. nov. *Zentralblatt fur Bakteriologie* **285**, 212-233. [1,2]

Uchida, K. & Aida, K. (1977). Acyl type of bacteria cell wall: its simple identification by colorimetric method. *Journal of General and Applied Microbiology* **23**,249-260. [2]

Uchida, K. & Aida, K. (1984). An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. *Journal of General and Applied Microbiology* **30**, 131-134. [2]

Ullmann, J. S. & McCarthy, B. J. (1973). The relationship between mismatched base pairs and the thermal stability of DNA duplexes. *Biochem Biophys Acta* **294**, 416-424. [1]

Van de Peer, Y., Neefs, J. & De Watcher, R. (1990). Small ribosomal subunit RNA sequences, evolutionary relationships among different life forms, and mitochondrial origins. *Journal of Molecular Evolution* **30**, 463-476. [1]

Van de Peer, Y., Caers, A., De Rijk, P. & De Wachter, R. (1998). Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* **26**, 179-182. [1]

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* **60**, 407-438. [1]

Vauterin, L., Swing, J. & Kersters, K. (1993). Protein electrophoresis and classification. In *Handbook of New Bacterial Systematics*, pp. 251-280. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press. [1]

Vauterin, L., Yang, P. & Swings, J. (1996). Utilization of fatty acid methyl esters for the differentiation of new *Xanthomonas* species. *International Journal of Systematic Bacteriology* **46**, 298-304. [1]

Veerkamp, J. H. (1971). Fatty acid composition of Bifidobacterium and Lactobacillus strains. *Journal of Bacteriology* **108**, 861-867. [1]

Verissimo, A., Morais, P. V., Diogo, A., Gomes, C. & Dacosta, M. S. (1996). Characterization of legionella species by numerical analysis of whole-cell protein electrophoresis. *International Journal of Systematic Bacteriology* **46**, 41-49. [1]

Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**, 6823-6831. [1]

Vincent, H. (1894). Étude sur le parasite du pied le madura. *Ann. Inst. Pasteur.* **8**, 129-151. [2]

Vobis, G. & Henssen, A. (1983) Ultrastructure of tuberculate spores in *Streptomyces thermoviolaceus*. *Archives of Microbiology* **134**, 295-298. [3]

Vylegzhanina, E. S., Dmitrieva, N. F., Polin, A. N., Naumova, I. B. & Epishin, IuN. (1986). Structure of teichoic acid of a poly(glycerophosphate) nature from *Streptomyces levoris* K-3053 and its biological properties (in Russian). *Antibiot. Med. Biotekhnol.* **31**, 584-587. [3]

Wagner, H., Horhammer, L. & Wolff, L. (1961). Dunnschicht-chromatographic von Phosphatiden und Glycolipiden. *Biochimische Zeitschrift* **334**, 175-184. [1,2]

Waksman, S. A. (1919). Cultural studies of species of *Actinomyces*. *Soil Science* **8**, 71-215. [3]

Waksman, S. A., Umbreit, W. W. & Cordon, T. C. (1939). Thermophilic actinomycetes and fungi in soils and in composts. *Soil Science* **47**, 37-61. [3]

Waksman, S. A. (1953). In S. A. Waksman, and H. A. Lechevalier, *Guide to the classification and identification of actinomycetes and their antibiotics.* Baltimore: Williams and Wilkins Co. [3]

Waksman, S. A. (1957). Family III. *Streptomycetaceae* Waksman and Henrici. In *Bergey's manual of determinative bacteriology*, pp. 744-825. Edited by Breed, R. S., Murray, E. G. D. & Smith, N. R., 7th ed. Baltimore: Williams & Wilkins Co. [3]

Waksman, S. A. (1961). *The Actinomycetes. Classification, identification and descriptions of genera and species.*, vol. 2. Baltimore: Williams & Wilkins . [1,3]

Waksman, S. A. (1967). *The Actinomycetales: A Summery of Current Knowledge.* The Ronald Press Company: New York. [1]

Waksman, S. A. & Curtis, R. E. (1916). The actinomycetes of the soil. *Soil Science* **1**, 99-134. [3]

Waksman, S. A. & Henrici, A. T. (1943). The nomenclature and classification of the actinomycetes. *Journal of Bacteriology* **46**, 337-341. [3]

Waksman, S. A. & Henrici, A. T. (1948). Family III. *Streptomycetaceae* Waksman and Henrici. In *Bergey's manual of determinative bacteriology*, 6th ed., pp. 929-980. Edited by R. S. Breed, E. G. D. Murray & P. Hichens. Baltimore: Williams & Wilkins . [2,3]

Waksman, S. A. & Woodruff, H. B. (1941). *Actinomyces antibioticus*, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. *Journal of Bacteriology* **42**, 231-249. [3]

Wallace, R. J. Jr., Brown, B. A.,Blacklock, Z.,Ulrich, R., Jost, K., Brown, J. M., McNeil, M. M., Onyi, G., Steingrube, V. A. & Gibson, J. L. (1995). New *Nocardia* taxon among isolates of *Nocardia brasiliensis* associated with invasive disease. *Journal of Clinical Microbiology* **33**, 1528-1533. [3]

Wang, Y., Zhang, Z. S. & Ruan, J. S. (1996a). Phylogenetic analysis reveals new relationships among members of the genera *Microtetraspora* and *Microbispora. International Journal of Systematic Bacteriology* **46**, 658-663. [1,2]

Wang, Y., Zhang, Z. & Ruan, J. (1996b). A proposal to transfer *Microbispora bispora* (Lechevalier 1965) to a new genus *Thermobispora* gen. nov. as *Thermobispora bispora* comb. nov. *International Journal of Systematic Bacteriology* **46**, 933-938. [1]

Wang, Y., Zwang, Z. S. & Ramanan, N. (1997). The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. *Journal of Bacteriology* **179**, 3270-3276. [1]

Ward, D. M. (1998). A natural species concept for prokaryotes. *Current Opinion in Microbiology* **1**, 271-277. [1]

Warwick, S., Bowen, T., McVeigh, H. & Embley, T. M. (1994). A phylogenetic analysis of the family *Pseudonocardiaceae* and the genera *Actinokineospora* and *Saccharothrix* with 16S rRNA sequences and a proposal to combine the genera *Amycolata* and *Pseudonocardia* in the emended genus *Pseudonocardia. International Journal of Systematic Bacteriology* **44**, 293-299. [2]

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. & Trüper, H. G. (1987). Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**, 463-464. [1,2,3]

Wayne, L. G., Good, R. C., Böttger, E. C., Butler, R., Dorsch, M., Ezaki, T., Gross, W., Jonas, V., Kilburn, J., Kirschner, P., Krichevsky, M. I., Ridell, M., Shinnick, T. M., Springer, B., Stackebrandt, E., Tarnok, I., Tarnok, Z., Tasaka, H., Vincent, V., Warren, N. G., Knott, C. A. & Johnson, R. (1996). Semantide- and chemotaxonomy-based analyses of some problematic phenotypic clusters of slowly growing mycobacteria, a cooperative study of the International Working Group on Mycobacterial Taxonomy. *International Journal of Systematic Bacteriology* **46**, 280-297. [1]

Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A. & Weiner, A. M. (1987). *Molecular Biology of the Gene*. Menlo Park, C.A.: The Benjamin/ Cummings Publishing Company. [1]

Wei, M.-Q., Groth, D. M., Mendis, A. H. W., Sampson, J., Wetherall, J. D. & Grubb, W. B. (1992). Typing of methicillin-resistant *Staphylococcus aureus* with an M13 repeat probe. *Journal of Hospital Infections* **20**, 233-245. [1]

Wellington, E. M. H. & Williams, S. T. (1978). Preservation of actinomycete inoculum in frozen glycerol. *Microbios Letters* **6**, 151-159. [2,3]

Wellington, E. M. H. & Williams, S. T. (1981). Host ranges of phages isolated to *Streptomyces* and other genera. In *Actinomycetes*, pp. 93-98. Edited by K. P. Schaal & G. Pulverer. Gustav Fischer Verlag, Stuttgart. [3]

Wellington, E. M. H., Stackebrandt, E., Sanders, D., Wolstrup, J. & Jorgensen, N. O. G. (1992). Taxonomic status of *Kitasatosporia*, and proposed unification with *Streptomyces* on the basis of phenotypic and 16S rRNA analysis and emendation of *Streptomyces* Waksman and Henrici 1943, 339^{AL}. *International Journal of Systematic Bacteriology* **42**, 156-160. [3]

Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213-7218. [1]

Weyland, H. (1969) Actinomycetes in North Sea and Atlantic Ocean sediments. *Nature* **233**, 851. [2]

White, D., Sharp, R. J. & Priest, F. G. (1993). A polyphasic taxonomic study of thermophilic bacilli from a wide geographical area. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **64**, 357-386. [1]

Wieczorek, J., Mordarska, H., Zakrzewska-Czerwinska, J., Gamian, A. & Mordarski, M. (1993). *Streptomyces spitsbergensis* sp. nov. *International Journal of Systematic Bacteriology* 43, 84-87. [3]

Williams, S. T., Sharples, G. P., Serrano, J. A., Serrano, A. A. & Lacey, J. (1976). The micromorphology and fine structure of nocardioform organisms. In *The Biology of Nocardiae*, pp. 102-140. M. Goodfellow, G. H. Brownell & J. A. Serrano, eds. London: Academic Press. [1]

Williams, S. T., Wellington, E. M. H., Goodfellow, M., Alderson, G., Sackin, M. & Sneath, P. H. A. (1981). The genus *Streptomyces* - a taxonomic enigma. In *Actinomycetes*, pp. 47-57. Edited by K. P. Schaal & G. Pulverer. Stuttgart: Gustav Fischer Verlag. [3]

Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983a). Numerical classification of *Streptomyces* and related genera. *Journal of General Microbiology* 129, 1743-1813. [1, 3]

Williams, S. T., Goodfellow, M., Wellington, E. M. H., Vicker, J. C., Alderson, G., Sneath, P. H. A., Sackin, M. J. & Mortimer, A. M. (1983b). A probability matrix for identification of some Streptomycetes. *Journal of General Microbiology* **129**, 1815-1830. [3]

Williams, S. T., Goodfellow, M. & Alderson, G. (1989). Genus *Streptomyces* Waksman and Henrici, 1943, 339AL. In *Bergey's manual of systematic bacteriology*, pp. 2452-2492. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. vol. 4. Baltimore: Williams & Wilkins . [1,2,3]

Williams, S. T., Sharpe, M. E. & Holt, J. G. (1989) *Bergey's Manual of Systematic Bacteriology*, vol. 4. Baltimore: Williams & Wilkins . [2]

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535. [1]

Williams, S. T., Locci, R., Beswick, A., Kurtboke, D. I., Kuznetsov, V. D., Lemonnier, F. J., Long, P. F., Maycroft, K. A., Palma, R. A., Petrolini, B., Quaroni, S. Todd, J. I. & West, M. (1993). Detection and identification of novel actinomycetes. *Research In Microbiology* 144, 653-656. [3]

Witt, D. & Stackebrandt, E. (1990). Unification of the genera *Streptoverticillium* and *Streptomyces*, and amendation of *Streptomyces* Waksman and Henrici 1943, 339^{AL}. *Systematic and Applied Microbiology* **13**, 361-371. [1,3]

Woese, C. R. (1987) Bacterial evolution. *Microbiological Review* **51**, 221-271. [1, 2]

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains *Archaea, Bacteria* and *Eucarya. Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576-4579. [1]

Wold, S. & Söderström, M. J. (1977). SIMCA: A method for analysing chemical data in terms of similarity and analogy, pp. 243-282. In *Chemometrics: Theory and Application.* Edited by B. Kowalski. Series no. 52, ACS Symposium. Washington, D. C.: American Chemical Society. [1]

Xu, L. H., Tiang, Y. Q., Zhang, Y. F., Zhao, L. X., Jiang, C. L. (1998). *Streptomyces thermogriseus*, a new species of the genus *Streptomyces* from soil, lake and hot-spring. *International Journal of Systematic Bacteriology* **48**, 1089-1093. [3]

Yamada, Y., Hou, C. F., Sasaki, J., Tahara, Y. & Yoshioka, H. (1982). The structure of the octahydrogenated isoprenoid side-chain menaquinone with nine isoprene units isolated from *Streptomyces albus. Journal of General and Applied Microbiology* **28**, 519-529. [1]

Yamamoto, S. & Harayama, S. (1998). Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of gyrB, ropD and 16S rRNA genes. IJSB **48**, 813-819. [1]

Yassin, A. F., Binder, C. & Schaal, K. P. (1993a). Identification of mycobacterial isolates by thin-layer and capillary gas-chromatography under diagnostic routine conditions. *Zentralblatt für Bakteriologie* **278**, 34-48. [1]

Yassin, A. F., Galinski, E. A., Wohlfarth, A., Jahnke, K.-D., Schaal, K. P. & Trüper, H. G. (1993b). A new actinomycete species, *Nocardiopsis lucentensis* sp. nov. *International Journal of Systematic Bacteriology* **43**, 266-271. [2]

Yassin, A. F., Rainey, F. A., Brzezinka, H., Jahnke, K. D., Eissbrodt, H., Budzikiewicz, H., Stackebrandt, E. & Schaal, K. P. (1995a). Lentzea gen. nov., a new genus of the order *Actinomycetales. International Journal of Systematic Bacteriology* **45**, 357-363. [2]

Yassin, A. F., Rainey, F. A., Brzezinka, H., Burghardt, J., Lee, H. J. & Schaal, K. P. (1995b). *Tsukamurella inchonensis* sp. nov. *International Journal of Systematic Bacteriology* **45**, 522-527. [1]

Yassin, A. F., Rainey, F. A., Brzezinka, H., Burghardt, J., Rifai, M., Seifert, P., Feldmann, K. & Schaal, K. P. (1996). *Tsukamurella pulmonis* sp. nov. *International Journal of Systematic Bacteriology* **46**, 429-436. [1]

Yassin, A. F., Rainey, F. A., Burghadrt, J., Brzezinka, H., Schmitt, S., Seifert, P. S., Zimmermann, O., Mauch, H., Gierth, D., Lux, I., & Shall, K. P. (1997a). *Tsukamurella tyrosinosolvens* sp. nov *International Journal of Systematic Bacteriology* **47**, 607-614. [1]

Yassin, A. F, Rainey, F. A, Burghardt, J., Gierth, D., Ungerechts, J., Lux, I., Seifert, P., Bal, C. & Schaal, K. P. (1997b). Description of *Nocardiopsis synnemataformans* sp. nov., elevation of *Nocardiopsis alba* subsp. *prasina* to *Nocardiopsis prasina* comb. nov., and designation of *Nocardiopsis antarctica* and *Nocardiopsis alborubida* as later subjective synonyms of *Nocardiopsis dassonvillei. International Journal of Systematic Bacteriology* **47**, 983-988. [2]

Yokota, A., Tamura, T., Takeuchi, M., Weiss, N. & Stackebrandt, E. (1994). Transfer of *Propionibacterium innocuum* Pitcher and Collins 1991 to *Propioniferax* gen. nov. as *Propioniferax innocua* comb. nov. *International Journal of Systematic Bacteriology* **44**, 579-582. [3]

Yoon, J. H., Kim, H., Kim, S. B., Kim, H. J., Kim, W. Y., Lee, S. T., Goodfellow, M. & Park, Y. H. (1996). Identification of *Saccharomonospora* strains by the use of genomic DNA fragments and rRNA gene probes. *International Journal of Systematic Bacteriology* **46**, 502-505. [1]

Zakrzewska-Czerwinska, J. (1989). *Organization of the ribosomal RNA genes* in *Streptomyces strains.* PhD Thesis, Ludwik Hirszfeld Institute, Wroclaw, Poland. [3]

Zhang, Z., Wang, Y. & Ruan, J. (1997) A proposal to revive the genus *Kitasatosporia* (Omura, Takahashi, Iwai, and Tanaka 1982) *International Journal of Systematic Bacteriology* **47**, 1048-1054. [1,3]

Zhang, Z. S., Wang, Y. & Ruan, J. S. (1998). Reclassification of *Thermomonospora* and *Microtetraspora*. *International Journal of Systematic Bacteriology* **48,** 411-422. [1,2]

Zillig, W., Klenk, H. P., Palm, P., Pühler, G., Gropp, F., Garrett, R. A. & Leffers, H. (1989). The phylogenetic relations of DNA-dependent RNA polymerase of archaebacteria, eukaryotes and eubacteria. *Canadian Journal of Microbiology* 35, 73-80. [1]

Zhou, Z-H., Lui, Z-H., Qian, Y-D., Kim, S. B. & Goodfellow, M. (1998). *Saccharopolyspora spinosporotrichia* sp. nov., a novel actinomycete from soil. *International Journal of Systematic Bacteriology* **48**, 53-58. [1]

Zolg, J. W. & Philippi-Schulz, S. (1994). The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. *Journal of Clinical Microbiology*. **32**, 2801-2812. [1]

Appendix 1

World Wide Web Sites

Biodiversity

Actinomycete-Streptomyces Internet Resource Center (ASIRC). This web site has been developed at the University of Minnesota with the aim of fostering interaction amongst actinomycete researchers in academia and industry. Address: http://molbio.cbs.umn.edu/asirc/

The Biodiversity and Biological Collections Web Server. This is designed to provide information of interest to systematists and whole-organism biologists. Information can be found about specimens in biological collections, taxonomic authority files, directories of biologists, reports by various standards bodies (e.g., IOPI, ASC, SA2000), an archive of the Taxacom, access to on-line journals (including Flora On-line) and information about MUSE and Delta. Address: http://biodiversity.uno.edu/

Convention on Biological Diversity (1992). The full text of the Convention on Biological Diversity, which was opened for signature at the 1992 Rio "Earth Summit", is provided. Address: http://www.unep.ch/bio/conv-e.html

List of Biodiversity World Wide Web Sites. This links many biodiversity-related sites. Address: http://www.biologie.unifreiburg.de/data/zoology/reide/taxalinks.html

The Microbial Strain Data Network (MSDN). This provides communication and information services on microbiology and biotechnology. Address: http://www.csa.ru:81/Inst/gorb_dep/inbios/msdn_co.htm

Species 2000 Project. The aim of this project is to prepare an inventory of all known species of plants, animals, fungi and microbes on Earth as the baseline dataset for studies of global biodiversity. Address: http://www.sp2000.org/

The Tropical Data Base (Base de Dados Tropical - BDT). This is an information centre housed at the Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas,

Brazil. The centre has established an interactive biodiversity/biotechnology information resource and users network. As major activities, the BDT is involved with the collection, analysis and dissemination of data relevant to biodiversity and biotechnology, and with the development of software for data management. Address: http://www.bdt.org.br/bdt/

The World Conservation Monitoring Centre (WCMC). This centre provides information services on conservation and sustainable use of the world's living resources, and helps others to develop information systems of their own. Address: http://www.wcmc.org.uk/

The World Resources Institute (WRI). This institute provides a valuable source for facts and figures on biodiversity. Address: http://www.wri.org/wri/biodiv/

Taxonomy-related sites

The American Type Culture Collection (ATCC) was established to acquire, authenticate, and maintain reference cultures, related biological materials, and associated data, and to distribute these to qualified scientists in education, industry and government. Address: http://www.atcc.org/atcc.htm

Bacterial Nomenclature UP-TO-DATE. This database has been compiled by the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Bacterial Nomenclature UP-TO-DATE includes all bacterial names which have been validly published since January 1 1980 together nomenclatural changes which have been validly published since then. The records are updated with the publication of each new issue of the *International Journal of Systematic Bacteriology*. Address: http://www.dsmz.de/DSMZ/bactnom/bactname.htm.

The List of Bacterial Names with Standing in Nomenclature also contains updated bacterial nomenclature. Address: http://www-sv.cict.fr/bacterio/

The DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) is an independent, non-profit organization dedicated to the acquisition, characterization, identification, preservation and distribution of animal cell lines, archaea, bacteria, fungi, phages, plant cell cultures, plant viruses and

plasmids. Address: http://www.dsmz.de/DSMZ/dsmzhome.htm

Phylogeny

Genetics Computer Group (GCG). This provides software for the analysis of genes and proteins. Address: http://www.gcg.com/

PHYLIP. This was constructed by Joe Felsenstein of the Department of Genetics at the University of Washington. Address: http://evolution.genetics.washington.edu/phylip.html

Phylogeny programs. This site contains some 120 of phylogeny packages and 5 free servers. Address: http://evolution.genetics.washington.edu/phylip/software.html

The Tree of Life. This is a project designed to contain information about the phylogenetic relationships and characteristics of organisms, to illustrate the diversity and unity of living organisms, and to link biological information available on the Internet in the form of a phylogenetic navigator. Address: http://ag.arizona.edu/tree/phylogeny.html

TreeBASE is a database of phylogenetic information sponsored by the National Science Foundation, Harvard University Herbaria, and the University of California at Davis. TreeBASE stores phylogenetic trees and the data matrices used to generate them from published research papers. Address: http://www.herbaria.harvard.edu/treebase/

TreeView. Tree drawing software for Apple Macintosh and Windows. Address: http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

Molecular sequence database

The International Nucleotide Sequence Database Collaboration involves the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory database (EMBL), and the GenBank database at the National Center for Biotechnology Information (NCBI). These organisations exchange data on a daily basis and share the same sequence accession numbers. Address: http://www. ncbi.nlm.nih.gov/collab/

DNA Data Bank of Japan (DDBJ), Mishima, Japan. <http://www.ddbj.nig.ac.jp/>

European Molecular Biology Laboratory (EMBL/EBI) Nucleotide Sequence

Database, Hinxton, UK. <http://www.ebi.ac.uk/ebi_home.html>

GenBank (National Center for Biotechnology Information) Bethesda, MD, USA. <http://www.ncbi.nlm.nih.gov/>

TIGR Microbial Database. This database contains a listing of the microbial genomes that have been published or are in the process of being sequenced. Address: http://www.tigr.org/tdb/mdb/mdb.html.

The Ribosomal Database Project. Large and small subunit rRNA sequence data are drawn from the major sequence databases (EBI and GenBank), other rRNA sequence collections and from direct submissions. The sequence data are stored and distributed in aligned form with entries arranged according to phylogenetic relationships. Additional information on the source and taxonomy of the respective organisms, the method of sequence determination, and other relevant data are provided with the sequences. The RDP electronic mail server offers data and software access. Some analytical functions are also included in the server. Full or partial rRNA sequence data (including oligonucleotide probe data) received on request are aligned, checked and phylogenetically analysed and the results returned *via* e-mail. Address: http://www.cme.msu.edu/RDP/

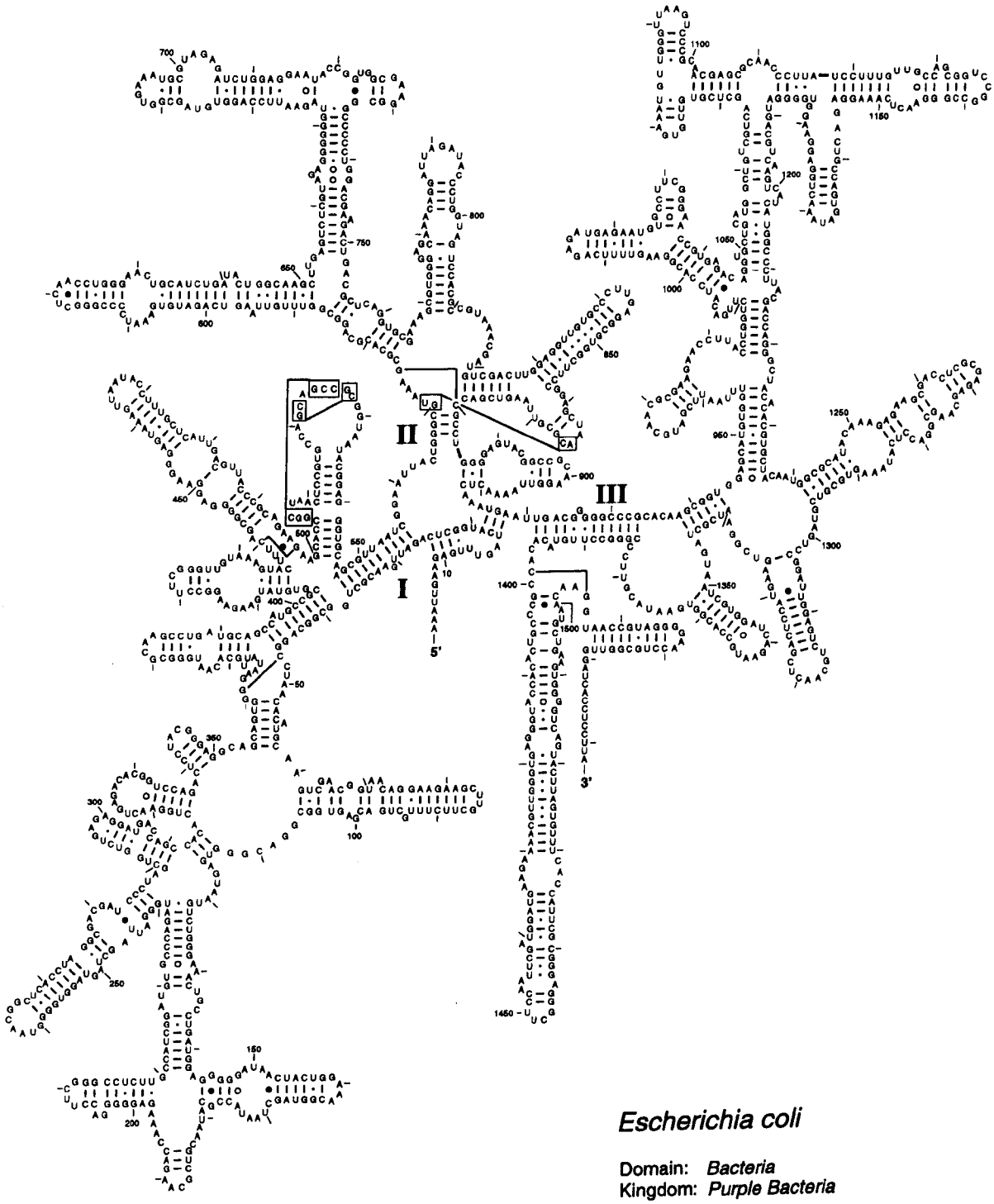
Small and Large Ribosomal Subunit RNA sequence databases. 16S and 23S rRNA sequences are collected weekly from the EBI and GenBank databases and processed by using a set of appropriate programs with respect to primary structural similarity and higher order structure predictions. The data are stored in the form of an alignment along with the postulated secondary structure pattern in encoded form. Software for sequence data editing and phylogenetic tree reconstruction (TREECON; Van de Peer & Wachter, 1994) are available for MS-DOS and VAX-VMS platforms. Address: http://rrna.uia.ac.be/lsu/ and http://rrna.uia.ac.be/ssu.

Appendix 2

Secondary structures of bacterial 16S ribosomal RNA

The secondary structures of the 16S rRNA of:: (a) *Escherichia coli* (GenBank Accession number J01695), (b) *Bacillus subtilis* strain W168 (GenBank Accession number K00637) and (c) *Streptomyces violaceoruber (coelicolor)* strain A3(2) (GenBank Accession number Y00411) are shown below. The diagrams were retrieved from the *RNA Secondary Structures* Web Site <http://pundit.icmb.utexas.edu>.

Secondary Structure: small subunit ribosomal RNA

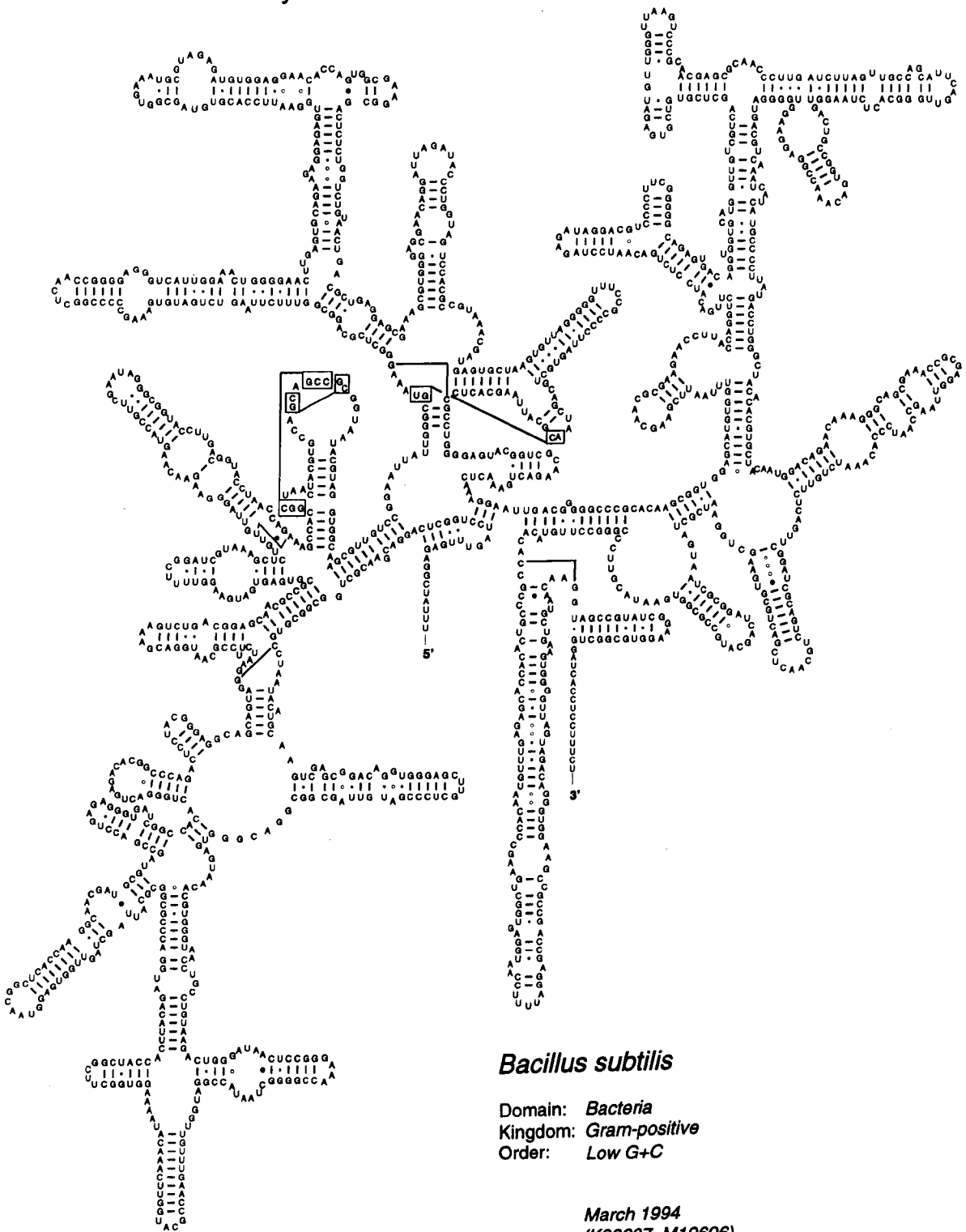


Escherichia coli

Domain: *Bacteria*
Kingdom: *Purple Bacteria*
Order: *gamma*

July 3, 1995 v4.0
(J01695)

Secondary Structure: small subunit ribosomal RNA

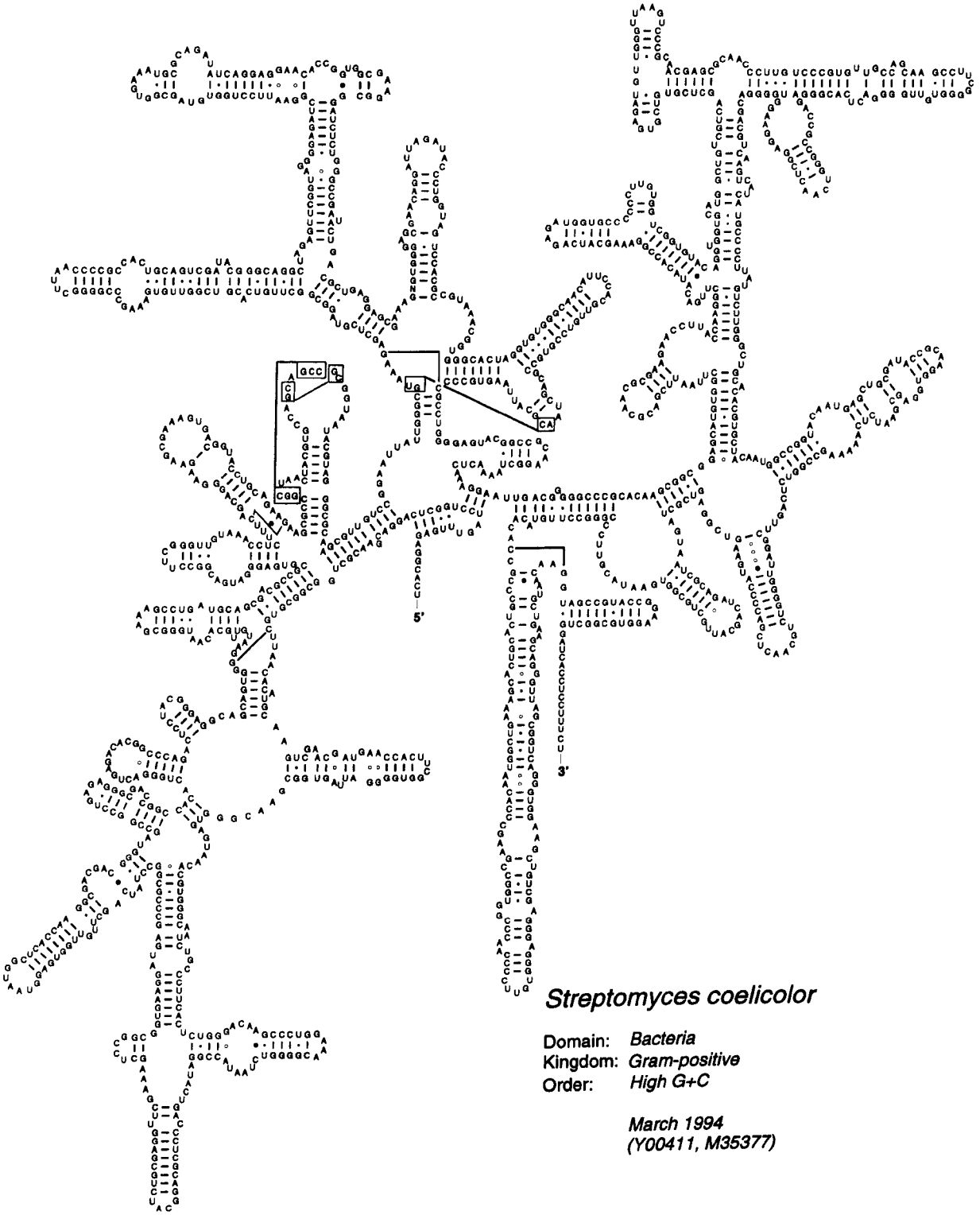


Bacillus subtilis

Domain: *Bacteria*
Kingdom: *Gram-positive*
Order: *Low G+C*

March 1994
(K00637, M10606)

Secondary Structure: small subunit ribosomal RNA



Streptomyces coelicolor

Domain: *Bacteria*
Kingdom: *Gram-positive*
Order: *High G+C*

March 1994
(Y00411, M35377)

Appendix 3

Formulae used for calculating oligonucleotide primer concentrations

Oligonucleotide primer concentrations were calculated and adjusted to reflect the molecular concentration rather than ng per µl or other expressions of concentration. Given the OD_{260} for the respective primer, the molar concentration can be calculated taking into account base composition and length according to the equation (Thein & Wallace, 1986):

$$M = \frac{OD_{260}}{\Sigma \text{ molar extinction coefficients of dNTPs}}$$

where the molar extinction coefficients of individual dNTPs, assuming a 1 cm pathlength cuvette, are: A = 15200; T = 8400; G = 12010; C = 7050.

Thus, given two 20mer oligonucleotide primers with different base compositions, but identical mol. % G+C contents (55 mol% G+C):

- P1: 5 A, 4 T, 9 G and 2 C, and

- P2: 5 A, 4 T, 2 G and 9 C.

The sum of the molar extinction coefficients can be calculated as:

- P1: $5 \times 15200 + 4 \times 8400 + 9 \times 12010 + 2 \times 7050 = 231790$, and

- P2: $5 \times 15200 + 4 \times 8400 + 2 \times 12010 + 9 \times 7050 = 197070$.

Assuming a hypothetical 1/100 dilution of each of the two stock solutions gives an OD_{260} = 0.25, the molar concentration of the primer stocks is:

- P1 = $\frac{0.25 \times 100}{231790} = 107.9\ \mu M$

- P2 = $\frac{0.25 \times 100}{197070} = 126.9\ \mu M$

Alternatively, the molar concentration of DNA oligonucleotides can be calculated by using the formulae of Sambrook *et al.* (1989). However, this approach is not recommended for calculating primer concentrations due to the resultant inaccuracy from using empirically determined average values. According to Sambrook *et al.* (1989) the OD_{260} value of 1 corresponds to approximately 33 μg ml-1 of oligonucleotides hence the primer concentration is equivalent to OD_{260} × dilution × 33. Thus, in the previous example, the primer concentration of both 20mer oligonucleotides can be calculated as:

Concentration = 0.25 × 100 × 33 = 825 μg ml-1

Given that the mean molecular weight of a base is 325 g per molecule, the molar concentration of both 20mer primers is equivalent to:

$$\frac{0.825 \text{ g } l^{-1}}{20 \times 325 \text{ g } ml^{-1}} = 126.9 \text{ μM}$$

The methods of Sambrook *et al.* (1989) and Thein and Wallace (1986) give the same result for the concentration of primer P2 but there is a 15% difference in the concentration of primer P1 using the two different approaches. These biases were found to be even greater with some of the primers used for PCR amplification of 16S rDNA and sequencing (results not shown). Consequently, the method of Sambrook *et al.* (1989) was not considered suitable for calculating oligonucleotide primer concentrations.

Appendix 4

Culture Media and Reagents

Aesculin/arbutin hydrolysis (Williams *et al.*, 1983)

Basal medium:

Ammonium ferrous citrate, 0.5 g; peptone, 10 g; sodium chloride, 1 g; Difco agar, 15 g; distilled water, 1 litre; pH 7.2. Autoclave at 121 °C for 20 minutes.

Test medium:

Supplement basal medium with 1.0 g aesculin/arbutin. Autoclave at 121 °C for 20 minutes.

Acid production from sugars (Gordon *et al.*, 1974)

Basal medium:

$(NH_4)_2HPO_4$, 1 g; KCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; agar, 15 g; distilled water, 1 litre; pH 7.0. Add bromocresol purple solution (0.04 %; 15 ml) and autoclave at 121 °C for 20 minutes.

Carbohydrate solutions:

Carbohydrate solutions (10 %, w/v) were prepared and separately autoclave at 121 °C for 20 minutes. These solutions were mixed with molten basal medium agar to give a final concentration of 1 % (w/v) before pouring Replidishes.

Carbohydrates: adonitol, L-arabinose, D-cellobiose, dextrin, *meso*-erythritol, D-fructose, D-galactose, *meso*-inositol, D-lactose, D-mannitol, D-melibiose, D-melezitose, α-methyl-D-glucoside, D-raffinose, L-rhamnose, salicin, D-sorbitol, sucrose and D-xylose.

Bennett's agar, modified after Jones (1949)

Yeast extract (Difco), 1.0 g; Lab-Lemco (Oxoid), 0.8 g; Bacto-casitone (Difco) , 2.0 g; glucose, 10.0 g; agar (Difco), 12.0 g; distilled water, 1 litre; pH 7.3.

Autoclave at 121°C for 20 minutes; the glucose solution is autoclaved separately.

Carbon source utilisation medium (Boiron *et al.*, 1993)

Ammonium sulphate, 2.64 g; potassium dihydrogen phosphate, 0.5 g; magnesium sulphate, 0.5 g; agar (Oxoid no. 1), 15.0 g; distilled water, 1 litre; pH 7.0. Autoclave at 121°C for 20 minutes. The carbon sources were autoclaved separately at 121°C for 20 minutes.

Carbon and nitrogen source utilisation medium (Boiron *et al.*, 1993)

Potassium dihydrogen phosphate, 0.5 g; magnesium sulphate, 0.5 g; agar (Oxoid no. 1), 15.0 g; distilled water, 1 litre; pH 7.0. Autoclave at 121 °C for 20 minutes. The carbon and nitrogen sources were autoclaved separately at 121 °C for 20 minutes.

Czapek Dox media (Weyland, 1969)

Ferrous sulphate, 0.01 g; magnesium glycerophosphate, 0.5 g; potassium chloride, 0.5 g; potassium sulphate, 0.35 g; sodium nitrate, 2.0 g; sucrose, 30.0 g; agar, 12 g; distilled water, 1 litre; pH 6.8. Autoclave at 121 °C for 20 minutes.

Degradation test media

Basal medium:

Yeast extract, 1 g; Bacto-casitone (Difco), 2 g; Lab-Lemco (Oxoid), 0.8 g; glycerol, 10 g; agar, 15 g; distilled water, 1 litre; pH 7.0. Autoclave at 121 °C for 20 minutes.

Organic substances:

Adenine (4.0 g), allantoin (5.0 g), casein (10 g), elastin (3 g), gelatin (4 g, soaked in cold distilled water), guanine (1 g), hypoxanthine (4 g), starch (1.0 g), testosterone (1.0 g), L-tyrosine (5.0 g), xanthine (4.0 g) and xylan (4.0 g) were each dissolved in 50 ml distilled water, sterilised by Tyndallisation at 100 °C for 30 minutes for three consecutive days and mixed with melted agar medium.

DNA/RNA degradation (Goodfellow *et al.*, 1979)

Tryptose, 20 g; deoxyribonucleic acid/ribonucleic acid, 3.0 g; sodium chloride, 5 g; agar (Lab M), 15 g; distilled water, 1 litre; pH 7.2. Autoclave at 121 °C for 20 minutes.

Glucose yeast-extract agar (GYEA; Gordon & Mihm, 1962)

Glucose, 10 g, yeast extract, 10 g; agar, 18 g; distilled water, 1 litre; pH 6.8. Autoclave at 121 °C for 20 minutes; the glucose solution was autoclaved separately.

Glycerol for storage of inocula

Glycerol (20%, v/v) dispensed in 1.5 ml amounts into cryo vials.

Glycerol-asparagine agar (ISP medium 5; Shirling & Gottlieb, 1966)

L-asparagine (anhydrous basis), 1.0 g; glycerol, 10.0 g; K_2HPO_4 (anhydrous basis), 1.0 g; agar, 20 g; distilled water, 1.0 litre; trace salts solution (page **), 1.0 ml; pH 7.0-7.4. Autoclave at 121 °C for 20 minutes.

Guanidine-sarkosyl solution (Pitcher *et al.*, 1989)

Guanidine thiocyanate (Sigma), 60.0 g; EDTA (0.5 mM) pH 8, 20 ml; deionised water, 20 ml. The preparation was heated at 65 °C until dissolved. After cooling, 5 ml of 10%, v/v sarkosyl solution (GES reagent) was added, the preparation made up to 100ml with deionised water, filtered using a disposable filters (0.45 µm; Acrodisc, Gelman Sciences, 600 South Wagner Road, Ann Arbor, Michigan, USA) and stored at room temperature.

Inorganic salts-starch agar (ISP medium 4; Shirling & Gottlieb, 1966)

Solution I: Soluble starch 10 g. Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml.

Solution II: K_2HPO_4 (anhydrous basis), 1.0 g; $MgSO_47H_2O$, 1.0 g; NaCl, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; $CaCO_3$, 2.0 g; agar, 20 g; distilled water, 500 ml; trace salts solution (page **), 1.0 ml.

Mix solutions I and II. The pH should be between 7.0 and 7.4. Autoclave at 121 °C for 20 minutes.

Nitrate reduction test medium (Gordon & Mihm, 1962)

Potassium nitrate, 1.0 g; peptone, 5 g; Lab lemco, 2.4 g; distilled water, 1 litre; pH 7.0. Autoclave at 121 °C for 20 minutes and dispensed aseptically in 3 ml amounts into sterile capped tubes.

Reagent A: 0.8 cm^3 of sulphanilic acid in 100 ml of 5N acetic acid.

Reagent B: (0.8%, w/v) 8-amino-2-naphthalene sulphonic acid in 100 ml of 5N acetic acid.

Non-sporulating agar (Sanglier *et al.*, 1992)

Casamino acids (Difco), 20.0 g; soluble starch (BDH), 20.0 g; yeast extract, 4.0 g; agar, 18.0 g; distilled water, 1 litre; pH 6.5. The starch, which was made into a paste with 100 ml of cold distilled water, was incorporated into the hot medium with constant stirring. Autoclave at 121 °C for 20 minutes.

Oatmeal agar (ISP medium 3; Shirling & Gottlieb, 1966)

Cook or steam 20 g oatmeal in 1000 ml distilled water for 20 minutes.

Filter through cheese cloth. Add additional distilled water to restore volume of filtrate to 1000 ml. Add agar (18 g) and trace salts solution (1.0 ml, page **). Adjust to pH 7.2. Autoclave at 121 °C for 20 minutes.

Peptone-yeast extract iron agar (ISP medium 6; Shirling & Gottlieb, 1966)

Bacto-peptone iron agar, dehydrated, 36 g; Bacto-yeast extract, 1.0 g; distilled water, 1.0 litre; pH 7.0-7.2. Autoclave at 121 °C for 20 minutes.

Bacto-peptone iron agar, dehydrated (36.58 g) contains Bacto-peptone, 15 g; proteose peptone, 5 g; ferric ammonium citrate, 0.5 g; dipotassium phosphate, 1 g; sodium thiosulphate, 0.08 g; Bacto-agar, 15 g.

Resistance to lysozyme (Gordon & Barnett, 1977)

A solution, made by dissolving 0.1 g lysozyme in 100 ml of sterile distilled water in a volumetric flask, sterilised by filtration using a disposable filter (0.45 μm; Acrodisc, Gelman Sciences, 600 South Wagner Road, Ann Arbor, Michigan, USA). This stock solution was stored at 3 to 4 °C for up to 2 weeks. A 5ml amount of the stock lysozyme solution was mixed with 95 ml of sterile glycerol broth (Lab M peptone, 5.0 g; Lab Lemco, 2.4 g; glycerol 70 ml; distilled water, 1litre, pH 7.0) and dispensed aseptically into sterile capped tubes. Tubes of lysozyme (300 to 500 U/ml) and glycerol broth (control) were seeded with a small amount of inoculum taken from plates incubated for less than 7 days. The cultures were observed for growth after a week of incubation at 45 °C.

Sauton's broth, modified (Mordarska *et al.*, 1972)

L-asparagine, 5.0 g; casamino acid, 2.0 g; glucose, 15 g; sodium citrate, 5.0 g; potassium dihydrogen phosphate, 5.0 g; magnesium sulphate, 0.5 g; potassium sulphate, 0.5 g; ferric ammonium citrate, trace; distilled water, 1 litre; pH 7.2. The medium and glucose were autoclaved separately at 121 °C for 20 minutes.

Trace salts solution (Pridham & Gottlieb, 1966)

$FeSO_4.7H_2O$, 0.1 g; $MnCl_2.4H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g; distilled water, 100 ml. Use as directed in ISP media 3, 4, 5 and 7.

Tryptic soy agar (Difco)

Bacto-tryptone, 15 g; Bacto-soytone, 5 g; NaCl, 5 g; Bacto-agar, 15 g; distilled water, 1 litre; pH 7.2. Autoclave at 121 °C for 20 minutes.

Tryptone-yeast extract broth (ISP medium 1; Shirling & Gottlieb, 1966)

Bacto-tryptone (Difco), 5.0 g; Bacto-yeast extract (Difco), 3.0 g; distilled water, 1 litre; pH 7.0-7.2. Autoclave at 121 °C for 20 minutes.

Tyrosine agar (ISP medium 7; Shirling & Gottlieb, 1966)

Glycerol, 15 g; L-tyrosine, 0.5 g; L-asparagine, 1.0 g; K_2HPO_4 (anhydrous

basis), 0.5 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; Bacto-agar, 20 g; distilled water, 1 litre; trace salts solution (page **), 1 ml; pH 7.2-7.4. Autoclave at 121 °C for 20 minutes.

Urease test medium (Rustigan & Stuart, 1941; Christensen, 1946)

Basal media:

Glucose, 1.0 g; monopotassium phosphate, 2.0 g; peptone, 1.0 g; sodium chloride, 5.0 g; agar (Lab M, MC2), 15 g; distilled water, 1 litre; pH 6.8. Phenol red (0.012 g) is added to this preparation. Autoclave at 121 °C for 20 minutes.

Urea: Urea (20 %, w/v) solution was filter sterilised using a disposable filter (0.45 µm; Acrodisc, Gelman Sciences, 600 South Wagner Road, Ann Arbor, Michigan, USA). The urea was added to the basal media and 2 ml amounts dispensed aseptically in bijoux bottles and slopes prepared.

Yeast extract-malt extract agar (ISP medium 2; Shirling & Gottlieb, 1966)

Bacto-yeast extract (Difco), 4.0 g; Bacto-malt extract (Difco), 10.0 g; Bacto-dextrose (Difco), 4.0 g; Bacto agar, 20 g; distilled water, 1 litre; pH 7.3. Autoclave at 121 °C for 20 minutes.