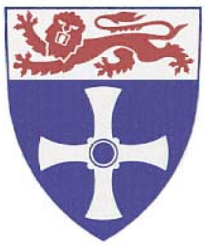
Biosystematics of the Genus *Dactylosporangium* and Some Other Filamentous Actinomycetes

Byung-Yong Kim
(BSc., MSc. Agricultural Chemistry, Korea University, Korea)



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School of Biology,
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Dedicated to my mother who devoted her life to our family,

and to my brother who led me into science

"Whenever I found out anything remarkable, I have thought it my duty to put down my discovery on paper, so that all ingenious people might be informed thereof." - *Antonie van Leeuwenhoek, Letter of June 12*, 1716

"Freedom of thought is best promoted by the gradual illumination of men's minds, which follows from the advance of science."- *Charles Darwin, Letter of October 13*, 1880

Abstract

This study tested the hypothesis that a relationship exists between taxonomic diversity and antibiotic resistance patterns of filamentous actinomycetes. To this end, 200 filamentous actinomycetes were selectively isolated from a hay meadow soil and assigned to groups based on pigments formed on oatmeal and peptone-yeast extract-iron agars. Forty-four representatives of the colour-groups were assigned to the genera *Dactylosporangium*, *Micromonospora* and *Streptomyces* based on complete 16S rRNA gene sequence analyses. In general, the position of these isolates in the phylogenetic trees correlated with corresponding antibiotic resistance patterns. A significant correlation was found between phylogenetic trees based on 16S rRNA gene and *vanHAX* gene cluster sequences of nine vancomycin-resistant *Streptomyces* isolates. These findings provide tangible evidence that antibiotic resistance patterns of filamentous actinomycetes contain information which can be used to design novel media for the selective isolation of rare and uncommon, commercially significant actinomycetes, such as those belonging to the genus *Dactylosporangium*, a member of the family *Micromonosporaceae*.

A culture-independent, nested PCR procedure based on genus-specific oligonucleotide primers detected the presence of *Dactylosporangium* strains in 14 out of 21 environmental samples. Clones generated from the 14 positive samples formed novel phyletic lines in the *Dactylosporangium* 16S rRNA gene tree. Presumptive dactylosporangiae were isolated from 7 of these samples using a medium designed to be selective for members of the genus *Dactylosporangium*. One hundred and two out of 219 representative presumptive dactylosporangiae were considered to be *bona fide* members of the genus *Dactylosporangium* as they gave PCR amplification products with primers specific for this taxon. Representatives of the *Dactylsporangium* isolates formed distinct phyletic lines in the *Dactylosporangium* 16S rRNA gene tree were designated as new species, namely *Dactylosporangium luridum* sp. nov. and *Dactylosporangium luteum* sp. nov., based on a polyphasic study. Similarly, "*Dactylosporangium salmoneum*" NRRL B-16294 was validly described as a new species, *Dactylosporangium salmoneum* sp. nov., nom. rev. In addition, "*Dactylosporangium variesporum*" NRRL B-16296 was transferred to the genus *Saccharothrix* as *Saccharothrix variisporea* corrig. (ex. Tomita *et al*. 1977) sp. nov., nom. rev. Some of the representative *Dactylosporangium* isolates inhibited the growth of *Bacillus subtilis*, *Kocuria rhyzophila* and *Staphylococcus aureus* strains, suggesting that novel *Dactylosporangium* strains might be a rich source of novel antibiotics.

Verrucosispora maris AB-18-032, another member of the family *Micromonosporacea*e, produces *atrop*-abyssomicin C, the first natural inhibitor of the *para*-aminobenzoic acid pathway. The self-protective mechanism of this strain was sought by conjugating an *atrop*-abyssomicin C sensitive *Streptomyces griseus* strain against a genomic DNA library prepared from *V. maris* AB-18-032. Seven resultant resistant exconjugants were screened for *atrop*-abyssomicin C resistance genes using four designed PCR primers. The failure to detect PCR amplification products suggests that the resistance shown by the exconjugants is conferred by mutation within the *S. griseus* strain or by cloning of unidentified resistance genes from the *V. maris* strain.

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I take deep pleasure in thanking my dear wife, Munja and our two lovely sons, Soo-Young and Junyoung for their love and patience during this tough journey. Finally, this study and all my achievements are dedicated to my mother, brother and four sisters who have always been my source of energy. It is through their selfless sacrifices that I have made it this far.

Author's Declaration

Except where acknowledgement has been given, this dissertation is the original work of the author. The material presented has never been submitted to the Newcastle University or to any other educational establishment for purposes of obtaining a higher degree.

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Publications Related to the Thesis

Publications:

Byung-Yong Kim, James E. M. Stach, Hang-Yeon Weon, Soon-Wo Kwon and Michael Goodfellow (2010). *Dactylosporangium luridum* sp. nov., *Dactylosporangium luteum* sp. nov. and *Dactylosporangium salmoneum* sp. nov., nom. rev., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* **60**, 1813-1823.

Byung-Yong Kim, Roselyn Brown, David P. Labeda and Michael Goodfellow (2010). Reclassification of '*Dactylosporangium variesporum*' as *Saccharothrix variisporea* corrig. (ex Tomita *et al*. 1977) sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology* (In press).

Byung-Yong Kim, Jenileima Devi Kshetrimayum and Michael Goodfellow (2010) Detection, selective isolation and characterisation of *Dactylosporangium* strains from diverse environmental samples. *Environmental Microbiology* (submitted).

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Byung-Yong Kim (2009). Detection, Selective Isolation and Characterisation of *Dactylosporangium* Strains. *15th International Symposium on Biology of Actinomycetes*, Shanghai, China.

Poster presentations:

Byung-Yong Kim, Roselyn Brown, James E. M. Stach and Michael Goodfellow (2009). Taxonomic Approach to the Soil Resistome. *Society of General Microbiology*, Harrogate, UK.

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Abbreviation

bp	Base pair
ng	Nanogram
µl	Microliter
10×	Tenfold concentration
no.	Number
M	Molar
nt	Nucleotide
v/v	Volume/volume
w/v	Weight/volume
rpm	Revolutions per minute
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine tetraacetic acid
FAME	Fatty acid methyl ester
%G+C	Percentage of guanine and cytosine
RNase	Ribonuclease
rRNA	Ribosomal RNA
PCR	Polymerase Chain Reaction
Taq DNA Polymerase	*Thermus aquaticus* DNA polymerase
S_{SM}	Simple matching coefficient
T_m	Melting temperature
T_{or}	Optimal renaturation temperature
UPGMA	Unweighted pair group method with arithmetic mean

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Chapter 1. General introduction

1.1 Aims of study

Actinomycetes are an integral part of the indigenous soil microflora (Goodfellow & Williams, 1983; Ul-Hassan & Wellington, 2009; Goodfellow *et al.*, 2010a) and are well known as the primary source of commercially significant secondary metabolites, notably antibiotics (Strohl, 2004; Bérdy, 2005). Many actinomycetes produce more than one antibiotic (Baltz, 2008) and show resistance to multiple antibiotics including those synthesised by other strains (D'Costa *et al.*, 2006). Evidence from numerous studies suggests that antibiotic-resistance genes probably originated from soil bacteria, notably antibiotic producing actinomycetes (Benveniste & Davies, 1973; Pang *et al.*, 1994; Davies, 1997; Mindlin *et al.*, 2006; Woo *et al.*, 2006; Wright, 2010), which means that soil may well be the reservoir of antibiotic resistance genes that have emerged or have the potential to be distributed amongst pathogenic microorganisms (Riesenfeld *et al.*, 2004; Wright, 2007; Dantas *et al.*, 2008). There is evidence that resistance genes of actinomycetes are genetically related to mechanisms of self-protection (Cundliffe, 1989; Hopwood, 2007; Cundliffe & Demain, 2010) and horizontal gene transfer (Wiener *et al.*, 2002; Laskaris *et al.*, 2010).

There may be a link between antibiotic resistance profiles and the production of secondary metabolites from soil bacteria, notably actinomycetes (Yamashita *et al.*, 1985; Bibikova *et al.*, 1989; Alderson *et al.*, 1993; Phillips *et al.*, 1994; Hotta & Okami, 1996; Hotta, 1999; Nodwell, 2007). This raises the possibility that antibiotic resistant patterns may contain useful information for the selective isolation of actinomycetes from microbial communities, which have the capacity to produce certain classes of biologically active compounds. Antibiotic resistance patterns of actinomycetes have been used both as taxonomic markers (Phillips *et al.*, 1994; Kumar & Goodfellow, 2008) and to choose antibiotics which are selective for the isolation of specific actinomycete genera or species from environmental samples when added to basal media (Suzuki *et al.*, 1994; Hayakawa, 2008; Goodfellow, 2010). Such selective agents are chosen to inhibit unwanted bacteria while showing little or no deleterious effects on target groups. However, it has still to be established whether there is a correlation between antibiotic resistance patterns and the taxonomy of indigenous soil bacteria (Shomura, 1993; Phillips *et al.*, 1994; Wright, 2007).

There is an urgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogens (Payne *et al.*, 2007; Spellberg *et al.*, 2008; Fischbach & Walsh, 2009) and to treat life threatening diseases such as cancer (Olano *et al.*, 2009). Amongst prokaryotes, members of the class *Actinobacteria*, notably the genus *Streptomyces*, remain a unique source of natural products (Bérdy, 2005; Newman & Cragg, 2007; Goodfellow & Fiedler, 2010). Actinobacteria account for about 45% of all bioactive secondary metabolites with 7,600 of them (30%) coming from *Streptomyces* strains (Bérdy, 2005) Even so, it has been predicted that only about 10% of the total number of natural products which can be produced by these organisms have been discovered (Watve *et al.*, 2001). It is evident from whole-genome sequencing studies that members of other actinomycete taxa, such as the genera *Amycolatopsis*, *Saccharopolyspora* and *Salinispora*, have the capacity to synthesise many unknown secondary metabolites (Oliynyk *et al.*, 2007; Udwary *et al.*, 2007; Zhao *et al.*, 2010). Consequently, actinomycetes are back in favour as a potential source of novel, clinically significant, natural products (Goodfellow & Fiedler, 2010).

It is becoming increasingly difficult to discover new bioactive compounds from representatives of well known actinobacterial species as this leads to the costly rediscovery of known metabolites (Busti *et al.*, 2006; Lam, 2007). This problem is being addressed by using standard procedures to selectively isolate novel actinomycetes from poorly studied habitats (Bredholdt *et al.*, 2007; Hong *et al.*, 2009; Okoro *et al.*, 2009), by developing new methods to selectively isolate uncommon and rare actinomycetes from soil (Suzuki *et al.*, 2001a, b; Tan *et al.*, 2006; Gontang *et al.*, 2007) and by devising innovative strategies for the cultivation of specific components of previously uncultivated actinomycetes detected in natural habitats using culture-independent techniques (Monciardini *et al.*, 2002; Mincer *et al.*, 2005; Hahn, 2009). These strategies have lead to the isolation of novel actinomycetes found to produce a range of novel bioactive compounds (Fiedler *et al.*, 2005; Jensen *et al.*, 2005, 2007; Goodfellow & Fiedler, 2010), as exemplified by the discovering of salinosporamide, an anticancer drug produced by *Salinispora tropica*, which is in clinical trials (Fenical *et al.*, 2009).

Improved strategies are needed to selectively isolate and characterize many of the 240 genera classified in the class *Actinobacteria*, including the genus like

Dactylosporangium which contains strains known to synthesise novel metabolites (Shomura *et al.*, 1980; Theriault *et al.*, 1987; Kizuka *et al.*, 2002; Tani *et al.*, 2004). This genus was proposed by Thiemann *et al*. (1967) and is one of the 27 genera classified in the family *Micromonosporaceae* (Zhi *et al.*, 2009; Xie *et al.*, 2010). It encompasses aerobic, filamentous actinomycetes which release motile spores from sporangia borne on short sporangiosphores on substrate mycelia and is characterised by the presence of *meso*-and/or hydroxy diaminopimelic acid in whole-organism hydrolysates and other chemical characters (Vobis, 1989, 2006). Little is known about either the taxonomic diversity or the ability of *Dactylosporangium* strains to produce novel bioactive compounds as such studies have been hindered by difficulties in isolating, growing and characterising members of this taxon (Hayakawa *et al.*, 1991a, b; Shomura, 1993; Hayakawa, 2008).

Resistance genes are generally associated with antibiotic biosynthetic gene clusters (Hopwood, 2007; Nodwell, 2007; Cundliffe & Demain, 2010; Laskaris *et al.*, 2010) as antibiotic-producing actinomycetes have resistance mechanisms for self-protection, as shown by the resistance of *Streptomyces* strains to spectinomycin (Lyutzkanova *et al.*, 1997; Kim *et al.*, 2008) and *Streptomyces hygroscopicus* to hygromycin (Dhote *et al.*, 2008). Little is known about the self-resistance mechanism(s) shown by *Verrucosispora maris* AB-18-032; this strain produces *atrop*-abyssomicin C, a polycyclic polyketide antibiotic which acts as an inhibitor of *para*-aminobenzoic acid (*p*ABA) biosynthesis (Bister *et al.*, 2004; Riedlinger *et al.*, 2004; Keller *et al.*, 2007a, b). This antibiotic shows activity against Gram-positive bacteria, including *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus* (MRSA), and was the first known natural inhibitor of the *p*ABA biosynthetic pathway.

The present study was designed to meet several objectives, notably to establish whether a correlation exists between antibiotic resistance patterns and the taxonomy of filamentous actinomycetes isolated from a hay meadow soil, to determine the taxonomic diversity of *Dactylosporangium* strains in a range of natural habitats using culture-dependent and culture-independent procedures, to establish the potential of representative dactylosporangiae to produce novel antibiotics, and to unravel the mechanism(s) whereby *Verrucosispora maris* AB-18-032 is protected against the action of *atrop*-abyssomicin C. The results of these investigation are presented as a series of papers. The thesis includes the following chapters:

Chapter 1. General introduction.

Chapter 2. Materials and methods.

Chapter 3. Comparative study of antibiotic resistance profiles and taxonomy of representative soil actinomycetes

- Selective isolation of filamentous actinomycetes from the hay meadow soil.
- Antibiotic resistance profiling of dereplicated isolates.
- Phylogenetic analyses of representative isolates.
- Correlation between antibiotic resistance and 16S rRNA gene similarity data.
- Comparison of phylogenies between *vanHAX* gene cluster and 16S rRNA gene sequences of vancomycin-resistant isolates.

Chapter 4. Detection, selective isolation and characterisation of *Dactylosporangium* strains from environmental samples.

- Molecular detection and culture-independent *Dactylosporangium* diversity.
- Selective isolation of *Dactylosporangium* strains from environmental samples.
- Culture-dependent *Dactylosporangium* diversity.
- Antimicrobial activity and detection of nonribosomal peptide synthetase and polyketide synthase genes in representative *Dactylosporangium* strains.

Chapter 5. Three new species of *Dactylosporangium* isolated from soil: *Dactylosporangium luridum* sp. nov., *Dactylosporangium luteum* sp. nov. and *Dactylosporangium salmoneum* sp. nov., nom. rev.

- Comparison of soil isolates assigned to the genus *Dactylosporangium* with one another and with the type strains of *Dactylosporangium* species and "*Dactylosporangium salmoneum* " NRRL B-16294.
- Description of *Dactylosporangium luridum* sp. nov. and *Dactylosporangium luteum* sp. nov.
- Proposal that "*Dactylosporangium salmoneum*" NRRL B-16294 be reclassified as *Dactylosporangium salmoneum* sp. nov.

Chapter 6. Reclassification of "*Dactylosporangium variesporum*" as *Saccharothrix variisporea* corrig. sp. nov., nom. rev.

- Polyphasic taxonomic study of "*Dactylosporangium variesporum*" NRRL B-16296.
- Comparison of "*Dactylosporangium variesporum*" NRRL B-16296 with the type strains of *Saccharothrix* species.
- Reclassification of "*Dactylosporangium variesporum*" NRRL B-16296 (Tomita *et al*. 1977) as *Saccharothrix variisporea* sp. nov., nom. rev.

Chapter 7. Search for a self-resistance mechanism of *Verrucosispora maris* AB-18-032 to *atrop*-abyssomicin C.

- Construction of resistant mutants by conjugation.
- Selection of *atrop*-abyssomicin C resistant exconjugants.
- PCR primer design of putative self-resistance genes
- Detection of target genes of exconjugants by PCR reactions.

Chapter 8. General discussion and perspectives for future work

1.2 Prokaryotic systematics

Prokaryotic systematics, the scientific study of the kinds and diversity of *Archaea* and *Bacteria,* is a scientific discipline which includes: *Classification, Nomenclature* and *Identification*. The initial step, classification, is the process of assigning organisms to taxonomic groups on the basis of similarities and differences. The outcome of this process is an orderly arrangement or system which is intended to show natural relationships between taxa and to serve as an information storage and retrieval system. The term classification includes both the process and the outcome of the exercise though outcomes are often referred to as taxonomies. Sound classification of prokaryotes is essential for stable nomenclature and for reliable identification.

Taxonomies based on genotypic and phenotypic properties are termed phenetic classifications. Such classifications involve the acquisition of measurable features of prokaryotes (e.g., biochemical, chemical, morphological and physiological properties), including genetic relationships (e.g., DNA-DNA homology values). Phenetic classifications show relationships between organisms as they exist now, that is, without reference to evolutionary pathways or ancestry. In contrast, phylogenetic classifications

express inferred evolutionary relatedness between organisms and reflect the extent of change over time. In practice, phylogenetic classifications are usually found to be phenetically coherent. Current approaches to prokaryotic classification based on 16S rRNA gene sequences claim to be phylogenetic, but many are in fact phenetic measures of affinity with homologous nucleotide sequences as characters.

The second step, nomenclature, deals with terms used to denote ranks in the taxonomic hierarchy (e.g., species, genera, families) and with the practice of giving correct, internationally recognised names to taxonomic groups according to rules laid out in successive editions of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1975, 1992). Two reforms of the "Bacteriological Code" edited by Lapage and his colleagues in 1975 have had a far reaching impact on the nomenclature of prokaryotes. Firstly, a definitive document and starting date for the recognition of names was introduced with the publication of the *Approved Lists of Bacterial Names* on January 1, 1980 (Skerman *et al.*, 1980); names published before this date and omitted from the Approved Lists lost their standing in nomenclature, a development that cleared away thousands of meaningless names. Secondly, it was established that names of new taxa could only be validly published in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM; formerly the *International Journal of Systematic Bacteriology*), but could be effectively published in appropriate international journals and then cited in Validation Lists published in the IJSEM. The correct use of names is important as microbiologists need to know which organisms they are studying before they can transmit information about them within and outside the scientific community. In other words, an organism's name is a key to its literature, an entry to what is known about it.

Identification, the final stage of the taxonomic trinity, is sometimes seen as the *raison d'etre* of prokaryotic systematics due to the importance of accurately identifying unknown organisms, not least pathogenic bacteria (Priest & Williams, 1993). It is both the act and the result of determining whether unknown organisms belong to established and validly named taxa (Krieg, 2005). It involves determining the key characteristics of unknown organisms and matching them against databases containing corresponding information on established taxa (Priest, 2004). Organisms found to fall outside known groups should be described and classified as new taxa.

Classifications of prokaryotes are data dependent and are in a continuous state of development as high quality information becomes available from the application of new and improved taxonomic methods. Such taxonomies are essentially pragmatic as they are driven by practical imperatives not by theoretical considerations, as exemplified by the biological species concept (Goodfellow *et al.*, 1997; Schleifer, 2009). Current approaches to prokaryotic taxonomy are based on the integrated use of genotypic and phenotypic features acquired through the application of chemotaxonomic, molecular systematic and numerical and non-numerical phenotypic methods. This practice, known as polyphasic taxonomy, was introduced by Colwell (1970) to signify successive or simultaneous studies on groups of prokaryotes using methods chosen to yield high quality data. The polyphasic approach has provided a sound basis for stable nomenclature and reliable identification, essential factors for a practical or utilitarian taxonomy designed to serve different end users (Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997; Gillis *et al.*, 2005; Schleifer, 2009).

The widespread application of polyphasic taxonomy led to significant improvements in the classification of prokaryotes, notably in groups like the *Actinobacteria* and *Cyanobacteria* where traditional approaches based on form and function proved unreliable (Goodfellow & Maldonado, 2006; Kroppenstedt & Goodfellow, 2006; Gupta, 2009). However, it has not been possible to draw up a recommended set of methods to be used in polyphasic studies as taxonomic toolkits are influenced by the biological properties and ranks of the taxa under study and by the equipment available to investigators. Nevertheless, sequencing highly conserved macromolecules, notably 16S rRNA genes, has provided valuable data for generating phylogenies at and above the genus level (Ludwig & Klenk, 2005). In contrast, DNA-DNA relatedness, molecular fingerprinting and phenotypic techniques are methods of choice for delineating taxa at and below the rank of species (Rosselló-Mora & Amann, 2001). A schematic overview of methods commonly used in polyphasic taxonomic studies is shown in Figure 1.1.

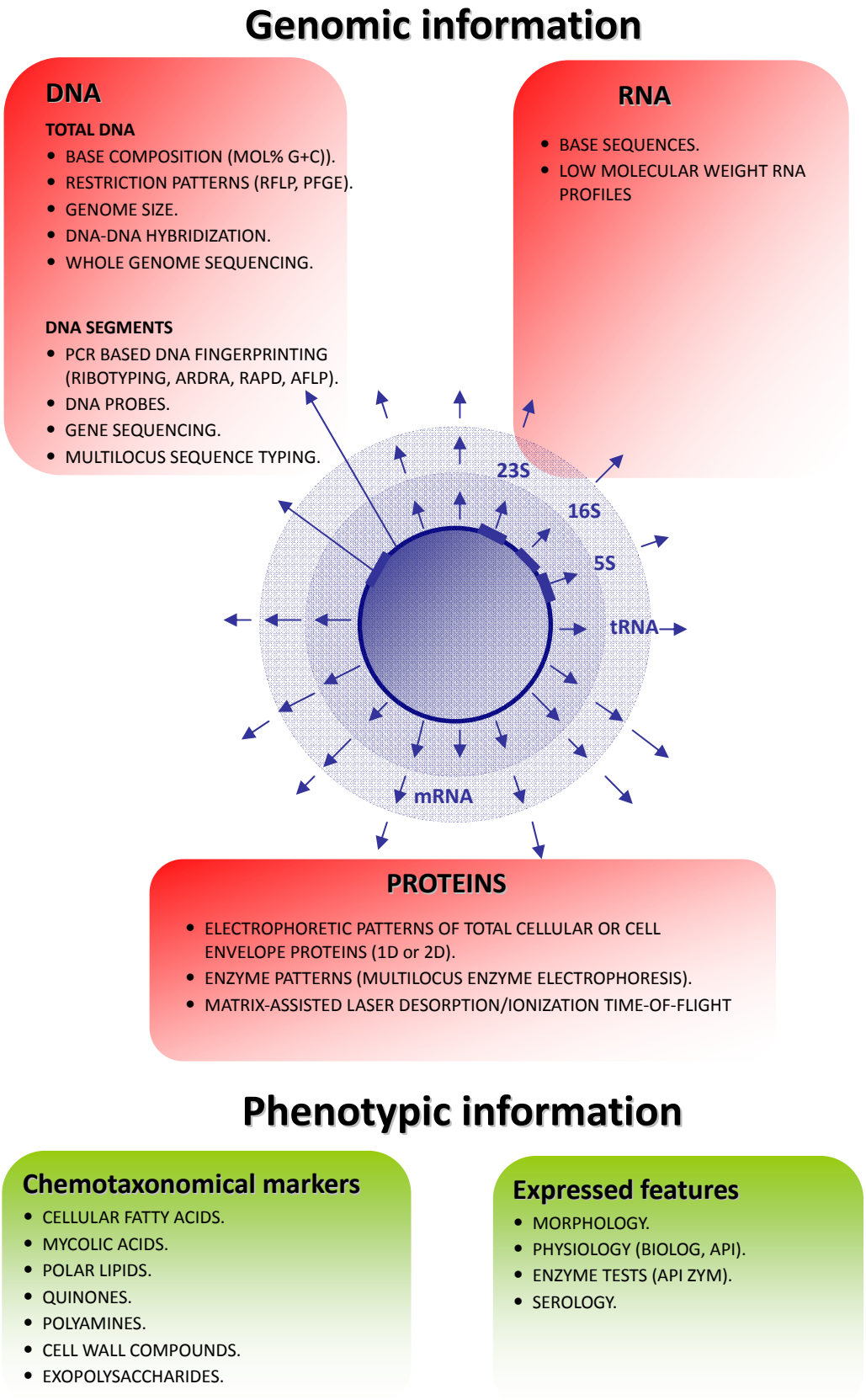


Fig. 1.1. Sources of taxonomic informations for polyphasic studies (modified from Vandamme *et al.*, 1996). Abbreviations: AFLP, amplified fragment length polymorphism; ARDRA, amplified rDNA restriction analysis; PFGE, pulsed field gel electrophoresis; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism.

The basic taxonomic unit in prokaryotic systematics is the species though the definition of species remains a source of controversy amongst microbiologists (Rosselló-Mora & Amann, 2001; Staley, 2006; Schleifer, 2009). Although there is not a universally accepted definition of species in prokaryotic systematics (Ward, 1998; Stackebrandt *et al.*, 2002b), an operational or utilitarian species concept has been proposed for cultivable bacteria on the basis of developments in classification and identification (Goodfellow *et al*., 1997; Schleifer 2009). Extensive taxonomic studies have led to the recommendation that genomic species should include strains with approximately 70% or more DNA-DNA relatedness with a difference of 5ºC or less in thermal stability (ΔTm; Wayne *et al.*, 1987).

Polyphasic taxonomic methods driven approaches to the circumscription of prokaryotic species are sound in an operational sense but are flawed from a theoretical perspective, as they do not take into account that species are products of evolutionary processes (Ward, 1998; Staley, 2006; Schleifer, 2009). Nevertheless, polyphasic studies which draw upon information acquired from chemotaxonomic, numerical phenetic and molecular systematic studies are of considerable practical value in applied microbiology (Priest & Goodfellow, 2000; De Vos *et al.*, 2009).

Chemotaxonomy. This is the study of the discontinuous distribution of chemical macromolecules, notably amino acids, lipids, polysaccharides and related polymers, proteins and isoprenoid quinones amongst members of different taxa and the use of such information for classification and identification (Goodfellow & O'Donnell, 1994; Schleifer, 2009). Chemotaxonomic analyses of macromolecules, particularly amino acids and peptides, lipids (e.g., fatty acids, mycolic acids and polar lipids), and polysaccarides and related polymers (e.g., sugars and teichoic acids) provide valuable data for the classification of prokaryotes at various ranks in the taxonomic hierarchy (Table 1.1).

The determination of amino acid and cell wall sugar composition and peptidoglycan structure led to a radical reappraisal of actinomycete systematics (Williams *et al.*, 1989; Goodfellow & Fiedler, 2010**).** Recently, analyses of proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Lanoot *et al.*, 2002) and matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF; Siegrist *et al.*, 2007) have provided valuable information for the classification of

diverse bacteria, including actinomycetes. In general, good congruence has been found between the discontinuous distribution of chemical markers and phyletic lines apparent in 16S rRNA gene trees (Chun & Goodfellow, 1995; Ward & Goodfellow, 2004; Goodfellow & Fiedler, 2010).

Table 1.1. Discriminatory power of taxonomic tools used to classify and identify prokaryotes

Cell Component	Analysis	Taxonomic Rank		
		Genus or above	Species	Subspecies or below
Chromosomal DNA	Base composition (mol %G+C)	✓	✓	
	DNA:DNA hybridisation		✓	✓
	Restriction patterns (e.g., RFLP, ribotyping)		✓	✓
DNA segments	DNA probes	✓	✓	✓
	DNA sequencing	✓	✓	✓
	Multilocus sequence typing		✓	✓
	PCR based DNA fingerprinting (e.g., PCR-RFLP, RAPD)		✓	✓
Proteins	Amino acid sequences	✓	✓	
	Electrophoretic patterns		✓	✓
	Multilocus enzyme electrophoresis			✓
	Serological comparisons	✓	✓	✓
	MALDI-TOF	✓	✓	✓
Chemical markers	Fatty acids	✓	✓	
	Isoprenoid quinones	✓	✓	
	Mycolic acids	✓	✓	
	Peptidoglycans	✓		
	Polar lipids	✓	✓	
	Polyamines	✓		
	Polysaccharides	✓	✓	
	Teichoic acids	✓	✓	
Whole organisms	Pyrolysis mass spectrometry		✓	✓
	Rapid enzyme tests		✓	✓

* Modifed from Goodfellow (2000). Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PCR-RAPD, PCR-random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism.

Numerical taxonomy. This is the classification by numerical methods of strains and taxonomic units into taxa based on many shared characters (Sneath, 1957). The primary aim of this method is to assign individual strains to homogeneous groups or clusters (taxospecies) using large sets of phenotypic data. The organisms to be classified are referred to as operational taxonomic units (OTUs; Sneath & Johnson, 1972). It is essential in such studies to use phenotypic characters that are genetically stable, hence not susceptible to environmental changes, and which are not particularly sensitive to experimental conditions or observational uncertainties. The usual practice is to take a selection of biochemical, cultural, morphological, nutritional and physiological characters to represent the phenome, that is, the genotype and phenotype. It is important in numerical taxonomic studies to have sufficient information to discriminate between taxa (Sneath & Sokal, 1973; Goodfellow *et al.*, 1997). Most unit characters tend to be binary or two-state; possession of a character is coded as 1 or + and absence by 0 or -. Test results, once obtained, need to be evaluated and unreliable information discarded.

Many resemblance coefficients are available to estimate relationships between pairs of organisms; the two most commonly used ones are the Jaccard (S_J; Jaccard, 1908) and simple matching (S_{SM}; Sokal & Michener, 1958) coefficients. The S_{SM} coefficient is used to calculate similarities based on both positive and negative similarities hence the ratio of the total number of matches to the total number of unit characters is determined. In the case of the S_J coefficient, negative matches are ignored which means that this coefficient measures the ratio of the total number of positive matches to the total number of characters minus the sum of negative matches. The S_J coefficient is generally used to ensure that relationships detected using the S_{SM} coefficient are not based on negative correlations, that is, OTUs are not grouped together because of having a high number of negative characters in common. The S_J coefficient is particularly useful in studies where relatively fast- and slow-growing organisms are compared (Whitham *et al.*, 1993; Trujillo & Goodfellow, 2003), as the latter may give a disproportionate number of negative results.

Hierarchical and non-hierarchical clustering methods are available to order OTUs into groups based on high overall phenetic similarity (Gevers *et al.*, 2006). Such methods are used to produce a ranked classification where strains are grouped into clusters (taxospecies) and aggregate groups (genera), though they are of little value in differentiating between taxa above the genus level (Goodfellow *et al.*, 1997; Castro *et*

al., 2002; Valera & Esteve, 2002). Conventional numerical taxonomic studies have tended to go out of fashion as they are seen to be time-consuming and laborious, but new high-throughput methods, such as commercially available 96 well phenotypic array plates, have been introduced to overcome this limitation (Bochner, 2003; Clemons, 2004; Bochner *et al.*, 2008).

The application of numerical taxonomic procedures led to significant improvements in the classification of actinomycetes. Numerical taxonomic studies have been used to circumscribe taxospecies, including those in complex actinomycete taxa such as the genera *Actinomadura* (Trujillo & Goodfellow, 2003), *Actinomyces* (Schofield & Schaal, 1981), *Actinoplanes* (Goodfellow *et al.*, 1990), *Corynebacterium* (Goodfellow *et al.*, 1982b), *Gordonia* (Goodfellow *et al.*, 1991), *Nocardia* (Goodfellow *et al.*, 1982a; Goodfellow, 1992), *Mycobacterium* (Wayne *et al.*, 1996), *Rhodococcus* (Goodfellow et al., 1998), *Streptomyces* (Williams *et al.*, 1983; Kämpfer *et al.*, 1991; Manfio *et al.*, 1995), *Streptosporangium* (Whitham *et al.*, 1993) and *Thermomonospora* (McCarthy & Cross, 1981). Phenotypic analyses of streptomycetes (Williams *et al.*, 1983; Kämpfer *et al.*, 1991; Manfio *et al.*, 1995) provided a sound base for selecting representative strains for more sophisticated taxonomic investigations, not least for comprehensive molecular systematic studies (Lanoot *et al.*, 2002; Lanoot *et al.*, 2005).

Molecular systematics. The most significant recent advances in prokaryotic systematics are based on the realisation that archaea and bacteria contain records of changes that have occurred since they diverged from a common ancestor around 3.5 billion year ago (Zuckerkandl & Pauling, 1965; Woese, 1987). Molecular-based systematics has a significant advantage over chemotaxonomic and numerical taxonomic apporaches as the acquisition of sequence data is independent of cultivation conditions. Molecular-based methods are currently the driving force in prokaryote systematics, partly as a consequence of technological changes but primarily because the end product of this approach reflects natural relationships between prokaryotes as encoded in DNA and protein sequences (Head *et al.*, 1998; Woese, 1998; Lerat *et al.*, 2005; Gevers *et al.*, 2006; Koonin, 2009; Schleifer, 2009; Alam *et al.*, 2010; Jensen, 2010).

16S rRNA gene sequencing. Data derived from sequencing 16S rRNA genes are used extensively for the classification of cultivated (Woese, 1987; Rosselló-Mora & Amann, 2001; Ludwig & Klenk, 2005) and uncultivated (Stach *et al.*, 2003a, b; Kumar *et al.*,

2007) prokaryotes, and to design oligonucleotide probes and primers for the identification of specific taxa (Yoon *et al.*, 1996; Shen & Young, 2005; Zhi *et al.*, 2006). However, 16S rRNA sequencing studies do not always allow delineation between closely related species (Fry *et al.*, 1991; Fox *et al.*, 1992), as exemplified by studies on the genera *Micromonospora* (Koch *et al.*, 1996), *Saccharomonospora* (Yoon *et al.*, 1997) and *Salinispora* (Jensen *et al.*, 2005).

16S rRNA gene sequence data held in the DNA Data Bank of Japan (DDBJ; Tateno *et al.*, 2002), the European Molecular Biology Laboratories Database (EMBL; Kanz *et al.*, 2005), the GenBank Database (Benson *et al.*, 2009) and by the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/index.jsp; Maidak *et al.*, 1997), are readily retrievable for comparative taxonomic studies. In general, good congruence has been found between phylogenetic trees based on 16S rRNA gene sequence data and corresponding trees generated from studies of other conserved molecules, such as elongation factors, protein-translocating ATPase subunits, and RNA polymerases (Ludwig & Klenk, 2005). Consequently, comparative 16S rRNA gene sequencing analyses are seen to be the most accurate and fast way of identifying unknown prokaryotes, including the recognition of putatively novel taxa (Goodfellow & Fiedler, 2010), despite their inherent limitations (Schleifer, 2009).

Analysis of sequence data and phylogenetic reconstruction. The alignment of rRNA gene sequences is very important for inferring phylogenetic relationships. The presence of insertions and deletions (indel sequences) may make alignments less accurate, especially when homologies are low. The use of secondary structural information becomes essential to localize indel sequences. It is customary to manually adjust alignments and to eliminate nucleotide positions considered to be uncertain (Brocchieri, 2001; Harayama & Kasai, 2006), procedures which rely on the judgment of the investigator.

Numerous tree-making methods are available to infer ancestry once nucleotide sequences have been aligned. The four major approaches are the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. The most frequently used method for calculating distances is the one-parameter model proposed by Jukes & Cantor (1969), this is based on the assumption that there are independent changes at all nucleotide positions, that is, there

is an equal probability of ending up with each of the other three bases.

The construction of trees from data in distance matrices is carried out by using available tree-making methods, such as neighbour-joining (Saitou & Nei, 1987) and weighted least-squares (Fitch & Margoliash, 1967) methods. The neighbour-joining method is theoretically related to clustering methods, such as the unweighted-pair-group method with arithmetic averages (UPGMA; Sneath & Sokal, 1973), but is not based on the assumption that data are ultra metric 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 least-squares method fits a given set of pairwise evolutionary distance estimates to an additive tree. The maximum-parsimony method is used to find the most parsimonious tree among all possible tree topologies, the tree with the minimal overall number of changes is the most parsimonious one and is taken as the one which infers evolution most closely (Felsenstein, 1981).

The maximum-likelihood method is the most statistically sound way of reconstructing phylogenies (Felsenstein, 1981). This approach to phylogenetic inference is used to determine an explicit model of evolution by analysing 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 statistical significance of the order of particular subtrees in a phylogenetic tree can be tested by resampling methods, such as the bootstrap procedure (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 and 1000 times with alternatively truncated or rearranged datasets.

DNA-DNA relatedness. A unique property of DNA and RNA is their capacity for reassociation or hybridization. Complementary strands of DNA, once denatured, can reassociate into native duplexes under appropriate experimental conditions. When comparing nucleic acids from any two closely related prokaryotes the extent of molecular hybrid and its thermal stability provide a measure of the nucleotide sequence similarity between them. These theoretically simple concepts are the basis of DNA-DNA relatedness studies. The importance of such studies in circumscribing species was underlined by recommendations from *ad hoc* committee on prokaryotic systematics

(Wayne *et al*., 1987; Stackebrandt *et al*., 2002). Wayne and his colleagues recommended that the phylogenetic definition of archaeal and bacterial species should be based on the assignment of strains to genomic species when they showed approximately 70% or more DNA relatedness values with 5˚C or less thermal stability (*ΔTm*). However, DNA-DNA relatedness values need to be interpreted with care as they may not reflect the actual degree of sequence similarity (Goodfellow *et al.*, 1997; Rosselló-Mora & Amann, 2001) and may be distorted by differences in genome size and by genomic rearrangements (Kang *et al.*, 2007). It is also well known that DNA-DNA relatedness data are prone to experimental error (Goodfellow *et al.*, 1997; Schleifer, 2009) and cannot be used to generate cumulative databases as they are based on pairwise comparisons (Stackebrandt *et al.*, 2002b; Mehlen *et al.*, 2004; Kang *et al.*, 2007).

Currently two experimental approaches are commonly used to measure the degree of DNA relatedness or similarity between prokaryotes. They are based on assessing the degree of binding by hybridization (Ezaki *et al.*, 1989; Jahnke, 1994) or by establishing differences in thermal denaturation midpoints (De Ley *et al.*, 1970; Mehlen *et al.*, 2004). The binding strategy involves fixing single-stranded, high-molecular-weight DNA on a solid support (generally nitrocellulose or nylon membranes) followed by incubation in the presence of single-stranded, low-molecular-weight, labelled DNA. The thermal denaturation temperature is used to estimate the thermal stability of hybrid DNA duplexes against homologous DNA. The temperature at which 50% of the initial double-stranded molecules have denatured into single-stranded DNA is the melting temperature or thermal denaturation midpoint (*Tm*).

A parameter used to estimate DNA-DNA relatedness, Δ*Tm*, is the differences between the *Tm* of the reference strain and that of the hybrid DNA. To estimate Δ*Tm,* purified total genomic DNA and mixtures of DNA from representatives of related species are denatured and allowed to renature at the optimal temperature for renaturation (T_{or}; De Ley *et al*., 1970). T_{or} can be estimated from the mol% G+C of the DNA of strains under study, as described by De Ley *et al.* (1970). The transition from double to single stranded DNA, DNA melting, can be measured by the change in absorbance at 260 nm. Alternatively, the shift in fluorescence of added SYBR Green I dye bound to double stranded DNA can be determined as DNA is ‘melted’ by progressive heating (Gonzalez & Saiz-Jimenez, 2005). This technique has several advantages over more established methods. It is rapid and inexpensive, and allows high-

throughput comparisons. Comparative studies show that results derived from estimating binding percentages and Δ*Tm* values are generally in good agreement (Roselló-Mora & Amann, 2001; Gonzalez & Saiz-Jimenez, 2005).

Despite limitations, DNA-DNA relatedness studies are still considered to provide the golden standard for the delineation of prokaryotic species (Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997; Rosselló-Mora & Amann, 2001; Kumar & Goodfellow, 2008). It is clear that such studies give greater resolution between closely related strains than corresponding 16S rRNA gene sequencing studies (Goodfellow *et al.*, 1997; Rosselló-Mora & Amann, 2001; Kumar & Goodfellow, 2008), as it is apparent from Figure 1.2. Organisms with almost identical 16S rRNA sequence similarities can be distinguished using corresponding DNA: DNA relatedness data.

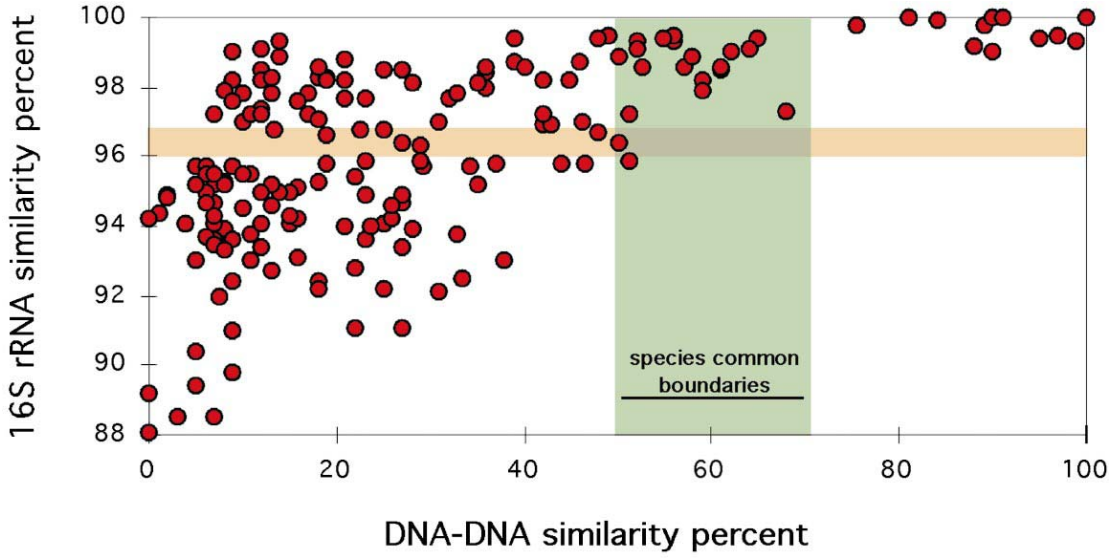


Fig. 1.2 Comparison of DNA-DNA and 16S rRNA gene similarities of *Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* and Gram-positive bacteria of high GC phyla. The vertical shaded zone indicates the range of cut-off values for DNA-DNA relatedness used for the delineation of genomic species while the horizontal shade zone indicates cut-off values for 16S rRNA gene sequence similarity (97%) previously used to delineate strains (taken from Rosselló-Mora & Amann, 2001).

The report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology recommended the use of DNA profiling (e.g., AFLP, PCR-RFLP, *rep*-PCR and ribotyping) and multilocus sequence typing (MLST) to discriminate between taxonomically closely related strains (Stackebrandt *et al.*, 2002a). These workers concluded that MLST was a potentially useful procedure, which might be used as an alternative to DNA-DNA relatedness studies in defining genomic species. MLST involves sequencing a minimum of five housekeeping or other protein coding genes and

presenting the resultant data in individual and/or concatenated trees (Enright & Spratt, 1999; Gevers *et al.*, 2005; Antony-Babu & Goodfellow, 2008). The choice of genes needs to be based on their loci; selected genes should be spread across the genome (Maiden *et al.*, 1998; Stackebrandt *et al.*, 2002a). MLST provides good resolution at and below the species level and greater clarity in genomic relatedness at inter- and intraspecific levels (Thompson *et al.*, 2005; Guo *et al.*, 2008; Martens *et al.*, 2008). Initially, MLST studies were restricted to epidemiological and population genetic studies (Enright & Spratt, 1999; Robinson & Enright, 2004; Miragaia *et al.*, 2007), but they are now being used to establish taxonomic relationships between closely related bacteria, as exemplified by studies on *Escherichia coli* (Adiri *et al.*, 2003), *Listeria monocytogenes* (Salcedo *et al.*, 2003), *Staphylococcus aureus*, (Grundmann *et al.*, 2002), *Streptomyces griseus* (Guo *et al.*, 2008; Rong & Huang, 2010; Rong *et al.*, 2010) and *Streptomyces albidoflavus* (Rong *et al.*, 2009).

1.3 Prokaryotic diversity and bioprospecting

It has been estimated that a gram of soil may contain between 2,000 and 8.3 million prokaryotic species (Torsvik *et al.*, 2002; Fierer & Jackson, 2006), while the total number of prokaryotic cells present in natural habitats has been considered to be 4 - 6 x 10^{30} cells (Whitman *et al.*, 1998). It is surprising in light of such figures that only about 10,000 species of prokaryotes have been formally described (http://www.bacterio.cict.fr; April, 2010), which means that the vast majority of prokaryotes (90-99%) present in natural habitats have still to be isolated and formally described (Zengler *et al.*, 2002; Rappé & Giovannoni, 2003; Schloss & Handelsman, 2004). Consequently, little is known of the role or distribution of individual prokaryotic species, including actinomycetes, in natural habitats (Bull *et al.*, 2000; Handelsman, 2004).

Biotechnological search and discovery generally starts with the assembly of appropriate biological material, moves through screening for desired attributes, selection of the best options from a short list of positive screened hits, and culminates with the development of a commercial product or process (Bull *et al.*, 2000; Goodfellow & Fiedler, 2010). The primary and secondary metabolism of prokaryotes has been exploited for the production of diverse natural products, notably secondary metabolites (Bérdy, 1995, 2005; Strohl, 2004), The best known secondary metabolites are antibiotics,

a new generation of which is needed to combat drug resistant bacteria and fungi and to provide safer and more potent compounds with improved pharmacological properties (Payne *et al.*, 2007; Fischbach & Walsh, 2009). Unknown, important products are generally found when high quality biological materials are examined within existing or new screening systems; it is, therefore, essential to foster these two aspects of novelty in drug discovery programmes (Nolan & Cross, 1988; Goodfellow & Fiedler, 2010).

Actinomycetes and natural products. Amongst prokaryotes, filamentous actinomycetes, notably streptomycetes, are the most prolific source of new antibiotics (Bérdy, 2005; Newman & Cragg, 2007; Goodfellow & Fiedler, 2010). It is likely that this trend will continue as full genome sequences of model actinomycetes have been found to contain over 20 natural product biosynthetic gene clusters for the production of known or predicted secondary metabolites, as shown by studies on *Amycolatopsis mediterranei* U32 (Zhao *et al.*, 2010), *Saccharopolyspora erythraea* NRRL 23338 (Oliynyk *et al.*, 2007), *Salinispora tropica* CNB 440 (Udwary *et al.*, 2007), *Streptomyces avermitilis* (Ikeda *et al.*, 2003) and "*Streptomyces coelicolor*" A3(2) (Bentley *et al.*, 2002). In contrast, few, if any, such gene clusters have been detected in whole genomes of other prokaryotes (Goodfellow & Fiedler, 2010).

Actinomycetes are currently known to produce over 10,000 bioactive compounds, 7,600 of which have been isolated from streptomycetes and 2,500 from non-streptomycetes, notably from the so called rare actinomycetes (Lazzarini *et al.*, 2000). Despite this amazing productivity, it has been estimated that only a tiny fraction of the total number of antimicrobial compounds which actinomycetes can produce have been found to date (Watve *et al.*, 2001). The number of antibiotics and "other bioactive" metabolites synthesised by different microbial groups are shown in Table 1.2.

Antibiotics produced by actinomycetes show a wide range of chemical diversity, as demonstrated in Table 1.3. It is clear from these data that streptomycetes synthesise a diverse range of chemical structures; it is also known that specific structural types of antibiotics occur commonly in these organisms, such as ansa-lactam rings, macrocyclic lactones, polyether and cyclopeptide skeletons (Bérdy, 1995, 2005). However, certain specific structures are produced more frequently by non-streptomycetes, such as *Actinoplanes* and *Amycolatopsis* strains, which are rich sources of vancomycin-like glycopeptides (Wink *et al.*, 2003).

Table 1.2. Approximate number of bioactive microbial metabolites according to their producers and bioactivities

Source	Antibiotics		Bioactive metabolites		Total bioactive metabolites
	Total antibiotics	(with other activity)	No antibiotic activity	(antibiotics plus other bioactivies)	
Bacteria:	**2900**	**(780)**	**900**	**(1680)**	**3800**
Eubacteriales	2170	(570)	580	(1150)	2750
Bacillus sp.	*795*	(235)	*65*	(300)	*860*
Pseudomonas sp.	610	(185)	185	(370)	795
Myxobacteria	400	(130)	10	(140)	410
Cyanobacteria	300	(80)	340	(420)	640
Actinomycetales:	**8700**	**(2400)**	**1400**	**(3800)**	**10100**
Streptomyces sp.	6550	(1920)	1080	(3000)	7630
Rare actinomycetes	2250	(580)	220	(800)	2470
Fungi:	**4900**	**(2300)**	**3700**	**(6000)**	**8600**
Microscopic fungi	3770	(2070)	2680	(4750)	6450
Penicillium/Aspergillus	1000	(450)	950	(1400)	1950
Basidiomycetes	1050	(200)	950	(1150)	2000
Yeasts	105	(35)	35	(70)	140
Slime moulds	30	(5)	20	(25)	60
Total	**16500**	**(5500)**	**6000**	**(11500)**	**22500**

* Taken from Bérdy (2005).

Rare actinomycetes. There is evidence that taxonomic diversity can be used as a surrogate for chemical diversity amongst actinomycetes, especially at the species level (Goodfellow *et al.*, 2007; Jensen *et al.*, 2007; Tan *et al.*, 2007). This means that novel taxa which populate gaps in actinomycete taxospace should be a rich source of new bioactive compounds (Ward & Goodfellow, 2004; Jensen, 2010). Consequently, it makes good sense to devise selective isolation and characterisation strategies to secure representatives of rare actinomycete genera for pharmaceutical screening programmes, a stragtegy which has been applied with some success (Lazzarini *et al*. 2000; Goodfellow & Fiedler, 2010).

Rare actinomycete genera include a plethora of taxa known to synthesise novel antibiotics, as exemplified by the genera *Actinokineospora, Acrocarpospora, Actinosynnema, Amycolatopsis, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kineosporia, Kutzneria, Microbispora, Microtetraspora, Nonomuraea, Thermomonospora, Pseudonocardia, Thermobifida, Saccharomonospora, Spirilliplanes, Streptosporangium* and *Virgosporangium* (Hayakawa, 2008). The

importance of rare actinomycetes can be demonstrated by the fact that some of them are the source of commercially important anti-microbial products, as exemplified by erythromycin produced by *Saccharopolyspora erythraea* (Oliynyk *et al.*, 2007), gentamicin by *Micromonopsora purpurea* (Weinstein *et al.*, 1963; Wagman & Weinstein, 1980), rifamycins by *Amycolatopsis mediterranei* (Jin *et al.*, 2002), teicoplanin by *Actinoplanes teichomyceticus* (Somma *et al.*, 1984; Jung *et al.*, 2009) and vancomycin by *Amycolatopsis orientalis* (Wink *et al.*, 2003).

Table 1.3. Antibiotics isolated from actinomycete genera, as described in the antibiotic database of the *Journal of Antibiotics* (http://www.antibiotics.or.jp)

Actinomycete	AG	ML	AML	BLA	PEP	GP	ANC	TC	NUC	POL	QN
Streptomyces	■	■	■	■	■	■	■	■	■	■	■
Other actinomycetes :											
Actinomadura	■	■					■			■	
Actinoplanes	■	■			■	■			■	■	■
Actinosynnema				■							
Ampullariella							■		■		
Amycolatopsis			■		■	■	■	■		■	■
Dactylosporangium	■	■					■		■	■	
Kibdelosporangium		■				■					
Kitasatospora		■			■						
Microbispora	■				■						■
Micromonospora	■	■	■		■		■		■	■	■
Microtetraspora		■			■						
Nocardia	■	■	■	■	■	■	■	■	■		■
Nocardiopsis	■	■									
Nonomuraea						■					
Pseudonocardia					■	■					■
Rhodococcus					■						■
Saccharomonospora						■					
Saccharopolyspora	■	■			■						
Saccharothrix	■	■			■	■					■
Streptoalloteichus						■					
Streptosporangium			■		■	■		■			
Thermomonospora					■						

* AG, aminoglycoside; ML, macrolide; AML, ansamacrolide; BLA, β-lactam; PEP, peptide; GP, glycopeptides; ANC, anthracycline; TC, tetracycline; NUC, nucleotide; POL, polyene; QN, quinine. ■, production (modified from Okami & Hotta, 1988).

Nonribosomal peptides and polyketides. Nonribosomal peptides and polyketides are very large classes of natural products which are assembled from simple acyl-coenzyme

A or amino acid monomers (Marahiel *et al.*, 1997; Shen, 2003). They have a broad range of biological activities and pharmacological properties and are a source of novel antibiotics, cytostatics, and immunosuppressants (Rascher *et al.*, 2003; Schwarzer *et al.*, 2003; Bergmann *et al.*, 2007). Non-ribosomal peptides and polyketides are synthesized by one or more large, specialized, multifunctional, modular enzymes, namely non-ribosomal polypeptide (NRPS) and polyketide (PKS) synthases, respectively (Schwarzer *et al.*, 2003). NRPS and PKS catalyse chain elongation from simple building blocks to generate diverse natural products (Staunton & Weissman, 2001; Donadio *et al.*, 2007). Three PKS types have been described though only types I and II are of major interest (Shen, 2003). Type I PKS's are common in bacteria, including actinomycetes (Staunton & Weissman, 2001) and type II PKS's, which are multi-enzymes, carry a single set of iteratively acting activities, as illustrated by the biosynthesis of aromatic polyketides, tetracenomycin (Shen, 2003). Type III PKS types, known as chalcone synthase-like PKS's, are homodimeric enzymes which are mainly acting condensing enzymes (Watanabe *et al.*, 2003).

Actinomycetes typically contain genes encoding PKS I, PKS II and NRPS (Metsa-Ketela *et al.,* 1999; Ayuso *et al.*, 2005; Ginolhac *et al.,* 2005) and hence are good vehicles for the discovery of novel secondary metabolites (Schwarzer *et al.*, 2003). PKS I and NRPS genes are usually organised into units or segments with similar gene segments repeated within a single gene cluster (Busti *et al.*, 2006). In contrast, PKS II systems do not have a modular organisation hence the individual biochemical steps of polyketide biosynthesis are catalyzed by discrete subunits which are used repeatedly in the biosynthetic cycle (Ayuso-Sacido & Genilloud, 2005). The utilization of PCR technology to amplify specific sequences from bacterial genomic DNA by using degenerate oligonucleotide primers which correspond to highly conserved motifs in peptide synthases has led to the establishment of a conventional approach for the identification and cloning of putative genes that encode these multi-enzymes (Metsa-Ketela *et al.*, 1999; Ayuso-Sacido & Genilloud, 2005; Savic & Vasiljevic, 2006; Hwang *et al.*, 2007).

Analysis of PKS and NRPS genes in putative bioactive compound producing actinomycetes helps not only to determine the evolutionary relationships of these genes but to relate them with the ecology and physiology of the organisms (Metsa-Ketela *et al.*, 2002; Ayuso *et al.*, 2005; Ginolhac *et al.*, 2005; Jenke-Kodama *et al.*, 2005;

Gontang *et al.*, 2010). Molecular screening of such genes is considered to be useful in dereplication and for screening systems with novel biosynthetic pathways thereby being of relevance to natural product screening programmes (Ayuso-Sacido & Genilloud, 2005; Ayuso *et al.*, 2005; Savic & Vasiljevic, 2006).

1.4 Actinomycete diversity in natural habitats

Culture-dependent and culture-independent approaches are widely used to study prokaryotic communities in natural habitats (Bull *et al.*, 2000; 2005). Culture-dependent approaches employ artificial media to culture or enrich for groups of microorganisms found in environmental samples. Until recently, this method was extensively used to determine the extent of prokaryotic diversity in natural habitats (Ward *et al.*, 1990; Hugenholtz *et al.*, 1998; Fierer & Jackson, 2006), but it is now known that the majority of microorganisms in the environment cannot be cultured by using conventional plate culture methods hence the vast majority of the soil microbiota remain undiscovered (Whitman *et al.*, 1998; Rappé & Giovannoni, 2003; Vartoukian *et al.*, 2010). One reason for the limited value of conventional isolation procedures is that the cultivation conditions used to isolate organisms do not reflect conditions in the environment hence fast-growing prokaryotes are selected that are best adapted to grow on individual growth media (Zengler *et al.*, 2002; Stevenson *et al.*, 2004; Vartoukian *et al.*, 2010). This limitation has promoted the use of culture-independent approaches to detect previously uncultured microbes and evaluate microbial diversity in natural habitats (Amann *et al.*, 1995; Stach *et al.*, 2003b; Kumar *et al.*, 2007; Xin *et al.*, 2008).

Culture-independent approaches. Culture-independent methods involve the extraction of community nucleic acids from cells present in environmental samples followed by the application of biomarker-based techniques, such as DNA analyses to trace the taxonomic diversity of microbial communities in the natural habitats (Pace, 1997; Head *et al.*, 1998; Hugenholtz *et al.*, 1998; Kirk *et al.*, 2004). Molecular approaches confirmed observations from direct microscopy that the number of prokaryotes which can be readily cultivated from environmental samples is only a small and skewed fraction of the diversity present (Pace, 1997; Rappé & Giovannoni, 2003; Vartoukian *et al.*, 2010). The inability to cultivate even the most numerous microorganisms from natural habitats has been referred to as the ‘great plate count anomaly’ (Pace, 1997; Hirsch *et al.*, 2010). Amann *et al.* (1995) considered that <1% of the bacteria present in

environmental samples formed colonies on nutrient agar and went on to stress the need to apply culture-independent approaches to unravel the full extent of prokaryotic diversity in natural habitats.

The use of 16S rRNA gene sequences as indicators of prokaryotic diversity is well established though other genes, including peptide elongation factor Tu RNA polymerase (*tuf*) and RNA polymerase (*rpoB*) genes, have been used for this purpose (Rintala *et al.*, 2001; Peixoto *et al.*, 2002; Kumar *et al.*, 2007; Tanaka *et al.*, 2010). In addition, various culture-independent techniques can be employed to target other genes such as those responsible for metabolic actions, for instance, polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes, which are widely used to check whether strains have the capacity to produce antibiotics (Wawrik *et al.*, 2005; Pang *et al.*, 2008). The use of such analytical strategies makes it possible to target cells, which actively express the specific metabolic functions.

Most culture-independent methods have been used to establish the extent of prokaryotic diversity in soil, including that of actinomycetes (Mincer *et al.*, 2005; Kumar *et al.*, 2007; Xin *et al.*, 2008; Hirsch *et al.*, 2010). Relatively harsh extraction methods are needed to extract community DNA from soil as indigenous prokaryotic cells tend to be tightly bound to soil particles (Roose-Amsaleg *et al.*, 2001; Feinstein *et al.*, 2009; Fitzpatrick *et al.*, 2010). Chemical and physical extraction methods are usually needed to lyse microbial cell walls. Chemical procedures tend to entail the use of lysozyme and sodium dodecyl (lauryl) sulphate to weaken and break open cell walls to release cellular DNA (Liles *et al.*, 2008); physical methods include freeze/thaw and/or bead beating protocols (Niemi *et al.*, 2001). Extraction kits designed specifically for the recovery of community DNA from soil are based on bead beating and chemical lysis methods. Following the disruption of microbial cells and the extraction of DNA/RNA, the resultant nucleic acids are purified using proteinase K and the phenol/chloroform extraction method. Nucleic acids carry a fixed negative charge per unit length of molecule hence for purification purposes nucleic acids are collected using a positively charged binding material followed by washing to discard unnecessary compounds.

Generation of gene clone libraries. Gene clone library construction is a widely used procedure which involves the extraction of purified nucleic acids from environmental

samples and the amplification of specific sequences of genomes present using the PCR, this, in turn, involves the use of oligonucleotide primers specific to the preferred amplicon gene sequence (Nakatsu *et al.*, 2000; Monciardini *et al.*, 2002; Kumar *et al.*, 2007). Amplified gene sequences are inserted into engineered plasmids, which contain an antibiotic resistant gene; this results in the generation of recombinant plasmids, which can be taken up by competent *Escherichia coli* host cells. The latter are grown on nutrient media containing the antibiotic in question hence only cells that contain the plasmid survive and replicate. The resultant colonies, or clones, contain copies of the same recombinant plasmid with inserts of foreign DNA. A range of methods can be used to determine colonies which contain DNA sequences of interest, such as white/blue differentiation or lacZ' selection (Ma *et al.*, 2009). Most 16S rRNA gene clone libraries consist of between 100 and 300 clones though the generation of additional clones has the advantage that it allows the detection of greater diversity in terms of community composition, richness and structure (Dunbar *et al.*, 2000; 2002).

1.5 Selective isolation of actinomycetes

Innumerable actinomycetes have been isolated and screened since Selman Waksman discovered that *Streptomyces griseus* produced streptomycin, a discovery that promoted the search for additional novel bioactive compounds of therapeutic value (Watve *et al.*, 2001). Early search and discovering programmes were focused on streptomycetes as these organisms were easy to isolate and grew quickly on isolation media. Intensive studies on members of the genus *Streptomyces* led to the discovery of many novel antibiotics, such as actinomycin from *S. antibioticus* (Waksman & Woodruff, 1941) and neomycin from *S. fradiae* (Waksman & Lechevalier, 1949).

It is becoming increasingly important to design new procedures for the selective isolation and characterisation of commercially significant actinomycetes from environmental samples to secure high quality biological material for pharmaceutical screening programmes (Bull *et al.*, 2000; Goodfellow & Fiedler, 2010). The introduction of such procedures show that actinomycetes once thought to be rare in natural habitats are widely distributed, as shown by studies on acidiphilic sporoactinomycetes (Kim *et al.*, 2003), motile actinomycetes (Hayakawa *et al.*, 2000; Suzuki *et al.*, 2001a), nocardiae (Orchard *et al.*, 1977; Maldonado *et al.*, 2000) and rhodococci (Colquhoun *et al.*, 1998). Targeted procedures are still needed for the

selective isolation and characterisation of commercially significant actinomycete taxa present in diverse environmental samples (Stach *et al*., 2003a, b; Kumar *et al*. 2007).

Most actinomycetes are saprophytes in aquatic and terrestrial ecosystems, notably in soils, freshwater and marine habitats (Bull *et al.*, 2005; Bull & Stach, 2007) . It is not possible to recommend a single procedure for the selective isolation of the multiplicity of the different kinds of actinomycetes present in environmental samples due to their diverse growth and incubation requirements. Consequently, numerous approaches have been recommended for the isolation of specific taxa (Nolan & Cross, 1988; Labeda & Shearer, 1990; Goodfellow, 2010). Most selective strategies have been focused on the isolation of particular taxonomic groups based on their biological properties (Cross, 1982; Wellington & Cross, 1983; Goodfellow & O'Donnell, 1989). Most procedures involve the extraction of propagules from selected environmental samples, pretreatment(s) of samples, design of selective media, incubation, and recognition of target colonies.

Extraction of actinomycete propagules. Physico-chemical interactions of bacterial propagules with particulate substrates affect their recovery from environmental samples. Traditional methods used to separate bacteria from organic matter, sediment and soil particles, include shaking in water or weak buffers (e.g., ¼ strength Ringer's solution), are not effective (Hopkins *et al.*, 1991). It is particularly important to thoroughly break up soil aggregates as many microorganisms, notably those showing mycelial growth, may be bound within them (Ramsay, 1984; Nishiyama *et al.*, 1992). Procedures used to promote the dissociation of microorganisms from particulate material include the use of chelating agents (MacDonald, 1986), buffered diluents (Niepold *et al.*, 1979), elutriation (Hopkins *et al*., 1991) and ultrasonication (Ramesy, 1984); all of these procedures address the problem of quantitative and representative sampling to varying degrees.

Pre-treatment of environmental samples. Several pre-treatment procedures are used to select different fractions of actinomycete communities present in environmental samples. In general, pre-treatment regimes select for target actinomycetes by inhibiting or eliminating unwanted microorganisms. Actinomycete spores are more resistant to desiccation than other bacterial cells hence simply air-drying soil/sediment samples at room temperature helps eliminate most unwanted Gram-negative bacteria which might otherwise overrun isolation plates (Williams *et al.*, 1984a; Labeda & Shearer, 1990). A

pre-treatment regime based on alternate drying and wetting of soil has been used to enrich for sporangia (spore vesicle) – forming actinomycetes (Makkar & Cross, 1982). Rare spore-forming actinobacteria (e.g., *Actinomadura*, *Microtetraspora*, *Pseudonocardia* and *Streptosporangium*) have been isolated from irradiated soil samples and soil suspensions (Bulina *et al.*, 1997; Terekhova, 2003).

Resistance of actinomycete propagules to desiccation is usually accompanied by some measure of resistance to heat. The basis of this resistance is not clear but it is apparent that many actinomycete spores (e.g., *Micromonospora* and *Microtetraspora*), spore vesicles (*Dactylosporangium* and *Streptosporangium*) and hyphal fragments (e.g., *Rhodococcus*) are more resistant to heat than Gram-negative cells. Actinomycetes are more sensitive to wet heat than dry heat, which means that much lower temperatures are used to isolate these organisms from suspensions of environmental samples. Heat pre-treatment protocols usually lead to a decrease in the ratio of bacteria to actinomycetes on isolation plates though actinomycete counts may be reduced (Williams *et al.*, 1984b).

Chemical pretreatments of mixed inocula are used to isolate specific actinomycete taxa, notably members of genera classified in the family *Streptosporangiaceae* (Hayakawa *et al.*, 1988, 1991a, 1995; Goodfellow, 2010). The selective chemical procedures introduced by Hayakawa and his colleagues are based on the differential ability of actinomycete spores to withstand treatment with chemical germicides, such as benzethonium chloride, chlorhexidine gluconate and phenol (Yamamura *et al.*, 2003). Treatment with these agents for 30 minutes at 30^{o}C kills vegetative cells of aerobic, endospore-forming bacilli and pseudomonads. The simultaneous use of more than one chemical germicide can further enhance selectivity, as exemplified by the use of chlorheximide gluconate and phenol for the isolation of *Microbispora* strains (Hayakawa *et al.*, 1991b).

Nutrient and baiting techniques are used to increase the populations of specific fractions of actinomycete communities in environmental samples to facilitate their isolation on selective media. Soil amendments with substrates such as chitin and keratin were first used by Jensen (1930) to boost the numbers of streptomycetes. This approach has fallen into neglect but does provide an effective way of enhancing specific components of actinomycete communities in soil (Williams *et al.*, 1971; Nonomura & Takagi, 1977).

Selective isolation media. Numerous media have been recommended for the isolation of broad range of actinomycetes from natural habitats and more specifically for selected families, genera and species (Kurtböke, 2003; Goodfellow, 2010). Most of the 'general' or 'non-selective' media were formulated without reference to either the nutritional or the tolerance preferences of the target organisms. It is now known that widely used 'non-selective' media, such as colloidal chitin-mineral salts (Hsu & Lockwood, 1975) and starch casein nitrate (Kuster & Williams, 1964) agars select for a relatively narrow range of *Streptomyces* species nor do they support the growth of actinomycetes with different nutritional requirements (Williams *et al.*, 1984a).

Selective isolation media can be formulated in an objective way by using phenotypic data held in taxonomic databases (Goodfellow & Haynes, 1984; Williams & Vickers, 1988; Goodfellow, 2010). One of the early successes of this approach was the formulation of a medium selective for the isolation of the *Nocardia* strains from soil (Orchard *et al.*, 1977; Orchard & Goodfellow, 1980; Maldonado *et al.*, 2000). The discovery that Diagnostic Sensitivity Agar supplemented with tetracycline was selective for *Nocardia* species was based on information held in a database of antibiotic sensitivities (Goodfellow *et al.*, 1989).

A logical extension of this work was the visual scanning of phenotypic databases to highlight antibiotics that might form the basis of selective isolation media. The number of verticillate streptomycetes (formerly members of the genus *Streptoverticillium*), for example, was increased by reducing the number of competing neutrophilic streptomycetes by supplementing isolation media with neomycin and oxytetracycline (Hanka & Schaadt, 1988); verticillate streptomycetes were known to be resistant and other neutrophilic streptomycetes sensitive to these antibiotics (Williams *et al.*, 1985).

Phenotypic databases generated in numerical taxonomic studies are ideal resources for the formulation of novel selective isolation media as they are rich in data on the antibiotic, nutritional and physiological properties of members of numerically defined clusters (Goodfellow & Haynes, 1984; Williams & Vickers, 1988; Goodfellow, 2010). Such databases are available for several industrially significant actinomyete taxa, including the genera *Actinomadura* (Athalye *et al.*, 1981; Trujillo & Goodfellow, 2003), *Gordonia* (Goodfellow *et al.*, 1998), *Nocardia* (Orchard & Goodfellow, 1980), *Rhodococcus* (Goodfellow & Williams, 1986), *Streptomyces* (Williams *et al.*, 1971;

Manfio *et al.*, 1995; Sembiring *et al.*, 2000; Duangmal *et al.*, 2005) and *Thermomonospora* (McCarthy & Cross, 1981), for members of the family *Pseudonocardiaceae* (Whitehead, 1989), but not for genera such as *Dactylosporangium*, *Microtetraspora* or *Nonomuraea*.

Antimicrobial agents, notably antibiotics, provide an effective means of increasing the selectivity of isolation media. It is standard practice to control or eliminate fungal containmants by using antifungal antibiotics, such as cycloheximide, nystatin and pimaricin (Porter *et al.*, 1960; Gregory & Lacey, 1963; Williams & Davies, 1965). Similarly, penicillin G and polymixin B have been used to select actinomycetes from competing soil inhabiting bacteria (Nonomura & Ohara, 1960; Williams & Davies, 1965), as have nalidixic acid and trimethoprin (Hayakawa *et al.*, 1996). Antibacterial antibiotics are widely used for the selective isolation for specific actinomycete taxa, as exemplified in Table 1. 4.

Table 1.4. Antibiotics used in the selective isolation of actinomycetes

Antibiotics	Target genera	References
Bruneomycin and streptomycin	*Actinomadura*	Preobrazhenskaya *et al.*(Preobrazhenskaya *et al.*)
Gentamicin	*Actinomadura* and *Streptosporangium*	Shearer (1987)
Josamycin, kanamycin and nalidixic acid	*Actinomadura viridis*	Hayakawa & Nonomura (1987)
Kanamycin, nalidixic acid and nofloxacin	*Microtetraspora*	Hayakawa *et al*. (1996)
Fradiomycin, kanamycin, nalidixic acid and trimethoprim	*Actinokineospora*	Otoguro *et al*. (2001)
Leucomycin and nalidixic acid	*Streptosporangium*	Hayakawa & Nonomura (1991) and Hayakawa *et al*. (1991a)
Nalidixic acid and penicillin	*Saccharothrix*	Shearer (1987)
Nalidixic acid and tunicamycin	*Micromonospora*	Nonomura & Takagi (1977)
Neomycin sulfate	*Amycolatopsis*	Tan *et al*. (2006)
Novobiocin and streptomycin	*Glycomyces*	Labeda & Kroppenstedt (2005)

* Adapted from Goodfellow (2010).

Incubation. Incubation conditions, including gaseous regimes, incubation times and temperature, contribute to selectivity. Incubation at 25 to 30°C favours mesophilic actinomycetes whereas their thermophilic counterparts require higher temperatures.

Thermophilic actinomycetes, such as *Pseudonocardia thermophila* and *Saccharopolyspora rectivirgula*, only require incubation for 2-3 days at 45 to 50°C to grow fully. In contrast, incubation times up to 5 weeks may be required to isolate some genera, such as those classified in the families *Micromonosporaceae* and *Streptosporangiaceae* (Labeda & Shearer, 1990; Goodfellow, 2010). Members of commonly isolated genera, such as *Micromonospora*, *Nocardia* and *Streptomyces*, may be selected from isolation plates after incubation for 14 days. Prolonged incubation was one of the factors which contributed to the initial isolation of the slow-growing, mutualistic symbiont, *Frankia* (Lechevalier, 1981). However, some slow-growing actinomycetes, such as members of the genus *Dactylosporangium* and slowly-growing mycobacteria, can be difficult to isolate.

Recognition and selection of colonies. The selection of colonies of target actinomycetes growing on isolation plates is a time-consuming and somewhat subjective stage in the isolation procedure. Colonies can be selected randomly or with some degree of choice. When selective isolation media are used the target organism(s) can sometimes be provisionally identified either on the basis of colony morphology or by examining colonies for distinctive morphological features, such as the presence of spore vesicles and the nature of spore chains, using a long working distance objective. However, it is not usually possible to distinguish between species of the same actinomycete genus on selective isolation plates. In such instances, the selection of large numbers of colonies is laborious and can lead to duplication of strains and hence effort, especially in low throughput screening systems. This problem can be overcome by grouping isolates based on pigmentation characters and molecular fingerprinting patterns (Tan *et al.*, 2006; Antony-Babu *et al.*, 2008).

1.6 The genus *Dactylosporangium*

Circumscription of the genus *Dactylosporangium*. The genus *Dactylosporangium* was proposed by Thiemann *et al*. (1967) to accommodate claviform sporangial actinomycetes which contained 3-hydroxy and/or *meso*-diaminopimelic acid, arabinose and glycine in the cell wall, but lacked mycolic acids. Members of the genus are Gram-positive, aerobic, catalase-positive, non-acid-fast actinomycetes, which form club-shaped, finger-shaped or pyriform sporangia, each containing a single row of three to four spores. The spores are oblong, ellipsoidal, ovoid, or slightly pyriform (0.4-1.3 ×

0.5-1.8 μm), and motile by means of flagella which are generally arranged in polar or lateral tufts. Large globose bodies (1.7-2.8 μm in diameter) are formed on the substrate hyphae (Sharples & Williams, 1974; Vobis, 1989, 2006). The substrate mycelium is pale to deep orange, rose or wine-coloured to brown with hyphae, which are 0.5-1.0 μm in diameter, branched, and rarely septate. Aerial mycelia are not formed. Colonies on agar media are compact, tough and leathery, mostly flat or elevated with a smooth to slightly wrinkled surface (Fig. 1.3). *Dactylosporangium* strains grow well on standard agar media, such as inorganic salt-starch, modified Bennet's, oatmeal, and yeast extract-malt extract agars (Shomura, 1993; Chiaraphongphon *et al.*, 2010; Seo & Lee, 2010).

The genus *Dactylosporangium* currently contains 8 validly described species (Table 1.5), which form a distinct phyletic line within the family *Micromonosporaceae* which encompasses 26 other genera (Xie *et al.*, 2010). These taxa can be distinguished using a combination of chemical and morphological properties (Table 1.6). Dactylosporangiae have a unique set of 16S rRNA nucleotides which can be used to differentiate them from other genera (Koch *et al.*, 1996). It can be seen from Figure 1.4 that *Dactylosporangium* species can be assigned to three distinct subclades with high bootstrap values (77-94%) and their nearest phylogenetic neighbour is the genus *Virgisporangium*. The type species of the genus is *Dactylosporangium aurantiacum* Thiemann *et al.*1967.

Dactylosporangiae contain 3-hydroxy and/or *meso*-diaminopimelic acid (*meso*-A_2pm), arabinose and xylose in whole-cell hydrolysates (wall chemotype II and sugar pattern D; Lechevalier & Lechevalier, 1970), N-acetylated muramic acid (Uchida & Seino, 1997), major amounts of branched *iso*-and *anteiso*-fatty acids (Vobis, 1989), and polar lipid patterns, which include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type II *sensu*; Lechevalier *et al*., 1977). Members of the genus are chemo-organotrophic, mesophilic (optimally growing between 25 to 37°C and between pH 6.0 to 7.0), and have a G+C ratio between 71 to 73 mol% (Vobis, 1989). Members of the genus *Dactylosporangium* can be distinguished from other genera classified in the family *Micromonosporaceae* using a combination of phenotypic properties as shown in Table 5.4.

Table 1.5. Validly described *Dactylosporangium* species

Species	Author	Type strain	Source
D. aurantiacum	Thiemann *et al.* (1967)	NRRL B-8018^T	Soil, Italy
D. darangshiense	Seo & Lee (2010)	DSM 45260^T	Rock surface soil, Korea
D. fulvum	Shomura *et al*. (1986)	DSM 43917^T	Soil, Japan
D. maewongense	Chiaraphongphon *et al.* (2010)	JCM 15933^T	Tropical forest soil, Thailand
D. matsuzakiense	Shomura (1983)	NRRL B-16293^T	Soil, Japan
D. roseum	Shomura (1985)	DSM 43916^T	Soil, Japan
D. thailandense	Thiemann *et al.* (1970)	DSM 43158^T	Soil, Thailand
D. vinaceum	Shomura (1983)	NRRL B-16297^T	Soil, Japan

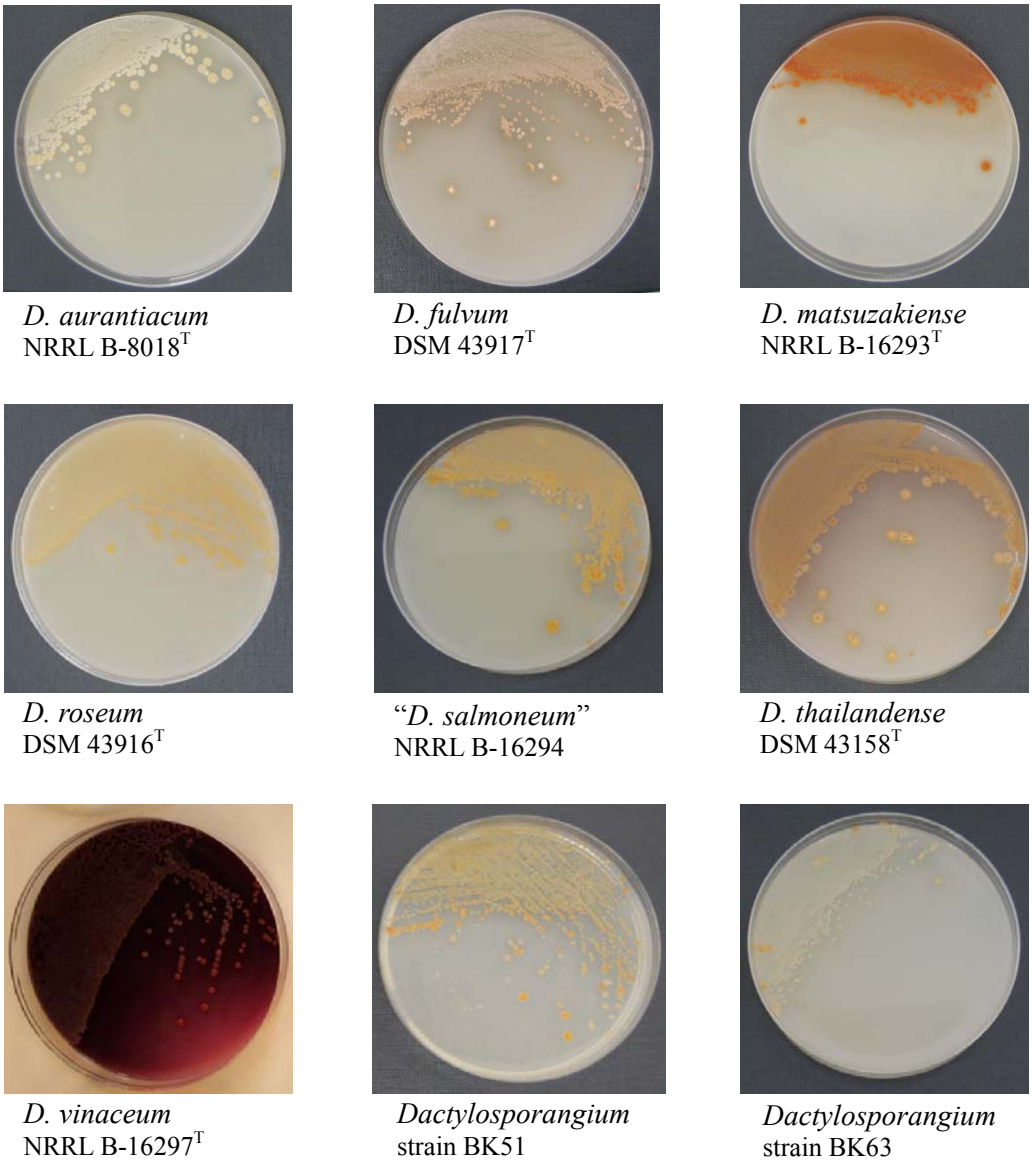


Fig. 1.3. Colonies of selected *Dactylosporangium* strains growing on oatmeal agar after incubation at 30˚C for 21 days.

Table 1.6. Chemical and morphological markers found in the members of the genera classified in the family *Micromonosporaceae*

Genus	Spore vesicles	Spore motility	Isomers of A_2pm	whole-cell sugars	Menaquinone(s) (MK-)[a]	Phospholipid type[b]	Fatty acid type[c]	DNA G+C content (mol%)
Actinaurispora	–	–	*meso*	Xyl, Man, Gal	9(H_6), 10(H_6)	II	3b	72
Actinocatenispora	–	–	*meso*	Ara, Gal, Xyl	9($H_{4,6}$)	II	3b	72
Actinoplanes	+	+	*meso*	Ara, Xyl	9(H_4), 10(H_4)	II	2d	72–73
Allocatelliglobosispora	–	–	3-OH	Glu, Rha, Rib, Xyl, Ara, Gal	10($H_{4,6}$), 9(H_4)	II	3b	70
Asanoa	–	–	*meso*	Ara, Gal, Xyl	10($H_{6,8}$)	II	2d	71–72
Catellatospora	–	–	*meso*	Ara, Gal, Xyl	10($H_{4,6}$), 9($H_{4,6}$)	II	3b	70-71
Catelliglobosispora	–	–	*meso*	Rha, Man, Xyl, Gal	10(H_4)	II	3b	70
Catenuloplanes	–	+	L-Lys	Xyl	9(H_8), 10(H_8)	III	2c	71–73
Couchioplanes	–	+	L-Lys	Ara, Xyl, Gal	9(H_4)	II	2c	70–72
Dactylosporangium	**+**	**+**	***meso***	**Ara, Xyl**	**9($H_{4,6,8}$)**	**II**	**3b**	**71–73**
Hamadaea	–	–	*meso,* 3-OH	Xyl, Gal, Man, Rib, Ara, Rha	9(H_6)	II	3b	70
Jishengella	–	–	*meso*	Xyl, Man, Ara, Rib, Glu	9($H_{4,6,8}$), 10(H_4)	II	3a	72
Krasilnikovia	–	–	*meso*	Gal, Man, Xyl, Ara, Rib	9($H_{6,4,8}$)	II	2d	71
Longispora	–	–	*meso*	Ara, Gal, Xyl	10($H_{4,6}$)	II	2d	70
Luedemannella	+	–	*meso*	Ara, Gal, Man, Rha, Rib, Xyl	9($H_{2,4,6,8}$)	II	2d	71
Micromonospora	–	–	*meso*	Ara, Xyl	10($H_{4,6}$), 9($H_{4,6}$)	II	3b	71–73
Phytohabitans	–	–	*meso,* L-Lys	Gal, Glu, Man, Rib, Xyl	9(H_6), 10($H_{4,6}$)	II	2d	73
Pilimelia	+	+	*meso*	Ara, Xyl	9($H_{2,4}$)	II	2d	ND
Planosporangium	+	+/–	*meso*	Ara, Xyl	9(H_4), 10(H_4)	II	3b	71
Plantactinospora	–	–	*meso*	Ara, Gal, Xyl	10($H_{6,8}$), 10(H_4)	II	2d	70
Polymorphospora	–	–	*meso*	Xyl	10($H_{4,6}$), 9($H_{4,6}$)	II	2a	71
Pseudosporangium	–	–	*meso,* 3-OH	Gal, Glu, Rib, Man, Xyl, Ara	9(H_6)	II	2d	73

Table 1.6. (*Continued*)

Genus	Spore vesicles	Spore motility	Isomers of A_2pm	whole-cell sugars	Menaqui-none(s) (MK-)[a]	Phospho-lipid type[b]	Fatty acid type[c]	DNA G+C content (mol%)
Rugosimonospora	–	–	3-OH	Ara, Gal, Xyl	9($H_{6,8}$)	II	2c	72-73
Salinispora	–	–	*meso*	Ara, Gal, Xyl	9(H_4)	II	3a	70-73
Spirilliplanes	–	+	*meso*	Ara, Xyl	10(H_4)	II	2d	69
Verrucosispora	–	–	*meso*	Man, Xyl	9(H_4)	II	2b	70
Virgisporangium	+	+	*meso*	Ara, Gal, Xyl	10($H_{4,6}$)	II	2d	71

* Data taken from Ørskov (1923), Couch (1950), Kane (1966), Thiemann *et al*. (1967), Asano & Kawamoto (1986), Yokota *et al*. (1993), Rheims *et al*. (1998), Kudo *et al*. (1999), Tamura *et al*. (1994, 1997, 2001, 2006), Lee & Hah (2002), Matsumoto *et al.* (2003), Maldonado *et al.* (2005), Thawai *et al*. (2006, 2010), Ara & Kudo (2006, 2007a, b), Ara *et al.* (2008a, b), Wiese *et al*. (2008), Monciardini, *et al*. (2009), Qin *et al.* (2009), Lee & Lee (2010), Inahash *et al.* (2010) and Xie *et al*. (2010). Abbreviations: A_2pm, diaminopimelic acid; L-Lys, L-lysine; Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose; +, Present; –, absent; ND, not determined.

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

[b], Characteristic phospholipids: II, only phosphatidylethanolamine; III, phosphatidylcholine (with phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidylglycerol variable, phospholipids containing glucosamine absent) (Lechevalier *et al.* 1977).

[c], Fatty acid classification after Kroppenstedt (1985). Numbers refer to the type of fatty acid biosynthetic pathway and letters to the type of fatty acid synthesised. Type 2 encompasses organisms which have terminally-branched fatty acids, notably, *iso*- and *anteiso*-branched fatty acids, including *iso*-14/16/18 (Type 2a), *iso*-15/17 (Type 2b), *anteiso*-15/17 (Type 2c), and all of them (Type 2d); Type 3 encompasses organisms which have complex branched fatty acid types, notably, both 10-methyl-branched (Type 1b) and *iso*- and/or *anteiso*-branched (Type 2) fatty acids, including *iso*-14/16/18 and 10-methyl-18(Type 3a), *iso*-15/17 and 10-methyl-17 (Type 3b), *iso*-14/16/18 and 10-methyl-17 (Type 3c), and *iso*-14/16/18, *anteiso*-15/17 and 10-methyl-18 (Type 3d).

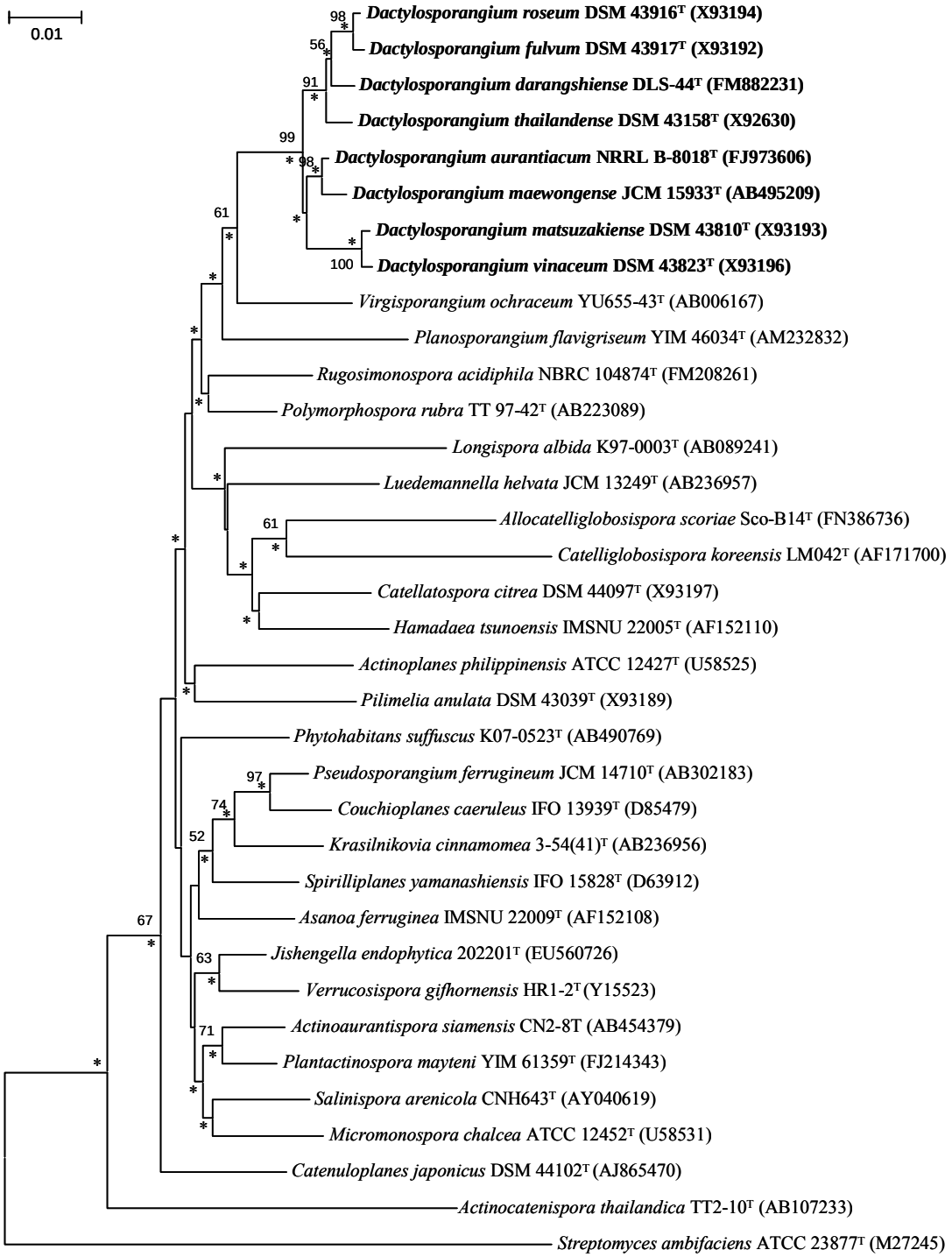


Fig. 1.4. Neighbour-joining tree showing relationships between *Dactylosporangium* species and type strains of type species classified in the family *Micromonosporaceae* based on nearly complete 16S rRNA gene sequences. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are shown. Asterisks indicate branches of the tree that were also recovered using the minimum-evolution and maximum-parsimony tree-making algorithms. Bar, 0.01 substitutions per site.

Biotechnological significance of the genus *Dactylosporangium*. Rare and uncommon actinomycetes are rich sources of novel secondary metabolites, notably antibiotics (Lazzarini *et al.*, 2000; Busti *et al.*, 2006). *Dactylosporangium* strains are a promising source of novel bioactive compounds of biotechnological interest (Table 1.7) though members of this genus have rarely featured in screening programmes as they are difficult to isolate and grow slowly on selective and standard growth media (Hayakawa *et al.*, 1991a). Indeed, only six species have been assigned to the genus *Dactylosporangium* in the past 40 years, hence little is known about either the taxonomic or the chemical diversity of these organisms.

Table 1.7. Bioactive compounds synthesised by *Dactylosporangium* strains

Organism	Metabolite	Compound class	Reference
D. aurantiacum SF-2185	SF-2185	Azetidine antibiotic	Matsumoto *et al.* (1985)
D. aurantiacum AB 718C-41	Tiacumicins A,B,C,D,E,F, bromo	Macrocyclic antibiotics	Theriault *et al*. (1987); Hochlowski *et al*. (1997)
D. aurantiacum SANK 61299	A-79197-2 and -3	Di-, tri- saccharides of streptol plant growth inhibitor	Kizuka *et al.* (2002)
D. fulvum SF-2113	Pyridomycin	12-membered ring compound	Shomura *et al*. (1986)
D. matsuzakiense SF-2052	Dactimicin	Pseudodisaccharide aminoglycoside	Shomura *et al*. (1980)
D. roseum SF-2186	Orthosomycin	Orthosomycin antibiotic group	*Shomura et al*. (1985)
D. salmoneum ATCC 31224	44161	Polycyclic ether antibiotic	Celmer *et al*. (1978)
D. thailandense G-367	G-367 S_1 (2'-N-formylsisomicin, $C_{20}H_{37}N_5O_8$)	Aminoglycoside antibiotic	Satoi *et al.* (1983)
"*D. variesporum* " D409-5	Capreomycin	Polypeptide antibiotic complex	Tomita *et al*. (1977)
D. vinaceum SF-2127	Dactimicin	Pseudodisaccharide aminoglycoside antibiotic	Shomura *et al*. (1983)
Dactylosporangium sp. FERM P-8502	AC7230	Polyether antibiotic	Yaginuma *et al*. (1987)
Dactylosporangium sp. ATCC 53693	Dactylocyclines A, B	Tetracycline glycoside antibiotic	Wells *et al*. (1992)
Dactylosporangium sp. RH1	Proline 4-hydroxylase	Monomer polypeptide enzyme	Shibasaki *et al*. (1999)
Dactylosporangium sp. SCC 1695	4a-hydroxy-8-methoxychlortetracycline (Sch 34164)	Tetracycline antibiotic	Patel *et al*. (1987)
Dactylosporangium sp. SF-2908	SF-2809 I, II, III, IV, V, VI	Chymase inhibitor alkaloid anti-allergy	Tani *et al.* (2004)

Dactylosporangium strains known to produce bioactive secondary metabolites include *D. aurantiacum* strain AB 718C-41 which produces tiacumicin B (Fidaxomicin, OPT-80, PAR-101), a novel group of 18-membered macrolide antibiotic discovered at the Abbott Laboratories, USA (Theriault *et al.*, 1987). Tiacumicin B inhibits RNA polymerase and shows good activity against pathogenic Gram-positive bacteria and selected anaerobes, notably *Clostridium difficile* (Finegold *et al.*, 2004; Biedenbach *et al.*, 2010). This antibiotic was recently put through phase III clinical trials for the treatment of *Clostridium difficile*-associated diarrhoea (Revill *et al.*, 2006; Miller, 2010). In turn, *D. matsuzakiense* strain SF-2052 produces dactimicin, a pseudodisaccharide aminoglycoside antibiotic which was first isolated at Meiji Seika Ltd., Japan (Shomura *et al.*, 1980). It inhibits *Citrobacter diversus*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Salmonella* spp., *Serratia marcescens* and *Yersinia* spp., and has a MIC for 90% of strains (MIC90) of less than or equal to 4 μg/ml (Bertelli & Bertelli, 1989; Gu & Neu, 1989).

Selective isolation of *Dactylosporangium* strains. *Dactylosporangium* strains have been found in small numbers in diverse types of soil, including cultivated field, forest and pasture soils (Hayakawa *et al.*, 1991a; Vobis, 2006). However, the lack of effective selective isolation procedures has limited the exploration of members of the genus for bioprospecting purposes.

Thiemann *et al*. (1970) recovered 140 dactylosporangiae from soils for the first time, but did not provide any information how they were isolated. Hayakawa and Nonomura (1987) isolated dactylosporangiae, microbisporae, micromonosporae and streptosporangiae on humic acid-vitamin (HV) agar, supplemented with cycloheximide. Hayakawa and his colleagues (1991a) later developed a more effective method for isolating dactylosporangiae from heat-preheated soil by using HV agar supplemented with benzenthonium chloride. However, in practice, the small colonies of the target organisms are difficult to see, and to isolate from the dark-coloured HV agar plates. They also devised a chemotactic method which employed collidine and D-xylose as chemo-attractants and isolated relatively large numbers of *Actinoplanes* and *Dactylosporangium* strains from soil (Hayakawa *et al.*, 1991c), though the identification of their isolates was mainly dependent on morphological characteristics. Indeed, dactylosporangiae are difficult to identify in this way as they form indistinct colonies which can easily be confused with other actinomycetes, such as other genera classified

in the family *Micromonosporaceae*. Consequently, improved methods are needed to selectively isolate *Dactylosporangium* strains from natural habitats prior to study their numbers, roles and taxonomic diversity.

The development of molecular techniques based on precise genotypic information offers an accurate, rapid, and practical way of evaluating the effectiveness of selective procedures. The use of taxon-specific oligonucleotide probes provides an effective way to identify rapidly large numbers of actinomycetes (Mehling *et al.*, 1995; Yoon *et al.*, 1996; Monciardini *et al.*, 2002) and to detect the presence of target organisms in natural habitats (Heuer *et al.*, 1997; Monciardini *et al.*, 2002; Blackwood *et al.*, 2005; Kumar *et al.*, 2007; Mühling *et al.*, 2008). For this purpose, oligonucleotide primers have been designed for the detection of members of the genera *Amycolatopsis* (Tan *et al*., 2006), *Dactylosporangium* (Monciardini *et al*., 2002), *Gordonia* (Shen & Young, 2005), *Micromonospora* (Qiu *et al*., 2008), *Nocardiopsis* (Salazar *et al*., 2002), *Pseudonocardia* (Morón *et al.*, 1999) *Saccharomonospora* (Salazar *et al*., 2000), *Saccharothrix* (Salazar *et al*., 2002), *Streptomonospora* (Zhi *et al.*, 2006) and *Streptomyces* (Rintala *et al.*, 2001), for the members of *Streptomyces violaceusniger* 16S rRNA gene clade (Kumar *et al*. 2007), and for the families *Micromonosporaceae*, *Streptomycetaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* (Monciardini *et al*., 2002).

1.7 Antibiotic resistance and self-protection mechanisms of actinomycetes

Antibiotics target many processes that are essential for a cell's growth, including cell wall synthesis, DNA duplication, folic acid metabolism, protein synthesis and transcription (Bush, 2004). The introduction of antibiotics for healthcare was rapidly accompanied by the appearance of microbial pathogens, which showed resistance to them. Some of the mechanisms used by pathogenic organisms to counter the effects of antibiotics are shown in Figure 1.5; some bacteria are intrinsically resistant to certain antibiotics as they have an impermeable membrane or lack the target of the antibiotic (Brown & Balkwill, 2009; Allen *et al.*, 2010). Others have multidrug resistance efflux pumps which excrete antibiotics from the cell into the periplasm, while genetic mutations can disable the antibiotic-binding sites (Nikaido, 1998; Poole, 2005; Reynolds & Courvalin, 2005). In addition, inactivation can occur by covalent modification of the antibiotic, such as that catalysed by acetyltransferases acting on

aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by β-lactamases acting on β-lactam antibiotics (Bush, 2001; Fischbach & Walsh, 2009).

There are two main mechanisms involved in the development of antibiotic resistance, namely mutation (Alekshun & Levy, 2007) and acquisition of resistance genes by horizontal gene transfer (Aminov & Mackie, 2007). Resistance genes can be transferred between members of distantly related taxonomic groups, including from Gram-positive to Gram-negative bacteria, through horizontal gene transfer, as exemplified by the transfer of a kanamycin resistance gene from *Streptococcus* to *Campylobacter* strains (Trieu-Cuot & Courvalin, 1986; Trieu-Cuot *et al.*, 1993; Mindlin *et al.*, 2006). The origin of antibiotic resistance determinants acquired by horizontal gene transfer must have resided in the non-pathogenic microbiosphere as human pathogens were susceptible to antibiotics before the widespread use of antibiotics (Davies, 1994; Davies, 1997).

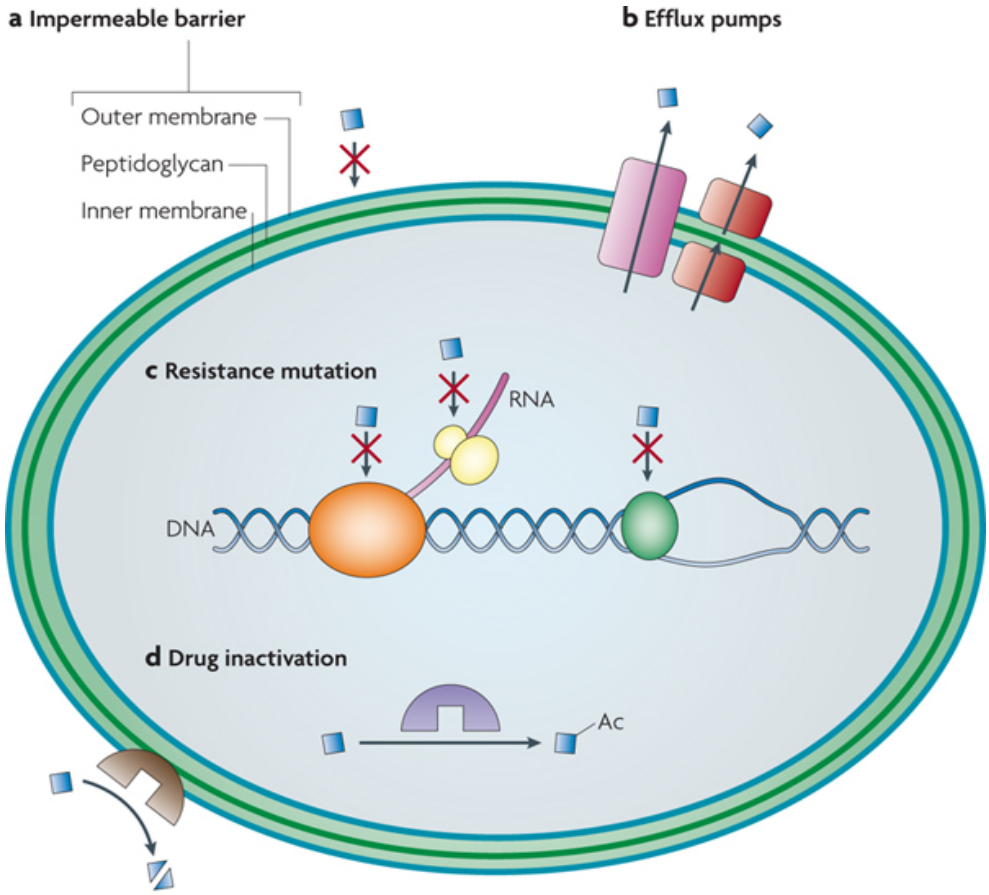


Fig. 1.5. Mechanisms of antibiotic resistance in bacteria (taken from Allen *et al.*, 2010)

It has been proposed that antibiotic resistance genes originated from the environmental microbiota, notably antibiotic-producing actinomycetes as these organisms need resistance mechanisms to protect them against the antibiotic(s) they produce (Benveniste & Davies, 1973; Roza *et al.*, 1986; Skeggs *et al.*, 1987; Cundliffe, 1995; Mindlin *et al.*, 2006; Hopwood, 2007; Cundliffe & Demain, 2010). Many resistance mechanisms, including antibiotic inactivation, alteration of permeability barrier or efflux system, and target modification, which are quite similar to antibiotic resistance mechanisms, could be involved in conferring self-resistance to the producer (Cundliffe, 1989; Dairi *et al.*, 1995; Hopwood, 2007; Cundliffe & Demain, 2010). It seems likely that resistance genes were mobilized through plasmids or transposable elements to other organisms thereby enabling them to grow in the presence of antibiotics (Davies, 1997; Poirel *et al.*, 2005; Mindlin *et al.*, 2006).

A well-studied example of horizontal transfer of resistance genes from producers to pathogens involves the *vanHAX* operon which encodes enzymes that synthesise peptidoglycans terminating in D-alanyl-D-lactate instead of D-alanyl-D-alanine (Hong *et al.*, 2004; Courvalin, 2006). This change decreases the binding affinity of glycopeptide antibiotics a 1000-fold rendering organisms resistant to teicoplanin and vancomycin (Reynolds & Courvalin, 2005). The *vanHAX* operon present in *Enterococcus* species has homologues in the glycopeptide-producing actinomycetes, such as *Actinoplanes teichomyceticus* ATCC 31131, *Amycolotopsis orientalis* M43-05865 and *Streptomyces toyocaensis* NRRL 15009 (Marshall *et al.*, 1998; Pootoolal *et al.*, 2002; Serina *et al.*, 2004; Beltrametti *et al.*, 2007). Enterococci and the glycopeptides producers share 54 to 61% amino acid sequence similarity for *VanH*, 59 to 63% for *VanA*, and 61 to 64% for *VanX* (Marshall *et al.*, 1998), while the gene orientation of the *vanHAX* operon is identical in both enterococci and the producers (Marshall *et al.*, 1998; Courvalin, 2006; Hong *et al.*, 2008).

Bacteria, which show antibiotic resistance, appear to be very widespread in the environment, as shown by D'Costa *et al*. (2006). These workers noted that multiple diverse mechanisms for resistance were associated with soil actinomycetes. They isolated 480 streptomycetes from agricultural, forest and urban soils and determined resistance of strains to 21 antibiotics, which represented all major chemical types and targets of activity. They found multiple mechanisms of resistance amongst the isolates to both naturally occurring and synthetic antibiotics, and many resistance mechanisms

were linked to enzyme inactivation.

Antibiotic resistance cannot just be attributed to the release of antibiotics into the environment, as bacteria isolated from multiple Siberian permafrost sediments dating back three million years have been found to be resistant to chloramphenicol, gentamicin, kanamycin, tetracycline and streptomycin (Mindlin *et al.*, 2008; Petrova *et al.*, 2009), indicating that indigenous soil bacteria have been under selective pressure to develop resistance to antibiotics for millions of years. It looks as though actinomycetes are an important source of new antibiotics, but are also the source of drug resistance. Consequently, analysing self-resistance mechanism of antibiotic-producing actinomycetes can help determine the resistance mechanisms of pathogenic microorganisms that could eventually emerge clinically, and provides strategies that can be applied in new antibiotic discovery programmes (Hotta *et al.*, 1983, 1992, 1999; Hotta & Okami, 1996; Wright, 2007, 2010).

Abyssomicins and self-resistance mechanisms. Abyssomicins are effective inhibitors of *para*-aminobenzoate (*p*ABA) biosynthesis in some Gram-positive bacteria. The *p*ABA, which is derived from the key-metabolite of chorismate, is an important metabolite in the folic acid pathway which is essential for bacterial metabolism (Nichols *et al.*, 1989; Dosselaere & Vanderleyden, 2001; Quinlivan *et al.*, 2006). Enzymes in *p*ABA and folic acid biosynthesis are present in many microorganisms, parasites, and plants, but not in humans, hence this pathway is an interesting target for the development of novel antibiotics, pesticidal and herbicidal agents (Alun & Jeremy, 2002). Prominent synthetic inhibitors of the folic acid pathway are sulphonamides and trimethoprim (Huovinen *et al.*, 1995; Sköld, 2001; Brochet *et al.*, 2008).

Abyssomicins, the first known natural inhibitors of the *p*ABA biosynthetic pathway, are synthesised by *Verrucosispora maris* AB-18-032, which was isolated from a marine sediment sample (Riedlinger *et al.*, 2004; Goodfellow *et al.*, 2010b). The abyssomicins are unique polycyclic polyketide-type 1- antibiotics (Fig. 1.6) in which the main compound, *atrop*-abyssomicin C, is active against Gram-positive bacteria, including *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus* strains (Riedlinger *et al.*, 2004; Keller *et al.*, 2007a).

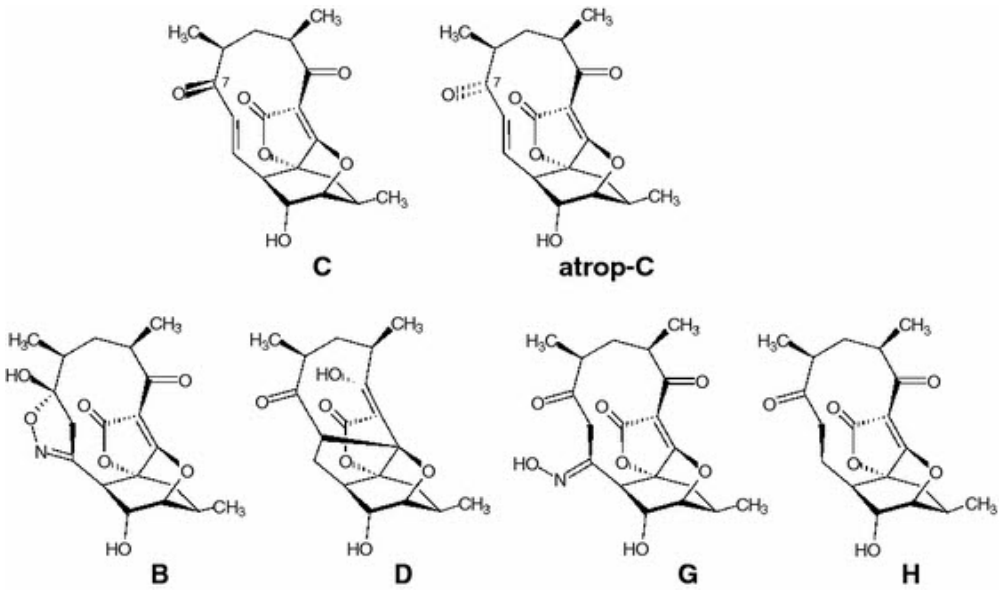


Fig. 1.6. Structures of abyssomicins isolated from *Verrucosispora maris* AB-18-032 (taken from Goodfellow & Fiedler, 2010)

All of the abyssomicins mimic the structure of chorismate, the natural substrate for the PabB subunit of 4-amino-4-deoxychorismate synthase, though only abyssomicin C and *atrop*-abyssomicin C bind covalently to PabB by a Michael addition mechanism (Keller *et al.*, 2007b). These two compounds bind the side chain of Cys263, located in the proximity of the active site of PabB (Fig. 1.7). However, the resistance mechanism is not clear hence it is important to determine whether *V. maris* AB-18-032, like other antibiotic-producing actinomycetes, can modify the highly conserved abyssomicin target site to make 4-amino-4-deoxychorismate synthase which is resistant to the drug.

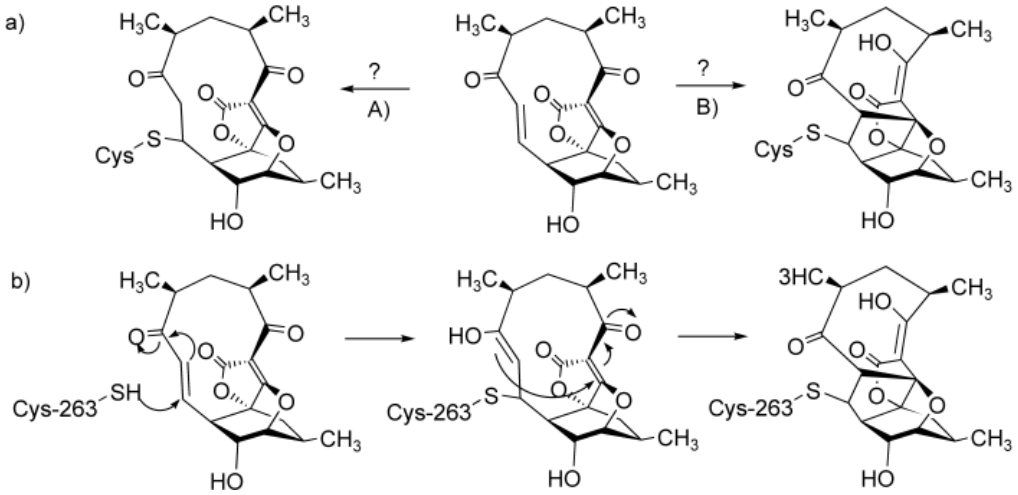


Fig. 1.7. Function of *atrop*-abyssomicin C as a Michael acceptor. a) Michael addition (pathway A) and dual Michael addition with subsequent rearrangement to an abyssomicin D derivative (pathwayB); b) Proposed reaction with Cys263 based on conversion with S nucleophiles 2-sulfanylethanol and *N*-acetylcysteine, respectively (taken from Keller *et al.*, 2007).

Chapter 2. Materials and Methods

2.1 Source and physico-chemical properties of samples

Source of environmental samples. Twenty-one samples were collected from diverse geographical locations (Table 2.1). They were sieved, air-dried at room temperature for 5-7 days and stored at 4˚C in plastic containers, apart from the marine sediment samples which were not dried.

Physico-chemical properties of environmental samples. The pH of the samples were determined using the procedure described by Reed and Cummings (1945). Two grams of each sample were added to 18 ml of deionised water, mixed thoroughly, and left for an hour when the pH was determined using a glass electrode pH meter (Model 320 Mettler-Toledo AG, CH.8603, Schwerzenbach, Switzerland). Each sample was examined in triplicate and the final pH values recorded as an average of the three readings. The percentage moisture contents of the triplicated samples were determined by drying known weights of sediment/soil to constant weight at 105˚C then calculating the average loss in weight between each set of samples. The dried samples were placed in a muffle furnace (Carbolite, Sheffield, UK), the temperature raised slowly to 700˚C and kept constant for 30 minutes to burn off organic matter. After cooling overnight in a desiccator, the average loss in weight for each set of samples was recorded as the organic matter content.

2.2 Selective isolation and enumeration

Inoculation of selective media. Two grams of each of the soil samples (Table 2.1) were resuspended in 18 ml of sterile ¼ strength Ringer's solution (Oxoid, UK) and blended in an Ultra-Turrax T25 homogeniser (Junke and Kunkel, Staufen, Germany) for a minute. The resultant 10^{-1} suspensions were agitated on a shaker incubator (Gallenkamp Orbital Incubator, UK) at 150 rpm at room temperature for 30 minutes, heat pre-treated at 55˚C for 20 minutes in a water bath and cooled at room temperature. The suspensions were serially diluted down to 10^{-5} in ¼ strength Ringer's solution and triplicate aliquots (100 µl) of the 10^{-2} to 10^{-5} dilutions spread, in triplicate, over plates of

selective media (Table 2.2) which had been dried for 15 minutes prior to inoculation, as recommended by Vickers and Williams (1987). Inoculated plates were incubated at 28˚C for 21 days.

Table 2.1. Source and physico-chemical properties of environmental samples

Code	Soil/Sediment samples	Location
ANT	Antarctic soil	Ross Dependency, Antarctica (78°01′S; 163°53′E)
AU96	Arid soil	Adelaide, Australia
BK	Hay meadow soil	Plot 6, Cockle Park Experimental Farm, Northumberland, UK (National Grid reference: NZ 200913)
CA	Rhizosphere soil	Izmir, Turkey
Ch6	Marine sediment	Bahia Tic Toc, Chilean Sea (43°53′ S, 72°87′W)
Ch27	Marine sediment	Estero Castro, Chilean Sea (42°30′S, 73°5′W)
CS	Chalk soil	Ambleside, Cumbria, UK (National Grid reference: NY-357978)
DR	Sediment	River Derwent, County Durham, UK (National Grid reference: NZ-055490)
ECU	Upland soil	Riobamba, Ecuador
KHA	Agricultural soil	Khartoum, Sudan
LS	Sediment	Shore of Loch Creran, UK (National Grid reference: NM-9124259)
MEG	Grassland soil	Syros, Greece
MS	Rhizosphere soil	Kuala Lumpur, Malaysia
NMS	Agricultural soil	Kuala Lumpur, Malaysia
NOR	Marine sediment	Østerfjord, Norwegian Sea (60°33′N, 5°19′E)
SAU	Arid soil	Nairobi, Kenya
SHO	Agricultural soil	Sholinar, Srinagar, India
TOR	Urban soil	Torremolinos, Spain
WAD	Sand dune soil	Borg El-Arab, Egypt
WS	Garden soil	Samsun, Turkey
ZIZ	Arid soil	Gazipur district, Bangladesh

Selection and colour grouping of actinomycetes. Organisms growing on the selective media were putatively identified as actinomycetes by their characteristic colonial morphology, notably by their ability to form leathery colonies which in many cases carried aerial hyphae. Two hundred actinomycete colonies were randomly selected from the isolation plates inoculated with the hay meadow soil suspensions and subcultured onto oatmeal agar (Shirling & Gottlieb, 1966) plates which were incubated at 28˚C for 21 days. The selected isolates were assigned to groups based on aerial spore mass,

colony reverse and diffusible pigment colours which were recorded using National Bureau of Standards (NBS) Colour Name Chart (Kelly, 1958).

Table 2.2. Media used for the selective isolation of dactylosporangiae

Media	Antifungal agents (25 μg/ml)	Antibacterial agents (4 μg/ml)	References
Humic acid-vitamin agar	Cycloheximide Nystatin	Gentamicin or Oxytetracycline	Hayakawa & Nonomura (1987)
Oatmeal agar	Cycloheximide Nystatin	Gentamicin or Oxytetracycline	Shirling & Gottlieb (1966)
Raffinose-histidine agar	Cycloheximide Nystatin	Gentamicin or Oxytetracycline	Vickers *et al*. (1984)
Starch casein agar	Cycloheximide Nystatin	Gentamicin or Oxytetracycline	Küster & Williams (1964)
Streptomyces Isolation Medium	Cycloheximide Nystatin	Gentamicin or Oxytetracycline	D'Costa *et al*.(2006)

Selection and enumeration of presumptive dactylosporangiae. Orange coloured colonies which lacked aerial hyphae were presumptively assigned to the genus *Dactylosporangium*. The numbers of presumptive dactylosporangiae, other filamentous actinomycetes and bacteria were expressed as the mean number of colony forming units (cfu's) per gram dry weight soil.

Maintenance of strains. All of the isolates were grown on oatmeal (Shirling & Gottlieb, 1966) and modified Bennett's (1949) agars at 28˚C for 3 weeks, and the reference strains on oatmeal or inorganic salts-starch agar plates under the same conditions (Table 2.3), as described in the relevant chapters. Suspensions of spores and mycelial fragments were scraped from the oatmeal agar plates and suspended in 1 ml aliquots of 20% (v/v) glycerol in cryo-vials (2 vials per strain) and the resultant preparations kept at -20˚C for short-term and at -80˚C for long-term storage.

2.3 Molecular taxonomic methods

2.3.1 *Genomic DNA extraction*

Total genomic DNA was extracted from all of the isolates and reference strains (Table 2.3), and grown on modified Bennett's agar (Jones, 1949) for 14 days at 28˚C. Two or three loopfuls of biomass of each strain were suspended in 200 μl of 1x TE buffer (pH 8.0; Appendix B), approximately 100 μg of sterile glass beads (≤ 106 μm; Sigma) added and the resultant preparations disrupted at a speed of 5.0 m/s for 30

seconds in a FastPrep® FP120 (Thermo Lab Systems, Waltham, USA). The preparations were then centrifuged at 13,000 rpm for 2 minutes, each supernatant transferred to a new tube and 20 µl of RNase (R6148, Sigma) added prior to incubation at room temperature for 2 minutes. DNA was precipitated from the resultant preparations by adding 400 µl of cold 100% ethanol and 20 µl of sodium acetate (3M, pH 5.2), rinsed with 70% ethanol, suspended in 100 µl of 1x TE buffer (pH 8.0), and quantified using a Nanodrop spectrophotometer (Thermo scientific, Wilmington, USA).

Table 2.3. Isolates and reference strains

Strains	Source	Tables
Isolates:		
BK-isolates	200 isolates from hay meadow plot6 soil, Cockle Park, Northumberland, UK	Table 3.1, Chapter 3
	61 isolates; hay meadow plot 6 soil, Cockle Park Northumberland, UK	Table 4.3, Chapter 4
CS-isolates	23 isolates; chalk soil, UK	Table 4.3, Chapter 4
DR-isolates	19 isolates; river sediment, County Durham, UK	Table 4.3, Chapter 4
ECU-isolates	25 isolates; Upland soil, Ecuador	Table 4.3, Chapter 4
LS-isolates	5 isolates; seashore sediment, UK	Table 4.3, Chapter 4
MEG-isolates	MEG-1, MEG-2; grassland soil, Greece	Table 4.3, Chapter 4
MS-isolate	MS-1; rhizosphere soil, Malaysia	Table 4.3, Chapter 4
NMS-isolates	20 isolates; agricultural soil, Malaysia	Table 4.3, Chapter 4
NOR-isolates	15 isolates; marine sediment (616m), Norway	Table 4.3, Chapter 4
SHO-isolates	29 isolates; agricultural soil, India	Table 4.3, Chapter 4
WAD-isolates	WAD-1, WAD-2; sand dune soil, Egypt	Table 4.3, Chapter 4
WS-isolates	12 isolates; garden soil, Turkey	Table 4.3, Chapter 4
ZIZ-isolates	5 isolates; arid soil, Bangladesh	Table 4.3, Chapter 4
Reference strains:		
D. aurantiacum	NRRL B-8018^T; ATCC 23491; DSM 43157; Thiemann D-748; soil, Pagani Gruppo Lepetit, Italy.	Table 5.3, Chapter 5
D. fulvum	DSM 43917^T; ATCC 43301; NBRC 14381; NRRL B-16292; Meiji Seika Kaisha SF-2113; soil, Chiba Prefecture, Japan.	Table 5.3, Chapter 5
*D. matsuzakiens*e	NRRL B-16293^T; ATCC 31570; NBRC 14259; Meiji Seika Kaisha SF-2052; Soil, Matsuzaki, Izu Peninsula, Japan. Produces dactimicin.	Table 5.3, Chapter 5

Table 2.3. (*Continued*)

Strains	Source	Tables
D. roseum	DSM 43916^T; NRRL B-16295; NBRC 14352; soil, Shizuoka, Japan.	Table 5.3, Chapter 5
"*D. salmoneum*"	NRRL B-16294; DSM 43910; ATCC 31222; NBRC 14103; soil, Japan. produces polycyclic ether antibiotic.	Table 5.3, Chapter 5
D. thailandense	DSM 43158^T; ATCC 23490, NBRC 12593; Thiemann D-449; soil, Thailand. Degrades natural rubber.	Table 5.3, Chapter 5
D. vinaceum	NRRL B-16297^T; DSM 43823; ATCC 35207; NBRC 14181; Meiji Seika Kaisha, SF-2127; soil, Japan. Produces dactimicin.	Table 5.3, Chapter 5
"*D. variesporum*"	NRRL B-16296; ATCC 31203, NBRC 14104; soil, Japan. Produces capreomycin.	Table 5.3, Chapter 5
"*S. coelicolor*"	A3(2); K.T. Charter, John Innes Institute, Norwich, UK.	Table 7.2 Chapter 7
S. griseus	NRRL B-2682^T; ATCC 23345; DSM 40236; NBRC 12875; soil. Produces streptomycin.	Table 7.2 Chapter 7
"*S. lividans*"	TK24; K.T. Charter, John Innes Institute, Norwich, UK.	Table 7.2 Chapter 7
S. venezuelae	ATCC 15439; M-2140; soil, Florence, Italy. Produces methymycin.	Table 7.2 Chapter 7
Verrucosispora maris	Isolate AB-18-032; marine sediment sample, Sea of Japan (East Sea).	Chapter 7

* ATCC, American Type Culture Collection Manassas, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NBRC, NITE Biological Resource Center, Chiba, Japan; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, US Department of Agriculture, Peoria, USA; ^{T}, Type strain.

2.3.2 *Sequencing and analysis of 16S rRNA genes*

PCR amplification and sequencing. Amplification of 16S rRNA genes from the strains (Table 2.3) was carried out using universal bacterial 16S rDNA primers 27F and 1525R (Lane, 1991; Table 2.4) in a *T*-gradient thermocycler® (Whatman-Biometra, Göttingen, Germany). All of the reagents for the PCR were purchased from New England Biolabs, Ipswich, UK. Reactions in a final volume of 50 µl contained 0.2 mM of each of the four dNTPs, 0.4 µm of primers 27f and 1525r, 1 µl (100 ng) of genomic DNA, 1.5 mM $MgCl_2$ and 1.25 units *Taq* DNA polymerase with 1x reaction buffer. Amplifications were performed under the following conditions: initial denaturation for 5 minutes at 95˚C, 30 cycles of denaturation at 94˚C for 1 minute, annealing at 55˚C for 1 minute, extension at 72˚C for 1 minute, and a final extension step of 10 minutes at 72˚C. The amplified products were analysed by agarose gel electrophoresis in agarose gels (1.0%, w/v) stained with ethidium bromide. PCR products were purified using a PCR purification kit (Sigma-Aldrich, UK), according to the manufacturer's protocol. The eluted products were stored at -20˚C until required. The sequences of the almost

complete length of the 16S rRNA genes were determined by either Macrogen Co. (Seoul, Korea) or Gene Vision Co. (Newcastle upon Tyne, UK).

Phylogenetic analyses. The chromatogram file in ABI was read in the Chromas program (Griffith University, Queensland, Australia) and converted into a FASTA format file. The probable nearest match of each new sequence was determined by performing a Basic Local Alignment Search Tool (BLAST) search at the National Center for Biotechnology Information (NCBI; Bethesda, USA) and pairwise sequence similarities calculated by using a global alignment algorithm, which was implemented at the EzTaxon web-server (http://www.eztaxon.org; Chun *et al.*, 2007). The almost complete 16S rRNA gene sequences were aligned manually using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit), and ambiguities and other discrepancies resolved by 16S rRNA secondary structure analysis and by examining the original chromatographic data.

Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Fitch, 1971) tree-making algorithms from the MEGA 3.0 program (Kumar *et al.*, 2004), and the maximum-likelihood method (Felsenstein, 1981) from the PHYLIP suite of programs (Felsenstein, 2004). The evolutionary distance model of Jukes & Cantor (1969) was used to generate evolutionary distance matrices for the neighbour-joining analysis. The topologies of the resultant trees were evaluated in bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset using the CONSENSE and SEQBOOT options from the PHYLIP package.

2.3.3 *Molecular fingerprinting*

PCR amplification. Repetitive extragenic palindromic PCR (*rep*-PCR) was carried out to dereplicate 40 dactylosporangiae strains (Fig. 5.1), as described by Versalovic *et al.* (1994), using three bacterial interspersed repetitive sequences, namely BOX elements (Martin *et al.*, 1992), enterobacterial repetitive intergenic consensus elements (ERIC; Hulton *et al.*, 1991) and repetitive extragenic palindromic elements (REP; Versalovic *et al.*, 1992); the primers generated the amplification of DNA fragments of various sizes. Genomic DNA and a negative control were used as template DNA in a 25 µl PCR mixture with the *rep*-PCR primers (BOX, ERIC and REP primer sets; Table 2.4). PCR was performed using the *T*-gradient Thermocycler® (Whatman-

Biometra) under the following conditions; initial DNA denaturation at 95˚C for 5 minutes, 30 cycles of DNA denaturation at 95˚C for 1 minute, annealing at 52˚C (BOX and ERIC-PCR) or 40˚C (REP-PCR) for 1 minute, extension at 65˚C for 8 minutes, and a final DNA extension at 65˚C for 16 minutes.

Analysis of DNA fingerprints. Agarose gels were prepared as 1.5% (w/v) gels in 0.5x TBE buffer supplemented with 0.5 μg/ml ethidium bromide. Mixtures of each DNA sample (7 μl) and loading dye (3 μl) were added to wells on the gels, and a Gene Ruler™ 100 bp DNA Ladders Plus (Fermentas Co, York, UK) used as the marker. Gel electrophoresis was performed at 70 V for 5 hours, and DNA fingerprints visualised and photographed under UV using a BIO-RAD Fluor-S™ MultiImager (BIO-RAD laboratories Ltd, Hertfordshire, UK). TIFF images were analysed by using BioNumerics software (Applied Maths, Kortrijk, Belgium). Three main steps were involved in this process: (1) definition of lanes and settings, (2) normalisation and designation of bands, and (3) cluster analysis. Raw data were normalised using a marker lane with clear banding patterns as the reference profile after conversion of the gel images to the standard track format. Gel patterns were analysed using a resolution of 1000 and bands manually assigned with reference to densitometric curves. Similarities between strains were calculated based on band positions using the Dice coefficient (Dice, 1945), and clustering achieved using the unweighted pair group method with arithmetic mean (UPGMA; Sneath & Sokal, 1973) algorithm. The results of the various analyses were presented as dendrograms.

Table 2.4. Oligonucleotide primers

Primer	Sequence (5' to 3')	Binding Site[a] (5'/3')	Reference
Bacterial 16S rRNA gene primers:			
27F	AGAGTTTGATCMTGGCTCAG	8/27	Lane (1991)
1525R	AAGGAGGTGWTCCARCC	1544/1525	Lane (1991)
***rep*-PCR primers:**			
BOXA1R	CTACGGCAAGGCGACGCTGACG	-	Koeuth *et al.* (1995)
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	-	Versalovic *et al.* (1991)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	-	
REP1R-I	IIIICGICGICATCIGGC	-	Versalovic *et al.* (1991)
REP2-I	ICGICTTATCIGGCCTAC	-	

[a] Numbering refers to the 16S rRNA gene sequence of *Escherichia coli* strain *rrn*B (GenBank accession number: JO1695).

2.4 DNA-DNA relatedness experiments

Determination of optimum temperature for renaturation. DNA-DNA relatedness values between the strains listed in Table 2.5 were determined by using the procedure described by Gonzalez & Saiz-Jamenez (2005). To determine the thermal denaturation midpoint of hybrid and reference DNA, purified genomic DNA and mixtures of DNA from the pairs of strains were denatured and renatured at the optimal temperature for renaturation (T_{or}; De Ley *et al*. 1970). T_{or} was estimated from mol % G+C contents, as described by De Ley and his colleagues (1970). The G+C contents of the strains were determined fluorimetrically by using the following procedure. Each genomic DNA preparation (2.5 µg) was added to a 0.5 ml tube, the preparation dried in a Speed vac-DNA 120 (Savant Speed Vac Systems, St. Paul, Minnesota, USA), resuspended in 90 µl resuspension solution (30% formamide, 0.1x SSC buffer [pH 8.0]) and transferred to 0.2 ml MJ clear tubes (MJ Research Inc, Waltham, USA) suitable for fluorescence measurements. 10 µl of 1x SYBR Green I (Molecular Probes) was added to each of the preparations and a thermal ramp run from 25 to 100˚C at 1˚C rise per minute in a PTC-200 DNA Engine Thermal Cycler (MJ Research Inc). The fluorescence from the tubes was measured at each step of the thermal ramp and the T_m of each strain calculated from the minimum value of the slope of the tangent to the melting curve of fluorescence versus temperature. The mol% G+C content of each strain was calculated using the equation: mol% G+C = $(1.99T_m)$ - 71.08. The optimal temperature for renaturation was determined from the G+C values using the equation: T_{or} = 0.51(mol % G+C) + 47.0, as described by De Ley *et al.* (1970).

Estimation of ΔT_m between pairs of strains. The generation of genomic DNA hybrids for estimation of ΔT_m values between the pairs of test strains was carried out by adding each genomic DNA (2.5 µg) from both the reference and the test strains to a 0.5 ml centrifuge tube; genomic DNA (5.0 µg) from the reference strain to another tube for control sample. The samples were first dried in a speed vac and the resultant dried preparations resuspended in 90 µl 0.1x SSC buffer (pH 8.0; Appendix B). The homologous DNA control and hybrid DNA samples were denatured and rehybridised in the *T*-gradient Thermocycler® (Whatman-Biometra) using the following conditions: 99˚C for 10 minutes, T_{or} for 8 hours followed by progressive steps in which the temperature was dropped by 10˚C then held for 1 hour until the temperature reached to

25˚C. The tubes were then held at 4˚C, 10µl of 1x SYBR Green I was added and the contents transferred to MJ clear tubes in an PTC -200 DNA Engine Thermal Cycler. Thermal denaturation was achieved as follows: 25˚C for 15 minutes, and a thermal ramp from 25 to 100˚C at 0.2˚C per second; fluorescence measurements were taken at each step of the ramp. The T_m of the homologous and hybrid DNA preparations was calculated by taking the temperature corresponding to a 50% decrease in fluorescence in the melting curve of fluorescence versus temperature. ΔT_m is the difference between these two temperatures; differences of 5˚C or more are considered to show that tested strains belong to different genomic species (Wayne *et al.*, 1987; Rosselló-Mora & Amann, 2001).

Table 2.5. *Dactylosporangium* strains examined in the DNA-DNA relatedness experiments

Test strains	Reference strains
Isolate BK51 *vs*:	Isolate BK53 Isolate BK69 Isolate BK63 *D. aurantiacum* NRRL B-8018^T *D. matsuzakiens*e NRRL B-16293^T *D. salmoneum* NRRL B-16294^T *D. vinaceum* NRRL B-16297^T
Isolate BK63 *vs*:	*D. aurantiacum* NRRL B-8018^T *D. matsuzakiens*e NRRL B-16293^T *D. salmoneum* NRRL B-16294^T *D. vinaceum* NRRL B-16297^T
"*D. salmoneum*" NRRL B-16294 *vs*:	*D. aurantiacum* NRRL B-8018^T *D. matsuzakiens*e NRRL B-16293^T *D. vinaceum* NRRL B-16297^T

2.5 Phenotypic characterisation

2.5.1 *Cultural and morphological characteristics*

The tested strains (Table 2.6) were grown on tryptone-yeast extract, yeast extract-malt extract, oatmeal, inorganic salts-starch, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1-7, respectively; Shirling & Gottlieb, 1966) for 21 days at 28˚C. The resultant colonies were examined by eye to determine substrate mycelial pigmentation and the colour of any diffusible pigments; colours were recorded using National Bureau of Standards (NBS) Centroid Colour Charts (Kelly, 1958).

Table 2.6. Sources and strain histories of dactylosporangiae examined in the phenotypic tests

Strains	Source
Dactylosporangium sp. BK51, BK63	Plot 6, hay meadow soil, Cockle Park, Northumberland, UK
D. aurantiacum	NRRL B-8018^T; ATCC 23491; DSM 43157; Thiemann D-748; soil, Pagani Gruppo Lepetit, Italy.
D. fulvum	DSM 43917^T; NBRC 14381; NRRL B-16292; Meiji Seika Kaisha SF-2113; soil, Chiba Prefecture, Japan.
*D. matsuzakiens*e	NRRL B-16293^T; ATCC 31570; NBRC 14259; Meiji Seika Kaisha SF-2052; soil, Matsuzaki, Izu Peninsula, Japan. Produces dactimicin.
D. roseum	DSM 43916^T; NRRL B-16295; NBRC 14352; soil, Shizuoka, Japan.
"*D. salmoneum*"	NRRL B-16294; DSM 43910; ATCC 31222; NBRC 14103; soil, Japan. Produces polycyclic ether antibiotic.
D. thailandense	DSM 43158^T; ATCC 23490, NBRC 12593; Thiemann D-449; soil, Thailand. Degrades natural rubber.
D. vinaceum	NRRL B-16297^T; DSM 43823; NBRC 14181; Meiji Seika Kaisha, SF-2127; soil, Japan. Produces dactimicin.
"*D. variesporum*"	NRRL B-16296; ATCC 31203, NBRC 14104; soil, Japan. Produces capreomycin.

Micromorphological properties of the strains were recorded from 3-week-old oatmeal agar plates using a Nikon Optiphot light microscope fitted with a long working-distance objective. In addition, the spore arrangement of isolates, BK51 and BK63, and "*D. salmoneum*" NRRL B-16294 were determined by scanning electron microscopy (SEM). Agar plugs, 2 per strain, were prepared from oatmeal agar plates using a sterile 6 mm cork borer, fixed using 2% (v/v) glutaraldehyde in Sorenson's phosphate buffer in small bijoux tubes, and the preparations kept at 4˚C overnight. The buffer was then discarded; the agar plugs dehydrated in an ethanol series (10, 20, 40, 60, 80 and 100% for 20 minutes) prior to SEM analysis (Electron Microscopy Unit, Newcastle University). Samples were dried using a Critical Point Dryer (SAMDRYR-780, Tousimis Research Corporation, USA) and liquid carbon dioxide, mounted on aluminum stubs coated with gold, using a Gold Sputter (E5100. "Cool" Sputter Coater, Polaron Equipment Limited, Walford, UK), and examined using a Stereoscan 240 scanning electron microscope (Cambridge Instruments Ltd., Cambridge, UK).

2.5.2 *Biochemical tests*

Test strains and inoculation of test media. The test strains (Table 2.6) were examined for 120 unit characteristics, as shown in Table 2.7. Most of the tests were carried out in Replidishes (Sterilin Ltd., Staffordshire, UK) at 28˚C unless stated otherwise, and results recorded after final readings. Sterile media were aseptically dispensed into each of the 25 compartments of the Replidishes and individual compartments inoculated with 10 µl aliquots of spore suspensions. To this end, spores from each of the strains were scraped from oatmeal agar plates, which had been incubated at 28˚C for 21 days, and transferred to individual 15 ml conical tubes which contained 5 ml of ¼ strength Ringer's solution. The resultant spore suspensions were filtered through sterile cotton wool, centrifuged and resuspended in 2 ml of ¼ strength Ringer's solutions to give a turbidity of 5 on the McFarland scale (BioMerieux® Co.). Each inoculum (1 ml) was pipetted into a sterile compartment in Replidishes and gently agitated to give an even suspension prior to inoculation. An automatic multipoint inoculator (Denley-Tech; Denley Instruments Ltd., Sussex, UK) was used to inoculate Replidishes containing each test media. All of the tests were carried out in duplicate.

Aesculin hydrolysis. This test was carried out using the method of Kutzner (1976) and results recorded after incubation for 7, 14 and 21 days. Strains with β-glucosidase activity hydrolyse aesculin to give 6, 7-dihydroxycoumarin which complexes with ferric ions in the medium to form a brown black melanin-like polymer. Blackening of the test medium indicated a positive result. It can be difficult to distinguish between a positive result and the production of dark pigments by some actinomycetes hence all strains were inoculated onto a negative control (without aesculin) which was compared to the corresponding aesculin test in order to prevent recording false positive results.

Allantoin hydrolysis. The hydrolysis of allantoin (0.33%, w/v) was detected using the basal medium of Gordon *et al.* (1974) supplemented with phenol red as the pH indicator. After incubation for 14 days, inoculated Replidishes were examined for the development of an alkaline reaction which was indicated by a colour change of the medium from orange to pink red. A positive test result, that is, the production of a pink-red colour, indicates the presence of two hydrolytic enzymes, one of which hydrolyses allantoin to allantoic acid and the other which catalyses the formation of urea and glycoxalate.

Table 2.7. Unit characters

A. Biochemical tests:
Aesculin hydrolysis
Allantoin hydrolysis
Arbutin hydrolysis
Catalase production
Hydrogen sulphide production*
Nitrate reduction*
Urease production*

B. Degradation tests (%, w/v):
Adenine (0.4)
Casein (1)
Cellulose (1.0)
Chitin (0.2)
DNA (0.2)
Elastin (0.3)
Gelatin (0.4)
Guanine (0.05)
Hypoxanthine (0.4)
RNA (0.3)
Starch (1.0)
Trybutyrin (0.1% v/v)
L-Tyrosine (0.5)
Tween 20 (1.0% v/v)
Tween 40 (1.0% v/v)
Tween 60 (1.0% v/v)
Tween 80 (1.0% v/v)
Uric acid (0.5)
Xanthine (0.4)
Xylan (0.4)

C. Nutritional tests:

Growth on sole carbon sources

At 1.0 %, w/v:
Monosaccharides:
Hexoses:
D-Fructose
D-Galactose
D-Glucose
D-Mannose
L –Sorbose
D- Xylose
Pentoses:
Amygdalin
L-Arabinose
L-Fucose
D-Furanose
L-Rhamnose
D-Ribose

Disaccharides:
D-Cellobiose
α- Lactose
D-Maltose
D-Melibiose
D-Sucrose
D-Trehalose

Tri & tetrasaccharides:
D (+) Melezitose
D (+) Raffinose

Polysaccharides:
Dextrin
Glycogen
Inulin
Pectin

Sugar alcohols:
Adonitol
D-Arabitol
Dulcitol
meso-Inositol
D-Mannitol
D-Sorbitol

Glycosides:
Aesculin
Arbutin
Salicin

At 0.1%, w/v:
Citric acid
L(+) Lactic acid
Malic acid
Oxalic acid
Propionic acid
Pyruvic acid
Succinic acid
L (+) Tartaric acid
Urea
Uric acid

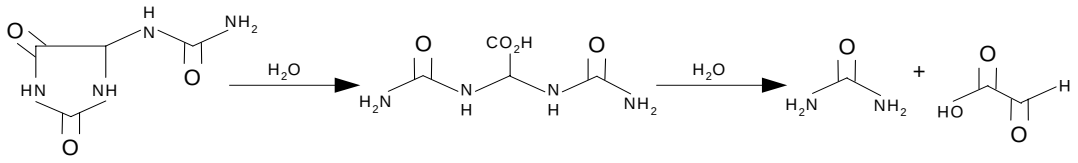
D. Tolerance tests:

Growth at:
pH 4.0, 5.0, 9.0 and 10.0
4˚C, 10˚C, 15˚C, 20˚C, 25˚C, 30˚C, 37˚C and 42˚C

Growth in presence of (%, w/v):
Sodium chloride (1.5, 3.0, 5.0 and 7.0)
Lysozyme (0.05)

Resistance to antibiotics (µg/ml):
Ampicillin (4):
Cephaloridine (2)
Chloramphenicol (8)
Ciprofloxacin (2)
Clindamycin (8)
Gentamicin (8)
Kanamycin (8)
Lincomycin (8)
Novobiocin (8)
Oxytetracycline (16)
Penicillin-G (2)
Rifampicin (16)
Streptomycin (4)
Tetracycline (8)
Tylosin (8)
Vancomycin (2)

*, tests carried out in sterilised Bijoux tubes. The remaining tests were carried out in Replidishes.



Allantoin Allantoic acid Urea Glycoxalate

Arbutin hydrolysis. The hydrolysis of arbutin (0.1%, w/v), a hydroquinone-α-glucopyranoside, was detected using the basal medium described by Williams *et al*. (1983). The hydrolysis of arbutin is catalysed by the hydrolase enzyme, β-glucosidase, resulting in the production of glucose and a hydroquinone, the latter complexes with the iron in the medium to form a black-brown melanin-like polymer. Blacking of the medium after 5, 10 or 15 days was recorded as a positive result.

Catalase production. The presence of catalase was detected by adding a few drops of 20% (v/v) hydrogen peroxide solution to 7 day old cultures grown on modified Bennett's agar (Jones, 1949). The production of oxygen bubbles from the reduction of hydrogen peroxide was recorded as a positive reaction.

Hydrogen sulphide production. The ability of the test strains to produce hydrogen sulphide was determined following the method described by Küster & Williams (1964). Tubes of nitrate broth (Appendix A) were inoculated and strips of sterile lead acetate paper hooked over their necks. Hydrogen sulphide converts lead acetate to lead sulphide resulting in blackening of the lead acetate paper. The blackening of lead acetate strips after incubation for 7 days was recorded positive.

Nitrate reduction. This test was performed to detect the presence of nitrate and nitrite reductases. Tubes containing 3 ml nitrate broth (0.1%, w/v; Gordon & Mihm, 1962) were inoculated and incubated for 14 days when a few drops of reagents A and B (Appendix B) were added. The sulfanilic acid in reagent A reacts with nitrite to form a diazonium salt which forms a stable red dye in the presence of α-naphthylamine in reagent B. The development of a red colour on addition of reagent B indicated the presence of nitrite and was recorded as positive for nitrate reductase activity. In the absence of a colour change traces of zinc dust were added to the broths. Zinc ions catalyse the same reaction as nitrate reductase hence when nitrate is still present the addition of zinc reduces it to nitrite with the formation of a characteristic red colour; such results were recorded as nitrate and nitrite reductase negative. The continued

absence of any colour change following the addition of zinc indicates that nitrate has been reduced to gaseous nitrogen; such reactions were recorded positive for nitrate and nitrite reductase.

$$\underset{\text{Nitrate}}{NO_3^- + 2\,H^+ + 2\,e} \xrightarrow{\text{Nitrate reductase}} \underset{\text{Nitrite}}{NO_2^- + H_2O} \xrightarrow{\text{Nitrite reductase}} \underset{\text{Nitrogen gas}}{N_2}$$

Urease production. The strains were tested for their ability to hydrolyse urea using the basal medium (Appendix A) of Gordon *et al.* (1974) supplemented with urea (1.76 %, w/v). Inoculated Replidishes were incubation for 14 days; and then examined for the development of a pink-red colour which was recorded as a positive result.

$$\underset{\text{Urea}}{NH_2CONH_2 + 2\,H_2O} \xrightarrow{\text{Urease}} \underset{\text{Ammonia}}{2NH_3 + CO_2 + H_2O}$$

2.5.3 *Degradation tests*

Adenine, casein, cellulose, elastin, guanine, hypoxanthine, L-tyrosine, uric acid, xanthine and xylan were incorporated into modified Bennett's agar (Jones, 1949) with care taken to ensure an even distribution of the insoluble compounds; each compound was sterilised by tyndalisation before adding to molten modified Bennett's agar. Inoculated plates were read after 7, 14 and 21 days. In all cases, disappearance of the substrate from under and around the area of growth was recorded as a positive result. Adenine, guanine, hypoxanthine and xanthine are nitrogenous purine bases involved in the biosynthesis of nucleic acids, L-tyrosine is an aromatic amino acid and elastin is a protein found in animal connective tissue. The degradation of uric acid and xylan indicate the production of uricase and xylanase enzymes, respectively.

Casein. The degradation of casein was detected in modified Bennett's agar (Jones, 1949) supplemented with skim milk powder (10 g per litre). Inoculated Replidishes were incubated for 15 days when the presence of clear zones from around and under areas of growth indicated a positive result. Similarly chitinolytic activity was observed on colloidal chitin agar (Hsu & Lockwood, 1975) after 14, 21 and 28 days and positive results recorded when a clear zone was detected around colonies.

Cellulose. Cellulose is composed of β-(1-4) linked glucose subunits. Carboxymethylcellulose plates inoculated with the test strains were incubated for 14 days then flooded with Congo red solution (0.1%, w/v) for 15 minutes at room temperature. Undigested cellulose stained a pinky red when excess reagent was removed; a pale orange to straw colour zone around areas of growth indicated the breakdown of cellulose, such results were recorded positive.

DNA and RNA. The degradation of DNA was detected using Bacto DNase test agar (Difco; Appendix A) which contains DNA (0.2%, w/v). Ribonucleic acid breakdown was examined in tryptose agar (Goodfellow *et al.*, 1979) supplemented with RNA (0.3%, w/v). In each case, inoculated 7-day-old plates were flooded with 1 N hydrochloric acid which causes nucleic acids to precipitate as a fibrous mass. Positive reactions were indicated by the presence of clear zones from under and around the growth of the tested strains.

Gelatin. The degradation of gelatin was detected using modified Bennett's agar (Jones, 1949) supplemented with gelatin (0.4%, w/v). Inoculated plates were incubated for 7 days then flooded with acidic mercuric chloride solution (Appendix B), the latter acts as a denaturing agent complexing with gelatin and causing it to precipitate. Extracellular proteases hydrolyse gelatin into small peptides and amino acids which do not react with mercuric chloride, positive reactions were indicated by the presence of clear zones from under and around the growth of the tested strains.

Starch. Starch, the main polysaccharide in plants, is composed of α-D-glucopyranose sub-units in two different structural configurations; amylose, an α-(1-4) linked polymer, and amylopectin, an α-(1-4) linked molecule with α-(1-6) branches. This test was carried out to detect the production of extracellular α-amylases which degrade starch. The test was performed by using modified Bennett's agar (Jones, 1949) supplemented with soluble starch (10 g/ litre). Inoculated plates were incubated for 15 days prior to flooding with Lugol's iodine (Appendix B); iodine complexes with left handed, α-helical, amylose molecules resulting in the formation of a dark blue starch-iodine complex. Extracellular α-amylases show endo-glycosidase activity and hence are able to hydrolyse starch molecules by random attack at points distant from the chain ends to form short polysaccharide chains (dextrins) and simple sugars which are unable to

complex with iodine. Positive results are indicated by the formation of clear zones from around the growth area following the addition of Lugol's iodine.

Tributyrin. The breakdown of glycerol tributyrate was carried out using trybutyrin agar (Appendix A; Sigma T3688). Inoculated plates were read after 7 and 14 days. Positive results were recorded when clear zones were observed from under and around colonies.

Tweens. Tweens 20, 40, 60 and 80 are a homologous series of water soluble, high molecular weight fatty acid esters of a polyoxyalkaline derivative of sorbitan which differ only in fatty acid components. Tweens 20, 40, 60 and 80 contain mono-laurate, palmitic, stearic and oleic acids, respectively and can be used to detect the production of specific esterases. Esterases produced by tested strains diffuse into the medium and hydrolyse ester linkages releasing free fatty acids which combine with calcium ions (Ca^{2+}) present in the medium to form insoluble calcium salts which precipitate out as white crystals characteristic for each of the Tweens. The basal medium of Sierra (1957) supplemented with individual Tweens (10 ml/litre), was inoculated, incubated and examined for the characteristic precipitate which indicated a positive response.

2.5.4 *Nutritional tests*

The ability of test strains to metabolise carbon compounds (Table 2.7) as sole sources of carbon for energy and growth was examined using carbon utilisation agar (ISP medium 9; Appendix A; Shirling & Gottlieb, 1966) as the basal medium. Solutions of each carbon source were sterilised by steaming at 101˚C for 30 minutes on three consecutive days then added to the basal medium to give the required concentration. Media dispensed into Replidishes were inoculated, incubated and examined after 7, 14 and 21 days. The test strains were also inoculated onto the basal media (the negative control) and onto the basal medium supplemented with 1%, glucose (the positive control). A positive result was recorded when growth on the test medium was equal or greater that on the positive control, but negative when growth on the test plate was equal to or less than that on the negative control plate.

2.5.5 *Tolerance tests*

Temperature. The test strains were examined for their ability to grow on modified Bennett's agar (Jones, 1949) at 4˚C, 10˚C, 20˚C, 25˚C, 30˚C, 37˚C and 42˚C. Inoculated Replidishes were incubated and read weekly for 6 weeks at 4˚C and 10˚C, and for 3

weeks at the other temperatures. In all cases, visible growth was recorded as a positive result. The Replidishes incubated at 37˚C and 42˚C were placed into plastic bags in order to prevent the inoculated medium from drying out.

pH. The ability of the test strains to grow over a range of pH regimes (Table 2.7) was examined on modified Bennett's agar adjusted to the appropriate pH using 0.1 M solutions of potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4). Growth was recorded as a positive result after incubation for up to 21 days.

Sodium chloride and lysozyme. The test strains were examined for their ability to grow on modified Bennett's agar (Jones, 1949) supplemented with sodium chloride at 1.5%, 3%, 5% and 7% (w/v) and lysozyme at 0.05% (v/v). Inoculated plates were read after 7, 14 and 21 days when the growth of strains was compared to that on control plates without the supplements; positive reactions were recorded when growth on the test plates was greater than that on the control plate and a negative one when growth was less or the same as on the control plate.

Resistance to antibiotics. The test strains were examined for their ability to grow in the presence of different antibiotics at various concentrations (Table 2.7). All of the antibiotic solutions were filter sterilised then added to sterile cooled modified Bennett's agar (Jones, 1949) to give the required concentrations before dispensing into Replidishes; control plates lacking antibiotic were also inoculated. Inoculated plates were incubated for up to 21 days. Organisms were recorded as resistant (+) where growth on the test plate was greater than or equal to that on the control plate and sensitive (-) when growth was less than that on the control plate.

2.6 Chemotaxonomic tests

Detection of diaminopimelic acid isomers. Test strains (Table 2.6) were grown on modified Bennett's agar (Jones, 1949) for 14 days at 28˚C. The isomeric forms of diaminopimelic acid (A_2pm) were determined by thin-layer-chromatography (TLC) of whole-organism hydrolysates on cellulose acetate sheets after Staneck & Roberts (1974). A loopful of biomass of each isolate was harvested, placed into a 2 ml cryotube which contained 500 µl of 6 N HCl, mixed and heated at 100˚C in an oven for 4 hours.

The resultant whole-organism hydrolysates were centrifuged at 6000 rpm for 4 minutes, and the supernatants collected and evaporated to dryness at 100˚C for 2 to 3 hours in a heating block held in a fume cupboard. Once most of the acid had evaporated, 1ml of distilled water was added to each preparation prior to evaporating at 100˚C. Finally, 100 µl of distilled water was added to each preparation to produce the extract needed for TLC.

The isomers of A_2pm were separated by one-dimensional TLC using glass backed cellulose TLC plates (20×20 cm; No.5716. Merck, Darmstadt, Germany). An aliquot (1 µl × 2) of each hydrolysate was applied as a spot along a line 2 cm from the base of the TLC plate. A standard solution (0.01 M) of A_2pm (Sigma), which contained a mixture of DL-, LL- and *meso*-A_2pm isomers, was prepared and 1 µl applied to each TLC plate on either side of the test samples. The plates were developed in glass tanks saturated with a solvent (methanol: water: 6 N HCl: pyridine; 80:26:4:10; v/v/v/v) for 4 hours or until the solvent front was approximately 5 cm from the top of the plate. Plates were air-dried, sprayed with freshly prepared ninhydrin solution (0.2%, w/v dissolved in acetone) and heated at 100˚C for 3 minutes in an oven. The isomers of A_2pm appeared as dark purple to brown spots that had a lower retention factor (R_f) value than amino acids, the latter yielded blue to purple coloured spots. The A_2pm isomers of the test strains were identified by comparison with the standard mixture. The R_f values of the A_2pm isomers run in the order: 3-hydroxy-A_2pm, *meso*-A_2pm, DD-A_2pm and LL-A_2pm (from lowest to highest).

Detection of mycolic acids. The alkaline methanolysis procedure, modified from Minnikin (1975), was used to examine the isolates (Table 2.6) for the presence of mycolic acids; *Nocardia brasiliensis* N318^T was used as the control. Two loopfuls of wet biomass per strain were suspended in 1 ml of 5% (v/v) aqueous tetrabutyl-ammonium hydroxide (diluted from a 40% commercial solution; Sigma) held in 2 ml vials which contained 100 µg glass beads (<106 µm diameter; Sigma). The samples were homogenised for 20 seconds (FastPrep FP120 Cell Disruptor, speed 5 m/s), incubated at 100˚C for 4 hours and cooled to room temperature prior to centrifugation at 4000 rpm for 5 minutes. The supernatants were transferred to clean tubes which contained a solution of dichloromethane (1 ml) and iodomethane (25 µl) and the resultant preparations shaken on a tumble shaker (Model TM1 Tumbler, speed No.5) for 30 minutes. The preparations were centrifuged (2000 rpm, 3 minutes), the upper

aqueous layers discarded, and the lower organic layers, which contained the methyl ester derivatives, were evaporated to dryness under a stream of nitrogen gas at 37˚C. Petroleum ether (50 µl) was added to reconstitute each sample for TLC analysis.

The mixtures of fatty acid methyl esters (FAME) and mycolic acid methyl esters (MAME) were separated by one-dimensional TLC using aluminium-backed silica gel TLC sheets (10×20 cm; No.5554, silica gel $60F_{254}$, Merck, Darmstadt, Germany). The samples (3 µl) were applied in 1 µl aliquots onto a line 1cm from the base of each sheet and the TLC plates developed in saturated glass tanks containing petroleum ether-acetone (95:5, v/v). The plates were air-dried when the solvent front approached the top of the plates; following air-drying the chromatograms were developed a second time in the same direction. The resultant chromatograms were sprayed with a 5% (w/v) solution of ethanolic molybdophosphoric acid (diluted from a 10% commercial solution; Sigma P-1518) and immediately dried in an oven at 180˚C for 5 minutes. The positions of the separated FAMEs and MAMEs were revealed as dark blue/black spots on a pale green background. The presence or absence of mycolic acids in the test strains was determined by comparison with the reference strain.

Determination of whole-organism sugar patterns. Whole-organism sugar patterns were determined according to the procedure described by Schaal (1983). Freeze-dried biomass (*ca.* 25 mg) of each isolate (Table 2.6) was hydrolysed with 1.5 ml of 1N H_2SO_4 in a sealed 2 ml vial held at 100˚C for 3 hours, the hydrolysate cooled to room temperature, neutralised (pH 5.0-5.5) by adding barium hydroxide (*ca.* 0.5 g) and shaken several times. The resultant preparation and the remaining $Ba(OH)_2$ were centrifuged at 6000 rpm for 5 minutes and the supernatant carefully removed and evaporated to dryness on a hot block at 40˚C. The residue was reconstituted with 0.1 ml distilled water and 0.25 ml pyridine, thoroughly mixed and the pyridine phase separated from the aqueous phase by centrifugation at 6000 rpm for 5 minutes. The upper pyridine layer, which contained the purified sugar extract, was transferred to a small glass vial and concentrated under a stream of nitrogen at room temperature to give a final volume of 0.3 ml.

The concentrated pyridine extracts (3 µl) were spotted in 1 µl aliquots onto a line 1 cm from the base of cellulose TLC aluminium sheets (10×20 cm; No.5552, Merck) and the sugars separated by ascending, one-dimensional TLC using n-butanol/water/

pyridine/toluene (10:6:6:1, v/v/v/v). Chromatograms were developed twice with intermediate air drying, and the sugars visualised by spraying chromatograms with an aniline-phthalate reagent (aniline, 2 ml; phthalic acid, 3.25 g in 100 ml water-saturated n-butanol), air drying and heating at 100˚C for 4 minutes when the hexoses, pentoses and 6-deoxy sugars gave brown, red and grey-green spots, respectively. The R_f values of the sugars increase in the order: galactose, glucose, mannose, arabinose, xylose, ribose, rhamnose and 6-deoxytalose. Mixtures of the sugars (arabinose, galactose, glucose, mannose, ribose; 1%, w/v in pyridine) were used as standards. The sugar patterns of the strains were identified by comparison with the standards.

Determination of muramic acid residues in the peptidoglycan. The *N*-acyl type of muramic acid in the cell wall peptidoglycan of the strains was determined using a modification of the glycolate test (Uchida & Seino, 1997). Dried biomass (*ca.* 10 mg) of each strain (Table 2.6) was hydrolysed with 500 µl of 6 N HCl at 100˚C in an oven for 2 hours and allowed to cool to room temperature before adding 1 ml of water-saturated diethyl ether. The resultant preparations were mixed on a tumble shaker (Model TM1 Tumbler) for 30 minutes, vortexed for two minutes and centrifuged at 5000 rpm for 2 minutes. The extracts of the upper layers were discarded to remove any interfering compounds from the glycolate fraction, and the extraction procedure repeated on the original hydrolysates using a further 1 ml of water-saturated diethyl ether. The upper layers of the samples were transferred to clean microcentrifuge tubes, using Pasteur pipettes, treated with 5 µl of 0.1 N sodium hydroxide, and the preparations inverted several times prior to evaporating to dryness at room temperature in a fume hood.

The residues were redissolved in 25 µl of sterile distilled water and 500 µl of freshly prepared DON reagent (0.02% w/v, 2,7-dihydroxynaphthalene dissolved in concentrated sulphuric acid). The DON reagent was prepared the day before use, stored overnight at 4˚C and was only considered stable if the colour had changed from yellow to colourlessness. The preparations were heated at 100˚C for 20 minutes, cooled to room temperature and vortexed briefly then allowed to stand for 10 minutes and the results recorded. The test strains were examined for *N*-acetylated muramic acid acyl groups (positive controls: *Amycolatopsis orientalis* KCTC 9412^T and *Saccharopolyspora rectivirgula* $F1^T$) and *N*-glycolated acyl groups (positive controls: *Gordonia bronchialis* $N654^T$ and *Nocardia brasiliensis* 318^T), a negative control lacking biomass was also used. Organisms characterised by the presence of *N*-

glycolated acyl groups produce a deep red to purple colour reaction whereas those which contain *N*-acetylated acyl groups remain clear or pale yellow. The colour reactions for the tested strains were compared with those of the control tubes.

Cellular fatty acid analysis. Cellular fatty acids (FAME) of the test strains (Table 2.6) were analyzed by gas-liquid chromatography using the Microbial Identification System (MIS; Sasser, 1990). The fatty acids were saponified, methylated, and extracted from whole-organisms according to the MIS operation manual (Sasser, 1990). Biomass (50mg) harvested from tryptic soy broth was added to 1ml of 30% NaOH/methanol (1:1, v/v), vortexed then heated at 100˚C for 5 minutes. The resultant preparations were mixed, heated at 100˚C for 25 minutes and allowed to cool at room temperature. Each reaction mixture was then added to 2 ml of 6 N HCl/methanol (3.25: 2.75, v/v), mixed, heated at 80˚C for 10 minutes and cooled quickly in a tray of cold tap water.

Fatty acid methyl esters were extracted from the acidic aqueous phase with 1.25 ml of hexane/*tert*-butylmethylether (1:1, v/v), transferred into a clean vial and analyzed using a model HP-5890II plus gas chromatograph (Hewlett-Packard Co.) equipped with a HP-Ultra 2, phenyl methyl silicone fused silica capillary column (25m × 0.2mm), an auto injector and a flame ionization detector (FID); the injector and detector temperatures were at 250˚C and 300˚C, respectively. The temperature was increased from 170˚C to 270˚C at a rate of 5˚C /minute using hydrogen as the carrier gas. Identification of the FAMEs was achieved by using the Sherlock system which compares the retention times of peaks with calibration standards (HP 19298-60500).

Menaquinone analysis. Menaquinones were extracted from the strains (Table 2.6) by using the small-scale integrated procedure of Collins *et al*. (1985). Freeze-dried biomass (100mg) of each strain was treated with 2 ml of 0.3% aqueous NaCl/methanol (1:10, v/v) and 2 ml of petroleum ether, mixed on a tube rotator for 30 minutes, and the upper layer transferred to a clean vial. The lower layer was treated with a further 1ml of petroleum ether, mixed for 30 minutes, and the upper layer added to the previous one. The combined extracts were transferred to small glass vials and evaporated to dryness by using nitrogen and stored at - 20˚C until required.

The extracted menaquinones were resuspended in 200 µl of isopropanol and analysed using a high performance liquid chromatography (HPLC; LC-10AS, Shimadzu

Co.) fitted with a reverse-phase C18 column (250 × 4.0 mm, 5 μm particle size; RP-18-Lichrosorb column; Capital Analytical, UK). Methanol/isopropanol (2:1, v/v) was used as the isocratic mobile phase with a flow rate of 1 ml/min at ambient temperature; aliquots of each sample (10 μl) were injected into the HPLC. The menaquinones were detected at a wavelength of 270 nm using a UV detector (SPD-10A, Shimadzu Co.), and the resultant chromatograms integrated using a C-R6A Chromatopac integrator (Shimadzu Co.). The menaquinones were identified by comparing their retention times with those of reference samples extracted from *Streptomyces indiaensis* JCM 3053^T which has a well known menaquinone profile (Kudo & Seino, 1987)

Polar lipid analysis. Polar lipids were extracted from the biomass remaining after the extraction of menaquinones (Collins *et al*., 1985). Each sample of cell debris was heated in a boiling water bath for 5 minutes, cooled to room temperature, and 2.3 ml of chloroform/methanol/0.3% aqueous NaCl (9:10:3, v/v/v) added. The resultant samples were mixed on a tube rotator for an hour, each supernatant was transferred to a clean tube to which 0.75 ml of chloroform/methanol/0.3% aqueous NaCl (5:10:4, v/v/v) was added prior to mixing for 30 minutes. Each of the resultant preparations was centrifuged; the supernatants combined with the corresponding previous one, 1.3 ml chloroform and 1.3 ml of 0.3% aqueous NaCl added and the preparations mixed thoroughly prior to centrifugation. Each of lower layers was transferred to a clean vial, evaporated to dryness in a nitrogen stream and stored at –20˚C.

The lipid extracts were dissolved in 50 μl of chloroform/methanol (2:1) and 10 μl of the resultant samples applied to the corners of five 10 x 10 cm silica-gels TLC plates (No. 1.05721, Merck Co.). Thin-layer-chromatography was carried out using chloroform/methanol/water (65:65:4, v/v/v) in the first direction and chloroform/acetic acid/methanol/water (80:15:12:4, v/v/v/v) in the second one. The resultant spots were characterized using differential stains after Komagata & Suzuki (1987). The first plate was sprayed with 5% (w/v) phosphomolybdic acid in ethanol (Sigma P-1518, Sigma Co.) and charred at 120˚C for 10 minutes to detect all of the lipids, the second one sprayed with ninhydrin (Sigma N-0507) and heated at 100˚C for 10 minutes to detect amino-lipids, and then cooled prior to spraying with Dittmer's reagent (Molybdenum Blue, Sigma M-3389) to detect phospholipids. The third plate was sprayed with α-naphtholsulfuric acid and heated at 100˚C for 10 minutes to detect glycolipids, and the fourth plate sprayed with Dragendorff reagent (Sigma D-7518) to detect choline-

containing lipids. Finally, the lipids on each of the TLC plates were identified by comparing their motilities with those of authentic lipids (Sigma PH-9) applied to the fifth plate.

2.7 Antibiotic resistance profiles

Forty four strains taken to represent the colour-groups were screened against 41 antibiotics (Table 2.8) which were mainly purchased from Sigma-Aldrich; ciprofloxacin and polymixin B were from Fluka Biochemika, and nalidixic acid from Duchefa Biochemie. Spores of each isolate were scrapped from oatmeal agar plates, which had been incubated 28˚C for 21 days, suspended in ¼ strength Ringer's solution, filtered through sterile cotton wool, centrifuged at 3000 rpm for 15 minutes, and resuspended in ¼ strength Ringer's solutions to give a turbidity of 5 on the McFarland scale (BioMerieux® Co.). Each inoculum (1 ml) was pipetted into a sterile compartment in Replidishes and gently agitated to give an even suspension prior to inoculation.

Table 2.8. Antibiotics used in the antibiotic profiling tests (20 μg/ml)

Amikacin	Doxycycline	Polymixin B
Amoxicillin	Erythromycin	Penicillin G
Ampicillin	Fosfomycin	Rifampicin
Apramycin	Fusidic acid	Streptomycin
Bacitracin	Gentamicin	Sulfadiazine
Carbenicillin	Kanamycin	Sulfamethoxazole
Cefoxitin	Lincomycin	Tetracycline
Cephalexin	Minocycline	Tobramycin
Cephradine	Nalidixic acid	Trimethoprim
Cephaloridine	Neomycin	Tylosin tartrate
Chloramphenicol	Novobiocin	Vancomycin
Chlortetracycline	Oleandomycin	
Clarithromycin	Oxacillin	
Ciprofloxacin	Oxytetracycline	
Clindamycin	Paromycin	

An automatic multipoint inoculator (Denley-Tech; Denley Instruments Ltd., Sussex, UK) was used to inoculate Replidishes containing modified Bennett's agar

(Jones, 1949) supplemented with 20 µg/ml of the appropriate antibiotic; control plates lacking antibiotics were also inoculated. The inoculated plates were incubated at 28˚C for 14 days. All of the tests were carried out in duplicate. Strains growing on modified Bennett's agar supplemented with an antibiotic were recorded as resistant (1) and those failing to grow as susceptible (0). The resultant data were examined using the simple matching coefficient (S_{SM}; Sokal & Michener, 1958), which includes both positive and negative similarities, and clustering achieved using the unweighted pair group methods with arithmetic averages (UPGMA) algorithm (Sneath & Sokal, 1973), using NTSYS software (Rohlf, 1998); the results were presented in a dendrogram.

2.8 Detection of dactylosporangial diversity using a culture-independent approach

Extraction of community DNA. Community DNA was extracted from 21 environmental samples (Table 2.1) using a method slightly modified from that of Griffiths *et al*. (2000). Briefly, each environmental sample (0.5 g wet weight) was added to a 1.5 ml screw-capped tube which contained 0.5 ml of hexadecyltrimethyl-ammonium bromide (CTAB) extraction buffer and 0.5 ml of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and 0.3 g of acid-washed sterile glass beads (≤ 106 µm; Sigma). The preparations were lysed twice at 5.5 m/s for 30 seconds using a ribolyser (Fastprep® FP220A Thermo Instrument; Qbiogene, France), cooled on ice for 2 minutes and the DNA containing aqueous phases separated by centrifugation (16,000 x g) for 5 minutes. Each supernatant was transferred to a fresh 1.5 ml tube and the phenol removed by adding an equal volume of chloroform-isoamyl alcohol (24:1, v/v) followed by centrifugation at (16,000x g) for 5 minutes. The supernatants were transferred to fresh 1.5 ml Eppendorf tubes and the nucleic acids precipitated by adding 1 ml of 30% (w/v) polyethelene glycol dissolved in 1.6 M NaCl (PEG). The resultant preparations were briefly vortexed, incubated at -80˚C for an hour, centrifuged at maximum speed for 5 minutes, the DNA pellets washed with 500 µl of 70% (v/v) ice-cold ethanol, air-dried and redissolved in 50 µl of TE buffer (pH 8.0). The DNA preparations were checked for purity by agarose-gel electrophoresis and the pure extracted DNA preparations stored at -20˚C.

Nested PCR for detection of 16S rRNA genes. A nested PCR approach was used to detect dactylosporangial 16S rRNA genes in each of the community DNA preparations. Bacterial 16S rRNA genes were amplified from each of the preparations by using

universal primers 27F and 1525R (Table 2.4), as described by Tan *et al*. (2006). The resultant primary PCR products were diluted tenfold as templates for the secondary PCR reactions. Amplification of 16S rRNA genes of members of the family *Micromonosporaceae* was achieved using the family-specific PCR primers M2F and A3R (Table 2.9) and the presence of dactylosporangiae by using the genus-specific PCR primers D3F and D2R (Table 2.9), as described by Monciardini *et al.* (2002). The nested PCR reaction mixture, prepared in a final reaction volume of 50 µl, contained 2.5 µl of each forward and reverse primer, 5 µl of 10x reaction buffer (Bioline), 2.5 µl of DMSO, 0.8 µl of 12.5 mM dNTPs (Bioline), 1.5 µl of 50 mM $MgCl_2$ and 0.5 µl *Taq* DNA polymerase (Bioline) and 1 µl of template DNA. The PCR reactions were carried out in the PCR thermocycler (Whatman Biometra Tgradient^{TM}) together with positive and negative controls.

Table 2.9. PCR primers used to detect the presence of *Dactylosporanium* and *Micromonosporaceae* clones in environmental samples

Primer	Sequence(5'-3')	Target genes*	Size of PCR product (bp)	Reference
M2F	SAGAAGAAGCGCCGGCC	Family *Micromonosporaceae*-specific 16S rRNA	900 – 1000	Monciardini *et al.* (2002)
A3R	CCAGCCCCACCTTCGAC			
D3F	GCGGCTTGTTGCGTCAG	Genus *Dactylosporangium*-specific 16S rRNA	500 – 600	Monciardini *et al.*(2002)
D2R	CCGCTGGCAACATCGAACA			

Cloning, sequencing and phylogenetic analysis of amplified PCR products. Cloning of the purified PCR products of *Dactylosporangium*-specific 16S rRNA genes amplified from the community DNA samples was performed using a StrataClone PCR Cloning Kit (Strategene, UK). Ligations were achieved by mixing 1.5 µl of cloning buffer, 1 µl of each PCR product (5-50 ng) and 0.5 µl StrataClone Vector. The resultant preparations were incubated at room temperature for 5 minutes, placed on ice and the transformations allowed to proceed by adding 1 µl of ligation mixture to thawed StrataClone SoloPack competent cells. The resultant preparations were mixed gently, placed on ice for 20 minutes, heated at 42˚C for 45 seconds and incubated on ice for 2 minutes. Pre-warmed Luria–Bertani (LB; Miller, 1972) broth (250 µl) was added to each tube prior to incubation at 37˚C in a shaker incubator (Gallenkamp Orbital Incubator, UK) for an hour at 160 rpm and centrifugation for 1 minute at 13,000 x g. The supernatants were discarded and the pellets redissolved in 100 µl of LB medium,

and the transformation mixtures (100 µl) plated onto pre-warmed LB-ampicillin plates which had been inoculated with 40 µl of 2% X-gal and incubated overnight at 37˚C.

Selected white colonies were streaked, using sterilised toothpicks onto LB-ampicillin plates, incubated overnight at 37˚C and transformants screened by using the colony-PCR technique with M13 forward and reverse primers (Hofmann & Brian, 1991). The purified colony PCR products were sequenced, and the partial 16S rRNA gene sequences of the clones aligned manually against corresponding sequences of the type strains of *Dactylosporangium* species retrieved from DDBJ/EMBL/GenBank databases using the pairwise alignment option. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Fitch, 1971) tree-making algorithms from the MEGA 3.0 program (Kumar *et al.*, 2004).

2.9 Antimicrobial potential and activity of isolates

Screening for non-ribosomal peptide and type I polyketide synthase genes. Fifty two representative *Dactylosporangium* isolates (Table 4.4) were screened for the presence of non-ribosomal peptide synthetase (NRPS) and type I polyketide synthase (PKS) genes using NRPS and PKS primers (Table 2.10), as recommended by Ayuso-Sacido & Genilloud (2005) and Courtois *et al* (2003), respectively. The amplification reactions were performed in a final reaction volumes of 25 µl, which contained 1 µl of 0.4 µM of either NRPS or PKS specific primers, 2.5 µl of 10x reaction buffer, 1 µl of DMSO, 1.6 µl of 2.5 mM dNTPs (Bioline), 1.5 µl of 50 mM $MgCl_2$, 0.25 µl *BioTaq* DNA polymerase (Bioline), 15.15 µl sterile distilled water and 1 µl of extracted DNA template. The PCR reaction temperature profile was initial denaturation at 95˚C for 5 minutes, 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 57˚C (PKS specific primers) or 61˚C (NRPS specific primers) for 30 seconds, extension at 72˚C for 1 minute with a final extension step at 72˚C for 10 minutes. The taxonomic relationships of the tested strains was established by constructing a 16S rRNA gene tree using the UPGMA algorithm from the MEGA 3.0 program (Kumar *et al.*, 2004) .

Table 2.10. PCR primers used to detect PKS and NRPS genes

Primer	Sequence(5'-3')	Target genes*	Size of PCR product (bp)	Reference
K1F	TSAAGTCSAACATCGGBCA	PKS-I	1200 – 1400	Ayuso-Sacido *et al.* (2005)
M6R	CGCAGGTTSCSGTACCAGTA			
A3F	GCSTACSYSATSTACACSTCSGG	NRPS	700 – 800	Ayuso-Sacido *et al.* (2005)
A7R	SASGTCVCCSGTSCGGTAS			

* NRPS, non-ribosomal peptide synthetase; PKS-1, type-I polyketide synthase.

Evaluation of antimicrobial activity. The antimicrobial activities of the 52 representative isolates (Table 4.4) were examined *in vitro* against a panel of microorganisms using a standard agar diffusion assay for the detection of antibacterial and antifungal activity (Barry & Thornsberry, 1991). The bacterial and yeast strains were grown on LB and YM media (1% yeast extract, 0.5% polypeptone, 1% glucose and 0.3% malt extract, pH 5.5), respectively at 30˚C for 2 days prior to seeding the test media. Agar plugs from each of isolates grown on oatmeal agar at 28˚C for 21 days were transferred to MH medium (Mueller–Hinton medium; Difco) plates which had been seeded with *Bacillus subtilis* DSM 10^T, *Escherichia coli* DSM 5698, *Kocuria rhizophila* ATCC 9341, *Pseudomonas fluorescens* DSM 50090^T and *Staphylococcus aureus* DSM 20231^T, or onto the YM medium inoculated with *Candida albicans* Tu 164 and *Saccharomyces cerevisiae* 6A. The resultant plates were incubated at 30˚C and the diameters of any inhibition zones were measured after 24 and 48 hours.

2.10 Self-resistance of *Verrucosispora maris* AB-18-032 to *atrop*-abyssomicin C

Antibiotic. A freeze-dried *atrop*-abyssomicin C sample obtained from Professor H-P Fiedler (University of Tübingen, Germany) was dissolved in 100% methanol (1 mg/ml).

Cultivation of Bacterial strains The *atrop*-abyssomicin C-producing strain, *V. maris* AB-18-032, and *S. griseus* NRRL B-2682^T were grown at 28˚C for 3 days either on inorganic salts-starch agar (ISP medium 4; Shirling & Gottlieb, 1966) or on tryptic soy broth (Oxoid). Abyssomicin-resistant exconjugants were grown on ISP 4 agar, supplemented with chloramphenicol (25 μg/ml), at 28˚C for 3 days. *Escherichia coli* DH5α and ET12567/pUB307 were cultured on Luria–Bertani (LB) broth (Miller, 1972) at 37˚C for 24 hours (Hanahan, 1983; Kieser *et al.*, 2000).

Sensitivity of *Streptomyces* strains to *atrop*-abyssomicin C. The sensitivity of, "*Streptomyces coelicolor*" A3(2), *Streptomyces griseus* NRRL B-2682^T, "*S. lividans*" TK24 and *S. venezuelae* ATCC 15439 to *atrop*-abyssomicin C was assayed in a minimal medium (glucose 0.5%, tri-sodium citrate·$2H_2O$ 0.05%, KH_2PO_4 0.3%, K_2HPO_4 0.7%, $MgSO_4 \cdot 7H_2O$ 0.01% and $(NH_4)_2SO_4$ 0.1%), as recommended by Riedlinger *et al*. (2004). Filter paper disks soaked in various concentrations of antibiotics (5 μg, 10 μg, 20 μg, 40 μg, 60 μg and 80 μg) were placed on each of the plates of minimal agar which were then inoculated with each of the *Streptomyces* strains. The resultant agar plates were incubated at 28˚C for 3 days and examined for the presence of any clear zones.

Conjugation of P1-derived artificial chromosomal library of *Verrucosispora maris* AB-18-032. A P1-derived artificial chromosome (PAC) library of genomic DNA of *V. maris* AB-18-032 was custom made by Bio S&T Co. (Montreal, Canada). An intergeneric conjugation standard protocol was carried out to introduce the PAC library of genomic DNA to the *atrop*-abyssomicin C sensitive strain, *S. griseus* NRRL B-2682^T, as described by Kieser *et al*. (2000). A culture of the donor *Escherichia coli* strain ET12567/pUZ307 containing pESAC13 (Sosio *et al.*, 2000) was grown in LB broth in the presence of kanamycin (25 μg/ml) to an OD_{600} of 0.2–0.6. The resultant cells were washed twice with an equal volume of LB broth to remove the antibiotic and then suspended in 0.1 volumes of LB broth. Spores (10^4-10^6) of the recipient strain, *S. griseus* NRRL B-2682^T, were suspended in 450μl of 2 × Yeast-Tryptone broth (yeast extract 1%, tryptone 1.6%, NaCl 0.5%) and incubated at 50˚C for 10 minutes. The spore solution was allowed to cool at room temperature prior to the addition of 0.5 ml of the *E. coli* donor cells (1.25×10^6-10^8 cells). The resultant preparations were mixed for 10 minutes at room temperature and 100 μl of the serial dilutions used to spread on Mannitol soya flour (MS) plates (Kieser *et al*. 2000) supplemented with 10 mM $MgCl_2$. Spore pellet from the remaining master dilution was recovered by centrifugation (13,000 rpm, 1 minute), resuspended in 100 μl of LB broth, and plated on MS plate. The conjugation plates were incubated at 28˚C for 16-18 hours, then overlaid with 1.5ml sterilised water containing kanamycin (50 μg/ml), and the resultant preparations were incubated at 28˚C for 3 days.

Selection of resistant conjugants. The selection of *atrop*-abyssomicin C resistant exconjugants was achieved using a double layer method (Kropinski *et al.*, 2009). The

Base plates contained 20 ml of minimal medium, amended with 1.5% agar. A spore suspension of *S. griseus* exconjugants (50 µl) mixed with 3 ml of minimal broth containing 0.8% soft agar supplemented with kanamycin (25 µg/ml) and *atrop*-abyssomicin C (50 µg/ml) was poured over basal agar plates. After incubation at 30˚C for 48 hours, resistant exconjugants were selected and transferred to inorganic salts-starch agar supplemented with kanamycin (25 µg/ml).

PCR primers. Four primers (Table 2.11) designed using Primer3 software (Rozen & Skaletsky, 2000) were used to detect putative abyssomicin resistance genes present in the exconjugants. Three primer pairs, AS, ICS and PAB, targeted chorismate converting enzymes, namely anthranilate synthase, isochorismate synthase and 4-amino-4-deoxychorismate synthase, respectively, and the remaining one primer, abyD, was designed to detect a putative abyssomicin transporter gene located in the abyssomicin biosynthetic gene cluster. DNA sequences of the genes were obtained from whole genome data of *V. maris* AB 18-032 by using a next-generation sequencing system (454 Life Sciences, Roche)(Jem Stach, personal communication); the primers were supplied by Eurogentec Co. (Seraing, Belgium).

Table 2.11. PCR primers designed for screening putative resistance genes of *Verrucosispora maris* AB-18-032

Primer name	Sequence (5'-3')	Target gene	Length of fragment (bp)
abyDf	ATCCTGCAGAACCTGACCTG	Abyssomicin synthetic gene cluster	400
abyDr	CAGGAACAACAGCGAGAACA		
ASf	AGACCACCTTCACCGAACTG	Anthranilate synthase	950
ASr	GCTCTCCTCGATTGTCACCT		
ICSf	CGAGAACATCGACAAGGTCA	Isochorismatase	750
ICSr	AAGTCCTGGTTGTTGCCATC		
PABf	GCAATCCAGGACTTCCTCAC	ADC synthase	1100
PABr	GTGTCTGGTTGCGGATCAT		

Isolation of genomic DNA. The selected abyssomicin-resistant exconjugants were inoculated into 10 ml TSB broth supplemented with kanamycin (25 µg/ml). The resultant preparation was incubated at 30˚C for 48 hours, the cells harvested by centrifugation (5,000 rpm × 20 minutes), resuspended in 200 µl of TEG buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0) containing 100 µg of lysozyme and

20 µg of RNaseA, and incubated at 37˚C for 30 minutes, 400 µl of 0.2 M NaOH, 10% (w/v) SDS were added and the mixture left at room temperature for 5 minutes prior to the addition of 300 µl of 3M potassium acetate (pH 5.2). The preparation was then vortexed, left on ice for 10 minutes, microcentrifuged for 10 minutes, the supernatant extracted with an equal volume of phenol : chloroform (1:1, v/v), and nucleic acids precipitated by the addition of an equal volume of isopropanol. The nucleic acids were harvested by centrifugation, washed in 70% (v/v) ethanol, dried, and dissolved in sterile water (Kieser *et al*., 2000).

PCR screening of putative resistance genes. The genomic DNA preparations of *S. griseus* NRRL B-2682^T and *V. maris* AB-18-032 were used as the negative and positive control samples, respectively in the PCR screening reaction. The standard PCR mixture (25 µl) contained 1.5 U of Phusion DNA polymerase (Finnzymes, Espoo, Finland) in the recommended buffer; 2.5 mM $MgCl_2$, 0.6 µM of each of the forward and reverse primers, 0.2 µM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), and 1 to 2 µl of DNA template (*ca*. 0.2 µg of genomic bacterial DNA). The PCR was performed using a GeneAmp PCR system 9600 thermocycler (PE Applied Biosystems, Foster City, USA) under the following conditions: initial hotstart at 98˚C for 3 minutes; 30 cycles of 98˚C for 20 seconds, 60˚C for 30 seconds, and 72˚C for 45 seconds; with a final extension at 72˚C for 10 minutes. The presence of amplified PCR products was confirmed by using a 1.5% agarose gel, followed by UV visualization after ethidium bromide staining.

Chapter 3. Comparative study of antibiotic resistance profiles and taxonomy of representative soil actinomycetes

3.1 Abstract

Two hundred colonies of filamentous actinomycetes randomly selected from starch-casein agar plates seeded with soil suspensions from a hay meadow soil were assigned to colour-groups based on aerial spore mass, substrate mycelial and diffusible pigments. Forty-four representatives of the colour-groups were clustered and compared based on antibiotic resistance profile and 16S rRNA sequence data. On average, each isolate showed resistance to 19 out of 41 antibiotics tested. All of the isolates were resistant to amoxicillin, fosfomycin and trimethoprim and most to nalidixic acid, oleandomycin, sulfadiazine and sulfamethoxazole. Most of the isolates were closely related to type strains of *Streptomyces* species though some were closely related to authentic *Actinomadura, Dactylosporangium, Micromonospora* and *Streptosporangium* strains. Partial congruence found between corresponding groups based on the antibiotic resistance profile and 16S rRNA sequence data, was reflected by a relatively low Pearson correlation coefficient based on an overall comparison between the two datasets. In contrast, complete congruence was observed between 16S rRNA and *vanHAX* gene sequences of nine vancomycin-resistant isolates. This study provides compelling evidence that filamentous actinomycetes in soil are a rich reservoir of resistance determinants which may spread into broader microbial communities, and hence could promote resistance of pathogenic microorganisms to clinically significant antibiotics.

3.2 Introduction

Antibiotic resistance genes probably originated from soil bacteria, notably from antibiotic-producing filamentous actinomycetes (Benveniste & Davies, 1973; Pang *et al.*, 1994; Davies, 1997; Marshall *et al.*, 1998; Mindlin *et al.*, 2006; Woo *et al.*, 2006; Wright, 2010) hence soil may serve as a reservoir of known and novel antibiotic resistance genes (Riesenfeld *et al.*, 2004; D'Costa *et al.*, 2006; Dantas *et al.*, 2008; Allen *et al.*, 2010; Wright, 2010). This problem was addressed by D'Costa *et al*. (2006) who screened a large number of soil streptomycetes against 21 antibiotics, including natural

products, semi-synthetic derivatives of antibiotics, and completely synthetic molecules, and found that they showed a remarkably level of resistance against both established and recently introduced clinically significant antibiotics.

Actinomycetes are an integral part of the indigenous soil microflora (Goodfellow & Williams, 1983; Ul-Hassan & Wellington, 2009; Goodfellow *et al.*, 2010) and are well known as the primary source of commercially significant antibiotics (Demain, 1998; Strohl, 2004; Bérdy, 2005; Goodfellow & Fiedler, 2010). The search for new metabolites from actinomycetes has been bedevilled by the view that antibiotic production is strain specific and highly variable among species (Strohl, 2004) and by the proposition that phylogenetically distant strains can synthesise identical metabolites (Metsa-Ketela *et al.*, 2002; Muramatsu *et al.*, 2005; Blodgett *et al.*, 2010). However, improvements in actinomycete systematics have underpinned the previously held concept that taxonomic diversity can be used as a surrogate for chemical diversity, especially at the species level (Ward & Goodfellow, 2004; Jensen, 2010). The coupling of taxonomic and chemical diversity in actinomycetes underpins the importance of screening novel actinomycetes in drug discovering programmes (Bull *et al.*, 2000; Busti *et al.*, 2006; Goodfellow & Fiedler, 2010). A comparison of the full genomic sequences of the type strains of *Salinispora arenicola* and *Salinispora tropica* helps explain the ability of these species to synthesise core sets of species-specific secondary metabolites (Penn *et al.*, 2009; Jensen, 2010). These findings are in line with reports that secondary metabolite profiles of filamentous fungi are species-specific (Larsen *et al.*, 2005; Frisvad *et al.*, 2008).

Many actinomycetes synthesise more than one antibiotic and show resistance to multiple ones (Fujisawa & Weisblum, 1981; Phillips *et al.*, 1994; Hotta & Okami, 1996; Hotta, 1999; D'Costa *et al.*, 2006; Mindlin *et al.*, 2006). Antibiotic biosynthetic genes and corresponding resistance genes may be genetically related through coevolution (Pootoolal *et al.*, 2002; Cundliffe & Demain, 2010; Laskaris *et al.*, 2010) or horizontal gene transfer (Davies, 1997; Wiener *et al.*, 2002; Wright, 2010). This link between resistance profile and secondary metabolite production means that information derived from studies on antibiotic resistant patterns of representatives of filamentous actinomycete taxa can be used to design media for selective isolation of specific members from complex actinomycete communities in natural habitats (Yamashita *et al.*, 1985; Bibikova *et al.*, 1989; Phillips *et al.*, 1994; Hotta & Okami, 1996; Hotta, 1999;

Goodfellow, 2010). Antibiotic resistance data of specific actinomycete taxa have been used for taxonomic purposes (Goodfellow & Orchard, 1974; De Boer *et al.*, 1990; Kageyama *et al.*, 2004) and for formulating novel selective isolation media (Hayakawa, 2008; Goodfellow, 2010).

Vancomycin is one of the few effective drugs available to treat infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and enterococci (Bush, 2004). It inhibits cell wall biosynthesis by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid-attached peptidoglycan precursors on the outside of the cytoplasmic membrane (Courvalin, 2006). This interaction blocks the formation of mature peptidoglycan, principally by denying transpeptidase access to its substrate thereby preventing the formation of peptide crosslinks between polysaccharide strands which give cell walls their rigidity (Hong *et al.*, 2004). Resistance in clinically significant bacteria, such as vancomycin-resistant enterococci (VRE), is due to the biosynthesis of a peptidoglycan terminating in D-alanine-D-lactate (D-Ala-D-Lac) rather than D-alanine-D-alanine (D-Ala-D-Ala), a process which leads to a low binding affinity to vancomycin. This remodelling of cell wall precursors requires three enzymes: VanH, which converts pyruvate into D-lactate; VanA, a D-Ala-D-Lac ligase; and VanX, a D-Ala-D-Ala dipeptidase that cleaves any residual circulating D-Ala-D-Ala dipeptide thereby ensuring that peptidoglycan precursors terminate uniformly in D-Ala-D-Lac (Walsh *et al.*, 1996; Healy *et al.*, 2000). These enzymes are encoded by a cluster of three genes, *vanH-vanA-vanX*, which can be readily identified by PCR analysis in resistant bacteria (Marshall *et al.*, 1998).

The *vanHAX* gene cluster is conserved in significant pathogens (e.g. VRE) and in glycopeptide-producing actinomycetes (Marshall *et al.*, 1998; Pootoolal *et al.*, 2002) and can also be found in the genomes of environmental bacteria which do not produce glycopeptides, such as *Desulfitobacterium hafniense* Y51 (Kalan *et al.*, 2009), *Paenibacillus apiarius* PA-B2B (Guardabassi *et al.*, 2004; 2005) and "*Streptomyces coelicolor*" A3(2) (Hong *et al.*, 2004). D'Costa *et al*. (2006) found that the 1% of actinomycete isolates were resistant to vancomycin and contained the *vanHAX* gene cluster. It seems likely, therefore, that phylogenies based on *vanH-vanA-vanX* sequences may mirror those derived from 16S rRNA gene sequences in vancomycin-resistant filamentous actinomycetes.

The present study was designed to determine whether a relationship exists between taxonomic diversity and corresponding antibiotic resistance patterns of filamentous actinomycetes isolated from a hay meadow soil which had not been exposed to antibiotics or fertilisers (Shiel & Rimmer, 1984; Hopkins *et al.*, 2009). To this end, the antibiotic resistance patterns of 44 representative actinomycetes were compared with the position of these organisms in 16S rRNA actinomycetes gene trees. In addition, a comparison was made between phylogenies based on 16S rRNA gene and *VanHAX* gene cluster sequences of 9 vancomycin-resistant streptomycetes. The resultant genotypic and phenotypic data provide significant evidences that soil actinomycetes are a rich reservoir of resistance determinants and relatively low correlation exists between the resistance profiles and taxonomic position of the tested strains.

3.3 Materials and methods

Sampling site. Soil samples were collected from Palace Leas hay meadow plot 6 at Cockle Park Experimental Farm, Northumberland, UK (National Grid Reference NZ 200913) in September 2005. The samples (100 g) were taken from the top 5cm of soil in the centre of the plot, using a sterile spatula, and placed into plastic bags. The samples were sieved, air-dried in sterile petri-dishes at room temperature for 7 days and mixed to obtain a composite sample which was stored at 4˚C.

Physico-chemical properties of the composite soil sample. The bulk pH of the composite soil sample was determined following the procedure described by Reed and Cummings (1945). The sample was examined in triplicate and the final pH recorded as an average of the three readings. The triplicate samples (*ca*. 1g) were dried to constant weight at 105˚C and the moisture content recorded as the percentage loss of weight of the three readings. The dried samples were placed in a muffle furnace (Carbolite, Sheffield, England, UK) and the temperature raised slowly to 700˚C and kept constant for 30 minutes to burn off organic matter prior to cooling overnight and reweighing. The average weight loss percentage of the triplicate samples was recorded as the organic matter content.

Selective isolation and enumeration of presumptive actinomycetes. Two grams of the composite soil sample were resuspended in 18 ml of sterile ¼ strength Ringer's solution (Oxoid, UK) and blended in an Ultra-Turrax T25 homogeniser (Junke and

Kunkel, Staufen, Germany) for a minute. The resultant 10^{-1} suspension was shaken on a shaker incubator (Gallenkamp Orbital Incubator, Loughborough, UK) at 150 rpm at room temperature for 30 minutes, heat pre-treated at 55˚C for 20 minutes in a water bath, and cooled at room temperature. The soil suspension was serially diluted in ¼ strength Ringer's solution down to 10^{-5} and the triplicate aliquots (100 µl) of each dilustion spread over starch-casein agar plates (Küster & Williams, 1964), supplemented with cycloheximide and nystatin (each at 25 µg/ml), which had been dried for 15 minutes prior to inoculation, as recommended by Vickers and Williams (1987). The inoculated plates were incubated at 28˚C for 21 days when organisms putatively identified as filamentous actinomycetes were distinguished by their characteristic colonial morphology, notably by their ability to form leathery colonies which usually carried abundant aerial hyphae. The numbers of presumptive actinomycetes and bacterial colonies were counted and the results expressed as the number of colony forming units (cfu) per gram dry weight of soil.

Selection, maintenance and colour-grouping. Two hundred colonies of filamentous actinomycetes were randomly selected from the starch-casein isolation plates and subcultured onto oatmeal agar (Shirling & Gottlieb, 1966) plates which were incubated at 28˚C for 21 days. The resultant plates were examined by eye to determine aerial spore mass colour, substrate mycelial pigmentation and the colours of any diffusible pigments using National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1958). The isolates were assigned to multi-membered and single-membered colour-groups based on pigments produced on oatmeal agar (Table 3.1), and were maintained on oatmeal agar slopes and as suspensions of spores and hyphal fragments in glycerol (20%, v/v) at -20˚C.

Antibiotic resistance profiling. Forty four isolates representing the multimembered and some of the single-membered colour-groups were screened against 41 antibiotics which were purchased from Sigma-Aldrich, apart from ciprofloxacin and polymixin B, which were bought from Fluka Biochemika, and nalidixic acid which was obtained from Duchefa Biochemie (Table 3.2). Each antibiotic was sterilised by filtration and the working concentration adjusted to 20 µg/ml. Spores of each of the isolates were scraped from oatmeal agar plates and suspended in ¼ strength Ringer's solution after incubation at 28˚C for 21 days. The resultant spore suspensions were filtered through sterile cotton wool, centrifuged at 3000 rpm for 15 minutes and resuspended in ¼ strength Ringer's

solutions to give a turbidity equivalent to McFarland no. 5 standard (BioMerieux). The inocula (1 ml) were pipetted into sterile compartments of Replidishes (Bibby-Sterlin Ltd; Stone, UK) and gently agitated to give an even suspension prior to inoculation. An automatic multipoint inoculator (Denley-Tech; Denley Instruments Ltd., Sussex, UK) was used to inoculate Replidishes containing modified Bennett's agar (Jones, 1949) supplemented with the appropriate antibiotics; control plates lacking antibiotics were also inoculated and incubated at 28˚C for 14 days; all of the tests were carried out in duplicate. Strains growing on modified Bennett's agar supplemented with the antibiotics were scored as resistant (1) and those failing to grow as susceptible (0). The resultant data were examined using the simple matching coefficient (S_{SM} ; Sokal & Michener, 1958), which includes both positive and negative similarities, and clustering achieved using the unweighted pair group method with arithmetic averages (UPGMA) algorithm (Sneath & Sokal, 1973), using NTSYS software (Rohlf, 1998), and the results presented in a dendrogram.

Phylogenetic analyses of 16S rRNA gene sequences. The phylogenetic positions of the 44 representative isolates were determined following 16S rRNA gene sequence analyses. Extraction of genomic DNA, PCR amplification and direct sequencing of the purified PCR products were carried out as described by Antony-Babu *et al*. (2008). The resultant, almost complete, 16S rRNA gene sequences of the isolates were examined using the EzTaxon server (http://www.EzTaxon.org) (Chun *et al.*, 2007) to determine their nearest validly described taxonomic neighbour. Phylogenetic trees of isolates provisionally assigned to specific genera were inferred using the UPGMA tree-making algorithm taken from the MEGA 3.0 program (Kumar *et al.*, 2004); the evolutionary distance model of Jukes & Cantor (1969) was used to generate evolutionary distance matrices (Sneath & Sokal, 1973).

Comparison of antibiotic resistance and 16S rRNA similarity data. The distance values acquired from the antibiotic resistance profiles and corresponding 16S rRNA gene sequences were converted to percentage similarity values using PHYDIT (Chun, 1995) and EXCEL (Microsoft Co. USA) software. The overall correlation between the resultant datasets was determined using the SAS program (version 9.2, SAS institute, USA) and the correlation factor calculated using the Pearson correlation coefficient (Rodgers & Nicewander, 1988). The resultant correlation values were presented as a plot using the EXCEL program.

Comparison of *vanHAX* gene cluster and 16S rRNA gene sequences. Nine out of the 200 strains (isolates BK14-BK22) that are resistant to vancomycin were selected in order to compare phylogenies based on their 16S rRNA gene and *vanH-vanA-vanX* gene sequences. PCR amplification of the genes in the *vanHAX* gene cluster were performed, as described by D'Costa *et al*. (2006), and specific PCR primers (forward primer, 5'-TCGG(A/G)GTG(A/G)TCGG(G/A)AC(G/A)GG-3'; reverse primer, 5'-CA(C/G/A)AG(G/C/A)AGCAG(C/G/A)CCGAA(G/C)CC-3') used to amplify the genes in the *vanHAX* gene cluster. Purified PCR products were cloned into a pCR2.1 TOPO cloning vector (Invitrogen, UK) and transformed into *Escherichia coli* TOP electrocompetent cells using a TOPO Cloining kit (Invitrogen, UK). Plasmid DNA from colonies with the correct insert was extracted using a Miniprep Plasmid DNA extraction kit (QIAGEN, UK), and extracted constructs confirmed by digestion of plasmid DNA with *Eco*R1 and sequenced. 16S rRNA gene sequence analyses of nine isolates were carried out as described by Antony-Babu *et al*. (2008).

Phylogenetic trees based on the *vanHAX* gene cluster sequences (*ca*. 1780 nucleotides) and on the corresponding 16S rRNA gene sequences (*ca*. 1450 nucletides) were generated by using the neighbour-joining algorithm (Saitou & Nei, 1987); the Jukes & Cantor (1969) model was used to calculate evolutionary distance matrices. The topologies of the resultant phylogenetic trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset.

3.4 Results

Composite soil sample: actinomycete and bacterial counts and physico-chemical properties. The physico-chemical properties of the composite soil sample were 5.4 ± 0.2 for pH, 4.0 ± 0.1 (%) for moisture content and 9.8 ± 0.3 (%) for organic matter content. The numbers of presumptive actinomycetes and total bacterial counts were 1.4 $\times$ 10^6 and 5.3 $\times$ 10^6 cfu/ gram dry weight composite soil, respectively.

Antibiotic resistance profiles of dereplicated isolates. The 200 randomly chosen actinomycetes were assigned to 17 major (5-37 isolates), 4 minor (2-4 isolates) and 5 single-membered colour-groups (Table 3.1). The percentages of the 44 dereplicated isolates resistant to each of the 41 antibiotics at a concentration of 20 μg/ml are shown

in Table 3.2. Identical antibiotic resistance profiles were obtained with the duplicated isolates. On average, each isolate showed resistance to 19 out of 41 antibiotics tested. All of the isolates were resistant to amoxicillin, fosfomycin and trimethoprim and sensitive to apramycin and minocycline. In addition, 80% or more were resistant to nalidixic acid, oleandomycin, oxacillin, sulfadiazine and sulfomethoxazole, and over 90% sensitive to amikacin, doxycycline, gentamicin, kanamycin, neomycin, paromomycin, tobramycin and vancomycin. The antibiotic resistance patterns of individual isolates are shown in Figure 3.1. Nearly 40% of the isolates were resistant to between 21 and 25 of the antibiotics. The isolate showing the most pronounced resistance, strain BK21, was resistant to 32 antibiotics. In contrast, isolate BK66 was resistant to only 4 of the antibiotics. It can be seen from Figure 3.2(a) that the isolates were assigned to 12 groups at a cut-off value of 70% in the S_{SM}, UPGMA analysis.

Table 3.1. Assignment of filamentous actinomycetes to multi- and single-membered colour-groups

Colour-group	Aerial spore mass colour	Reverse colour	Colour of diffusible pigment	Number of strains	Strains[a] (BK-)
Multimembered groups					
1	Dark gray	Black	Brown	49	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, **13**, 14, 15, 16, 17, 18, 19, 20, **21**, 22, 23, 24, 25, 26, 27, **28**, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, **49**
2	Gray	Gray	Brown	13	89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, **101**
3	Dark brown	Black	Light brown	12	102, 103, 104, 105, 106, 107, **108**, **109**, 110, 111, 112, 113
4	Grayish brown	Brown	Light brown	8	**160**, **161**, 162, 163, 164, 165, **166**, 167
5	Gray	Brown	None	5	186, 187, 188, **189**, **190**
6	White	Dark brown	Brown	5	114, **115**, 116, 117, 118
7	White	Brown	None	4	153, 154, 155, **178**

Table 3.1. (*Continued*)

Colour-group	Aerial spore mass colour	Reverse colour	Colour of diffusible pigment	Number of strains	Strains[a] (BK-)
8	White	Purple	Brown	4	156, **157**, 158, 159
9	Light green	Brown	Brown	5	**119,** 123, 124, **125**, 126
10	Light yellow	Light brown	Light brown	6	127, **128**, **129**, 130, 131, 132,
11	Yellow	Light brown	Light purple	6	133, **134**, 135, 136, 137, **138**
12	Light gray	Light yellow	Light green	3	**120**, 121, **122**
13	Light gray	Light yellow	Yellowish brown	8	**168**, 169, 171, 172, 173, 174, 175, 176
14	Light gray	Gray	Light yellow	5	**192**, 193, 194, 195, 196
15	Light gray	Yellow	Yellow	8	79, 82, 80, 81, 83, **180**, 181, 182
16	Light gray	Brown	Brown	4	183, 184, **185**, 191
17	Light gray	Greyish-yellow	Light green	6	84, 85, 86, 88, 87, **179**
18	White	Light yellow	None	6	**139**, 140, 141, 142, **143**, 144
19	White	Light yellow	Light yellow	8	145, 146, **147**, **148**, 149, 150, 151, 152
20	Light orange	Orange	None	9	50, **51**, 52, **53**, 54, 55, 56, **57**, **63**
21	Dark orange	Orange	Yellow	21	**58**, 60, 59, **61**, **62**, 64, **65**, **66**, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 78, 77, **177**
Single-membered groups					
22	White	Black	Yellow	1	**170**
23	White	Dark orange	Orange	1	197
24	Light yellow	Orange	Yellow	1	198
25	Light yellow	Yellow	None	1	**199**
26	Gray	Light brown	None	1	200

[a] The code numbers of the representative strains included in the 16S rRNA sequencing and antibiotic resistance profiling studies are given in bold and underlined.

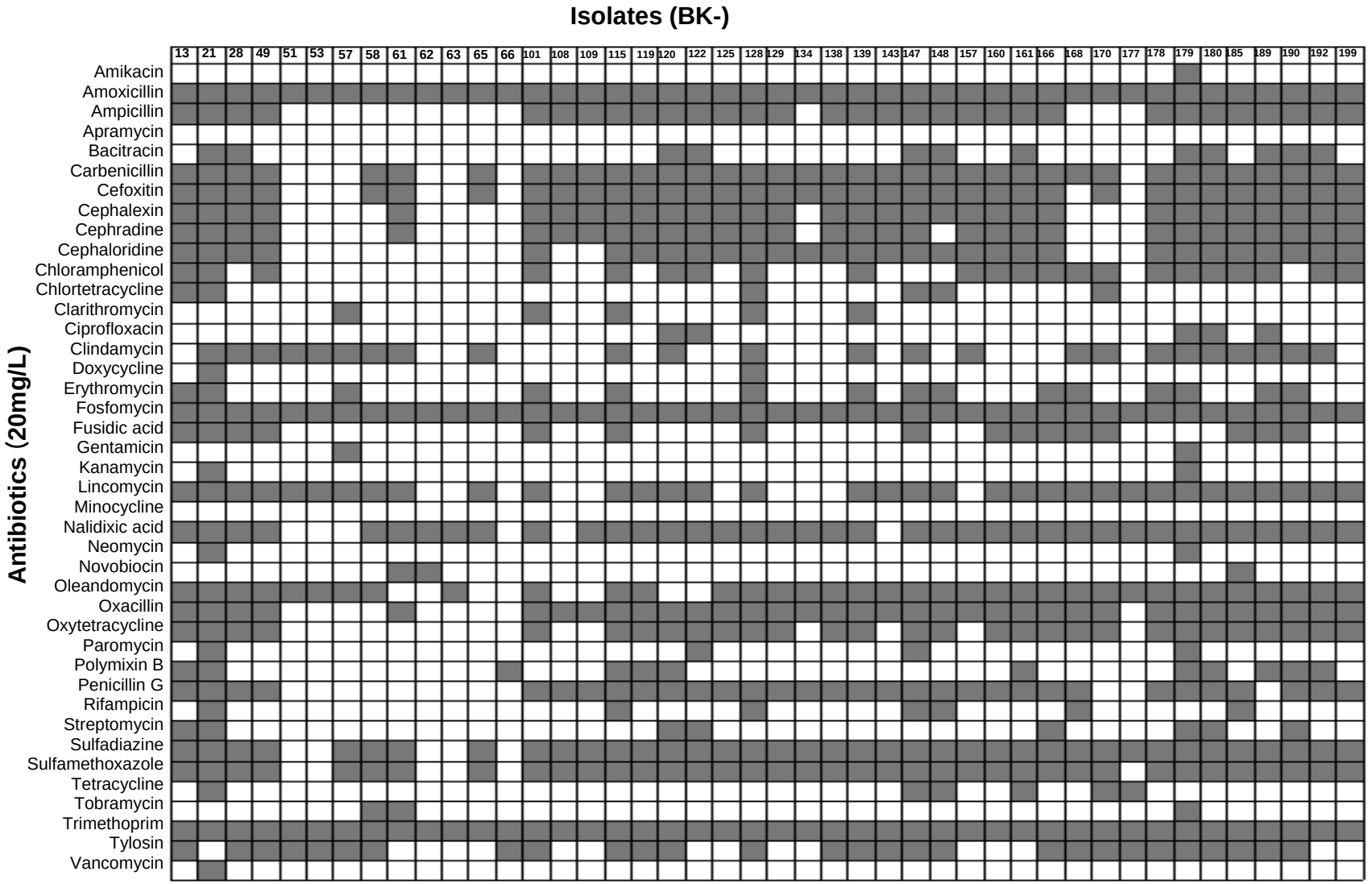


Fig. 3.1. Heat map showing the antibiotic-resistance profiles of the 44 representative filamentous actinomycetes (■, Resistant; □, Susceptible)

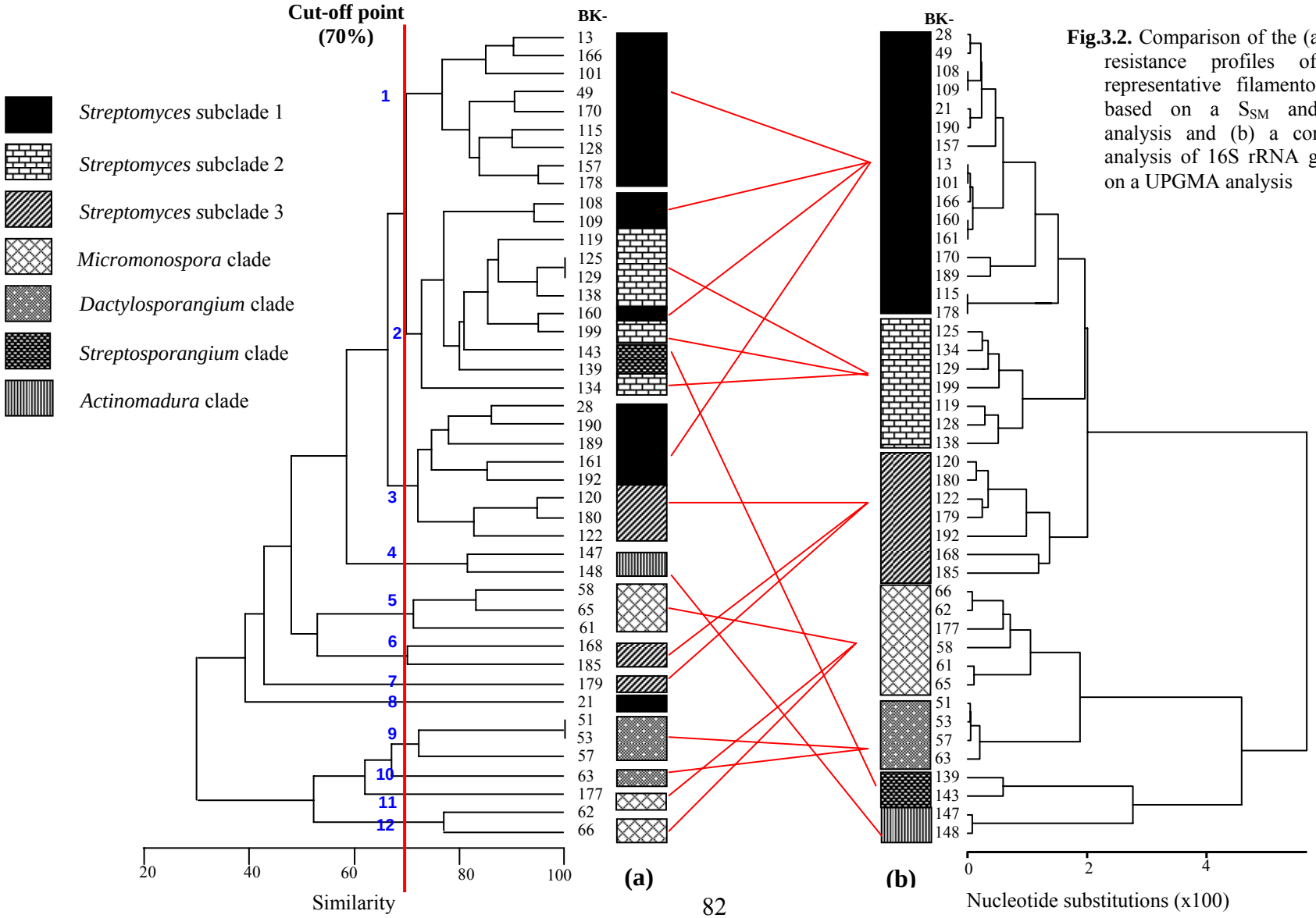


Fig.3.2. Comparison of the (a) antibiotic resistance profiles of the 44 representative filamentous isolates based on a S_{SM} and UPGMA analysis and (b) a corresponding analysis of 16S rRNA genes based on a UPGMA analysis

Table 3.2. Percentage of the representative 44 isolates resistant to the antibiotics

Antibiotics	Percentage of resistance (%)	Antibiotics	Percentage of resistance (%)
Aminoglycosides **:**		***Macrolides*** **:**	
Amikacin	2	Clarithromycin	11
Apramycin	0	Erythromycin	34
Gentamicin	5	Oleandomycin	82
Kanamycin	5	Tylosin tartrate	64
Neomycin	5	***Quinolone*** **:**	
Paromomycin	9	Ciprofloxacin	11
Streptomycin	18	Nalidixic acid	86
Tobramycin	7	***Sulfonamides*** **:**	
Beta-lactams **:**		Sulfadiazine	89
Amoxicillin	100	Sulfamethoxazole	86
Ampicillin	71	***Tetracylines*** **:**	
Carbenicillin	84	Chlortetracycline	14
Cefoxitin	82	Doxycycline	5
Cephalexin	73	Minocycline	0
Cephradine	71	Oxytetracycline	66
Cephaloridine	68	Tetracycline	14
Oxacillin	80	***Others*** **:**	
Penicillin G	73	Chloramphenicol	50
Cyclic polypeptides **:**		Novobiocin	7
Bacitracin	27	Rifampicin	16
Polymyxin B	27	Fosfomycin	100
Lincosamides**:**		Fusidic acid	36
Clindamycin	51	Trimethoprim	100
Lincomycin	77	Vancomycin	2

Phylogenetic analyses. Almost complete 16S rRNA gene sequences were obtained for the 44 representative filamentous actinomycetes. The percentage similarities and number of nucleotide differences found between each isolate and its nearest phylogenetic neighbour are shown in Table 3.3. Most of the isolates shared their highest 16S rRNA similarity with type strains of *Streptomyces* species, the remainder with validly described *Actinomadura, Dactylosporangium*, *Micromonospora* and *Streptosporangium* species. It can be seen from Figure 3.2(b) that the isolates can be assigned to five 16S rRNA gene clades which correspond to the genera *Actinomadura*, *Dactylosporangium*, *Micromonospora*, *Streptomyces* and *Streptosporangium*.

Isolates BK147 and BK148 were most closely related to the type strains of *A. bangladeshensis* (98.5% similarity, 22 nt difference at 1431 locations), and isolates BK51, BK53, BK57 and BK63 to *D. aurantiacum* DSM 43157^T, sharing similarities with the latter within the range 98.7 to 99.2 %, values equivalent to 12 to 18 nt difference at 1434 locations. Similarly, isolates BK62, BK66 and BK177 were most closely related to the type strain of *M. matsumotoense* showing similarities with this organism within the range 98.8 to 99.7, values corresponding to between 5 and 17 nt differences at 1443 locations. In contrast, isolates BK58, BK61 and BK65 were most closely related to the type strain of *M. saelicesensis* (99.7% similarity, 4 nt differences at 1433 locations), *M. echinaurantiaca* (99.1% similarity, 13 nt differences at 1437 locations) and *M. chaiyaphumensis* (99.3% similarity, 10 nt differences at 1422 locations), respectively. Isolate BK143 had an identical 16S rRNA gene sequence with *S. album* DSM 43023^T; the corresponding score between isolate BK143 and this strain was 98.8 %, a value equivalent to 17 nt differences at 1423 locations. All of these isolates were recovered in clades corresponding to these generic assignments (Fig. 3.2b).

The 30 *Streptomyces* isolates shared 16S rRNA gene similarities of 98.4–100% with their closest phylogenetic neighbours (Table 3.3). These organisms can be assigned to three subclades (Fig. 3.2b). Six of the subclade 1 isolates were most closely related to *S. sanglieri* NBRC 100784^T (99.5-100% similarity) and 5 isolates to *S. drozdowiczii* NBRC 101007^T (99.4-99.5% similarity). Isolate BK21 shared an identical 16S rRNA gene sequence with the type strain of *S. sanglieri*. In addition, isolates BK115 and BK 178 have identical 16S rRNA gene sequences; they were most closely related to *S. gelaticus* NRRL B-2928^T, sharing a 98.9% similarity with the latter (16 nt differences at 1435 sites).

Table 3.3 Nearest neighbours of the 44 representative filamentous actinomycetes

Isolate number (BK -)	Nearest type strain	Similarity (%)	Number of nucleotide differences	16S rRNA sequence accession number
	Genus *Actinomadura*			
147	*A. bangladeshensis* 3-46-b(3)^T	98.5	22/1431	FR692101
148	*A. bangladeshensis* 3-46-b(3)^T	98.5	22/1431	FR692102
	Genus *Dactylosporangium*			
51	*D. aurantiacum* DSM 43157^T	99.1	13/1434	FJ973604
53	*D. aurantiacum* DSM 43157^T	99.0	14/1434	FN662848
57	*D. aurantiacum* DSM 43157^T	99.2	12/1434	FN662851
63	*D. aurantiacum* DSM 43157^T	98.7	18/1433	FJ973605
	Genus *Micromonospora*			
65	*M. chaiyaphumensis* MC5-1^T	99.3	10/1422	FR692085
61	*M. echinaurantiaca* DSM 43904^T	99.1	13/1437	FR692083
62	*M. matsumotoense* IMSNU 22003^T	98.8	17/1443	FR692084
66	*M. matsumotoense* IMSNU 22003^T	99.0	15/1443	FR692086
177	*M. matsumotoense* IMSNU 22003^T	99.7	5/1443	FR692109
58	*M. saelicesensis* Lupac 09^T	99.7	4/1433	FR692082
	Genus *Streptomyces*			
157	*S. badius* NRRL B-2567^T	99.7	4/1442	FR692103
129	*S. bambergiensis* NBRC 13479^T	98.8	17/1430	FR692096
134	*S. bambergiensis* NBRC 13479^T	98.6	20/1429	FR692097
199	*S. bambergiensis* NBRC 13479^T	99.2	11/1430	FR692117
192	*S. catenulae* ISP 5258^T	98.4	23/1446	FR692116
13	*S. drozdowiczii* NBRC 101007^T	99.5	7/1434	FR692070
101	*S. drozdowiczii* NBRC 101007^T	99.5	7/1434	FR692087

Table 3.3 (*Continued*)

Isolate number (BK -)	Nearest type strain	Similarity (%)	Number of nucleotide difference	16S rRNA sequence accession number
160	*S. drozdowiczii* NBRC 101007^T	99.4	9/1434	FR692104
161	*S. drozdowiczii* NBRC 101007^T	99.4	9/1434	FR692105
166	*S. drozdowiczii* NBRC 101007^T	99.4	8/1434	FR692106
178	*S. gelaticus* NRRL B-2928^T	98.9	16/1435	FR692110
115	*S. gelaticus* NRRL B-2928^T	98.9	16/1435	FR692090
119	*S. hirsutus* NBRC 12786^T	98.6	20/1437	FR692091
128	*S. hirsutus* NBRC 12786^T	98.4	23/1435	FR692095
138	*S. hirsutus* NBRC 12786^T	98.7	21/1435	FR692098
122	*S. lydicus* NBRC 13058^T	99.8	3/1435	FR692093
179	*S. lydicus* NBRC 13058^T	99.2	11/1435	FR692111
170	*S. mirabilis* NBRC 13450^T	99.7	4/1433	FR692108
120	*S. nigrescens* NBRC 12894^T	99.6	7/1439	FR692092
168	*S. paucisporeus* NBRC 102072^T	98.8	17/1402	FR692107
125	*S. prasinus* NBRC 12810^T	98.8	18/1430	FR692094
189	*S. olivochromogenes* NBRC 3178^T	99.9	2/1432	FR692114
21	*S. sanglieri* NBRC 100784^T	100	0/1434	FR692078
28	*S. sanglieri* NBRC 100784^T	99.6	6/1434	FR692080
49	*S. sanglieri* NBRC 100784^T	99.5	7/1434	FR692081
108	*S. sanglieri* NBRC 100784^T	99.6	6/1434	FR692088
109	*S. sanglieri* NBRC 100784^T	99.6	6/1434	FR692089
180	*S. sioyaensis* NRRL B-5408^T	99.5	7/1438	FR692112
185	*S. sporoclivatus* NBRC 100767^T	99.9	2/1435	FR692113
190	*S. sanglieri* NBRC 100784^T	99.9	1/1434	FR692115
	Genus *Streptosporangium*			
139	*S. album* DSM 43023^T	100	0/1424	FR692099
143	*S. album* DSM 43023^T	98.8	17/1423	FR692100

The remaining subclade 1 strains, isolates BK157, BK170 and BK189, were most closely related to the type strains of *S. badius* (99.1% similarity), *S. mirabilis* (99.7% similarity) and *S. olivochromogenes* (99.9%), respectively. The seven subclade 2 strains, isolates BK119, BK125, BK138, BK129, BK134, BK138 and BK199, were most closely related to the type strains of *S. hirsutus* (98.6% similarity), *S. prasinus* (98.8% similarity), *S. hirsutus* (98.7% similarity), *S. bambergiensis* (98.8% similarity), *S. bambergiensis* (98.6% similarity), *S. hirsutus* (98.7% similarity) and *S. bambergiensis* (99.2% similarity), respectively. Two of the subclade 3 strains, isolates BK122 and BK179, shared their highest 16S rRNA gene similarity to *Streptomyces lydicus* NBRC 13058^{T} (99.8 and 99.2%, respectively). The remaining strains in this subclade, isolates BK120, BK168, BK180 and BK185, were most closely related to the type strains of *S. nigrescens* (99.6% similarity) *S. paucisporeus* (98.8% similarity), *S. sioyaensis* (99.5% similarity) and *S. sporoclivatus* (99.9% similarity), respectively.

Comparison of antibiotic resistance and 16S rRNA gene sequence data. Some of the isolates were assigned to corresponding groups in the analyses based on the antibiotic resistance profiles and 16S rRNA gene sequence data, notably the isolates which were most closely related to *bona fide* members of the genera *Actinomadura, Dactylosporangium,* and *Streptosporangium* (Fig. 3.2). In contrast, the isolates closely related to the *Micromonospora* strains were assigned to two multimembered and one single-membered cluster based on their antibiotic resistance profiles; one of the multimembered clusters was composed of isolates BK62 and BK66, which are phylogenetically close to *M. matsumotoense* IMSNU 22003^{T}. It is also interesting that the putative *Actinomadura, Dactylosporangium, Micromonospora* and *Streptosporangium* isolates belonged to colour-groups 19, 20, 21 and 18, respectively.

All but 4 of the 30 isolates found to be closely related to *Streptomyces* type strains were recovered in three aggregate groups defined at the 70% similarity level in the dendrogram based on the antibiotic resistance data (Fig. 3.2a). The most pronounced congruence was found between isolates which shared high 16S rRNA similarity values, as exemplified by isolates BK13, BK101 and BK166, isolates BK108 and BK109, isolates BK125 and BK129, isolates BK120 and BK180, and isolates BK168 and BK185. There was little evidence of a correlation between the membership of colour-groups and taxa defined in the 16S rRNA gene sequence and antibiotic-resistance

pattern analyses though isolates BK108 and BK109 clustered together in all three analyses.

The partial congruence found between corresponding groups in the dendrograms (Fig. 3.3) was reflected in the relatively low value of Pearson correlation coefficient, 0.543 ($p=0.001 < 0.01$) in the overall comparison between the two sets of data presented as similarity values. It can be seen from Figure 3.3 that pairs of isolates with 16S rRNA gene similarities between 96 and 100% showed relatively high resistance profile similarities of between 50 and 100%. In contrast, pairs of isolates with 16S rRNA gene similarities between 88 and 92% showed a wide-range resistance profile similarities, namely from 25 to 92% .

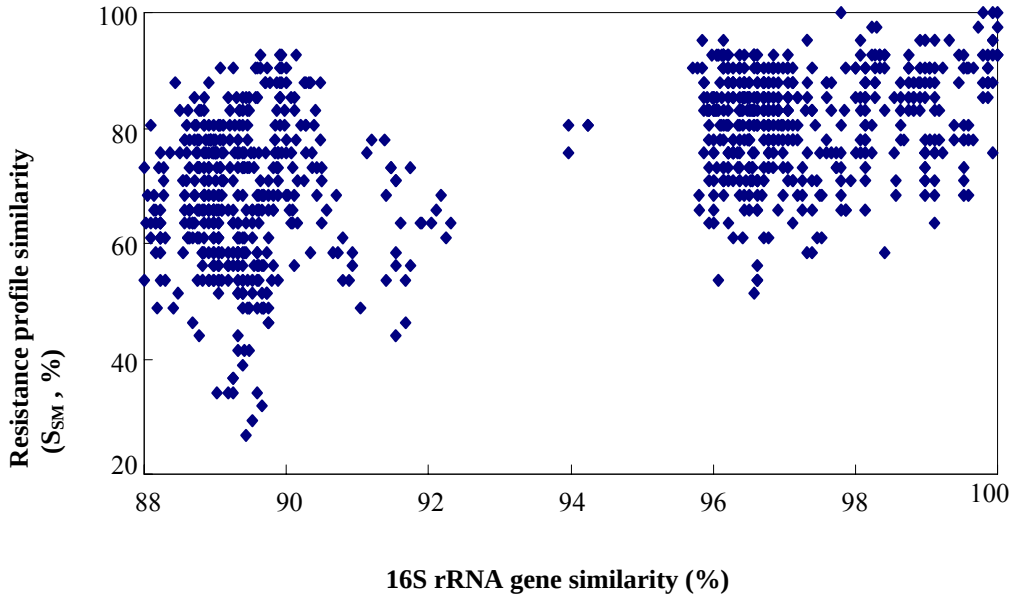


Fig. 3.3. Correlation of 16S rRNA gene similarities and antibiotic resistance profiles of the 44 isolates.

Comparison of phylogenies based on *vanHAX* gene cluster and 16S rRNA gene sequences. All 9 isolates which were resistant to vancomycin shared identical 16S rRNA gene sequences (*ca.* 1434 bp) with one another and with the type strain of *S. sanglieri*. The sequences of the genes comprising the *vanHAX* gene cluster (*ca*. 1780 bp) were also identical. It can be seen from Figure 3.4 that the vancomycin-resistant strains formed a distinct branch in each of the phylogenetic trees.

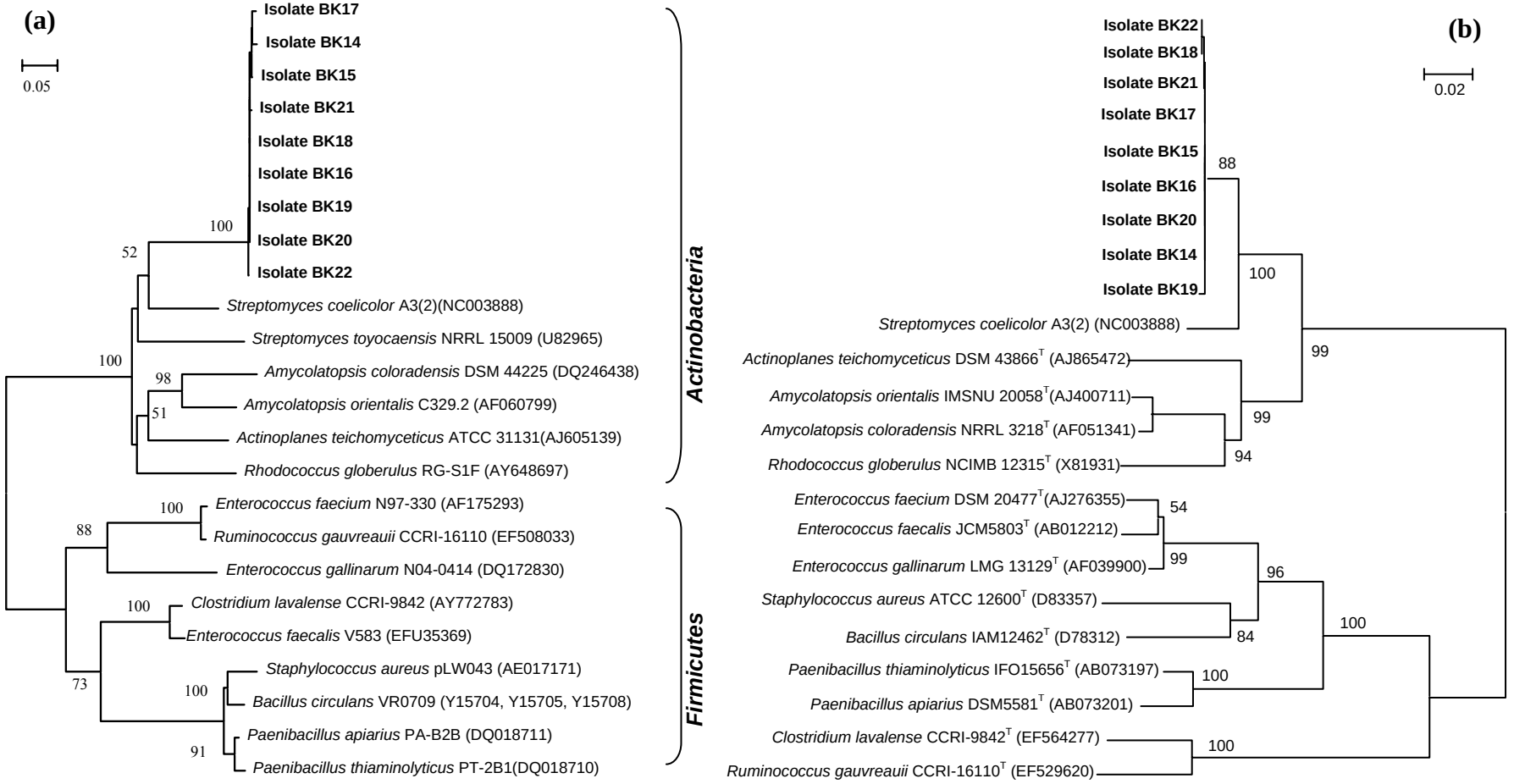


Fig. 3.4. Position of the vancomycin-resistant isolates in phylogenetic trees based on (a) *VanHAX* gene cluster and (b) 16S rRNA gene sequence data

3.6 Discussion

The present study confirms and extends the pioneering work of D'Costa *et al.* (2006) who found that large numbers of streptomycetes isolated from several soils grew in the presence of one or more of a panel of 21 antibiotics. On average, the organisms were resistant to 7 or 8 of the antibiotics. In this investigation, 44 out of 200 dereplicated actinomycetes, notably streptomycetes, were resistant to 18 or 19 antibiotics which included representatives of 41 structural types. Isolates BK21, which had a 16S rRNA gene sequence identical to that of the type strain of *S. sanglieri*, was resistant to 32 of the antibiotics. All of the isolates were resistant to amoxicillin, fosfomycin and trimethoprim and a further 35 isolates to nalidixic acid, oleandomycin, oxacillin, sulfadizine and sulfamethoxazole. The isolates were more resistant to antibiotics, which targeted the folic acid pathway, such as sulphonamides and trimethoprim (86~100%), and cell wall biosynthesis, such as β-lactam antibiotics (68~100%). In contrast, less resistance was shown to aminoglycosides (0~18%) and tetracyclines (0~14%) apart from oxytetracyline (66%). These data provide further compelling evidence that soil actinomycetes are a rich reservoir of resistance determinants which may be spread into broader microbial communities, and hence could promote antibiotic resistance amongst pathogenic bacteria (Riesenfeld *et al.*, 2004; D'Costa *et al.*, 2006; Dantas *et al.*, 2008; Laskaris *et al.*, 2010; Wright, 2010).

All of the dereplicated filamentous actinomycetes were isolated from a hay meadow soil which had not be exposed to either antibiotics or fertilizers (Shiel & Rimmer, 1984; Hopkins *et al.*, 2009) This result provide further evidence that substantial populations of antibiotic-resistant bacteria are present in such habitats even though direct evidence for the production of antibiotics in soil is limited (Anukool *et al.*, 2004). However, there is convincing evidence for the coexistence and coevolution of antibiotic resistance and biosynthetic genes in soil streptomycetes (Laskaris *et al.*, 2010).

The representative set of isolates for the antibiotic resistance studies were selected from the colour-groups as it is known that streptomycete group based on aerial spore mass, substrate mycelial and diffusible pigment colours are predictive; strains taken to represent such groups key out to novel and established species (Goodfellow & Haynes, 1984; Williams & Vickers, 1988; Atalan *et al.*, 2000; Antony-Babu *et al.*, 2008) or to groups of closely related species (Sembiring *et al.*, 2000; Goodfellow *et al.*, 2007). It is

encouraging that the isolates found to be closely related to established members of the genera *Actinomadura*, *Dactylosporangium*, *Micromonospora* and *Streptosporangium* were recovered in corresponding colour-groups. In contrast, isolates found to be closely related to the type strains of *S. drozdowiczii* and *S. sanglieri* belonged to more than one colour-group, a result which can be attributed to the fact these organisms produce gray to grayish brown, and brown to light brown aerial spores and diffusible pigments, respectively on oatmeal agar. This deduction needs to be tested using the computer-based system introduced by Antony-Babu *et al*. (2010), which highlights relationship between colour-groups and corresponding *rep*-PCR data of alkaliphilic streptomycetes.

It is not clear from the present study that the extent to which a relationship exists between the antibiotic resistance profiles and the taxonomic positions of filamentous actinomycetes. However, the relatively low correlation coefficient (0.543) found between the corresponding datasets may be more apparent than real due to the small number and taxonomic diversity of dereplicated filamentous actinomycetes which featured in this study. This interpretation is supported by the observations that greater correlation was found between the corresponding datasets for isolates found to share high 16S rRNA gene similarities. Indeed, this interpretation is strongly endowed given the complete congruence found between the phylogenetic trees based on the analyses of the 16S rRNA gene and *vanH-vanA-vanX* gene sequences of the vancomycin-resistant strains identified as *Streptomyces sanglieri*.

Chapter 4. Detection, selective isolation and characterisation of *Dactylosporangium* strains from diverse environmental samples

4.1 Abastract

A culture-independent, nested PCR procedure based on genus-specific oligonucleotide primers detected the presence of members of the genus *Dactylosporangium* in 14 out of 21 diverse environmental samples. Clones generated from the 14 positive environmental samples formed distinct phyletic lines in the *Dactylosporangium* 16S rRNA gene tree. *Dactylosporangium* strains isolated from 7 of these samples using a medium designated to be selective for members of the genus *Dactylosporangium*, namely *Streptomyces* Isolation Medium supplemented with gentamicin and antifungal antibiotics. One hundred and two out of 219 representative presumptive dactylosporangiae were considered as authentic members of the genus *Dactylosporangium* as they gave PCR amplification products using the genus-specific primers and had chemical features typical of dactylosporangiae. Representatives of the *Dactylosporangium* isolates formed distinctive phyletic lines in the 16S rRNA dactylosporangial gene tree, contained the non-ribosomal peptide or type-I polyketide synthase genes and inhibited the growth of *Bacillus subtilis*, *Kocuria rhizophila* or *Staphylococcus aureus* strains. It is evident from these results that the genus *Dactylosporangium* is underspeciated, widely distributed in natural habitats and is a potentially rich source of novel secondary metabolites.

4.2 Introduction

There is an urgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogens (Payne *et al.*, 2007; Spellberg *et al.*, 2008; Fischbach & Walsh, 2009) and to treat life-threatening diseases such as cancer (Olano *et al.*, 2009). Amongst prokaryotes, filamentous actinomycetes, notably streptomycetes, remain a unique source of new antibiotics (Bérdy, 2005; Newman & Cragg, 2007; Goodfellow & Fiedler, 2010) and this is likely to continue as full genome sequences of model actinomycete strains contain over 20 natural product biosynthetic gene clusters for the production of known or predicted secondary metabolites, as exemplified by

studies on *Amycolatopsis mediterranei* U32 (Zhao *et al.*, 2010), *Saccharopolyspora erythraea* NRRL 23338 (Oliynyk *et al.*, 2007), *Salinispora tropica* CNB 440 (Udwary *et al.*, 2007), *Streptomyces avermitilis* (Ikeda *et al.*, 2003) and "*Streptomyces coelicolor*" A3(2) (Bentley *et al.*, 2002). In contrast, few, if any, such gene clusters have been detected in whole genomes of other prokaryotes (Goodfellow & Fiedler, 2010).

It is becoming increasingly difficult to discover new antibiotics from common actinomycetes as screening such organisms tends to lead to the costly rediscovery of known secondary metabolites (Busti *et al.*, 2006; Lam, 2007). This problem can be addressed by using standard procedures for the selective isolation of novel actinomycetes from poorly studied habitats (Bredholdt *et al.*, 2007; Hong *et al.*, 2009; Okoro *et al.*, 2009), by applying novel procedures for the selective isolation and characterisation of uncommon and rare actinomycetes (Suzuki *et al.*, 2001a, b; Tan *et al.*, 2006; Gontang *et al.*, 2007) and by detecting the presence of novel, rare and target actinomycetes in environmental samples using culture-independent methods prior to the application of selective isolation procedures (Stach *et al.*, 2003; Maldonado *et al.*, 2005). These strategies have lead to the isolation of novel actinomycetes with the capacity to produce diverse new bioactive compounds (Fiedler *et al.*, 2005; Jensen *et al.*, 2005, 2007; Goodfellow & Fiedler, 2010),

Effective taxon-specific strategies are required for the selective isolation of neglected filamentous actinomycetes, not least for the isolation of members of the genus *Dactylosporangium* which encompasses strains known to produce anti-allergic drugs (Tani *et al.*, 2004), antibiotics, such as dactimicin (Shomura *et al.*, 1980) and tiacumicins (Theriault *et al.*, 1987), and a plant growth inhibitor (Kizuka *et al.*, 2002). The genus *Dactylosporangium* encompasses aerobic, filamentous actinomycetes which form motile spores in spore vesicles borne on short sporangiophores on substrate hyphae and have hydroxy and/or *meso*- diaminopimelic acids in whole-organism hydrolysates, but lack mycolic acids (Vobis, 1989; Kim *et al.*, 2010). Dactylosporangiae have been isolated from a range of terrestrial habitats (Hayakawa *et al.*, 1991a; Hayakawa, 2008) but have rarely featured in pharmacological screening programmes as they are difficult to isolate in large numbers and grow slowly.

The present study was designed to establish the extent of dactylosporangial taxonomic diversity in diverse environmental samples using a culture-independent

procedure prior to developing a reliable strategy for the selective isolation and characterisation of dactylosporangiae known to be present in environmental samples. In addition, representative cultivable isolates were examined to determine their phylogenetic diversity and whether they contained non-ribosomal peptide and type-I polyketide synthase genes, and to evaluate their ability to inhibit the growth of a panel of pathogenic microorganisms.

4.3 Materials and methods

Sources and physico-chemical properties of environmental samples. Twenty-one environmental samples from diverse geographical locations (Table 4.1) were sieved, air-dried at room temperature for 5-7 days and stored at 4˚C in plastic containers, apart from the marine sediment samples which were not dried. The pH of the samples was determined using the procedure described by Reed and Cummings (1945). Two grams of each sample were added to 18 ml of deionised water, mixed thoroughly, and left for an hour when the pH were determined using a glass electrode pH meter (Model 320 Mettler- Toledo AG, CH.8603, Schwerzenbach, Switzerland). Each sample was examined in triplicate and the final pH values recorded as an average of the three readings. The percentage moisture content of the triplicated samples was determined by drying known weights of sediment/soil to constant weight at 105˚C then calculating the average loss in weight between each set of samples. The dried samples were placed in a muffle furnace (Carbolite, Sheffield, UK) and the temperature raised slowly to 700˚C and kept constant for 30 minutes to burn off organic matter. After cooling overnight in a desiccator, the average loss in weight for each set of samples was recorded as the organic matter content.

Extraction of community DNA. Community DNA was extracted from all 21 environmental samples using a method slightly modified from that of Griffiths *et al*. (2000). Briefly, each sample (0.5 g wet weight) was added to a 1.5 ml screw-capped tube which contained 0.5 ml of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5 ml of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and 0.3 g of acid-washed sterile glass beads (≤ 106 μm; Sigma). The preparations were lysed twice at 5.5 m/s for 30 seconds using a ribolyser (Fastprep® FP220A Thermo Instrument; Qbiogene, France), cooled on ice for 2 minutes and the DNA containing aqueous phases separated by centrifugation (16,000 x g) for 5 minutes.

Table 4.1. Sources and physico-chemical properties of environmental samples

Code	Soil/Sediment samples	Location	pH	Moisture content (%)	Organic matter content (%)
ANT	Antarctic soil	Ross Dependency, Antarctica (78°01′S; 163°53′E)	7.3	0.3	0.4
AU96	Arid soil	Adelaide, Australia	5.5	0.2	1.7
BK	Hay meadow soil	Plot 6, Cockle Park Experimental Farm, Northumberland, UK (National Grid reference: NZ 200913)	5.5	4.0	9.5
CA	Rhizosphere soil	Izmir, Turkey	7.4	1.0	8.2
Ch6	Marine sediment	Bahia Tic Toc, Chilean Sea (43°53′ S, 72°87′W)	7.8	19.6	2.5
Ch27	Marine sediment	Estero Castro, Chilean Sea (42°30´S, 73°5´W)	7.9	20.6	3.0
CS	Chalk soil	Ambleside, Cumbria, UK (National Grid reference: NY-357978)	7.2	2.4	3.2
DR	River sediment	River Derwent, County Durham, UK (National Grid reference: NZ-055490)	6.9	5.0	4.5
ECU	Upland soil	Riobamba, Ecuador	6.7	2.5	5.6
KHA	Agricultural soil	Khartoum, Sudan	5.8	0.2	6.3
LS	Sediment	Shore of Loch Creran, UK (National Grid reference: NM-9124259)	7.7	25.0	4.5
MEG	Grassland soil	Syros, Greece	7.3	0.4	2.5
MS	Rhizosphere soil	Kuala Lumpur, Malaysia	7.4	1.2	4.1
NMS	Agricultural soil	Kuala Lumpur, Malaysia	5.7	0.3	7.1
NOR	Marine sediment	Østerfjord, Norwegian Sea (60°33′N, 5°19′E)	8.7	21.2	4.2
SAU	Arid soil	Nairobi, Kenya	5.9	1.0	2.0
SHO	Agricultural soil	Sholinar, Srinagar, India	5.5	0.7	6.1
TOR	Urban soil	Torremolinos, Spain	6.5	4.0	3.5
WAD	Sand dune soil	Borg El-Arab, Egypt	7.8	0.8	1.0
WS	Garden soil	Samsun, Turkey	6.7	2.8	11.3
ZIZ	Arid soil	Gazipur district, Bangladesh	6.5	3.5	4.0

Each supernatant was transferred to a fresh 1.5 ml tube and phenol removed by adding an equal volume of chloroform-isoamyl alcohol (24:1, v/v) followed by centrifugation (16000 x g) for 5 minutes. Each supernatant was transferred to a fresh 1.5 ml eppendorf tube and the nucleic acids precipitated by adding 1 ml of 30% (w/v) polyethylene glycol (PEG) dissolved in 1.6 M NaCl. The resultant preparations were briefly vortexed, incubated at −80˚C for an hour, centrifuged at maximum speed for 5 minutes, the DNA pellets washed with 500 µl of 70% (v/v) ice-cold ethanol, air-dried and redissolved in 50 µl of TE buffer (pH 8.0). The DNA preparations were checked for purity by agarose-gel electrophoresis and the purified extracted DNA preparations stored at -20˚C.

Nested PCR for detection of 16S rRNA dactylosporangial genes. A nested PCR approach was used to detect dactylosporangial 16S rRNA genes in each of the community DNA preparations. Bacterial 16S rRNA genes were amplified from these preparations by using universal primers 27F and 1525R (Table 2), as described by Tan *et al*.(2006). The resultant primary PCR products were diluted tenfold as templates for the secondary PCR reactions. Amplification of the 16S rRNA genes specific for members of the family *Micromonosporaceae* was performed using the PCR primers M2F and A3R (Table 4.2) and the presence of dactylosporangiae sought using genus-specific PCR primers D3F and D2R (Table 4.2), as described by Monciardini *et al*. (2002). The nested PCR reaction mixture, prepared in a final reaction volume of 50 µl, contained 2.5 µl of each forward and reverse primer, 5 µl of 10x reaction buffer (Bioline), 2.5 µl of DMSO, 0.8 µl of 12.5 mM dNTPs (Bioline), 1.5 µl of 50 mM $MgCl_2$ and 0.5 µl *Taq* DNA polymerase (Bioline) and 1 µl of template DNA. The PCR reactions were carried out in a PCR thermocycler (Whatman Biometra Tgradient^{TM}, Germany) together with positive and negative controls.

Cloning, sequencing and phylogenetic analysis of amplified PCR products. Cloning of the purified PCR products of *Dactylosporangium*-specific 16S rRNA genes amplified from community DNA samples was performed using a StrataClone PCR Cloning Kit (Strategene, UK). Ligations were achieved by mixing 1.5 µl of cloning buffer, 1 µl of each PCR product (5-50 ng) and 0.5 µl StrataClone Vector and the resultant preparations incubated at room temperature for 5 minutes then placed on ice, the transformations were performed by adding 1µl of ligation mixture to thawed StrataClone SoloPack competent cells. The resultant preparations were mixed gently, placed on ice for 20 minutes, heated at 42˚C for 45 seconds and incubated on ice for 2

minutes. Pre-warmed Luria-Bertani (LB; Miller, 1972) broth (250 µl) was added to each tube prior to incubation at 37˚C in a shaker incubator (Gallenkamp Orbital Incubator, UK) for an hour at 160 rpm and centrifugation for 1 minute at 13,000 x g. The supernatants were discarded and the pellets redissolved in 100 µl of LB medium, and the transformation mixtures (100 µl) plated onto pre-warmed LB-ampicillin plates which had been inoculated with 40 µl of 2% X-gal and incubated overnight at 37˚C. Randomly selected white colonies were streaked, using sterilised toothpicks, onto LB-ampicillin plates, incubated overnight at 37˚C, transformants screened by using the colony-PCR technique with M13 forward and reverse primers (Hofmann & Brian, 1991), and purified colony PCR products sequenced. The partial 16S rRNA gene sequences of the clones were aligned manually against corresponding sequences of the type strains of *Dactylosporangium* species retrieved from DDBJ/EMBL/GenBank databases using the pairwise alignment option. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Fitch, 1971) tree-making algorithms from the MEGA 3.0 program (Kumar *et al.*, 2004).

Table 4.2. PCR primers

Primer	Sequence(5'-3')	Target genes*	Size of PCR product (bp)	References
27F	AGAGTTTGATCMTGGCTCAG	Universal 16S rRNA	1400 – 1500	Lane (1991)
1525R	AAGGAGGTGWTCCARCC			
M2F	SAGAAGAAGCGCCGGCC	Family *Micromonosporaceae*-specific 16S rRNA	900 – 1000	Monciardini *et al.* (2002)
A3R	CCAGCCCCACCTTCGAC			
D3F	GCGGCTTGTTGCGTCAG	Genus *Dactylosporangium*-specific 16S rRNA	500 – 600	Monciardini *et al.*(2002)
D2R	CCGCTGGCAACATCGAACA			
K1F	TSAAGTCSAACATCGGBCA	PKS-I	1200 – 1400	Ayuso-Sacido *et al.* (2005)
M6R	CGCAGGTTSCSGTACCAGTA			
A3F	GCSTACSYSATSTACACSTCSGG	NRPS	700 – 800	Ayuso-Sacido *et al.* (2005)
A7R	SASGTCVCCSGTSCGGTAS			

* NRPS, non-ribosomal peptide synthetase; PKS-1, type- I polyketide synthase.

Selective isolation and enumeration of dactylosporangiae. A pilot experiment was carried out to determine whether representatives of seven validly described *Dactylosporangium* species (*D. aurantiacum* NRRL B-8018^T, *D. fulvum* DSM 43917^T, *D. matsuzakiens*e NRRL B-16293^T, *D. roseum* DSM 43916^T, *D. salmoneum* NRRL B-16294^T, *D. thailandense* DSM 43158^T and *D. vinaceum* NRRL B16297^T) grew on five media designed to be selective for members of the genus *Dactylosporangium*; namely, humic acid-vitamin agar (Hayakawa & Nonomura, 1987), oatmeal agar (Shirling & Gottlieb, 1966), raffinose-histidine agar (Vickers *et al.*, 1984), starch casein agar (Küster & Williams, 1964) and *Streptomyces* Isolation Medium (D'Costa *et al.*, 2006) supplemented with antifungal antibiotics and either gentamicin or oxytetracycline. The putative selective media were also evaluated for their effectiveness in isolating members of the genus *Dactylosporangium* from a soil sample taken from Palace Leas hay meadow plot 6 at Cockle Park Experimental Farm, Northumberland (Atalan *et al.*, 2000), using the procedure described below.

Two grams of the soil sample were suspended in 18 ml of ¼ strength Ringer's solution (Oxoid), mixed by vortexing (Vortex Genie 2 Model G.560E, USA) and blended for a minute using an ULTRA TUREX ®-Antried T 25 Blender. The resultant suspension was shaken in a Gallenkamp Orbital Incubator at 150 rpm for 30 minutes at room temperature, pre-treated at 55˚C for 20 minutes in a water bath, and cooled at room temperature prior to the preparation of a tenfold serial dilution in ¼ Ringer's solution. Aliquots (100 µl) of the 10^{-2} to 10^{-4} dilutions were spread, in triplicate, over plates of the five selective media supplemented with antifungal antibiotics (25 µg/ml of both cycloheximide and nystatin) and an antibacterial antibiotic (4 µg/ml of either gentamicin or oxytetracycline), and the plates incubated at 28˚C for 21 days; in all cases the isolation plates were dried for 15 minutes prior to inoculation, as recommended by Vickers *et al*. (1984). Orange coloured colonies which lacked aerial hyphae were presumptively assigned to the genus *Dactylosporangium*. The numbers of presumptive dactylosporangiae, other filamentous actinomycetes and bacteria were expressed as the mean number of colony forming units (cfu's) per gram dry weight soil. The *Streptomyces* Isolation Medium supplemented with cycloheximide, nystatin and gentamicin supported the growth of all of the *Dactylosporangium* type strains and the highest number of putative dactylosporangiae isolated from soil suspensions prepared from Palace Leas hay meadow, plot 6. In light of these results, the 13 environmental

samples found to contain dactylosporangiae in the culture-independent experiments (Table 4.3) were examined using the isolation procedure described above.

Selection, purification and maintenance of dactylosporangiae. Over 200 presumptive *Dactylosporangium* strains (Table 4.3) representing different colonial morphologies were taken from the selective isolation plates, using sterile toothpicks, and transferred onto oatmeal agar plates which were incubated at 28˚C for up to 21 days. The isolates were checked for purity by streaking onto another set of oatmeal plates which were incubated as before. Biomass from each of the purified isolates was scraped from the oatmeal agar plates and suspended in 1 ml glycerol tube (20%, v/v). The resultant glycerol stocks were preserved at −20˚C and served as a source of inoculum.

16S rRNA gene analysis and chemical characterisation of *Dactylosporangium* isolates. The 219 isolates were tested for their ability to produce the *Dactylosporangium*-specific PCR amplification product. Total genomic DNA was extracted from the isolates using Gen Elute^{TM} Bacterial Genomic Kits (Sigma-Aldrich Co.), following the manufacturer's protocol, stored at -20˚C, and PCR reactions performed with the genus-specific primers, as described by Monciardini *et al*. (2002). The 102 isolates which gave the genus-specific amplification product (Table 4.4) were the subject of 16S rRNA gene sequencing studies using the universal primers, 27F and 1525R (Table 4.2), as described by Tan *et al.* (2006). Phylogenetic analyses were carried out on the resultant sequences using the tree making algorithms mentioned earlier.

The 102 isolates were also examined for the presence of diaminopimelic acid (A_2pm) isomers in whole-organism hydrolysates by using the procedure described by Staneck & Roberts (1974), albeit with the minor modifications introduced by Tan *et al.* (2006). Briefly, two loopfuls of wet biomass of each isolate grown on oatmeal agar for 21 days at 28˚C were suspended in 500 µl of 6 N HCl containing 100 µg sterile glass beads (≤ 106 µm; Sigma), homogenised at 5.0 m/s for 20 seconds using the ribolyser (Fastprep® FP220A Instrument), incubated at 100˚C for 4 hours and centrifuged at 13,000 rpm for 5 minutes. The resultant supernatants were transferred to fresh tubes, evaporated to dryness on a hotblock (90-100˚C), and the hydrolysates reconstituted with distilled water (100 µl) prior to thin-layer-chromatographic (TLC) analysis. The isolates

were also examined for the presence of mycolic acids by using the alkaline methanolysis procedure described by Minnikin *et al*. (1980).

Table 4.3. Numbers of presumptive and confirmed *Dactylosporangium* strains isolated from environmental samples*

Code	Soil/Sediment samples	Presumptive isolates	Confirmed isolates
BK	Hay meadow soil Plot 6, UK	61	54
CA	Rhizosphere soil, Turkey	0	0
CS	Chalk soil, UK	23	19
DR	River sediment, UK	19	4
ECU	Upland soil, Ecuador	25	1
LS	Seashore sediment, UK	5	0
MEG	Grassland soil, Greece	2	0
MS	Rhizosphere soil, Malaysia	1	0
NMS	Agricultural soil, Malaysia	20	11
NOR	Marine sediment, Norway	15	0
SHO	Agricultural soil, India	29	6
WAD	Sand dune soil, Egypt	2	0
WS	Garden soil, Turkey	12	7
ZIZ	Arid soil, Bangladesh	5	0
	Total	219	102

* The remaining samples, ANT, AU96, Ch6, Ch27, KHA, SAU and TOR, were not examined using the selective isolation procedure as community DNA extracted from them gave negative reactions using the genus-specific PCR primers. In contrast, *Micromonosporaceae* strains were detected in all of the samples.

Non-ribosomal peptide and type I polyketide synthase genes. Fifty two representative *Dactylosporangium* isolates (Table 4.4) were screened for the presence of non-ribosomal peptide (NRPS) and type I polyketide synthase (PKS) genes using NRPS and PKS primers, as recommended by Ayuso-Sacido & Genilloud (2005) and Courtois *et al.* (2003), respectively. The amplification reactions were performed in a final reaction volume of 25 µl, which contained 1 µl of 0.4 µM of either NRPS or PKS specific primers (Table 4.2), 2.5 µl of 10x reaction buffer, 1 µl of DMSO, 1.6 µl of 2.5 mM dNTPs (Bioline), 1.5 µl of 50 mM $MgCl_2$, 0.25 µl *BioTaq* DNA polymerase

(Bioline), 15.15 µl sterile distilled water and 1 µl of extracted DNA template. The PCR reaction temperature profile was initial denaturation at 95˚C for 5 minutes, 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 57˚C (PKS specific primers) or 61˚C (NRPS specific primers) for 30 seconds and extension at 72˚C for 1 minute with an extension step at 72˚C for 10 minutes. The taxonomic relationships of the strains were established by constructing a 16S rRNA gene tree using the unweighted-pair-group method with arithmetic mean (UPGMA; Sneath & Sokal, 1973) algorithm from the MEGA 3.0 program (Kumar *et al.*, 2004) .

Table 4.4. *Dactylosporangium* strains isolated from the environmental samples

Code	Soil/Sediment samples	Isolation Media†	Isolates
BK	Hay meadow soil, UK	HV-GM	BK-5, **BK-10**, BK-11
		HV-OTC	BK-12, **BK-14**
		OA-GM	BK-13, **BK-15**, BK-16
		OA-OTC	BK-6, **BK-43**, BK-65
		RH-GM	BK-19, BK-31**, BK-56**
		RH-OTC	BK-26, BK-27, **BK-61**
		SCA-GM	BK-29, BK-36, BK-37, BK-45, BK-46 BK-64, **BK-66, BK-67**
		SCA-OTC	BK-17, **BK-68**, BK-71, BK-72, BK-91, **BK-92**
		SIM-GM	BK-18, BK-22, BK-41, BK-42, BK-49, BK-50, **BK-53**, BK-60, BK-62, BK-77, BK-78, **BK-93,** BK-94, **BK-97**
		SIM-OTC	BK-32, BK-33, BK-43, **BK-54, BK-57**, BK-66, BK-73, BK-76, BK-79
CS	Chalk soil, UK	SIM+GM	**CS-1, CS-2, CS-3, CS-4, CS-5, CS-7, CS-8, CS-9, CS-10, CS-13, CS-14, CS-15, CS-16, CS-17, CS-19, CS-20, CS-21, CS-23, CS-24**
DR	River sediment, UK	SIM-GM	**DR-11, DR-12, DR-16, DR-20**
ECU	Agricultural soil, Ecuador	SIM-GM	**ECU-2**
NMS	Agricultural soil, Malaysia	SIM-GM	**NMS-1, NMS-2, NMS-3, NMS-6, NMS-7, NMS-9, NMS-10, NMS-11, NMS-12, NMS-14, NMS-18**
SHO	Agricultural soil, India	SIM-GM	**SHO-9, SHO-10, SHO-11, SHO-12, SHO-13, SHO-22**
WS	Garden soil, Turkey	SIM-GM	**WS-1, WS-2, WS-3, WS-5, WS-6, WS-7, WS-10**

***** Isolates given in bold were included in the 16S rRNA sequencing studies. All of the isolates from the samples CS, DR, ECU, NMS, SHO and WS, and 4 isolates from the BK sample (BK-51, BK-53, BK-57 and BK-63) were examined for NRPS and PKS genes and included in the antibiotic assays. The selective isolation media were supplemented with antifungal (25 µg/ml of both cycloheximide and nystatin), and antibacterial antibiotics (4 µg/ml of either gentamicin or oxytetracycline).

† Media; HV, Humic acid-Vitamin agar; OA, Oatmeal agar; RH, Raffinose-Histidine agar; SCA, Starch Casein agar; SIM, *Streptomyces* Isolation Medium. Antibiotics; GM, gentamicin; OTC, oxytetracycline.

Evaluation of antimicrobial activity. The antimicrobial activities of 52 representative isolates (Table 4.4) were examined *in vitro* against a panel of microorganisms using a standard agar diffusion assay for the detection of antibacterial and antifungal activity (Barry & Thornsberry, 1991). The bacterial and yeast strains were grown on LB (Miller, 1972) and YM media (1% yeast extract, 0.5% polypeptone, 1% glucose and 0.3% malt extract, pH 5.5), respectively at 30˚C for 2 days prior to seeding the test media. Agar plugs from each of the isolates, grown on oatmeal agar at 28˚C for 21 days, were transferred to MH medium (Mueller–Hinton medium; Difco) plates which had been seeded with *Bacillus subtilis* DSM 10, *Escherichia coli* DSM 5698, *Kocuria rhizophila* ATCC 9341, *Pseudomonas fluorescens* DSM 50090 and *Staphylococcus aureus* DSM 20231, or onto the YM medium inoculated with *Candida albicans* Tu 164 and *Saccharomyces cerevisiae* 6A, and the resultant plates incubated at 30˚C. The diameters of any inhibition zones were measured after 24 and 48 hours.

4.4 Results

Culture-independent *Dactylosporangium* diversity. Dactylosporangiae were detected in 14 out of the 21 environmental samples and members of the family *Micromonosporaceae* in all of them (Table 4.3). Purified PCR products from the 14 environmental samples were cloned and sequenced. A BLAST search of the sequences of the 26 clones from the hay meadow plot 6 sample and 1-4 clones from the other samples showed that they belonged to the 16S rRNA *Dactylosporangium* gene tree (Fig. 4.1) and shared similarities with the *Dactylosporangium* type strains within the range of 95.6-99.9 %, values corresponding to 1-31 nucleotide differences over 549 locations. It is evident from Figure 4.1 that the clones can be assigned to three clades, the taxonomic integrity of which was supported by all of the tree-making algorithms and high bootstrap values.

The largest group, clade I, contained 41 clones (60 % of total) which were assigned to 4 multimembered and one single-membered subclade (Fig. 4.1). The largest taxon, subclade 1, contained 17 clones from 6 environmental samples together with the type strains of *D. matsuzakines*, *D. salmoneum* and *D. vinaceum*. The latter formed a distinct group together with individual clones from agricultural (NMS), arid (ZIZ), grassland (MEG) and rhizosphere soils (MS); this taxon was supported by all of the tree-making algorithms and a 64 % bootstrap value.

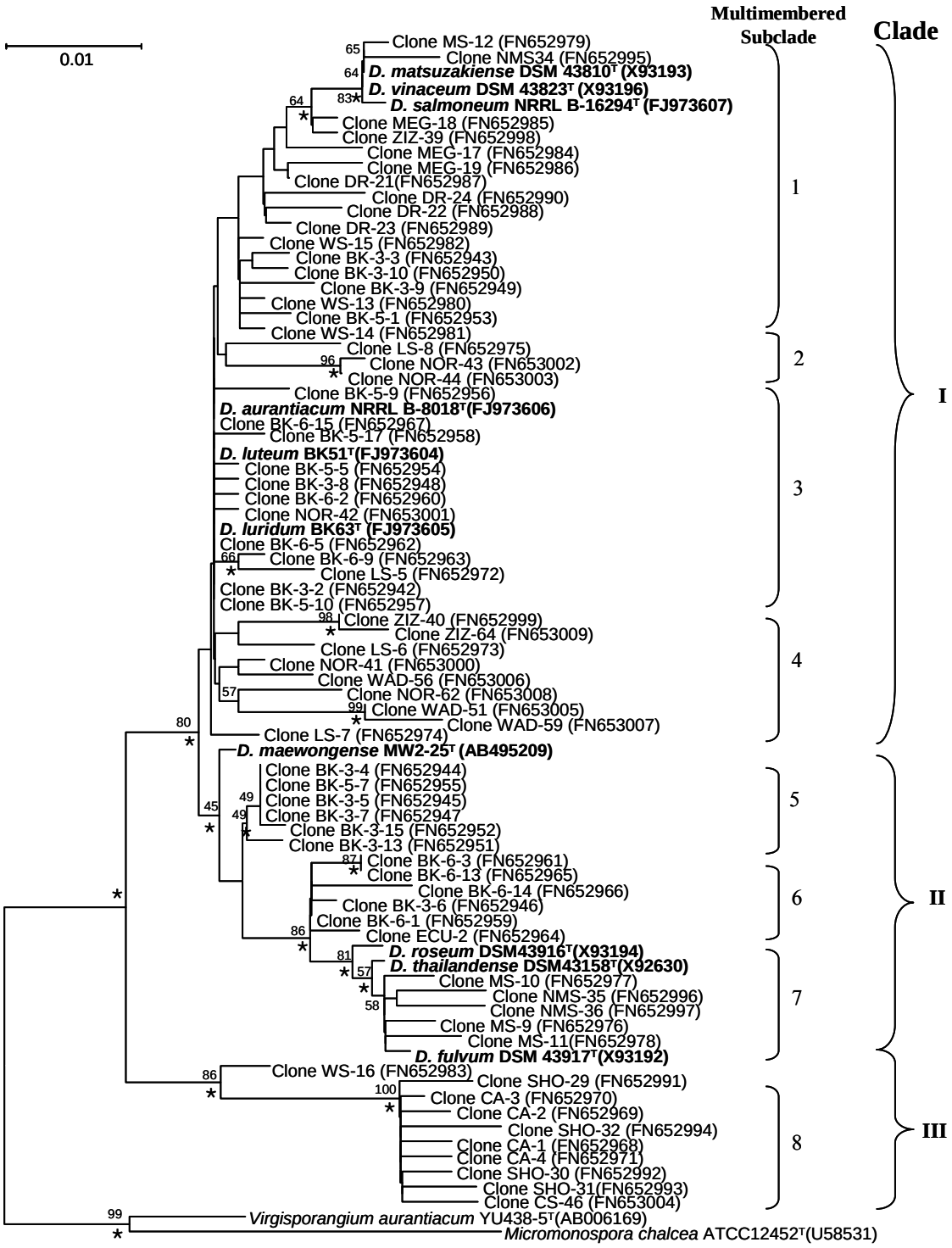


Fig. 4.1. Neighbour-joining tree based on about 545 bp of 16S rRNA gene sequences showing relationships between clones generated from 14 environmental samples using nested PCR amplification. Asterisks indicate branches of the tree that were also recovered using the minimum-evolution and maximum-parsimony tree-making algorithms. Numbers at the nodes are bootstrap values based on 1,000 resamplings of the neighbour-joining dataset; only values above 50% are shown. Bar, 0.01 substitutions per nucleotide. The codes showing the source of the clones are explained in Table 4.3

Four of the13 remaining subclade 1 clones were from hay meadow plot 6 soil (BK clones), a further 6 clones from this soil formed subclade 3 together with the type strains of *D. aurantiacum, D. luridum* and *D. luteum* and a clone from the marine sediment sample (NOR) taken from the Norwegian Sea. The remaining clones were assigned to subclades 2, 3 and 5. Three pairs of clones recovered in subclade 4 formed groups supported by all of the tree-making algorithms and by high bootstrap values (Fig. 4.1); one of these pairs comprised 2 out of the 3 clones from the Bangladeshian soil sample (ZIZ).

Clade II contained 17 clones (25% of total) which were assigned to three multimembered subclades, the integrity of which was supported by all of the tree-making algorithms and by high bootstrap values (Fig. 4.1). The type strain of *D. maewongense* was included in, but only loosely associated, with clade II. Subclade 5 contained 6 clones from hay meadow plot 6 (BK) and subclade 6 another 6 clones from this source and a single clone from a upland soil (ECU). Subclade 7 encompassed clones from the Malaysian soil samples (MS and NMS) and the type strains of *D. fulvum, D. roseum* and *D. thailandense.* The third clade contained remaining 10 clones (16%) of total, these were assigned to one single-membered and one multimembered subclade; the latter, subclade 8, contained clones from agricultural (SHO), chalk (CS) and rhizosphere (CA) soils.

Selective isolation of presumptive dactylosporangiae. All of the *Dactylosporangium* type strains grew well on humic acid-vitamin (HV), oatmeal (OA), raffinose-histidine (RH), starch-casein (SCA) and *Streptomyces* Isolation Medium (SIM) agars supplemented with the antifungal antibiotics and with either gentamicin or oxytetracycline. They formed compact, tough colonies with slightly wrinkled surfaces which lacked aerial hyphae. The substrate mycelia were pale orange or yellow on the OA, SCA and SIM agar plates, but red or brown coloured on the corresponding HV and RH plates. Presumptive dactylosporangial colonies were detected on all of the selective media inoculated with suspensions prepared from hay meadow plot 6 soil (Fig. 4.2). The highest count, 8.3×10^4 cfu/g dry weight soil, was recorded on SIM agar supplemented with gentamicin; these isolates accounted for 56 % of the total number of colonies growing on the isolation plates.

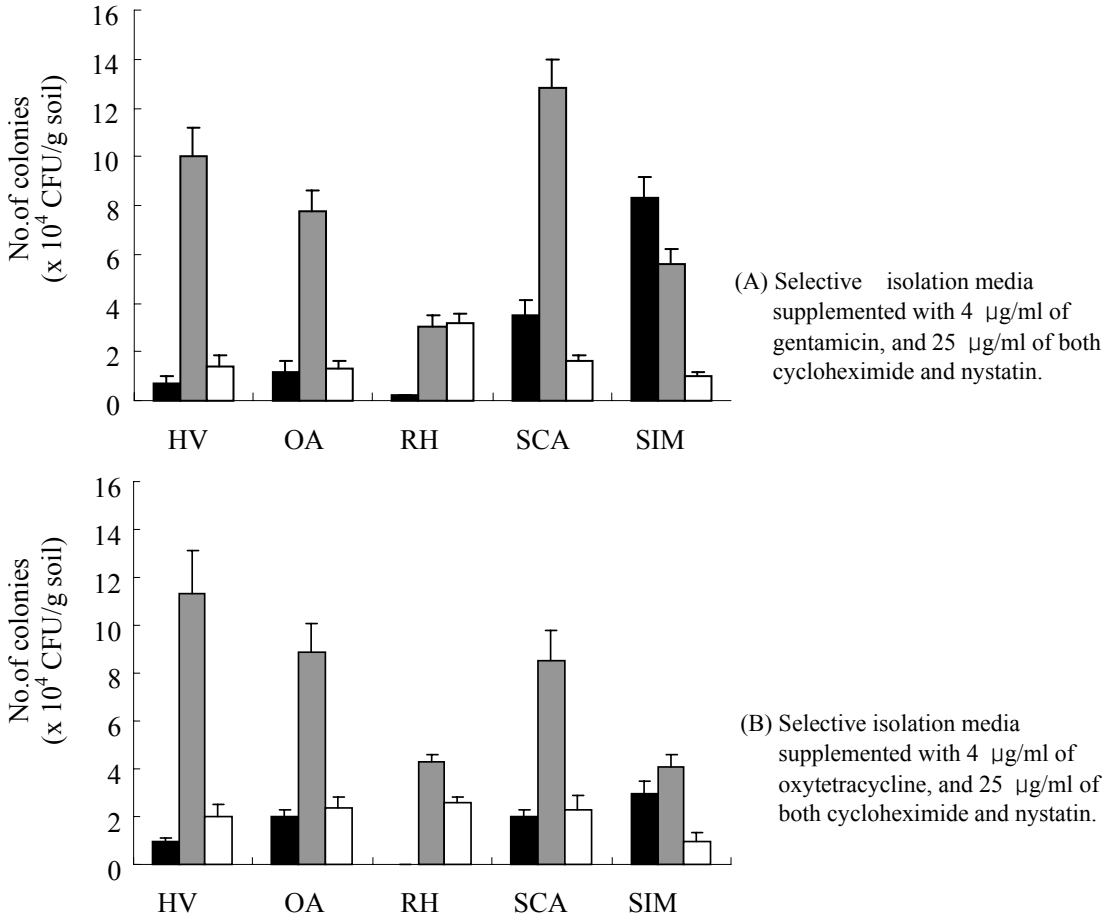


Fig. 4.2. Average number of colonies (104 cfu/g dry weight soil) of presumptive dactylosporangiae (black bar, ■), other actinomycetes (grey bar, ▒) and other bacteria (white bar, □) detected on the five selective isolation media supplemented with antibacterial and antifungal compounds. Error bars show standard deviations.

Media; HV, Humic acid-Vitamin agar; OA, Oatmeal agar; RH, Raffinose-Histidine agar; SCA, Starch Casein agar; SIM, *Streptomyces* Isolation Medium.

Relatively high numbers of presumptive dactylosporangiae, 3.5×10^4 cfu/g dry weight soil, were detected on the corresponding isolation plates supplemented with oxytetracyline, these isolates accounted for 38 % of the total number of colonies. In contrast, relatively few presumptive dactylosporangiae , $0.2\text{-}2.0\times10^4$ cfu/g dry weight soil, grew on the HV, OA or RH plates, these isolates accounted for between 3-15 % of the total number of colonies.

In general, lower numbers of presumptive dactylosporangiae were seen on the selective isolation plates supplemented with oxytetracycline; none grew on the RH plates. Consequently, the *Streptomyces* Isolation Medium supplemented with gentamicin and the antifungal antibiotics was used to try and isolate dactylosporangiae from the 14 environmental samples found to contain members of this taxon in the culture-independent study. Dactylosporangiae were isolated from all but one of these samples, the exception was the rhizosphere soil from Turkey, though, in general, fewer presumptive dactylosporangiae were detected compared with those growing on isolation media inoculated with suspensions from the hay meadow soil.

Characterisation of presumptive *Dactylosporangium* strains. Two hundred and nineteen presumptive dactylosporangiae, selected from the isolation plates inoculated with suspensions of the 14 environmental samples known to contain members of the genus (Table 4.3), were transferred to oatmeal agar plates which were incubated at 28˚C for 21 days. Nearly 50% of the isolates (102 out of 219) from 7 of the environmental samples gave the characteristic PCR amplification product with the genus-specific primers and hence were considered to be *bona fide* members of this taxon. Whole-cell hydrolysates of these isolates contained 3-hydroxy and/or *meso*-A_2pm and lacked mycolic acids, properties consistent with their assignment to the genus *Dactylosporangium*.

Most of the isolates were taken from media inoculated with suspensions prepared from the agricultural (NMS), chalk (CS), garden (SHO) and hay meadow (BK) soils, the remainder from the upland soil (ECU) and the river sediment (DR). When almost complete 16S rRNA gene sequences (about 1405 nucleotides) of the 63 isolates taken to represent different colony types, environmental samples and isolation media (Table 4.4) were compared with representatives of the other genera classified in the family *Micromonosporaceae*, they were found to belong to the genus *Dactylosporangium* (data

not shown). These isolates were distributed throughout the 16S rRNA *Dactylosporangium* gene tree (Fig. 4.3), and shared similarity values within the range of 97.1% and 100% with the *Dactylosporangium* type strains.

The isolates were assigned to 4 multimembered and one single-membered clade defined at a 16S rRNA gene sequence similarity level of 99.0% (Fig. 4.3), a value which corresponds to 14 nucleotide differences over 1396 locations. It can be seen from the Figure 4.3 that 12 subclades representing separate phyletic lines can be recognised though the bootstrap values of 30% is low. The taxonomic status of the largest group, clade I, was supported by all of the tree-making algorithms. This taxon contained 27 out of the 63 isolates (43% of total) and 2 *Dactylosporangium* type strains, these organisms were recovered in three multimembered and one single-membered subclade. Subclade 1 contained 5 isolates from the hay meadow plot (BK) and 2 from the river sediment (DR). Subclade 2 encompassed an additional 5 isolates from the hay meadow soil, three of which, isolates BK-15, BK-92 and BK-93, had identical 16S rRNA gene sequences with the type strain of *D. luteum*; the status of this taxon was supported by all of the tree-making algorithms and by a bootstrap value of 61%. Subclade 3, the largest group, contained 13 out of the 18 strains isolated from the chalk soil (CS). The type strain of *D. luridum* formed a single-membered subclade at the periphery of clade I.

The clade II contained 19 isolates (30 % of total) and two *Dactylosporangium* type strains which were assigned to four multimembered and one single-membered subclade. The taxonomic status of all of the subclades was supported by all of the tree-making algorithms and in three cases by high bootstrap values (Fig. 4.3). The type strain of *D. maewongense* formed a single-membered taxon which was loosely associated with subclade 4, the latter contained 7 strains isolated from three of the environmental samples. Subclade 5 was composed of two isolates from the river sediment (DR) and subclade 6 of an isolate from the upland soil (ECU) and three strains from the chalk soil (CS). The final taxon, subclade 7, encompassed all six isolates from the Indian agricultural soil (SHO). These isolates shared 16S rRNA gene similarities of 99.7 to 99.9% with the type strain of *D. aurantiacum*, values which corresponded to between 1 and 4 nucleotide differences at 1397 locations.

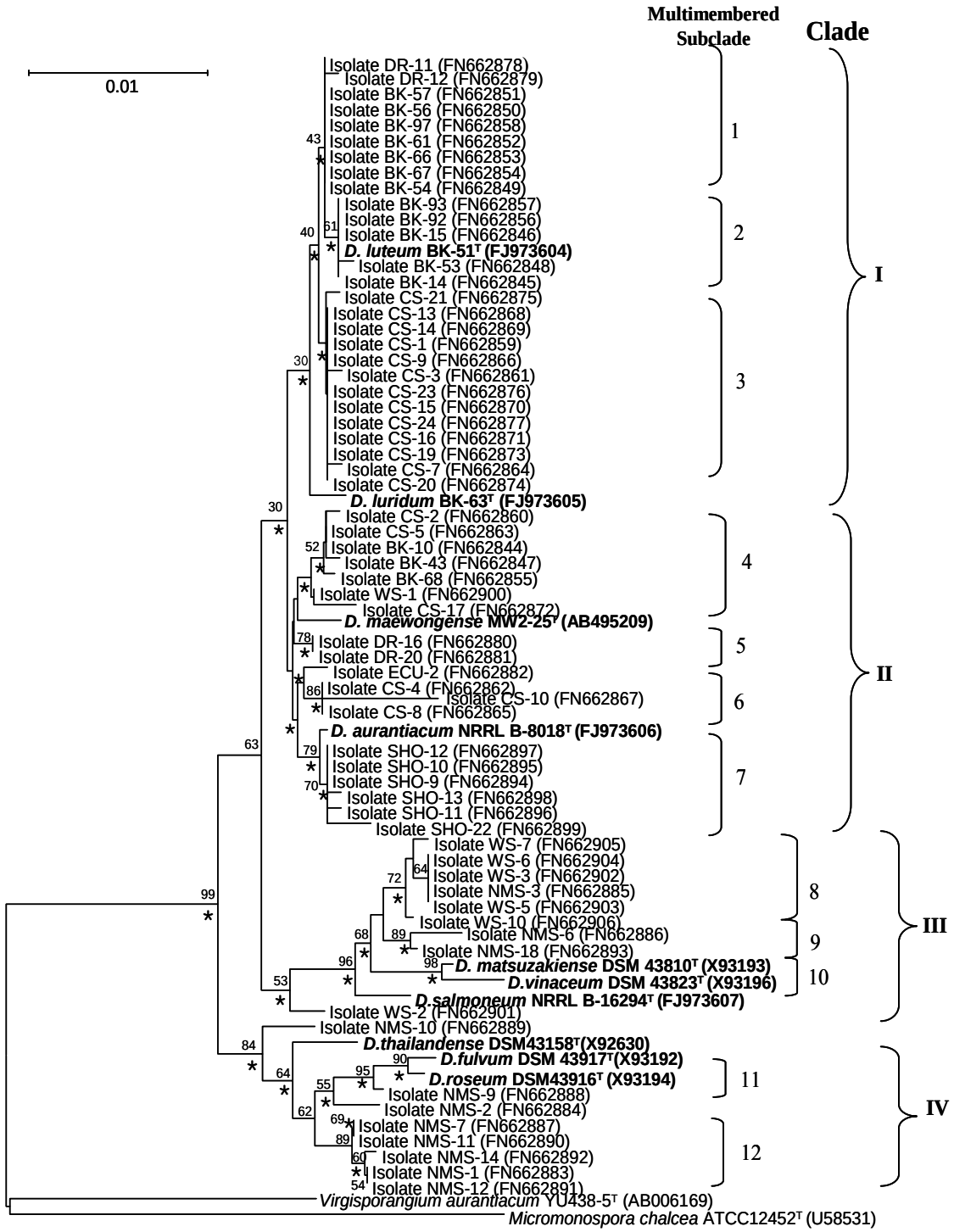
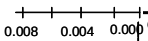


Fig. 4.3. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between the isolates and between them and the type strains of *Dactylosporangium* species. Asterisks indicate branches of the tree that were also recovered using the minimum-evolution and maximum-parsimony tree-making algorithms. Numbers at the nodes are bootstrap values based on 1,000 resamplings of the neighbour-joining dateset; only values at or above 30% are shown. Bar, 0.01 substitutions per nucleotide. The codes showing the source of the isolates are explained in Table 4.3.

The 9 isolates and 3 *Dactylosporangium* type strains assigned to clade III formed a well circumscribed taxon (Fig. 4.3). These organisms were assigned to three multimembered and two single-memebered subclades, the stratus of which was supported by all of the tree-making algorithms and by high bootstrap values. Subclade 8 encompassed 5 isolates from the Turkish garden soil (WS) and a single isolate from the Malaysian agricultural soil (NMS); two isolates from this latter source constituted subclade 9. Subclade 10 was contained the type strains of *D. matsuzakiense* and *D. vinaceum*, these strains shared a 16S rRNA gene similarity of 99.6%, a value equivalent to 6 nucleotide differences at 1396 locations. Two organisms, the type strain of *D. salmoneum* and an isolate from the Turkish garden soil (isolate WS-2), formed single-membered subclades at the periphery of clade III.

Seven of the 11 Malaysian soil isolates (NMS) and 3 *Dactylosporangium* type strains were assigned to two multimembered and two single-membered subclades in clade IV. Subclade 11 contained the type strains of *D. fulvum* and *D. roseum*, and isolate NMS-9. The type strains shared a 16S rRNA gene similarity of 99.7%, a value equivalent to 4 nucleotide differences at 1396 locations. Isolate NMS-9 was closely related to *D. fulvum*; these organisms shared a similarity of 99.5%, a value corresponding to 7 nucleotide differences at 1396 locations. The final multimembered taxon, subclade 12, contained five isolates which shared 16S rRNA gene similarities within the range 99.1 to 99.4% with the closest type strain, *D. thailandense*, these values are equivalent to 9-13 nucleotide differences at 1388 locations. The two remaining organisms, the type strains of *D. thailandense* and isolate NMS-2, formed single-membered subclades. Isolate NMS-10, which formed the single-membered clade, was recovered at the periphery of clade IV. This strain was most closely related to *D. thailandense* DSM 43158^{T}, these organisms shared a similarity of 99.1%, a value equivalent to 13 nucleotide differences at 1388 locations.

Antimicrobial activity and detection of the PKS and NRPS genes. It can be seen from Figure 4.4 that 24 out of the 52 representative *Dactylosporangium* isolates inhibited the growth of one or more of the 7 strains included in the agar diffusion assays. Eleven of these isolates inhibited the growth of *B. subtilis* DSM 10, 22 that of *K. rhizophila* ATCC 9341, and 6 *S. aureus* DSM 20231.



Isolate Code	Detection of gene[a]		Activity against[b]:		
	NRPS	PKS-I	*B. subtilis* DSM 10	*K. rhizophila* ATCC 9341	*S. aureus* DSM 20231
CS-19	+	+	-	-	-
CS-15	+	+	++	+++	++
CS-20	+	+	-	-	-
CS-23	+	+	-	+	-
CS-16	+	+	-	+	-
CS-13	-	+	-	++	+
BK-57	+	-	-	+	-
DR-11	+	+	-	-	-
CS-3	-	+	+	++	+
CS-9	+	+	++	+++	-
CS-7	-	-	+	+	+
DR-12	-	+	-	-	-
BK-15	+	-	-	+	-
BK-53	+	-	-	++	-
BK-68	-	-	-	-	-
CS-21	+	+	-	+	-
CS-1	+	+	+	+	-
CS-14	+	+	++	+	+
CS-24	+	+	-	+	-
SHO-10	+	-	-	-	-
SHO-12	+	+	-	-	-
SHO-9	+	+	-	-	-
SHO-13	+	-	-	-	-
SHO-11	-	-	-	-	-
SHO-22	+	-	-	-	-
CS-8	+	+	-	-	-
CS-4	+	+	-	-	-
ECU-2	-	+	+	+	-
CS-17	+	-	-	-	-
DR-16	-	-	-	-	-
DR-20	+	-	-	+	-
CS-2	+	-	-	-	-
CS-5	+	+	-	+	+
WS-1	+	+	-	-	-
WS-2	+	+	-	-	-
CS-10	+	+	-	-	-
NMS-6	-	+	-	-	-
NMS-18	+	-	-	-	-
WS-7	+	-	-	-	-
WS-10	-	-	-	-	-
WS-6	+	-	-	-	-
WS-5	+	+	-	+	-
NMS-3	-	+	-	++	-
WS-3	-	-	-	-	-
NMS-2	-	+	+	-	-
NMS-10	-	+	-	-	-
NMS-9	+	+	+	++	-
NMS-14	-	-	-	-	-
NMS-11	+	-	+	+	-
NMS-7	+	-	+	-	-
NMS-1	-	-	-	-	-
NMS-12	-	-	-	++	-

Fig. 4.4. Antibiotic activity and detection of NRPS/PKS genes in representative *Dactylosporangium* isolates compared with their position in a 16S rRNA gene tree based on almost complete sequences. a: +, present; −, absent. b: Estimated by measuring the diameter of the zone of growth inhibition.; +, ++ and +++, weak, moderate and strong activity, respectively; −, no activity

Four of the strains isolated from the chalk soil, namely isolates CS-3, CS-7, CS-14 and CS-15, showed activity against all three of these strains. In contrast, none of the isolates from the Indian agricultural soil (SHO) and few from the Turkish garden soil (WS) showed any antimicrobial activity. None of the isolates inhibited the growth of *C. albicans* Tu 164, *E. coli* DSM 5698, *P. fluorescens* DSM 50090 or *S. cerevisiae* 6A.

Thirty five of the 52 representative *Dactylosporangium* isolates contained NRPS genes and 29 PKS-I genes (Fig. 4.4). Twenty one of these isolates (40% of the total) contained both sets of genes. However, there was no clear correlation between the distribution of NRPS and PKS-I genes and the capacity of isolates to show antibacterial activity. Thus, while isolates CS-14, CS-15 and NMS-9 contained both sets of genes and showed antibacterial activity, other isolates, such as CS-7 and NMS-12, exhibited antibacterial activity even though NRPS and PKS-I genes were not detected.

4.5 Discussion

The primary aim of this study was to determine the distribution and taxonomic diversity of members of the genus *Dactylosporangium* in diverse natural habitats using culture-independent and culture-dependent procedures. A nested PCR approach was used to detect the presence of dactylosporangial 16S rRNA genes in community DNA extracted from 27 diverse environmental samples. Bands of the expected size were generated from all of the samples using the universal and *Micromonosporaceae*-specific primer sets. The final round of PCR, using the *Dactylosporangium*-specific primers of Monciardini *et al*. (2002), showed that members of this genus were present in 14 of the environmental samples. These results provide further evidence that nested PCR primers provide on effective way of detecting specific components of actinobacterial communites present in natural habitats (Rheims & Stackebrandt, 1999; Mincer *et al.*, 2005; Shen *et al.*, 2007; Xin *et al.*, 2008; Lefebvre *et al.*, 2009; Sun *et al.*, 2010).

Phylogenetic analysis of the small clone library generated from 14 community DNA preparations using the genus-specific primers showed that all of the phylotypes fell within the *Dactylosporangium* 16S rRNA gene tree. Most of clones showed between 95 and 99% 16S rRNA sequence similarity to their closest phylogenetic neighbour though a few shared identical, or almost identical sequences to the type strains of either *D. luridum* or *D. luteum*. Consequently, it seems highly likely that most

of the clones represent novel species, as formally described members of the clade with 16S rRNA gene similarities above 99.4% have DNA:DNA relatedness values below 70% (Kim *et al.*, 2010), that is, under the cut-off point recommended for delineation of bacterial species (Wayne *et al.*, 1987). The clones were distributed throughout the dactylosporangial gene tree indicating that the genus-specific primers of Monciardini *et al*. (2002) can be used to detect unknown members of the clade; this diversity is likely to be genuine as care was taken in the culture-independent studies to detect chimeras.

The results of the culture-independent study indicate that taxonomically diverse members of the genus *Dactylosporangium* are present in diverse natural habitats. The clones generated from community DNA extracted from the hay meadow soil (BK) showed a particularly high degree of taxonomic diversity. However, there was little evidence that clones from specific habitats clustered together though this may reflect the small size of the clone library. Nevertheless, it has been shown for the first time that taxonomically diverse representatives of the *Dactylosporangium* 16S rRNA gene clade are present in freshwater and marine habitats; these findings are of both ecological and industrial interest, not least because actinomycetes from marine sediments are proving to be an especially rich source of novel secondary metabolites, notably antibiotics (Bull & Stach, 2007; Baltz, 2008; Olano *et al.*, 2009; Goodfellow & Fiedler, 2010; Jensen, 2010).

The *Dactylosporangium* type strains formed characteristic orange or brown colonies, which lacked aerial hyphae, on all of the media designed to selectively isolate dactylosporangiae from natural habitats. Similar characteristic colonies were detected on all of the putative selective media following inoculation with suspensions prepared from the hay meadow plot 6 soil and subsequent incubation at 28˚C for 21 days. The highest counts of presumptive dactylosporangial were recorded on plates of *Streptomyces* Isolation Medium supplemented with gentamicin and the antifungal antibiotics hence this medium was chosen for the subsequent isolation experiments.

Presumptive dactylosporangiae were isolated from 13 of the 14 environmental samples found to contain members of the genus *Dactylosporangium* in the culture-independent studies though counts of these organisms were fewer than those found on plates inoculated with suspensions of hay meadow plot 6 soil. Counts from this latter source compare favourably with those based on more rigorous procedures

recommended for the selective isolation of *Dactylosporangium* and *Actinoplanes* (Hayakawa *et al.*, 1991b; Hayakawa, 2008). The current procedure has several advantages over the earlier ones notably that the presumptive dactylosporangiae are easy to recognise and account for a high proportion of colonies growing on the isolation plates.

The generic assignment of 102 out of the 211 isolates taken from the selective isolation plates (48.3%) and provisionally assigned to the genus *Dactylosporangium* was underpinned when it was shown that they produced a characteristic amplification product of the expected size with the genus specific primers D3F and D2R (Monciardini *et al.*, 2002). All of these isolates formed stable orange or brown coloured mycelia devoid of aerial hyphae, produced whole-organism hydrolysates rich in hydroxyl and/or *meso*-diaminopimelic acid, but lacked mycolic acids, properties consistent with their assignment to the genus *Dactylosporangium* (Vobis, 1989, 2006). The genetic location of 63 of these isolates was confirmed by the 16S rRNA gene sequencing studies.

The 63 representative isolates formed a phylogenetically diverse group within the 16S rRNA *Dactylosporangium* gene tree. The type strains of the two most closely related species, *D. fulvum* and *D. roseum*, share a 16S rRNA gene similarity of 99.7%, which corresponds to 4 nucleotide differences at 1396 locations, while those of *D. luridum* and *D. aurantiacum* share a 16S rRNA gene similarity of 99.4% (7 nucleotide differences at 1435 sites) and have a DNA:DNA relatedness value of 53% (Kim *et al.*, 2010), a value well below the recommend cut-off point for assigning strains to the same genomic species (Wayne *et al.*, 1987). Clearly on these measures, the isolates from the chalk (CS) and hay meadow (BK) soils can be assigned to, or are very closely related to *D. luteum* though they do fall into different subclades in clade I. In contrast, most of the remaining isolates (57% of the total) fall into new centres of taxonomic variation which can be equated with presumptive novel species. There is also additional evidence that isolates from specific habitats tend to cluster together, as exemplified by isolates from the two agricultural soils (NMS, SHO). The isolation of 3 strains from the River Derwent sediment sample (DR) is in line with results from the culture-independent study.

It can be concluded from this study that the genus *Dactylosporangium* is grossly underspeciated as many presumptively novel dactylosporangial species were detected in

diverse environmental samples using both culture-dependent and culture-independent approaches. It is particularly encouraging that the isolation and characterisation strategy proved to be effective as rare and presumptively novel dactylosporangiae were isolated from diverse soil samples. These findings provide further evidence that innovative isolation procedures are needed if members of specific actinomycete taxa are to be isolated from natural habitats known, from culture-independent studies, to contain them (Goodfellow, 2010; Goodfellow & Fiedler, 2010). Such studies are particularly relevant with respect to commercially significant actinomycete taxa as they facilitate the judicious selection of isolates for low throughout pharmacological screens (Bull *et al.*, 2000; Goodfellow & Fiedler, 2010). Results from the present study also suggest that *Dactylosporangium* strains may be a rich source of novel secondary metabolites as representative strains contained both NRPS and type-I PKS genes and showed evidence of antibacterial activity.

It is also evident from this study that improved selective isolation procedures together with the identification of phylotypes provides an effective way of tackling the great plate count anomaly (Staley & Konopka, 1985), whereby only a small and skewed fraction of prokaryotic diversity present in natural habitats is detected using traditional cultural and characterisation procedures. Indeed, both innovative culture-dependent and culture-independent methods are needed to unravel the full extend of prokaryotic diversity in natural habitats, not least because each of these approaches is flawed by biases which can distort the richness and structure of microbial communities in nature (Hugenholtz *et al.*, 1998; Bull *et al.*, 2000; Mincer *et al.*, 2005; Xin *et al.*, 2008; Hirsch *et al.*, 2010).

It is also becoming clearer that rapid and accurate procedures are required to characterise and formally describe the many kinds of actinomycetes which grow on selective isolation plates, a point highlighted in both the present investigation and in comparable studies on amycolatopsis (Tan *et al.*, 2006), gordoniae (Goodfellow *et al.*, 1998), rhodococci (Colquhoun *et al.*, 1998) and alkaliphilic streptomycetes (Antony-Babu & Goodfellow, 2008).

Chapter 5. Three new species of *Dactylosporangium* isolated from soil: *Dactylosporangium luridum* sp. nov., *Dactylosporangium luteum* sp. nov. and *Dactylosporangium salmoneum* sp. nov., nom. rev.

5.1 Abstract

Forty strains isolated from soil taken from a hay meadow were assigned to the genus *Dactylosporangium* on the basis of colonial properties. 16S rRNA gene sequencing showed that the isolates formed a group that was most closely related to the type strain of *Dactylosporangium aurantiacum,* but well separated from other *Dactylosporangium* type strains and from "*Dactylosporangium salmoneum*" NRRL B-16294. Twelve out of thirteen representative isolates had identical 16S rRNA gene sequences and formed a subclade that was distinct from corresponding phyletic lines composed of the remaining isolate, strain BK63^T, the "*Dactylosporangium salmoneum*" strain and each of the *Dactylosporangium* type strains. DNA:DNA relatedness data showed that representatives of the multimembered 16S rRNA subclade, isolate BK63^T and the "*Dactylosporangium salmoneum*" subclade formed distinct genomic species; all of these organisms had chemotaxonomic and morphological properties consistent with their classification in the genus *Dactylosporangium*. They were also distinguished from one another and from the *Dactylosporangium* type strains using a range of phenotypic properties. The combined genotypic and phenotypic data showed that isolate BK63^T, isolates BK51^T, BK53 and BK69, and strain NRRL B-16294 should be classified in the genus *Dactylosporangium* as novel species. The names proposed for these taxa are *Dactylosporangium luridum* sp. nov., *Dactylosporangium luteum* sp. nov. and *Dactylosporangium salmoneum* sp. nov. ; the respective type strains of these species are BK63^T(= DSM 45324^T = KACC 20933^T = NRRL B-24775^T), BK51^T (= DSM 45323^T = KACC 20899^T = NRRL B-24774^T) and NRRL B-16294^T (= DSM 43910^T).

5.2 Introduction

The genus *Dactylosporangium* was proposed by Thiemann *et al*. (1967) to accommodate aerobic, filamentous actinomycetes which released motile spores formed

in sporangia borne on short sporangiosphores carried on substrate mycelia. At the time of writing, the genus comprises six recognised species: *Dactylosporangium aurantiacum* (the type species; Thiemann *et al*., 1967), *D. fulvum* (Shomura *et al*., 1986), *D. matsuzakiense* (Shomura *et al*., 1980), *D. roseum* (Shomura *et al*., 1985), *D. thailandense* (Thiemann *et al*., 1967) and *D. vinaceum* (Shomura *et al*., 1983); two additional taxa, "*D. salmoneum*" and "*D. variesporum*", have been cited as *species incertae sedis* (Vobis, 1989). Dactylosporangiae form a distinct phyletic branch in the *Micromonosporaceae* 16S rRNA gene tree (Koch *et al*., 1996; Thawai *et al*., 2008), and can be distinguished from other genera classified in the family *Micromonosporaceae* by using a combination of chemotaxonomic, morphological and phylogenetic data (Vobis, 2006; Ara *et al*., 2008). In contrast, members of *Dactylosporangium* species show minimal differences in chemical, morphological and physiological properties and are distinguished mainly on the basis of diffusible pigment and substrate mycelial colours (Vobis, 1989, 2006).

The primary aim of the present investigation was to determine the taxonomic status of a group of filamentous soil actinomycetes which had colonial and morphological properties characteristic of members of the genus *Dactylosporangium*. To this end, the isolates were compared with the type strains of *Dactylosporangium* species and with "*D. salmoneum*" NRRL B-16294 in a polyphasic taxonomic study. The resultant genotypic and phenotypic data showed that the isolates represented two novel *Dactylosporangium* species, namely *Dactylosporangium luridum* sp. nov. and *Dactylosporangium luteum* sp. nov. It is also proposed that "*D. salmoneum*" NRRL B-16294 be recognized as a novel species.

5.3 Materials and methods

Selective isolation, purification, maintenance of dactylosporangiae. Filamentous actinomycetes were isolated on plates of *Streptomyces* Isolation Medium (per litre distilled water: 0.4 g casein, 1.0 g starch, 0.1 g $CaCO_3$, 0.2 g KH_2PO_4, 0.5 g KNO_3, 0.1 g $MgSO_4$, 15 g agar), supplemented with cycloheximide (20 μg ml^{-1}) and oxytetracycline (20 μg ml^{-1}), following inoculation with suspensions of soil taken from Palace Leas meadow hay plot 6 (Atalan *et al*., 2000) at Cockle Park Experimental Farm, Northumberland, UK (National Grid Reference NZ 200913). Forty isolates which produced orange coloured substrate mycelia characteristic of *Dactylosporangium* strains

were purified and maintained on oatmeal agar (ISP medium 3; Shirling & Gottlieb, 1966) at room temperature and as suspensions of mycelial fragments in glycerol (20%, v/v) at -20˚C.

DNA extraction, molecular fingerprinting and phylogenetic analyses. DNA extracted from the 40 isolates was screened using BOX, ERIC and REP-PCR primers (Versalovic *et al*., 1991, 1994). The resultant band patterns were analysed using Pearson's correlation coefficient and clustering achieved by the UPGMA algorithm using BIONUMERICS software (Applied Maths) and the data presented as a consensus dendrogram . Thirteen strains were taken to represent the taxonomic variation shown by the isolates based on their *rep*-PCR profiles. These isolates, together with the *Dactylosporangium* type strains and "*D. salmoneum*" NRRL B-16194, were grown in shake flasks of modified Bennett's broth (Jones, 1949) at 28˚C for 14 days at 150 rpm to obtain biomass for the molecular systematic studies. The cultures were checked for purity and harvested by centrifugation. The biomass preparations were washed in NaCl/EDTA buffer (0.1M EDTA, pH 8.0, 0.1M NaCl) and stored at -20˚C until needed.

The phylogenetic positions of the strains were determined by 16S rRNA gene sequence analysis. Chromosomal DNA, PCR and direct sequencing of the purified products were carried out as described by Tan *et al*. (2006). The resultant, almost complete, 16S rRNA gene sequences (1430-1439 nucleotides) were aligned manually, using the jPHYDIT program (Jeon *et al*., 2005) against corresponding sequences of representatives of genera classified in the family *Micromonosporaceae*, retrieved from the GenBank database. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms from the MEGA version 3 program (Kumar *et al*., 2004), and the maximum-likelihood method (Felsenstein, 1981) from the PHYLIP suite of programs (Felsenstein, 1993). The evolutionary distance model of Jukes & Cantor (1969) was used to generate an evolutionary distance matrix for the neighbour-joining algorithm. The topologies of the resultant trees were evaluated in a bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset using the CONSENSE and SEQBOOT options from the PHYLIP package.

Partial RNA polymerase β–subunit (*rpoB*) gene sequences, generated for four representative isolates, strains BK51^T, BK53, BK63^T and BK69, "*D. salmoneum*"

NRRL B-16294 and the *Dactylosporangium* type strains were compared with corresponding genes of members of genera classified in the family *Micromonosporaceae*, drawn from GenBank, by using the same tree-making algorithms as above, but with "*Streptomyces coelicolor*" A3(2) as the outgroup.

Determination of DNA:DNA relatedness and DNA G+C contents. DNA:DNA relatedness studies were carried out to determine the finer taxonomic relationships between isolates BK51^T, BK63^T and "*D. salmoneum*" NRRL B-16294 and their closest phylogenetic neighbours. The levels of DNA:DNA relatedness between the tested strains was established by measuring the divergence between the thermal denaturation midpoints of homologous and heterologous DNA (ΔTm) following the procedure developed by Gonzalez & Saiz-Jimenez (2005). Confidence can be placed in the resultant data as comparable results have been obtained when the same strains have featured in thermal denaturation and spectrophotometric studies (Jurado *et al*. 2005; Goodfellow *et al* 2007). ΔTm values of 5.0 and 6.0˚C correspond to DNA:DNA relatedness levels of 60 and 70%, respectively (Rosselló-Mora & Amann, 2001). DNA G+C contents were determined for all of the strains included in the DNA:DNA experiments using the procedure described by Gonzalez & Saiz-Jimenez (2005).

Chemotaxonomical analysis. Biomass for the chemotaxonomic studies was prepared by growing isolates BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294 and the *Dactylosporangium* type strains in modified Bennett's broth at 150 rpm for 21 days at 28˚C; cells were harvested by centrifugation, washed in distilled water, re-centrifuged and freeze-dried. Standard procedures were used to extract and analyse the isomers of diaminopimelic acid (A_2pm; Staneck & Roberts, 1974), isoprenoid quinones (Collins, 1994), muramic acid type (Uchida *et al*., 1999), polar lipids (Minnikin *et al*., 1984) and whole-organism sugars (Schaal, 1985), using appropriate controls. Cellular fatty acids were extracted, methylated and analyzed by GC using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990) and mycolic acids sought by using the TLC procedure introduced by Minnikin *et al*. (1975).

Biochemical, cultural and morphological studies. Isolates BK51^T, BK63^T and "*D. salmoneum*" NRRL B-16294 were grown on tryptone-yeast extract, yeast extract-malt extract, oatmeal, inorganic salts-starch, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1-7, respectively; Shirling and Gottlieb, 1966) for 21

days at 28°C. Colonies growing on these media were examined by eye to determine substrate mycelial pigmentation and the colour of any diffusible pigments; colours were recorded using National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1958). The peptone- yeast extract- iron and tyrosine agar plates were examined to determine whether the strains produced melanin pigments. The strains were also examined to establish whether they grew on Luria-Bertani (LB), nutrient agar (NA) and trypticase soy agar (TSA) (Sambrook *et al*. 1989) after incubation at 28°C for 3 weeks.

Isolates BK51^T and BK63^T together with "*D. salmoneum*" NRRL B-16294 were examined for micromorphological properties on three week-old inorganic salts-starch and oatmeal agar plates by using a Nikon Optiphot light microscope fitted with a long-working-distance objective. In addition, gold coated, dehydrated preparations from the oatmeal agar plates were examined using a Cambridge Stereoscan 240 scanning electron microscopy following the procedure described by O'Donnell *et al.* (1993). Preparations from the oatmeal agar plates were flooded with sterile water and left for 60 minutes then examined for the presence of motile spores using a light microscope. Isolate BK51^T, BK53, BK63^T and BK69, "*D. salmoneum*" NRRL B-16294 and the *Dactylosporangium* type strains were examined for a broad range of phenotypic properties using media and methods described by Goodfellow *et al.*(1979).

5.4 Results and discussion

Molecular fingerprinting. It is evident that all but one of the isolates can be assigned to a homogeneous cluster that was well separated from all of the marker strains (Fig. 5.1). The remaining isolate, strain BK63^T, formed a distinct branch in the dendrogram, as did "*D. salmoneum*" NRRL B-16294 and each of *Dactylosporangium* the marker strains.

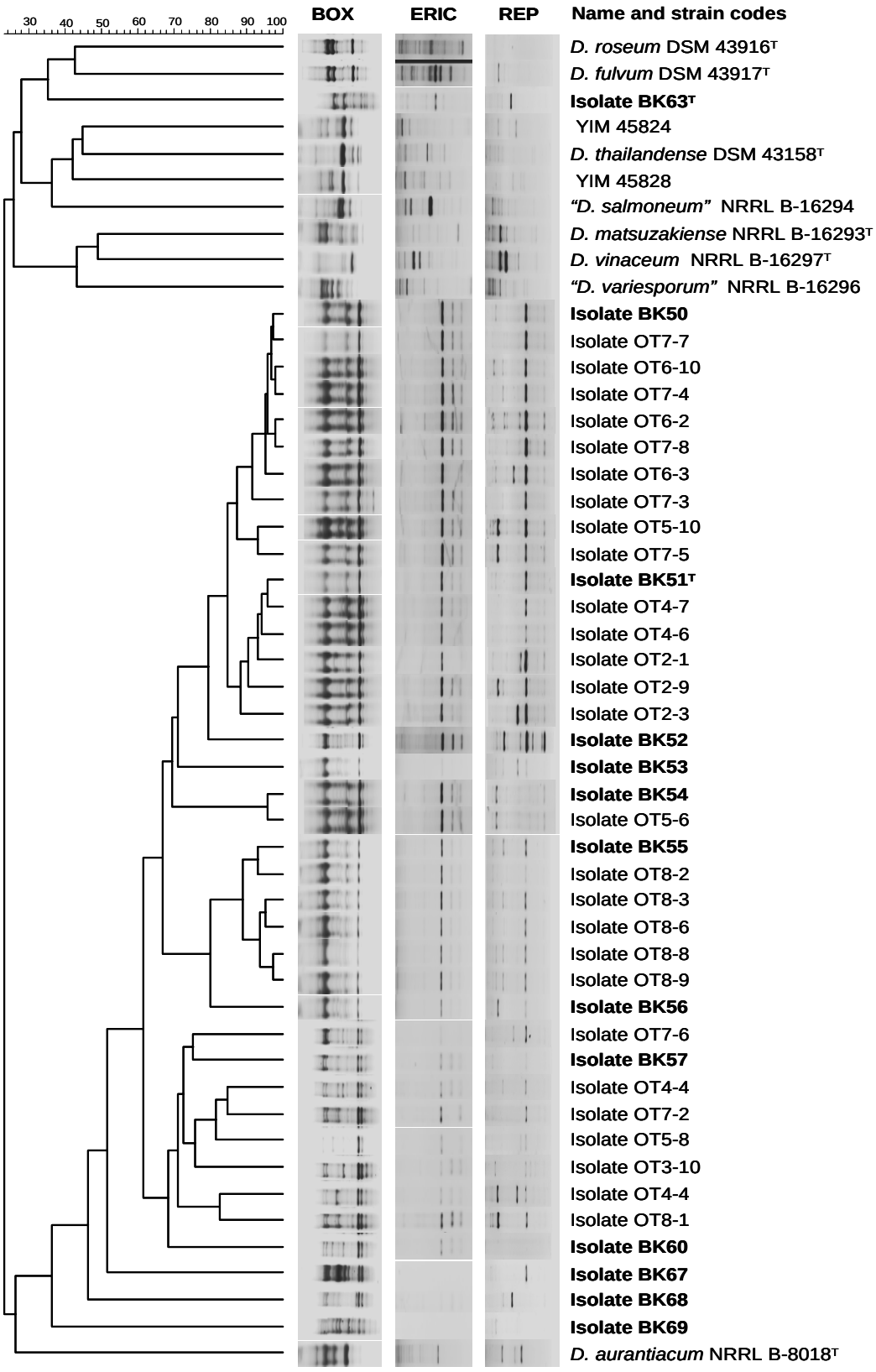


Fig. 5.1. A consensus dendrogram showing relationships between the isolates and reference *Dactylosporangium* strains based on similarity values derived by using Pearson's correlation coefficient and the UPGMA algorithm in an analysis of *rep*-PCR fingerprints generated by using BOX, ERIC and REP-PCR primers. Isolates given in bold were included in the 16S rRNA gene sequencing studies.

Phylogenetic analysis of 16S rRNA and *rpoB* genes. In the 16S rRNA *Micromonosporaceae* gene tree, the isolates, "*D. salmoneum*" NRRL B-16294 and the *Dactylosporangium* type strains formed a well delineated clade (Fig. 5.2). The members of the taxon were most closely related to representatives of the genus *Virgisporangium*, a relationship which was supported by all of the tree-making algorithms (Fig. 5.3). All but one of the isolates had identical 16S rRNA gene sequences and formed a distinct subclade together with the type strain of *D. aurantiacum*; they shared a similarity of 99.4% with the latter, a value that corresponded to 9 nucleotide differences over 1435 locations. Relatively high 16S rRNA gene similarities were shown with the type strains of *D. fulvum* (98.1%), *D. matsuzakiense* (98.3%), *D. roseum* (98.2%), *D. thailandense* (98.5%) and *D. vinaceum* (98.2%), as well as with the "*D. salmoneum*" strain (98.2%). The remaining isolate, strain BK63^T, also shared its highest affinity with *D. aurantiacum* NRRL B-8018^T; these organisms had a similarity of 98.9%, a value equivalent to 16 nucleotide differences across 1436 sites. The isolates together with the type strain of *D. aurantiacum* formed a subclade that was supported by all of the tree-making algorithms.

A second 16S rRNA subclade contained "*D. salmoneum*" NRRL B-16294 and the type strains of *D. matsuzakiense* and *D. vinaceum*. The close relationship between this taxon, the *D. matsuzakiense* subclade, and the *D. aurantiacum* subclade is underpinned by all of the tree-making algorithms. "*D. salmoneum*" NRRL B-16294 and the type strains of *D. matsuzakiense* and *D. vinaceum* shared similarities of 98.9% and 98.8%, respectively, values that corresponded to 16 and 17 nucleotide differences over 1432 locations.

D. matsuzakiense DSM 43810^T and *D. vinaceum* DSM 43823^T showed a 16S rRNA gene similarity of 99.6%, a value equivalent to 6 nucleotide differences over 1468 locations. *D. fulvum* DSM 43917^T and *D. roseum* DSM 43916^T, the two most closely related type strains showed a similarity of 99.7%, this is equivalent to 5 nucleotide differences across 1471 sites. These strains, together with *D. thailandense* DSM 43158^T, formed a well delineated taxon, the *D. thailandense* subclade, the integrity of which was underscored by all of the tree-making algorithms and by a bootstrap value of 94%.

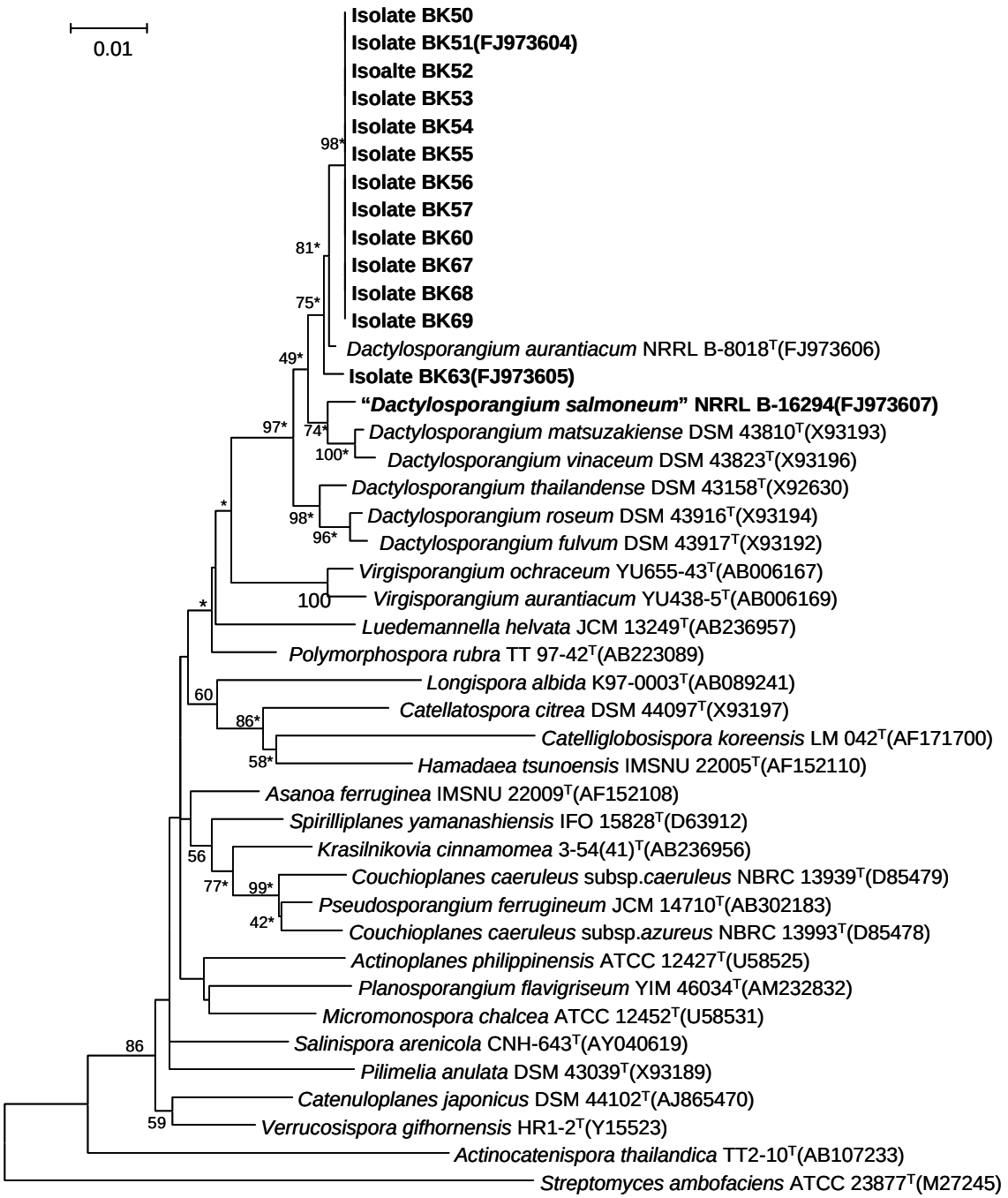


Fig. 5.2. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between the isolates, "*D. salmoneum*" NRRL B-16294, the type strains of recognised *Dactylosporangium* species and members of genera classified in the family *Micromonosporaceae*. Asterisks indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at the nodes indicate the levels of bootstrap support (%), only values above 50% are shown. Bar, 0.01 substitutions per site.

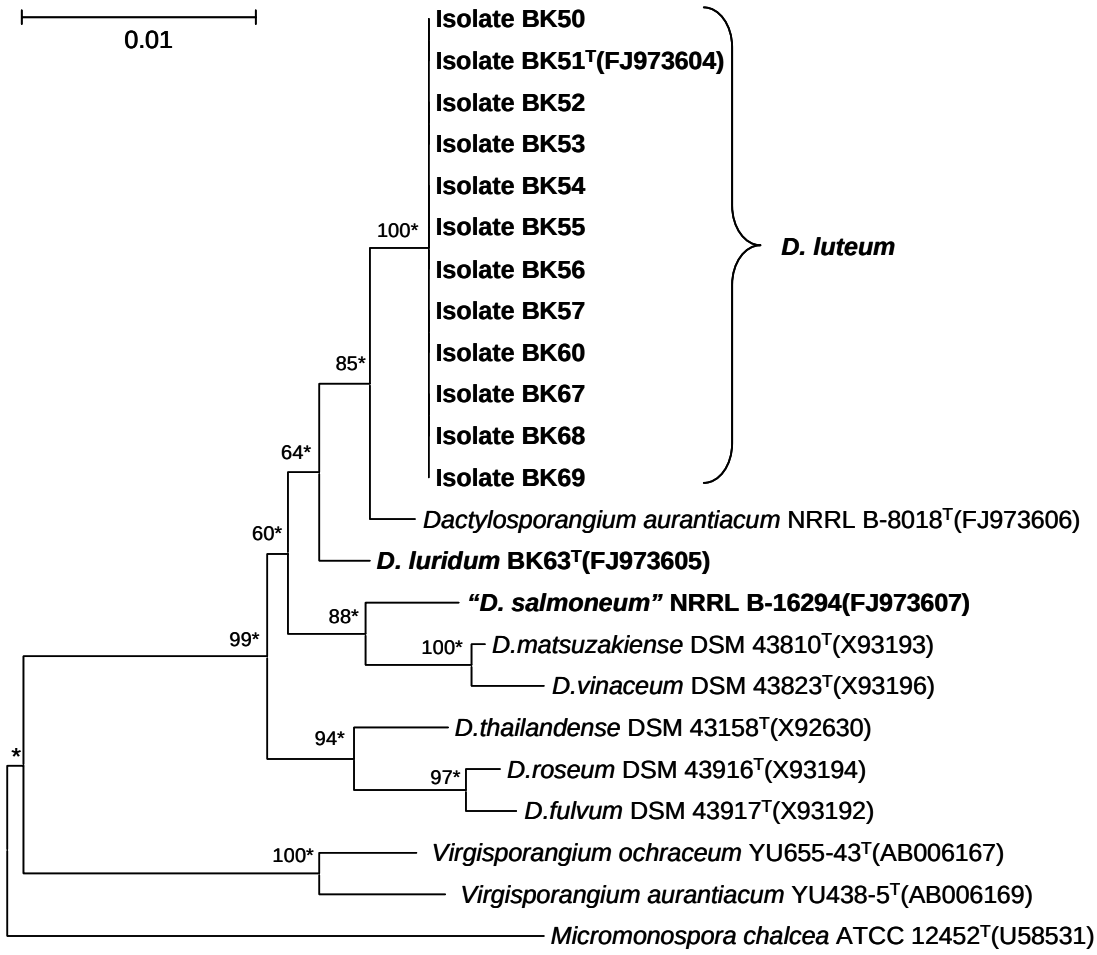


Fig. 5.3. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between strains BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294 and the type strains of *Dactylosporangium* and *Virgisporangium* species. The root position of the neighbour-joining tree was obtained using *Micromonospora chalcea* ATCC 12452^T as the outgroup. The same tree-making algorithms and criteria were used as detailed in Figure 5.2.

Phylogenetic analyses based on partial RNA polymerase β–subunit (*rpoB*) gene sequences have provided valuable data in polyphasic studies designed to clarify relationships within and between genera of actinomycetes (Kim *et al*., 1999, 2004; Goodfellow *et al*., 2007). Isolates BK51^T, BK53 and BK69 had identical *rpoB* sequences and formed a subclade with *D. aurantiacum* NRRL B-8018^T, the integrity of this taxon was underpinned by all of the tree-making algorithms and by a 95% bootstrap value. The remaining isolate, strain BK63^T, formed a distinct single membered subclade at the periphery of the *Dactylosporangium rpoB* gene tree. The "*D. salmoneum*" strain also formed a distinct subclade that was associated with *D. roseum* DSM 43917^T. These results are in good agreement with those from the 16S rRNA analyses as they show that the isolates fall into one distinct multimembered and one single membered clade though discrepancies are apparent between the topologies of the two trees (see Figures. 5.3 and 5.4).

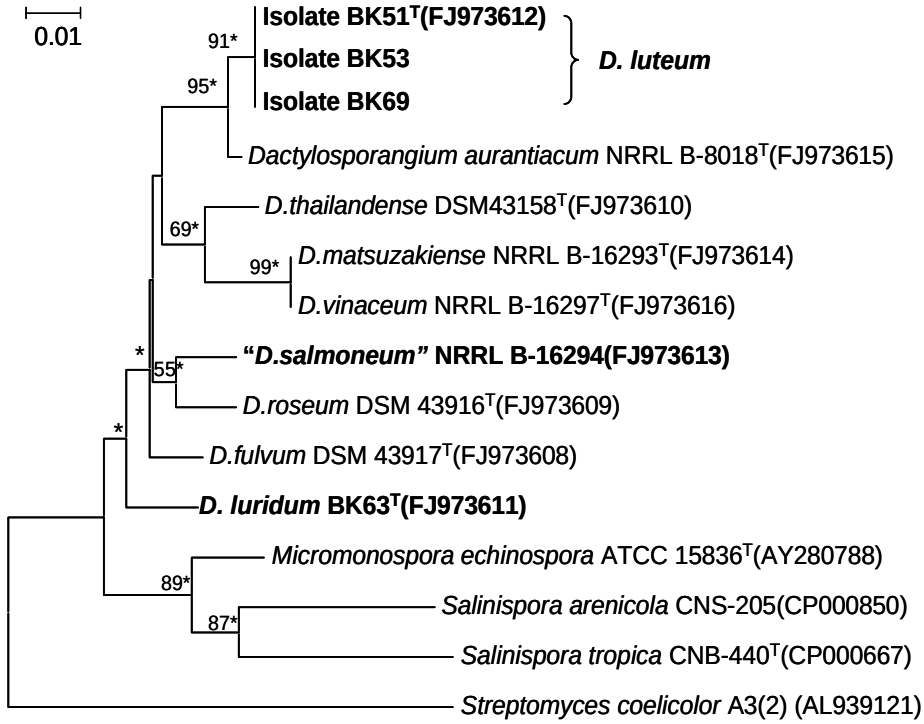


Fig. 5.4. Neighbour-joining tree based on partial *rpoB* gene sequences showing relationships between strains BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294, the type strains of recognised *Dactylosporangium* speices and members of genera classified in the family *Micromonosporaceae*. The same tree making algorithms and criteria were used as cited in Figure 5.2.

DNA:DNA relatedness. Isolate BK51^T showed very low ΔTm values (0.2-1.6°C) with isolates BK53 and BK69 (Table 5.1), values well below the cut-off point recommended for the delineation of genomic species (ΔTm > 5.0°C; Wayne *et al*. 1987). In contrast, isolate BK51^T shows ΔTm levels well above this cut-off point with isolate BK63^T, "*D. salmoneum*" NRRL B-16294 and the type strains of *D. aurantiacum*, *D. matsuzakiense* and *D. vinaceum*. Isolate BK63^T also exhibits relatively high ΔTm values (5.8-6.2°C) with the type strains of related *Dactylosporangium* species and with "*D. salmoneum*" NRRL B-16294, indicating that it belongs to a distinct genomic species. "*D. salmoneum*" NRRL B-16294 can also be assigned to a new genomic species as it exhibits high ΔTm values (5.8-7.5°C) with its nearest neighbours, namely *D. matsuzakiense* NRRL B-16293^T and *D. vinaceum* NRRL B-16297^T. The strains had DNA G+C contents within the range 70-74 mol % (see Table 5.4).

Table 5.1. Levels of DNA-DNA relatedness between isolates BK51^T, BK63 ^{T} and "*D. salmoneum*" NRRL B-16294, and their closest phylogenetic neighbours based on Δ*Tm* values

Strains	Δ*Tm* (°C)	DNA-DNA relatedness (%)	Nucleotide base differences/ total number of 16S rRNA nucleotides
Isolate BK51^T vs:			
Isolate BK53	1.6	82	0/1444
Isolate BK69	0.2	89	0/1444
Isolate BK63^T	7.2	53	11/1439
D. aurantiacum NRRL B-8018^T	7.2	53	9/1435
*D. matsuzakiens*e NRRL B-16293^T	7.6	51	24/1430
D. salmoneum NRRL B-16294^T	5.8	61	21/1431
D. vinaceum NRRL B-16297^T	9.5	42	26/1430
Isolate BK63 vs:			
D. aurantiacum NRRL B-8018^T	6.2	59	16/1436
*D. matsuzakiens*e NRRL B-16293^T	5.8	61	22/1432
"*D. salmoneum*" NRRL B-16294	6.0	60	18/1433
D. vinaceum NRRL B-16297^T	5.8	61	23/1432
"*D. salmoneum*" NRRL B-16294 vs:			
D. aurantiacum NRRL B-8018^T	7.5	52	23/1407
*D. matsuzakiens*e NRRL B-16293^T	6.0	60	16/1468
D. vinaceum NRRL B-16297^T	6.5	57	17/1476

Chemotaxonomical properties. All of the organisms contained mixtures of 3-hydroxy and *meso*-diaminopimelic acid, *N*-glycolylmuramic acid, hexahydrogenated and octahydrogenated menaquinones with nine isoprene units (MK-9[H_6] and MK-9[H_8]) as predominant menaquinones, and arabinose, galactose, glucose, mannose and xylose in whole-organism hydrolysates, mycolic acids were not found. The isolates contained 13-methyltetradecanoic acid (*iso*-$C_{15:0}$) and 14-methylpentadecanoic acid (*iso*-$C_{16:0}$) as predominant fatty acids with varying kinds and proportions of minor compounds, and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol as major polar lipids (phospholipid pattern type 2 *sensu* Lechevalier *et al*., 1977) with a discontinous distribution of phosphatidylinositol mannoside, an unknown aminolipid and an unknown phospholipid (Table 5.2). These results are in good agreement with those from previous studies (Kroppenstedt, 1985; Vobis, 2006).

Table 5.2. Chemotaxonomic properties of strains BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294 and the type strains of recognised *Dactylosporangium* species

Strains: 1, BK51^T; 2, BK63^T; 3, *D. aurantiacum* NRRL B-8018^T, 4, *D. fulvum* DSM 43917^T; 5, *D. matsuzakiense* NRRL B-16293^T; 6, *D. roseum* DSM 43916^T; 7, "*D. salmoneum*" NRRL B-16294; 8, *D. thailandense* DSM 43158^T; 9, *D. vinaceum* NRRL B-16297^T. All data were acquired in the present study. −, Not detected.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Fatty acids (% of total)									
iso-$C_{14:0}$	3.3	4.0	4.9	2.8	1.5	1.2	1.4	1.9	6.8
anteiso-$C_{15:0}$	8.1	9.7	3.5	4.2	3.4	2.2	4.7	4.0	10.0
iso-$C_{15:0}$	38.7	45.0	27.1	32.0	18.4	27.8	33.5	30.1	17.5
$C_{16:0}$	2.0	0.7	1.6	4.6	1.1	3.3	0.4	0.5	-
iso-$C_{16:0}$	21.1	13.9	42.3	25.6	43.0	32.9	31.3	36.7	54.6
iso-$C_{16:1}$ G	2.2	2.0	0.3	-	7.9	-	3.0	7.1	-
$C_{17:0}$	2.1	5.8	1.4	1.8	1.1	-	0.3	0.9	2.4
10-methyl $C_{17:0}$	2.1	4.0	1.8	0.6	2.7	4.8	1.2	1.8	-
anteiso-$C_{17:0}$	5.8	3.4	4.1	5.7	7.2	4.7	9.1	6.5	8.8
iso-$C_{17:0}$	4.0	2.0	4.3	5.1	3.5	4.4	7.0	4.0	-
$C_{17:1}$ *w*8*c*	1.9	5.4	0.5	-	2.1	-	0.2	0.6	-
$C_{18:0}$	1.4	0.2	1.6	5.7	1.1	2.4	0.5	0.4	-
$C_{18:1}$ *w*9*c*	2.7	0.4	2.4	2.1	1.9	5.4	0.4	0.4	-
Menaquinones (% of total)									
MK-9 (H_2)	-	15	-	-	-	-	-	-	-
MK-9 (H_4)	10	25	10	-	-	-	14	-	-
MK-9 (H_6)	23	50	20	28	10	15	47	10	16
MK-9 (H_8)	66	5	69	71	90	82	36	88	80
Major polar lipids*	DPG, PE, PG, PI, PL	DPG, PE, PG, PI, PL	DPG, PE, PG, PI, PIM, PL	DPG, PE, PG, PI	DPG, PE, PG, PI, PL	DPG, PE, PG, PI	DPG, PE, PG, PI, PL	DPG, PE, PG, PI, PIM, PL	DPG, PE, PG, PI, PL

*DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine;
PG, phosphatidylglycerol; PI, phosphatidylinositol;
PIM, phosphatidylinositol mannosides; PL, unknown phospholipid.

Biochemical, cultural and morphological properties. All of the strains grew well on inorganic salts-starch, oatmeal and tyrosine agar plates producing a range of substrate mycelial colours but relatively few diffusible pigments (Table 5.3). The type strains of *D. roseum*, *D. thailandense* and *D. vinaceum* together with "*D. salmoneum*" NRRL B-16294 grew well on all of the media except glycerol-asparagine agar. In contrast, isolates BK51^T and BK63^T grew poorly on the glycerol-asparagine, peptone-yeast extract-iron, tryptone-yeast extract and yeast extract-malt extract agar plates. In general, few of the organisms produced soluble pigments on the agar media though *D. vinaceum* NRRL B-16297^T formed soluble pigments on all of these media. It is also interesting that the two isolates can be distinguished by the colour of the substrate mycelia they produced on the various media.

All of the strains formed irregular branched substrate hyphae (0.3-0.7 μm in diameter), which penetrated the agar. Similarly, all three strains formed sporangia which released motile spores. Motile spores were formed in finger-like, short, narrow sporangia produced directly on substrate mycelium (Fig. 5.5). They also formed globose bodies with smooth surfaces at the top of short sporophores on substrate hyphae.

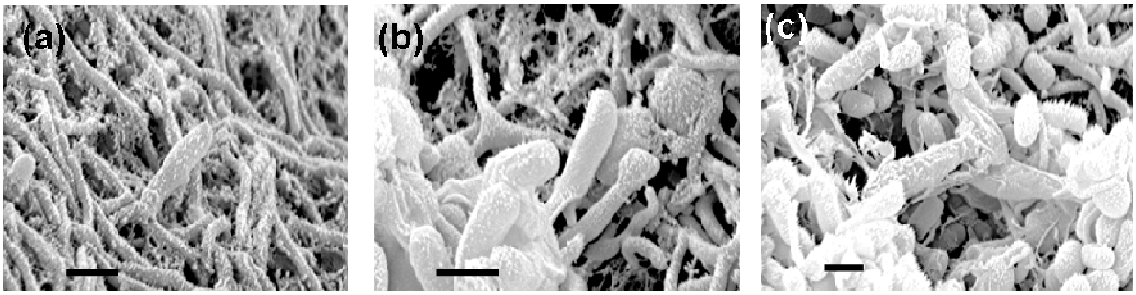


Fig. 5.3. Scanning electron micrographs of isolate BK51^T grown on ISP 2 medium for 2 weeks (a), isolate BK63^T grown on ISP 7 medium for 2 weeks (b) and strain "*D. salmoneum*" NRRL B-16294 grown on ISP 7 medium for 8 weeks (c). Bar, 1.0 μm.

All of the strains were positive for catalase, degraded aesculin and xylan, used glycogen, D(-)-glucose and sucrose as sole carbon sources, and were sensitive to novobiocin (8 μg ml^{-1}) and rifampicin (16 μg ml^{-1}). All were negative for nitrite reduction, did not degrade adenine, cellulose, chitin, guanine, hypoxanthine, pectin, tributyrin, Tween 20, uric acid or xanthine, did not use L(+)- lactic acid, L(-)-sorbose, oxalic acid or D(-)-ribose as sole carbon sources and did not grow in the presence of 3% NaCl (w/v) or 0.05% lysozyme (Table 5. 4).

Table 5.3. Growth and cultural characteristics of the strains, BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294 and the type strains of recognised *Dactylosporangium* species

Strains: 1, BK51^T; 2, BK63^T; 3, *D. aurantiacum* NRRL B-8018^T; 4, *D. fulvum* DSM 43917^T ; 5, *D. matsuzakiens*e NRRL B-16293^T; 6, *D. roseum* DSM 43916^T; 7, "*D. salmoneum*" NRRL B-16294; 8, *D. thailandense* DSM 43158^T; 9, *D. vinaceum* NRRL B-16297^T. Data were obtained in this study.

Culture medium	1	2	3	4	5	6	7	8	9
Glycerol-asparagine agar :									
Growth	Poor	Poor	Poor	Poor	Poor	Poor	Moderate	Moderate	Poor
Substrate mycelium	Orange	Light yellow	Light yellow	Light yellow	Orange	Light yellow	Light yellow	Light yellow	Orange
Soluble pigment	None	None	None	None	None	None	None	None	Light brown
Inorganic salts-starch agar :									
Growth	Abundant	Moderate	Abundant	Abundant	Abundant	Abundant	Abundant	Abundant	Abundant
Substrate mycelium	Orange	Light yellow	Light yellow	Brown	Reddish brown	Light yellow	Light orange	Light brown	Deep purple
Soluble pigment	None	None	None	Light brown	None	None	None	Light brown	Black purple
Oatmeal agar :									
Growth	Abundant	Moderate	Abundant	Abundant	Abundant	Abundant	Abundant	Abundant	Abundant
Substrate mycelium	Orange	Light yellow	Orange	Yellow	Reddish orange	Orange	Deep orange	Light brown	Purple
Soluble pigment	None	None	None	Light brown	None	None	None	Light brown	Black purple
Peptone-yeast extract-iron agar :									
Growth	Poor	Poor	Poor	Poor	Poor	Moderate	Moderate	Moderate	Moderate
Substrate mycelium	Orange	Light yellow	Yellow	Yellow	Orange	Orange	Yellow	Orange	Reddish orange
Soluble pigment	None	None	None	None	None	None	None	None	Brown
Tryptone-yeast extract agar :									
Growth	Poor	Poor	Moderate	Moderate	Moderate	Moderate	Abundant	Abundant	Moderate
Substrate mycelium	Orange	Light yellow	Orange	Light brown	Orange	Orange	Yellow	Orange	Reddish brown
Soluble pigment	None	None	None	None	None	None	None	None	Brown
Tyrosine agar :									
Growth	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Substrate mycelium	Orange	Light yellow	Orange	Reddish yellow	Light brown	Light yellow	Light brown	Yellow	Reddish orange
Soluble pigment	Light brown	None	Light brown	None	Light brown	None	None	None	Light brown
Yeast extract-malt extract agar :									
Growth	Poor	Poor	Moderate	Abundant	Moderate	Moderate	Abundant	Abundant	Moderate
Substrate mycelium	Orange	Light yellow	Orange	Light brown	Orange	Orange	Yellow	Orange	Reddish brown
Soluble pigment	None	None	None	None	None	None	None	Light yellow	Light brown

Table 5.4. Differential phenotypic characteristics of strains BK51^T and BK63^T, "*D. salmoneum*" NRRL B-16294 and the type strains of recognized *Dactylosporangium* species.

Strains: 1, BK51^T (identical results for strains BK53 and BK69); 2, BK63^T; 3, *D. aurantiacum* NRRL B-8018^T; 4, *D. fulvum* DSM 43917^T; 5, *D. matsuzakiens*e NRRL B-16293^T; 6, *D. roseum* DSM 43916^T; 7, "*D. salmoneum*" NRRL B-16294; 8, *D. thailandense* DSM 43158^T; 9, *D. vinaceum* NRRL B16297^T. +, positive; –, negative. All data were obtained in the present study.

Characteristics	1	2	3	4	5	6	7	8	9
Biochemical tests :									
Allantoin hydrolysis	-	-	-	-	-	+	-	+	-
Coagulation and peptonization of milk	-	-	-	-	-	-	-	-	+
Hydrogen sulphide production	-	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	-	+	+	-	-
Urease production	-	-	+	+	-	-	-	+	+
Degradation tests :									
Arbutin	+	-	+	+	+	+	+	+	+
Casein	-	-	-	-	-	+	+	-	+
DNA	-	-	+	+	-	-	+	-	-
Elastin	-	-	+	-	-	+	-	-	-
Gelatin	-	-	+	+	-	+	+	+	+
RNA	-	-	-	-	-	-	+	-	-
Starch	+	+	-	+	+	-	+	+	+
Tween 40	-	-	-	+	+	+	+	+	+
Tween 60	-	+	+	+	+	+	+	+	+
Tween 80	-	-	-	+	+	-	+	+	-
Sole carbon sources (1.0%, w/v) :									
Adonitol	+	-	-	+	-	-	+	-	-
L(+)-Arabinose	+	-	-	-	-	-	+	-	-
D(+)-Arabitol	+	-	-	-	-	-	-	-	-
D(+)-Cellobiose	+	-	+	+	-	+	+	+	-
Dextrin	+	-	-	+	-	-	+	-	-
D(-)-Fructose	+	-	-	-	-	-	+	+	-
D(+)-Galactose	+	-	-	+	-	-	+	+	-
Glycerol	+	-	+	+	-	-	+	-	-
Myo-inositol	-	-	-	-	-	-	+	-	-
Inulin	+	-	+	+	-	+	+	+	+
D(+)-Lactose	+	-	+	-	-	-	+	+	-
D(+)-Maltose	+	-	-	-	-	+	+	+	-
D(+)-Mannitol	+	-	-	+	-	-	+	+	-
D(+)-Mannose	+	-	-	-	-	+	+	+	-
D(+)-Melibiose	+	-	+	-	-	-	+	-	-
α-Methyl D-glucoside	+	-	+	+	-	-	+	+	-
D(+)-Raffinose	+	-	+	+	-	+	+	+	-
L(-)-Rhamnose	+	-	+	-	-	-	+	+	-
Salicin	-	-	+	+	-	-	+	-	-
Starch	+	+	+	+	+	-	+	+	+
D(+)-Trehalose	+	+	+	+	-	-	+	+	-
D(+)-Xylose	+	+	+	+	-	-	+	+	-

Table 5.4 (*Continued*).

Sole carbon sources (0.1%, w/v) :									
Citric acid	+	-	-	-	-	-	+	+	-
L(+)-Lactic acid	+	-	-	+	-	-	-	+	-
Malic acid	+	-	-	+	-	+	+	+	-
Propionic acid	+	-	-	+	-	+	+	-	-
Pyruvic acid	+	-	+	+	+	+	+	+	-
L(+)-Tartaric acid	+	-	-	+	-	-	+	+	-
Growth at :									
15°C	+	-	+	-	+	-	-	+	-
37°C	-	-	+	+	+	+	+	+	-
pH4.0	-	+	-	-	+	+	+	+	-
pH10.0	+	-	-	+	-	-	+	+	-
Production of melanin pigments on tyrosine agar	+	+	+	-	-	-	-	-	+
Growth in presence of :									
NaCl 1.5%, w/v	+	+	-	-	+	-	+	-	-
Resistance to antibiotics (μg ml^{-1}) :									
Ampicillin (4)	-	-	-	-	+	-	+	+	+
Cephaloridine (2)	-	-	-	-	+	-	+	+	-
Chloramphenicol (8)	-	-	-	+	-	-	-	+	-
Ciprofloxacin (2)	-	-	+	+	+	+	+	+	-
Clindamycin (8)	+	-	+	+	+	-	+	+	-
Gentamicin (8)	+	-	+	+	+	-	+	+	+
Kanamycin (8)	-	-	-	-	+	-	+	-	+
Lincomycin (8)	+	-	+	+	+	-	+	+	+
Oxytetracycline (8)	-	-	+	+	+	-	+	+	-
Penicillin G (2)	+	-	-	-	+	+	+	+	+
Streptomycin (4)	-	-	-	+	-	-	-	-	-
Tetracycline (8)	-	-	+	+	-	-	-	-	-
Tylosin (8)	-	-	+	+	+	-	+	+	-
Vancomycin (2)	-	-	-	-	+	-	-	+	+
DNA G+C content (mol%)	74	70	73	71	73	73	73	71	73

Isolates $BK51^T$, BK53 and BK69 had identical phenotypic profiles which served to distinguish them from isolate $BK63^T$, "*D. salmoneum*" NRRL B-16294 and the type strains of recognised *Dactylosporangium* species. Indeed, the three isolates were the only strains to use D(+)-arabitol as a sole carbon source and to be unable to produce H_2S or degrade Tween 80. Similarly, unlike most of the other strains they used adonitol, L(+)-arabinose, dextrin, D(-)-fructose and D(+)-melibiose as sole carbon sources, but were unable to hydrolyse gelatin or grow at 37°C. Isolate $BK63^T$ and "*D. salmoneum*" NRRL B-16294 were also readily separated from one another and from the *Dactylosporangium* type strains, notably by the inability of strain $BK63^T$ to metabolise

most of the sole carbon sources and by the activity of the "*D. salmoneum*" NRRL B-16294 on the degradation tests.

5.5 Conclusions

The genotypic and phenotypic data clearly show that isolates BK51^T, BK53 and BK69, isolate BK63^T and "*D. salmoneum*" NRRL B-16294 represent three new centres of taxonomic variation in the genus *Dactylosporangium*. It is therefore proposed that these strains be classified in the genus *Dactylosporangium* as representing *Dactylosporangium luteum* sp. nov., *Dactylosporangium luridum* sp. nov., and *Dactylosporangium salmoneum*, respectively. The genotypic data acquired on isolates BK50, BK52, BK54-BK57, BK60, BK67 and BK68 are consistent with their assignment to *D. luteum*.

Description of *Dactylosporangium luridum* sp. nov.

Dactylosporangium luridum (lu'ri.dum. L. neut. adj. *luridum*, pale yellow).

Aerobic, Gram-positive, motile actinomycete which forms branched, pale yellow coloured mycelia on inorganic salts-starch and oatmeal agars. Short and narrow finger-like sporangia (1.0-1.2 × 2.0-2.5 μm), which release motile spores, are formed on glycerol-asparagine and tyrosine agars. Globose bodies with smooth surfaces are produced on short sporophores arising from substrate hyphae. Melanin pigments are not formed on either peptone-yeast extract-iron or tyrosine agars. Grows well on inorganic salts-starch, oatmeal and tyrosine agars, but poorly on glycerol-asparagine, peptone-yeast extract-iron, tryptone-yeast extract and yeast extract-malt extract agars. Grows well between 20 and 30°C (optimally around 28°C), and from pH 4-9 (optimally around pH 7). Additional phenotypic properties are cited in the main text and in Table 5.4. Chemotaxonomic properties are consistent with classification in the genus *Dactylosporangium*. The organism forms a distinct phyletic line in the *Dactylosporangium* 16S rRNA gene sequence tree. The G+C content of the DNA of the type strain is 70 mol%.

The type strain, BK63^T (= DSM 45324^T =KACC 20933^T = NRRL B-24775^T), was isolated from a soil sample taken from Palace Leas hay meadow plot 6 at Cockle Park Experimental Farm, Northumberland, UK.

Description of *Dactylosporangium luteum* sp. nov.

Dactylosporangium luteum (lu'te.um. L. neut. adj. *luteum*, orange-yellow, flame-coloured).

Aerobic, Gram-positive, motile actinomycetes which forms branched, orange-yellow coloured mycelia on oatmeal and inorganic salts-starch agars. Short and narrow finger-like sporangia (1.0-1.2 × 2.0-2.5 μm), which release motile spores, are formed on glycerol-asparagine, tyrosine and yeast extract-malt extract agars. Globose bodies with smooth surfaces are produced on short sporophores arising from substrate hyphae. Melanin pigments are not formed on either peptone-yeast extract-iron or tyrosine agars. Grows well on inorganic salts-starch, oatmeal and tyrosine agars, but poorly on glycerol-asparagine, peptone-yeast extract-iron, tryptone-yeast extract and yeast extract-malt extract agars. Grows well between 15 and 30°C (optimally around 28°C), and from pH 5-10 (optimally around pH 7). Additional phenotypic properties are cited in the main text and in Table 5.4. Chemotaxonomic properties are consistent with classification in the genus *Dactylosporangium*. The organism forms a distinct phyletic line in the *Dactylosporangium* 16S rRNA gene sequence tree. The G+C content of the DNA of the type strain is 74 mol%.

The type strain, BK51^T (= DSM 45323^T= KACC 20899^T = NRRL B-24774^T) , was isolated from a soil sample taken from Palace Leas hay meadow plot 6 at Cockle Park Experimental Farm, Northumberland, UK.

Description of *Dactylosporangium salmoneum* sp. nov.

Dactylosporangium salmoneum (sal.mo.ne'um. L. n. *salmo -onis*, salmon; L. adj. suff. *-eus -a -um*, suffix used with various meanings; N.L. neut. adj. *salmoneum*, salmon-coloured).

The description is based on data from the present study and from Celmer *et al*. (1978). Aerobic, Gram-positive, motile actinomycete that forms branched, orange

coloured mycelia on inorganic salts-starch and oatmeal agars. Numerous sporangia are formed on calcium malate plates. Dactyloform sporangia are enlarged slightly towards the apex (1.5 × 5.5 μm) and contain 3 to 4 spores, which are mainly elliptical (1.1-1.6 × 2.2-2.7 μm) and are motile. Globose bodies with smooth surfaces are formed at the top of short sporophores on substrate hyphae. Melanin pigments are not produced on either peptone-yeast extract-iron or tyrosine agars. Good growth is shown on glycerol-asparagine, inorganic salts-starch, oatmeal, peptone-yeast extract-iron, tryptone-yeast extract, tyrosine and yeast extract-malt extract agars; grows poorly on Luria-Bertani, nutrient and trypticase soy agars. Grows well between 20 and 37 °C (optimally around 28°C), and at pH 4-10 (optimally around pH 7). Additional phenotypic properties are cited in the main text and in Table 5.4. Chemotaxonomic properties are consistent with classification in the genus *Dactylosporangium*. The organism forms a distinct phyletic line in the *Dactylosporangium* 16S rRNA gene sequence tree. The G+C content of the DNA of the type strain is 73 mol%.

The type strain, NRRL B-16294^T (=ATCC 31222^T =DSM 43910^T = JCM 3272^T = NBRC 14103^T), was isolated from soil in Japan.

Chapter 6. Reclassification of "*Dactylosporangium variesporum*" as *Saccharothrix variisporea* corrig. (ex Tomita *et al*. 1977) sp. nov., nom. rev.

6.1 Abstract

In the course of a polyphasic study it was observed that "*Dactylosporangium variesporum*" NRRL B-16296 is misclassified in the genus *Dactylosporangium* as it exhibits properties consistent with its assignment to the genus *Saccharothrix*. Phylogenetic analyses based on 16S rRNA gene sequences show that the strain falls within the evolutionary radiation of the genus *Saccharothrix*, a result which is supported by corresponding chemotaxonomic and morphological markers. The strain is phylogenetically most closely, albeit loosely, related to *Saccharothrix espanaensis,* but can be readily distinguished from this and other validly described *Saccharothrix* species by using a range of phenotypic properties. The combined genotypic and phenotypic data demonstrate conclusively that this strain should be classified as a new species in the genus *Saccharothrix* for which the name *Saccharothrix variisporea* sp. nov. is proposed. The type strain is NRRL B-16296^T(= ATCC 31203^T = DSM 43911^T = JCM 3273^T = NBRC 14104^T).

6.2 Introduction

"*Dactylosporangium variesporum*" sp. nov. was proposed by Tomita *et al*. (1977) to accommodate a strain isolated from a soil sample collected in India which they considered to have chemical and morphological properties consistent with its assignment to the genus *Dactylosporangium*. The microorganism, strain D409-5, produces capreomycin and was reported by Tomita and his colleagues to form finger-shaped sporangia, singly, in pairs or in clusters on substrate hyphae, to contain *meso*-diaminopimelic acid (*meso*–A_2pm) in the cell wall peptidoglycan, and galactose, mannose and rhamnose in whole-cell hydrolysates. Uchida & Seino (1997), however, questioned the placement of this strain in the genus *Dactylosporangium* as they were unable to observe sporangia and found that the peptidoglycan contained N-glycolated as opposed to N-acetylated muramic acid moieties.

When "*D. variesporum*" NRRL B-16296 was included in a study designed to clarify the taxonomy of invalidly described *Dactylosporangium* species, it was found to have properties consistent with its assignment to the genus *Saccharothrix*. In the present investigation, strain NRRL B-16296 (= strain D409-5) was the subject of a polyphasic study which showed that it formed a novel *Saccharothrix* species for which the name *Saccharothrix variisporea* sp. nov. is proposed.

6.3 Materials and methods

Cultivation and maintenance of the strains. "*D. variesporum*" NRRL B-16296 and the equivalent strain DSM 43911 were maintained at −20°C as glycerol suspensions (20%, v/v). Biomass for 16S rRNA gene sequence analyses of these strains and for the chemotaxonomic analyses of strain NRRL B-16296 was prepared in modified Bennett's broth (Jones, 1949) at 150 rpm in shake flasks for 21 days at 28°C. Cells were checked for purity and harvested by centrifugation. Biomass for chemotaxonomic analyses of the strain was washed twice in distilled water and freeze-dried. Cells collected for DNA isolation and sequencing of 16S rRNA genes were washed in NaCl/EDTA buffer (0.1M EDTA, 0.1M NaCl, pH8.0) and stored at −20°C.

Molecular sequencing and phylogenetic analysis. Preparation of genomic DNA, amplification and direct sequencing of the purified PCR products of 16S rRNA genes prepared from strains NRRL B-16296 and DSM 43911 were performed after Tan *et al*. (2006). The resultant, almost complete, 16S rRNA gene sequences (1458 nucleotides) were aligned manually, using the jPHYDIT program (Jeon *et al*., 2005), against corresponding sequences of the type strains of *Saccharothrix* species retrieved from the GenBank database. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony algorithms (Takahashi & Nei, 2000) from the MEGA 3.1 program (Kumar *et al*., 2004). The evolutionary distance model of Jukes & Cantor (1969) was used to generate an evolutionary distance matrix for the neighbour-joining algorithm. The topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset. The root position of the neighbour-joining tree was obtained using *Pseudonocardia thermophila* IMSNU 20112^T as the outgroup.

Chemotaxonomical analyses. Chemotaxonomic studies were undertaken to confirm that strain NRRL B-16296^T exhibited a chemical profile which supported its classification in the genus *Saccharothrix*. Standard procedures were used to extract and analyse the isomers of diaminopimelic acid (Staneck & Roberts, 1974), isoprenoid quinones (Collins, 1994), muramic acid type (Uchida *et al*., 1999), polar lipids (Minnikin *et al*., 1984) and whole-organism sugars (Schaal, 1985), using appropriate controls. Cellular fatty acids were extracted, methylated and analyzed by GC using the standard Sherlock MIDI (Microbial Identificatin) system (Sasser, 1990) and mycolic acids sought using the TLC procedure described by Minnikin *et al*. (1975).

Biochemical, cultural and morphological analysis. Strain NRRL B-16296^T was examined for a broad range of phenotypic properties using standard media and methods described by Goodfellow *et al.* (1979). The gross morphological properties of the strain were observed on plates of tryptone-yeast extract, yeast extract-malt extract, oatmeal, inorganic salts-starch, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agar plates (ISP media 1-7, respectively; Shirling and Gottlieb, 1966) which were incubated for 21 days at 28°C. Morphological characteristics were observed by examining gold-coated, dehydrated preparations from a 14-day-old culture of the strain grown on inorganic salts-starch agar at 28°C, using a Cambridge Stereoscan 240 scanning electron microscope and the procedure described by O'Donnell *et al*. (1993).

6.4 Results and discussion

Phylogenetic analysis of 16S rRNA gene sequences. Identical 16S rRNA gene sequences were obtained for "*D. variesporum*" NRRL B-16296 and DSM 43911 hence only the sequence for strain NRRL B-16296 was deposited in GenBank (GQ917213) and is considered further. It can be seen in Figure 6.1 that this strain falls within the evolutionary radiation of the genus *Saccharothrix* based on the phylogenetic analysis of 16S rRNA genes. A subclade supported by all of the tree-making algorithms and by a bootstrap value of 61% is formed by this strain and the type strains of *S. espanaensis*, *S. mutabilis* subsp. *capreolus* and *S. mutabilis* subsp. *mutabilis*.

Strain NRRL B-16296^T and *S. mutabilis* subsp. *capreolus* NRRL 2773^T are reported to produce the antibiotic capreomycin though NRRL B-16296^T was found to be most closely related to *S. espanaensis* NRRL 15764^T; the two strains shared a 16S

rRNA gene similarity of 98.9 %, a value which corresponded to 16 nucleotides differences at 1446 locations. The corresponding values between strain 'NRRL B-16296^T' with the type strains of *S. mutabilis* subsp. *capreolus* and *S. mutabilis* subsp. *mutabilis* were 98.5% (21 nucleotides differences at 1425 locations) and 98.8% (18 nucleotides differences at 1430 locations), respectively. The 16S rRNA gene sequence similarities between strain NRRL B-16296^T and the type strains of the remaining *Saccharothrix* species ranged from 97.5% (with *S. violaceirubra*) to 98.5% (with *S. australiensis*). The 16S rRNA of strain NRRL B-16296^T contains the unique oligonucletide signature characteristic for members of the genus *Saccharothrix* (Labeda & Kroppenstedt, 2007).

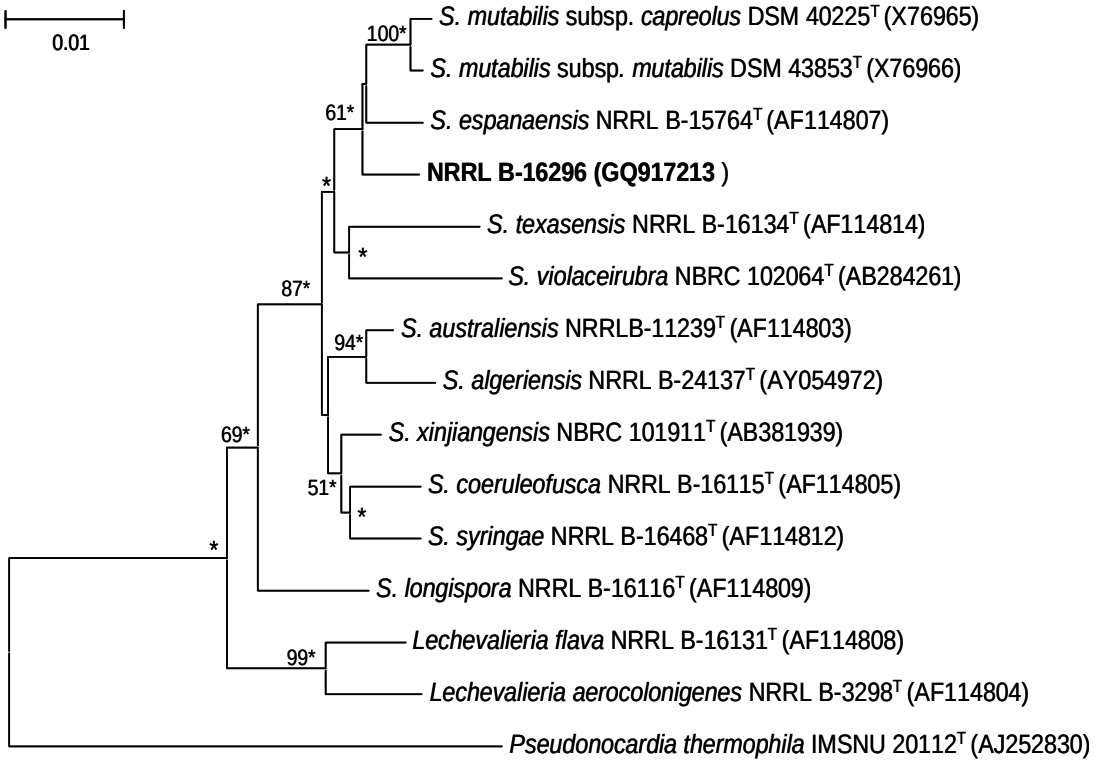


Fig. 6.1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the position of *Saccharothrix variisporea* NRRL B-16296^T in the *Saccharothrix* gene tree. Numbers on branches indicate percentage bootstrap values from 1000 replicates (only values > 50% are shown). Asterisks indicate branches that were also recovered in the minimum-evolution and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

Determination of G+C content (mol%) and DNA–DNA relatedness. The DNA G+C composition of strain NRRL B-16296^T was determined by using the procedure described by Gonzalez & Saiz-Jimenez (2005) and found to be 74 mol %. DNA–DNA relatedness studies were not carried out between strain NRRL B-16296^T and its closest phylogenetic neighbours as type strains of *Saccharothrix* species with similar 16S rRNA

gene sequences have DNA–DNA hybridization values well below the 70% cut-off point recommended by Wayne *et al*. (1987) for the circumscription of strains that belong to the same genomic species. Thus, the type strains of *S. espanaensis* and *S. mutabilis*, have a 16S rRNA gene similarity of 99.0%, but a DNA-DNA relatedness value of 0 % (Labeda & Lechevalier, 1989) whereas the type strains of *S. syringae* and *S. xinjiangensis* share a 16S rRNA gene similarity of 99.1 % but have a DNA–DNA relatedness value of only 50 % (Hu *et al*., 2004). Similarly, *S. algeriensis* NRRL B-24137^T and *S. australiensis* NRRL 11239^T have a 16S rRNA gene similarity of 98.8 % and a DNA–DNA relatedness of 55.9 % (Zitouni *et al*., 2004).

Chemotaxonomic properties. The strain contained *meso*-diaminopimelic acid (wall chemotype Ⅲ *sensu* Lechevalier & Lechevalier 1970), N-acetylated muramic acid moieties, galactose, mannose and rhamnose as diagnostic whole-organism sugars, tetrahydrogenated menaquinones with nine isoprene units (MK-9[H_4]) as the predominant menaquinone, and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol as major polar lipids (phospholipid pattern type Ⅱ *sensu*; Lechevalier *et al*. 1977) together with a trace of phosphatidylinositol mannoside and single unknown aminolipid and phopholipid components. The predominant fatty acids were 13-methyltetradecanoic (*iso*-$C_{15:0}$), 14-methylpentadecanoic (*iso*-$C_{16:0}$) and 14-methylhexadecanoic (*anteiso*-$C_{17:0}$) acids; mycolic acids were not present. All of these properties are in line with the classification of the strain in the genus *Saccharothrix* (Labeda *et al*. 1984; Otoguro *et al*., 2009).

Biochemical, cultural and morphological properties. It can be seen from Table 6.1 that strain B-16296^T can be clearly distinguished from the type strains of *Saccharothrix* species, including its nearest neighbours, *S. espanaensis* NRRL 15764^T and the type strains of the two subspecies of *S. mutabilis*, by using a combination of phenotypic properties. Strain B-16296^T, unlike the type strain of *S. espanaensis*, grows at 45°C and degrades starch and tyrosine, and utilizes sugars such as L(+)-arabinose, dextrin, *myo*-inositol, D(+)-lactose, D(+)-melibiose, D(+)-raffinose, and D(+)-xylose as sole sources of carbon for energy and growth. Additional phenotypic properties of the strain are given in Table 1 and in the species description.

Table 6.1. Phenotypic properties which separate strain NRRL B-16296^T from the type strains of *Saccharothrix* species.

Taxa: 1, strain NRRL B-16296^T; 2, *S. algeriensis* NRRL B-24137^T; 3, *S. australiensis* NRRL 11239^T; 4, *S. coeruleofusca* DSM 43679^T; 5, *S. espanaensis* NRRL 15764^T; 6, *S. longispora* DSM 43749^T; 7, *S. mutabilis* subsp. *capreolus* DSM 40225^T; 8, *S. mutabilis* subsp. *mutabilis* NRRL B-16077^T; 9, *S. syringae* DSM 43886^T; 10, *S. texasensis* NRRL B-16134^T; 11, *S. violaceirubra* KCTC 19326^T; 12, *S. xinjiangensis* NBRC 101911^T. Data for the reference strains were taken from Labeda (2002), Zitouni *et al*. (2004), Hu *et al*. (2004) and Otoguro *et al*. (2009). +, Positive; –, negative; ND, not determined; w, weakly positive; v, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Decomposition of :												
Adenine	–	–	–	–	–	+	–	–	–	–	–	ND
Hypoxanthine	+	–	–	–	+	–	+	+	–	+	–	ND
Starch	+	–	–	+	–	+	+	+	+	+	–	+
Tyrosine	+	+	+	+	–	+	–	+	+	+	+	+
Nitrate reduction	–	+	+	–	w	+	–	+	–	+	+	–
Assimilation of :												
Citrate	–	+	–	–	v	+	+	+	–	–	–	–
Lactate	–	ND	w	–	+	+	–	+	–	+	–	–
Malate	+	ND	+	–	+	+	+	+	+	+	–	ND
Utilization of :												
Arabinose	+	–	–	+	–	+	+	+	+	+	–	+
Dextrin	+	–	+	+	–	+	+	+	+	+	–	ND
Inositol	+	–	–	–	–	–	+	+	–	+	–	ND
Lactose	+	–	–	+	–	+	–	+	+	+	–	+
Melibiose	+	–	–	–	–	–	+	+	+	+	–	+
α-Methyl-D-glucoside	+	–	–	+	–	–	–	+	–	+	–	+
Raffinose	+	–	–	+	–	–	–	+	+	–	–	+
Rhamnose	+	–	–	+	–	+	–	–	+	+	–	+
Salicin	+	ND	–	+	–	ND	+	+	w	+	–	ND
Sorbitol	–	–	+	+	–	–	–	–	–	–	–	–
Sucrose	+	–	–	+	+	+	–	+	+	+	+	+
D-Xylose	+	–	–	+	v	+	+	+	+	+	–	+
Growth at :												
4 % NaCl (w/v)	+	+	+	+	+	+	+	–	+	–	–	–
5 % NaCl (w/v)	–	–	–	+	–	+	+	–	+	–	–	–
37°C	+	+	+	+	+	+	+	+	+	+	–	+
45°C	w	+	+	+	–	–	+	+	+	–	–	+

Strain NRRL B-16296 ^{T} grew well on all of the media, apart from peptone-yeast extract-iron agar, producing a light yellow to light reddish brown substrate mycelium and rudimentary aerial hyphae on glycerol-asparagine, oatmeal and tyrosine agars. Melanin pigments were formed on tyrosine agar, but not on peptone-yeast extract-iron agar. The organism produced a branched substrate mycelium and spores on synnemata-like structures, but there was no evidence of spore vesicles (Figure 6.2).

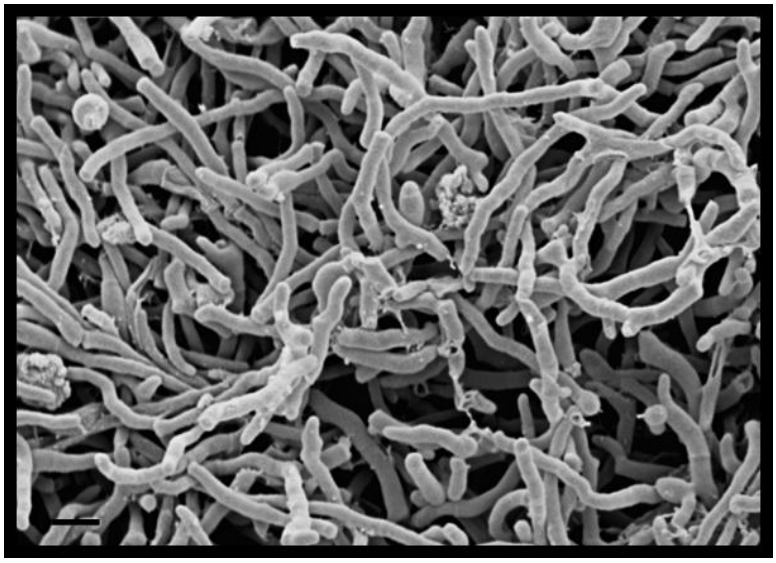


Fig. 6.2. Scanning Electron Micrograph of strain NRRL B-16296^{T} grown on inorganic salts-starch agar (ISP4) for 2 weeks at 30°C. Bar, 1µm.

6.5 Conclusions

The genotypic and phenotypic data clearly demonstrate that strain NRRL B-16296^{T} is misclassified in the genus *Dactylosporangium* as its phylogenetic position and chemotaxonomic profile are typical of members of the genus *Saccharothrix*. Moreover, the phylogenetic data show convincingly that the strain forms a new centre of taxonomic variation within this genus. It is, therefore, proposed that strain NRRL B-16296^{T} be classified in the genus *Saccharothrix* as a new species, *Saccharothrix variispora* sp. nov.

Description of *Saccharothrix variispora* sp. nov.

Saccharothrix variispora (va.ri.i.spo're.a. L. adj. *varius*, diverse, different, various;

Gr. n. *spora*, a seed, in microbiology, a spore; L. suff. *eus, -a, -um*, suffix with various meanings, but in general, made of or belongs to; N.L. fem. adj. *variisporea*, with different spores).

The description is based on data taken from the present study and from the earlier work of Tomita *et al*. (1977). Aerobic, Gram-positive actinomycete which forms branched, light orange coloured mycelia on inorganic salts-starch and oatmeal agars. Grows well between 30 and 37°C, from pH 4 to 10, and optimally around pH 7. Positive for aesculin and allantoin hydrolysis, catalase, coagulation and peptonization of milk, and for hydrogen sulfide and urease production, but is negative for nitrate reduction. Degrades arbutin, casein, DNA, elastin, gelatin, Tweens 40, 60 and 80, uric acid, and xylan, but not cellulose, chitin, guanine, pectin, RNA, tributyrin, Tween 20 or xanthine. D(+)-cellobiose, D(+)-galactose, D(-)-glucose, glycerol, glycogen, inulin, mannitol, D(+)-maltose, D(+)-mannose, D(-)-ribose, starch and D(+)-trehalose (at 1.0%, w/v), and L(+)-tartaric acid, L(-)-malic acid, propionic acid, pyruvic acid and uric acid (at 0.1%, w/v) are used as sole carbon sources, but not adonitol, D(+)-arabitol, dulcitol, D(-)-sorbitol, L(-)-sorbose or xylitol (at 1.0%, w/v) or L(+)-lactic acid, oxalic acid, or urea (at 0.1%, w/v). Resistant (μg ml^{-1}) to ampicillin (4), cephaloridine (2), clindamycin (8), gentamicin (8), kanamycin (8), lincomycin (8), oxytetracycline (8), penicillin G (2), tylosin (8) and vancomycin (2), but is sensitive to ciprofloxacin (2), chloramphenicol (8), novobiocin (8), rifampicin (16), streptomycin (4) and tetracycline (8). Growth occurs in the presence of 4% (w/v) NaCl and 0.05% lysozyme, but not in the presence of 5% NaCl. Additional phenotypic properties are shown in Table 1 and in the main text. The predominant fatty acids are *iso*-$C_{16:0}$ (39.2%), *anteiso*-$C_{17:0}$ (11.9%), *iso*-$C_{15:0}$ (9.9%), 9-methyl $C_{16:0}$ (8.4%), *iso*-$C_{17:0}$ (6.9%), iso-$C_{16:1}$H (6.7%), $C_{17:1}$ω6c (5.3%), $C_{16:1}$ 2-OH (1.8%), *cis* 9-$C_{16:1}$ (1.7%), *anteiso*-$C_{15:0}$ (1.3%), *cis* 9-$C_{17:1}$ (1.1%) and *anteiso*-$C_{17:1}$C (1.0%). The G+C content of the type strain is 74 mol%.

The type strain, NRRL B-16296^T (=ATCC 31203^T =DSM 43911^T =JCM 3273^T =NBRC 14104^T), was isolated from a soil sample from India.

Chapter 7. Search for a self-resistance mechanism of *Verrucosispora maris* AB-18-032 to *atrop*-abyssomicin C

7.1. Abstract

P1-derived artificial chromosome (PAC) library of genomic DNA of the *Verrucosispora maris* AB-18-032 was introduced into an abyssomicin-sensitive strain, *Streptomyces griseus* NRRL B-2682^T (MIC 5-10 μg/ml) by conjugation. Five mutants which showed resistance to *atrop*-abyssomicin C were isolated. Exconjugants were screened by PCR reactions for putative abyssomicin resistance genes (*abyD*, *isochorsmatase, pabB* and *trpE*) identified from the abyssomicin biosynthetic gene cluster (abyD) and the whole genome of *V. maris* AB-18-032 (*isochorismatase, pabB* and *trpE*), respectively. All of the exconjugants were negative for the putative resistance genes, indicating that the *atrop*-abyssomicin C resistance shown by the mutants is not conferred by the putative resistance genes from *V. maris* AB-18-032 hence the resistance observed in the exconjugants is probably conferred by mutations in the target protein (PabB) or through decreased uptake or increased efflux of *atrop*-abyssomicin C.

7.2. Introduction

Global antibiotic resistance is becoming an increasing public health problem with the emergence of multi-drug resistant bacterial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Spellberg *et al.*, 2008). Every new generation of older antibiotics and new compounds approved eventually face the emergence of resistance (Wright, 2010). Actinomycetes produce two-thirds of the known natural products, notably antibiotics (Bérdy, 2005), and have evolved self-resistant mechanisms to protect themselves from the toxicity of these molecules, thereby making them 'hot spots' for studying the evolution of antibiotic resistance (Cundliffe, 1989; Davies, 1997; D'Costa *et al.*, 2006; Mindlin *et al.*, 2006; Hopwood, 2007; Wright, 2010). It has been suggested that a number of mechanisms, including target modification, antibiotic inactivation, permeability barrier alterations or an efflux system could be involved in conferring the self-resistance to the producer (Mindlin *et al.*, 2006; Hopwood, 2007; Cundliffe & Demain, 2010). Analysis

of the molecular basis of resistance in antibiotic-producing actinomycetes can help determine the resistance mechanisms of pathogenic organisms which could eventually emerge clinically, and provides strategies which can be applied in new antibiotic discovery programmes (Hotta *et al.*, 1983, 1992, 1999; Hotta & Okami, 1996; Wright, 2007, 2010). Therefore, if the self-resistance mechanisms of newly discovered antibiotics are elucidated, it could be possible to identify and screen the resistance determinants from environmental samples, environmental isolates and clinical pathogens (Guardabassi *et al.*, 2004, 2005; Guardabassi & Agerso, 2006; D'Costa *et al.*, 2007; Wright, 2010).

New drug compounds have been sought from actinomyetes isolated from extreme habitats and sources of which target new biosynthetic processes (Bull & Stach, 2007). *Atrop*-abyssomicin C, the first known natural inhibitor of the *para*-aminobenzoate (*p*ABA) biosynthesis pathway, is produced by *Verrucosispora maris* AB-18-032, a strain isolated from a marine sediment sample (Riedlinger *et al.*, 2004). It is a polycyclic polyketide-type antibiotic which shows antibiotic activity against Gram-positive bacteria, including *B. subtilis* and methicillin-resistant *Staphylococcus aureus*. This compound is the main component of the abyssomicin complex, which also includes abyssomicin B, C, D, G and H (Keller *et al.*, 2007a).

Recently, the mode of action of *atrop*-abyssomicin C has been elucidated. Keller *et al.* (2007b) showed that the highly conserved PabB subunit of 4-amino-4-deoxychorismate (ADC) synthase from *Bacillus subtilis* is the molecular target of *atrop*-abyssomicin C. This compound acts as a covalent binder to the side chain of Cys263, located in the proximity of the active site of PabB. However, the resistance mechanism still remains unclear although it has been suggested that resistance may be through the modification of target (PabB) or the presence of a PabB homologue that may be able to complement inhibition of the PabB enzyme (Keller *et al.*, 2007b). It is important, therefore, to see whether the strain, *Verrucosispora maris* AB-18-032, like other antibiotic-producing actinomycetes, can modify or complement the abyssomicin target site to make ADC synthase resistant to the compound. Recently, the whole genome of the strain, *Verrucosispora maris* AB-18-032, has been partially sequenced and the putative resistance genes have been identified (Jem Stach, personal communication). This work is designed to elucidate the role and identification of putative resistance genes which are responsible for self-resistance. To this end, a P1-

derived artificial chromosome (PAC) library of genomic DNA of strain *V. maris* AB-18-02 was introduced into *Streptomyces griseus* NRRL B-2682^T, a strain known to be susceptible to *atrop*-abyssomicin C by conjugation. Resistant exconjugants were isolated and screened for the presence of putative abyssomicin resistance genes. The results indicate that resistance in the exconjugants is conferred either by mutation within the *S. griseus* strain or by cloning of unidentified resistance genes from *V. maris* AB-18-032.

7.3. Materials and methods

The cultivation of test strains, selection of *atrop*-abyssomicin C sensitive strains, conjugation of genomic DNA library, selection of exconjugants, PCR primer design, extraction of genomic DNA from exconjugants, and PCR screening of putative resistance genes, were carried out using procedures described earlier in section 2.10.

7.4. Results

Sensitivity of *Streptomyces* strains to abyssomicin. The sensitivity of, "*S. coelicolor*" A3(2), *S. griseus* NRRL B-2682^T, "*S. lividans*" TK24 and *S. venezuelae* ATCC 15439 to abyssomicin was tested by agar plate diffusion assay using minimal media (Table 7.1). All of four stains were sensitive to abyssomicin at ≥10 μg with "*S. coelicolor*" A3(2), "*S. lividans*" TK24 and *S. venezuelae* ATCC 15439 showing similar sensitivities to abyssomicin. The remaining strain *S. griseus* NRRL B-2682^T showed higher sensitivity and was chosen for further studies.

Table 7.1. Antibacterial activity of *atrop*-abyssomicin C, determined by the agar plate diffusion assay (inhibition zone in mm)

Concentration	"*S. coelicolor*" A3(2)	*S. griseus* NRRL B-2682^T	"*S. lividans*" TK24	*S. venezuelae* ATCC 15439
5 μg	0	0	0	0
10 μg	1	3	2	2
20 μg	5	10	5	5
40 μg	10	20	10	10
60 μg	15	25	15	15
80 μg	20	30	20	20

Selection of resistant exconjugants. *Streptomyces griseus* NRRL B-2682^T was conjugated with a genomic DNA library of *V. maris* AB-18-032 in plasmid pESAC13. Seven exconjugants were initially selected as they grew on agar plates containing 40 μg/ml abyssomicin. The parental *S. griseus* was totally inhibited with 40 μg/ml *atrop*-abyssomicin C under similar conditions. When spores prepared from the resistant clones were allowed to grow again in the presence of *atrop*-abyssomicin C, all of five clones, SG-19, SG-26, SG-27, SG032 and SG-33, showed resistance to abyssomicin, as well as conserving the kanamycin resistance marker of the pESAC13 vector (Fig. 7.1).

Three exconjugants, SG-19, SG-26 and SG-32 were particularly resistant to *atrop*-abyssomicin C showing very reduced inhibition zones. In contrast, two exconjugants, SG-27 and SG-33, were moderately resistant to *atrop*-abyssomicin C showing larger inhibition zones than the other exconjugants. A control culture of *S. griseus* strain carrying the empty pESAC13 did not show any resistance to the drug in the absence of kanamycin.

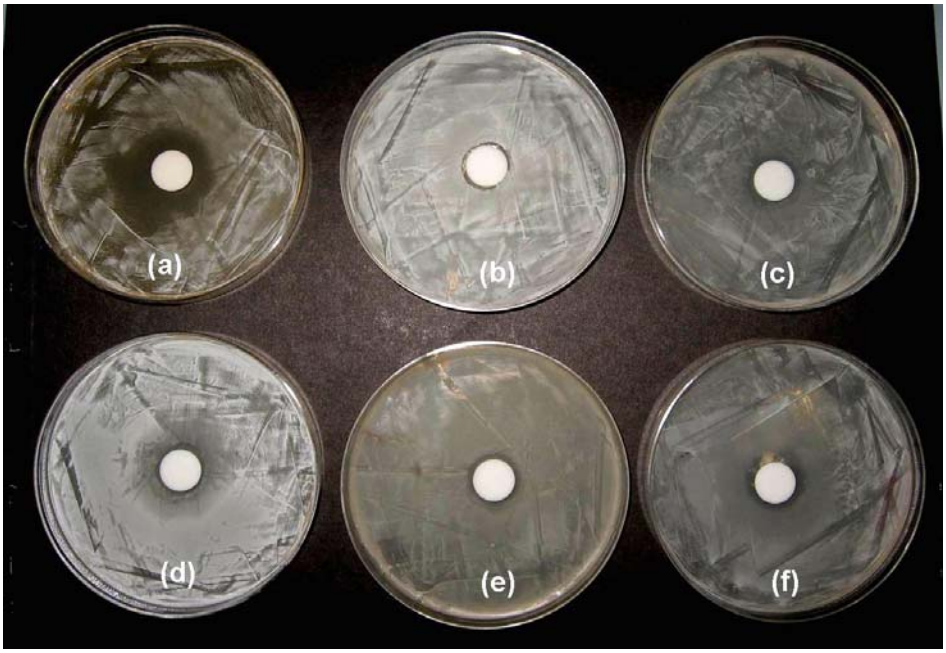


Fig. 7.1. Sensitivity of 5 exconjugants and the wild type strain of *S. griseus* to 40 μg (disk) of *atrop*-abyssomicin C after culturing at 30˚C for 48 hours. (a), wild type strain; (b), exconjugants SG-19; (c), SG-26; (d), SG-27; (e), SG-32; (f), SG-33.

Putative resistant gene screening. Four PCR primers designed from target genes thought to be responsible for self-resistance in the *V. maris* AB-18-032, were used to

screen the exconjugants. The primers were tested on the genomic DNA of both *V. maris* AB-18-032 and *S. griseus* NRRL B-2682^{T}, as the positive and negative controls, respectively. The primers were found to be specific to the target genes of the strain. PCR amplified product was not observed when these primers were tested on the five abyssomicin-resistant exconjugants.

Two PCR primers designed from the *abyD* gene of the biosynthetic gene cluster and *pabB* gene of the 4-amino-4-deoxychorismate synthase gene cluster, showed weak bands. However, the amplified PCR products were different to the expected size and were also observed in the negative control DNA (Fig. 7.2).

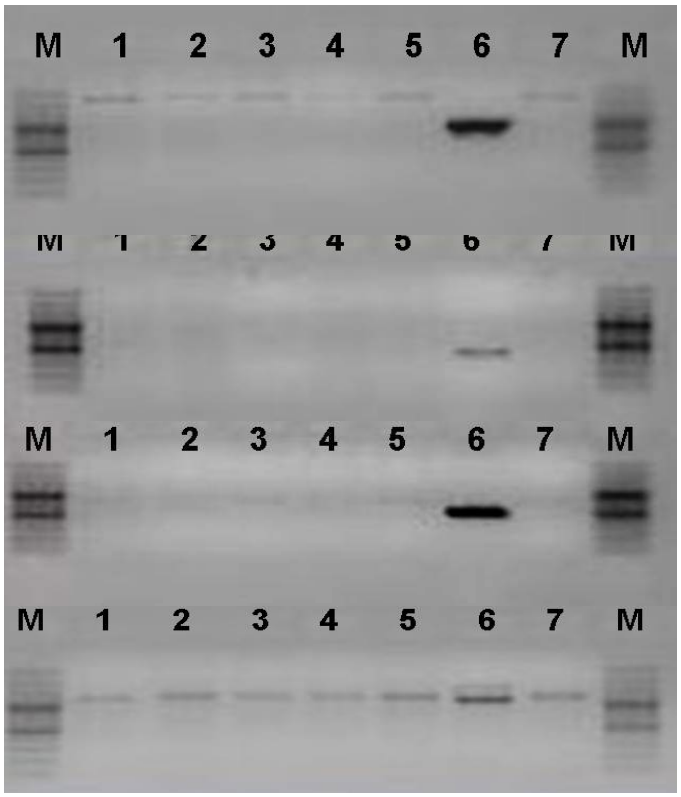


Fig. 7.2. Agarose gel electrophoresis of PCR amplifications derived from PCR reactions using target gene primers (Table 2.11). (a), abyD; (b), AS; (c), ICS; (d), PAB.

Lane: 1, SG-19; 2, SG-26; 3, SG-27; 4, SG-32; 5, SG-33; 6, *V. maris* AB-18-032; 7, *S. griseus* NRRL B-2682^{T}; M, molecular marker (100bp ladder; MBI Fermentas, UK)

7.5 Discussion

The *para*-aminobenzoic acid (*p*-ABA) derived from the key-metabolite of chorismate, is an important metabolite in the folic acid pathway which is essential to bacterial metabolism (Nichols *et al.*, 1989). The enzymes of the *p*ABA and folic acid biosynthetic pathway are conserved in many microorganisms, parasites and plants, but not in humans hence this pathway is an interesting target for the development of novel antibiotics, pesticidal and herbicidal agents (Alun & Jeremy, 2002). Sulfonamides and trimethoprim are prominent synthetic inhibitors of the folic acid pathway (Huovinen *et al.*, 1995). The natural antibiotic, *atrop*-abyssomicin C is a very efficient inhibitor of the *p*ABA) biosynthesis in the Gram-positive bacteria.

A selection of chorismate-utilising enzymes, including 4-amino-4-deoxychorismate (ADC) synthase, anthranilate synthase and isochorismate synthase have been considered to be evolutionarily related on the basis of sequence homology, structural similarity, and a unified reaction mechanism (He *et al.*, 2004). In all three enzymatic reactions, the C4 hydroxy group of chorismate is replaced by the addition of a nucleophile at the C2 position (Fig. 7.3). *p*ABA biosynthesis from chorismate requires two enzymes: ADC synthase, which converts chorismate and glutamine into ADC and glutamate, and ADC lyase which catalyses an elimination reaction of ADC to produce *p*ABA. ADC synthase is a heterodimer composed of two dissimilar subunits, PabA and PabB. PabA functions as a glutamine amidotransferase that hydrolyses glutamine (Gln) to glutamate (Glu) and NH_4^+. PabB catalyses the formation of 4-amino-4-deoxychorismate from chorismate and ammonia provided by the amidotransferase of PabA (He & Toney, 2006).

It is evident from Table 7.1 that *atrop*-abyssomicin C showed a strong inhibitory activity against the four *Streptomyces* strains. The minimal inhibitory concentration (MIC) of *atrop*-abyssomicin C against these strains was 5-10 µg. The inhibitory activity of the antibiotic was significantly stronger when the test organism, *S. griseus* NRRL B-2682^T was grown in minimal media. These results indicate that the *S. griseus* with the highest susceptibility to the *atrop*-abyssomicin, is the most appropriate strain to investigate the mechanism of self-resistance of *V. maris* AB-18-032 .

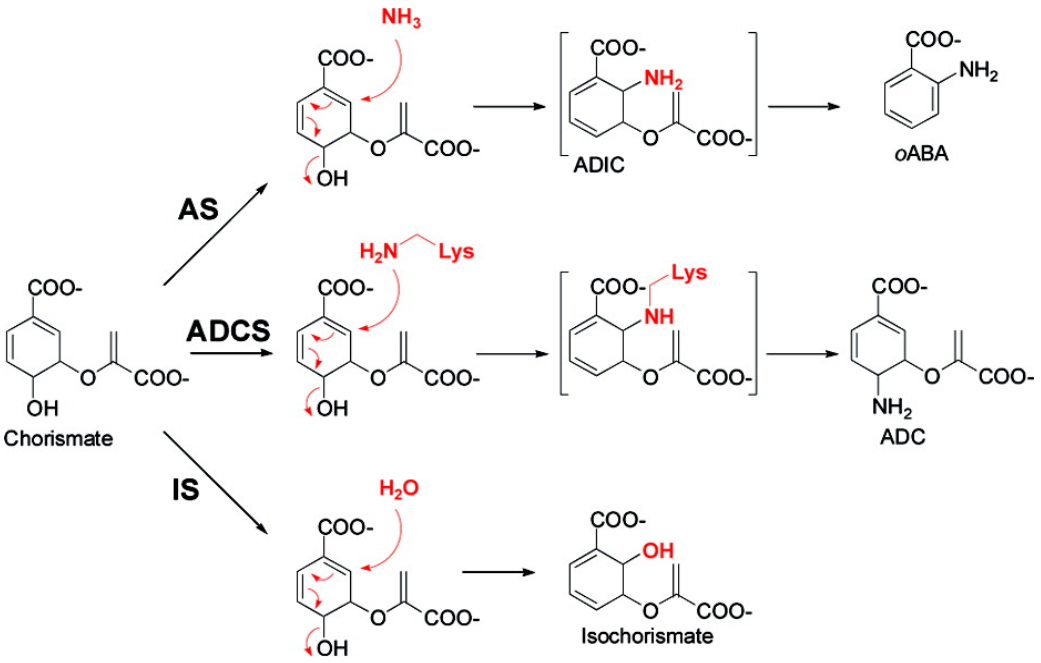


Fig. 7.3. Three chorismate-utilizing enzymes of *E. coli* sharing a common reaction mechanism: anthranilate synthase (AS), 4-amino-4-deoxychorismate synthase (ADCS), and isochorismate synthase (IS) (adapted from He *et al.*, 2004) .

A number of studies have shown that biosynthetic and resistance genes of antibiotic-producing actinomycetes are genetically related due to the mechanism of self-protection (Cundliffe, 1989; Pootoolal *et al.*, 2002; Mindlin *et al.*, 2006; Hopwood, 2007). It was for this reason that five of the mutants were selected for PCR screening of the target gene, which might be involved in self-resistance to abyssomicin. The three primers were used to detect the *isochorismatase, pabB* and *trpE(G)* genes coding for three chorismate utilising enzymes, IS, ADCS and AS, respectively (Table 2.11). The remaining primer was used to detect the *abyD* gene which was located in the *atrop*-abyssomicin C biosynthetic gene cluster. However, none of these genes were detected by PCR screening of the exconjugants indicating that the resistance mechanism working in the mutants of *S. griseus* does not involve any of these genes hence further work is needed to identify the mechanism(s) of resistance in the exconjugants.

Sequencing of the *pabB* gene of the exconjugants will reveal whether or not mutatons in the target protein confer resistance to *atrop*-abyssomicin C in *Streptomyces* strains. If this were not the case, then determination of the genes transferred from *V. maris* AB-18-032 to *S. griseus* NRRL B-2682^T might allow identification of the *atrop*-abyssomicin C resistance genes. Cloning of genomic DNA from the exconjugants back

into *atrop*-abyssomicin C sensitive *S. griseus* strains would enable any mutations that confer resistance to abyssomicin, which were not the result of *pab*B mutations, to be identified. Once identified, these resistance genes could be used to detect the resistance in either environmental isolates or clinical pathogens, where *atrop*-abyssomicin C was to be used clinically.

Chapter 8. General discussion and perspectives for future work

8.1. General discussion

The importance of filamentous actinomycetes in biotechnology is a function of their remarkable metabolic diversity, their long association with natural habitats and the needs of human beings (Goodfellow & O'Donnell, 1989; Bull *et al.*, 2000; Ul-Hassan & Wellington, 2009; Goodfellow & Fiedler, 2010). However, the overall value of these organisms to humankind is difficult to measure as they exhibit both beneficial and detrimental roles, notably in healthcare. Thus, on one hand, they are the richest source of clinically significant antibiotics (Strohl, 2004; Bérdy, 2005; Goodfellow & Fiedler, 2010), but on the other hand, they are the likely source of antibiotic-resistance genes present in pathogenic microorganisms (Davies, 1997; D'Costa *et al.*, 2006; Mindlin *et al.*, 2006; Woo *et al.*, 2006; Wright, 2010). The battle between filamentous actinomycetes as friends and foes was the underlying theme of this project which was designed (a) to establish whether a correlation exists between antibiotic resistance patterns and the taxonomy of dereplicated strains of filamentous actinomycetes isolated from a hay meadow soil, (b) to determine the taxonomic diversity and biotechnological potential of members of the genus *Dactylosporangium*, and (c) to try and establish the mechanism(s) which protects *Verrucosispora maris* strain AB-18-032 against the potent antibiotic it produces, namely *atrop*-abyssomicin C. Most of objectives were achieved to a greater or lesser extent.

An important indicator of antibiotic production in soil is the widespread occurrence of antibiotic-resistance amongst indigenous soil bacteria, especially streptomycetes (D'Costa *et al.*, 2006; Laskaris *et al.*, 2010; Wright, 2010). In the present investigation, a small, but reasonably representative, set of filamentous actinomycetes, notably streptomycetes, showed an astonishing capacity to grow in the presence of a panel of antibiotics. Indeed, on average, these organisms showed resistance to 19 of the 41 tested antibiotics. These results support the view that filamentous actinomycetes present in soil are a reservoir of resistant determinants which can be spread into the broader microbial community (D'Costa *et al.*, 2007; Wright, 2007, 2010).

The 16S rRNA gene sequencing studies showed that most of the dereplicated filamentous actinomycetes belonged to the genus *Streptomyces* though some were

phylogenetically close to authentic *Actinomadura, Dactylosporangium, Micromonospora* and *Streptosporangium* strains. The weak correlation found between the antibiotic resistance patterns and the taxonomy of the dereplicated strains may reflect that small and heterogeneous nature of the complement of strains as a higher correlation was found with isolates which showed high levels of 16S rRNA gene similarity. This somewhat generous interpretation of the data is supported by the discovery that all of the vancomycin-resistant isolates keyed out as *Streptomyces sanglieri* (Manfio *et al.*, 2003).

Improved selective isolation and characterisation procedures show that filamentous actinomycetes once thought to be rare in natural habitats are widely distributed and may be integral parts of microbial communities, as shown by studies on the genera *Amycolatopsis* (Tan *et al.*, 2006), *Micromonospora* (Qiu *et al.*, 2008), nocardiae (Orchard *et al.*, 1977; Maldonado *et al.*, 2000), *Planobispora* (Suzuki *et al.*, 2001), *Rhodococcus* (Colquhoun *et al.*, 1998) and *Salinispora* (Mincer *et al.*, 2005; Jensen & Mafnas, 2006). Effective taxon-specific isolation procedures are needed for the selective isolation and characterisation of members of most filamentous actinomycete taxa of commercial interest (Goodfellow & Fiedler, 2010), not least the genus *Dactylosporangium*, which encompass strains known to produce antibiotics (Hayakawa *et al.*, 1991; 2000; Hayakawa, 2008).

One of the major achievements of the project was the discovery that *Streptomyces* Isolation Medium (SIM) supplemented with gentamicin and antifungal antibiotics supported the growth of relatively large numbers of presumptive novel *Dactylosporangium* species. The novelty of selected isolates was confirmed in a polyphasic study which led to the formal recognition of two species, *Dactylosporangium luteum* and *Dactylosporangium luridum*. A third species, *Dactylosporangium salmoneum* was proposed for an organism which had no formal nomenclatural standing. The application of the new selective isolation procedure showed that *Dactylosporangium* strains are widely distributed in natural habitats, notably soil. Preliminary studies also showed that novel members of the genus may prove to be a source of new antibiotics as some isolates contained non-ribosomal peptide and type-I polyketide synthase genes and inhibited the growth of Gram-positive bacteria.

A key aim of the present study was to gain an insight into the distribution and taxonomic diversity of members of the genus *Dactylosporangium* in a number of environmental samples using culture-independent and culture-dependent procedures. Although it was not the intention to estimate the full extent of dactylosporangial diversity in the environmental samples, an unexpectedly high degree of taxonomic variation was achieved in a small clone library. It was also interesting that the range of diversity detected using the two approaches was comparable indicating that the genus-specific primers of Monciardini *et al.* (2002) can be used to detect new centres of taxonomic diversity in dactylosporangial gene tree. This means that the primer set together with other appropriate molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP), could be used to prescreen environmental samples before selective isolation studies. Such a twin-track strategy would allow broader ecological issues to be addressed, such as unraveling the geographical distribution, abundance, diversity and roles of *Dactylosporangium* species in diverse habitats, as well as providing novel biological material for pharmaceutical screens.

The twin-track strategy outlined above is labour intensive which may account for the fact that it has rarely been used in the search for novel secondary metabolites, notably antibiotics. However, studies along these lines have shown that members of the obligate marine actinomycete *Salinispora* are a rich source of novel antibiotics (Jensen *et al.*, 2005; Jensen *et al.*, 2007; Udwary *et al.*, 2007), including the anticancer drug, salinsporamide which is currently in clinical trials (Fenical *et al.*, 2009). Indeed, the species-specificity of *Salinispora* biosynthetic pathways (Jensen *et al.*, 2007) strongly supports the view of a coupling between taxonomic and chemical diversity, as has been inferred from studies on *Amycolatopsis regifaucium* (Tan *et al.*, 2007), *Streptomyces clavuligerus* (Ward & Goodfellow, 2004) and *Stretomyces violaceusniger*-like strains (Goodfellow *et al.*, 2007).

It was disappointing that the search for a mechanism whereby the *Verrucosispora maris* AB-18-032 strain protects itself against *atrop*- abyssomcin C was not successful though the work is only a preliminary study. Further studies using the resistant exconjugants would be required to find the responsible gene or protein located in the cell and to obtain advance insight into the real mechanism(s) involved in resistance.

8.2 Perspectives for future work

Ecology:

- Selective isolation and identification of representatives of common *Streptomyces* present in plot 6 of the hay meadow soil to establish whether a strain correlation exists between their position in the 16S rRNA gene tree and ability to grow in the presence of antibiotics.
- Comparison of *Streptomyces* Isolation medium (SIM), supplemented with gentamicin and antifungal antibiotics agents, with other media recommended for the selective isolation of members of the genus *Dactylosporangium*.
- Determination of the full extent of dactylosporangial diversity in natural habitats using appropriate molecular procedures (e.g., PCR-DGGE, PCR-SSCP and Pyrosequencing method).
- Biogeographical studies on the selected *Dactylosporangium* species to determine whether they show site specific endemism.
- Ecophysiological studies on representatives of common *Dactylosporangium* species to foster an understanding of their role in natural habitats.

Systematics:

- Formal description of additional *Dactylosporangium* species following polyphasic studies on presumptively novel strains isolated in this study.
- Establishment of minimal standards for the recognition of new *Dactylosporangium* species.
- Characterisation of representatives of the putative *Dactylosporangium* isolates which gave negative results against the genus-specific primers.
- Formal description of the putatively novel *Actinomadura, Micromonospora, Streptomyces* and *Streptosporangium* strains included in the 16S rRNA gene sequencing studies.

Bioprospecting:

- Screening of metabolites from representatives of dactylosporangiae for novel antibacterial, antifungal, antitumor and nematocidal activity.
- Identification of the compounds giving interesting hits in the antibacterial assays using appropriate chemical procedures.

- Application of genome mining and genome scanning methods to representatives of the *Dactylosporangium* strains to detect cryptic biosynthetic gene clusters that can be activated by genetic manipulation and/or non-standard fermentation conditions.
- Whole-genome sequencing of representative dactylosporangiae to establish their potential to produce novel antibiotics.
- Establishment of the degree of coupling between the taxonomic and chemical diversity of representatives of *Dactylosporangium* species.

Resistance:

- Search for other putative resistance genes transported to exconjugants which are resistant to *atrop*-abyssomicin C.
- Investigation of genetic mechanism whereby *Verrucosispora maris* strain AB18-032 is protected and regulated against *atrop*-abyssomicin C.
- Screening of resistance determinant of *atrop*-abyssomicin C in other bacteria, including environmental isolates and environmental samples.

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Appendix A. Media formulations

All media formulations were prepared with reagents of high purity (BDH Chemicals Ltd., Dorset, UK; Difco, Difco Laboratories, Michigan, USA; Oxoid, Oxoid Ltd., Cambridge, UK; Sigma, Sigma-Aldrich, Dorset, UK) when a specific product was stated in the formulation. Autoclave was carried out at 121°C for 20 minutes except some physiological test media.

1) Culture media

Glucose-yeast extract-malt extract (ISP medium 2; Shirling & Gottlieb, 1966)

Glucose……	4 g
Yeast extract…….....	4 g
Malt extract…….……	10 g
CaCO3…….…….....	2 g
Agar…….	15 g
Distilled water……..	1000 ml
pH…….	7.2 ± 0.2

Glycerol-asparagine agar (ISP medium 5; Shirling & Gottlieb, 1966)

L-asparagine ……...	1 g
Glycerol……..	10 g
KH_2PO_4.....	1 g
Trace salt solution …….	1 ml
Agar…….....	15 g
Distilled water…….	1000 ml
pH……	6.8 ± 0.2

Inorganic salt starch agar (ISP medium 4; Shirling & Gottlieb, 1966)

Distilled water……	500 ml
K_2HPO_4 (anhydrous basis)…….	1.0 g
$MgSO_4 \cdot 7H_2O$……..	1.0 g
$(NH_4)_2SO_4$……...…….	2.0 g
NaCl……	1.0 g
$CaCO_3$…….	2.0 g
Soluble starch solution……..……	500 ml
Agar……..	15.0 g
pH……	7.2 ± 0.2

Preparation of soluble starch solution: 10 g of soluble starch were added to 500 ml of distilled water and the preparation mixed well to prevent clumping.

Luria-Bertani (LB) medium (Miller, 1972)

Tryptone…….	10 g
Yeast extrct……	5 g
NaCl……..	10 g

Modified Bennett's agar (Jones, 1949)

Yeast extract …….	1 g
Lab-LEMCO…….	0.8 g
Bacto-Casitone…….....	2 g
Glycerol……	10 g
Agar……..	15 g
pH…….	7.2 ±0.2

Oatmeal agar (ISP medium 3; Shirling & Gottlieb, 1966).

Oatmeal (mixture)……	1000 ml
Trace salt solution stock…….....	1.0 ml
Agar…….	15.0 g
pH……	7.2 ±0.2

Oatmeal mixture: Add 20 g of oatmeal into 1000 ml of distilled water and cook for 20 minutes. The mixture was then filtered through cheese cloth and restored to 1000 ml by adding distilled water.

Trace salt solution stock:

$FeSO_4 \cdot 7H_2O$ ……	0.1 g
$MnCl \cdot 4H_2O$……..	0.1 g
$ZnSO_4 \cdot 7H_2O$……	0.1 g
Distilled water ……..	100 ml

The solution was filter-sterilised using cellulose acetate membrane filters (pore size 0.45 μm) and stored at 4°C.

Peptone-yeast extract-iron agar (ISP medium 6; Shirling & Gottlieb, 1966)

Bacto-peptone iron agar……	36 g
Bacto-yeast extract……	1.0 g
Distilled water……..	1000 ml
pH…….	7.0 ±0.2

Tryptone-yeast extract agar (ISP medium 1; Shirling & Gottlieb, 1996)

Bacto-Tryptone ……..	5 g
Bacto-Yeast extract…….....	3 ml
Agar……	15 g
Distilled water……	1000 ml
pH……..	7.0 -7.2

Tyrosine agar (ISP medium 7; Shirling & Gottlieb, 1966)

Glycerol…….	15 g
L-Tyrosine (Difco)……. .	0.5 g
L-Asparagine (Difco)……..	1.0 g
K_2HPO_4 (anhydrous basis)…….…….	0.5 g
$MgSO_4 \cdot 7H_2O$…….…….	0.5 g
NaCl……..	0.5 g
$FeSO_4 \cdot 7H_2O$…….. …….	0.01 g
Distilled water……..	1000 ml
Trace salt solution (see ISP3) …..……...	1.0 ml
Agar…….....	20.0 g
pH……	7.2 - 7.4

Modified Bennett's agar (Jones, 1949)

The composition of the Bennett's Agar followed the original formulation of Jones (1949) with the substitution of beef-extract (1g per litre) and N-Z-Amine A' (2g per litre) by Lab-Lemco (0.8g per litre; Oxoid) and Bacto-Casitone (2g per litre; Difco), as recommended by P. Agrawal (unpublished data).

Gucose ……	10.0 g
Bacto-Casitone (Difco) ……	2.0 g
Yeast-extract ……	1.0 g
Lab-Lemco (Oxoid) ……..	0.8 g
Agar ……	15.0 g

The medium components were dissolved in 1 litre of cold distilled water and the pH adjusted to 7.3 ± 0.2 with dilute (0.1M) NaOH or HCl. The agar was then added and the medium liquefied by steaming prior to autoclaving. Alternatively, the medium was aliquoted to glass bottles and the agar (1.5%, w/v) added to each bottle before autoclaving directly.

Vitamin Stock Solution.

The following vitamins were dissolved in 1 litre of cold distilled water and the pH adjusted to 3.0 ± 0.2 with 0.1M HCl prior to sterilising by vacuum membrane filtration.

p-aminobenzoic acid ……10 mg
Biotin …… 10 mg
Inositol ……. 10 mg
Nicotinamide …… 10 mg
Pantothenic acid (B_5) …… 10 mg
Pyridoxine (B_6) ……. 10 mg
Riboflavin Na choline (B_2) …… 10 mg
Thiamin (B_1) ……. 10 mg
Cyanocobalamin (B_{12}) …… 0.5 mg
Folic acid …… 0.5 mg

2) Phenotypic tests media

Aesculin/ Arbutin degradation (Kutzner, 1976)

Basal medium

Yeast extract……...	3 g
Ferric ammonium citrate ……..	0.5 g
Agar……..	7.5 g
pH…….	7.2

Autoclaved at 121°C for 20 minutes. The aesculin or arbutin was tyndallised in distilled water then added to the melted basal medium to give a final concentration of 0.1% (w/v), respectively.

Allantoin degradation (Gordon *et al*., 1974)

Basal medium

KH_2PO_4…….	9.1 g
Na_2HPO_4……...	9.5 g
Yeast extract…….	0.1 g
Phenol red…….....	0.01 g
Distilled water……..	1000 ml
pH…….	6.8

Autoclaved at 121°C for 20 minutes. The allantoin was tyndallised in distilled water then added to the basal medium to give a final concentration of 0.33 %, w/v. Three ml amounts of the broth were dispensed into test tubes.

Buffer media for pH tolerance test

Potassium phosphate buffers (0.2 M) were prepared at pH 4.0, 5.0, 9.0 and 10.0 by combining the volume of buffers A (0.2 M potassium dihydrogen phosphate) and buffer B (0.2 M dipotassium hydrogen phosphate) in the ratio of 199.7: 0.3; 197.3: 2.7; 1.4: 198.6 and 0.1: 199.9, respectively. Buffer at pH 11.0 was prepared by combining 0.2 M dipotassium hydrogen phosphate with 1 N NaOH to reach the pH. Each 200 ml of warm buffer was added aseptically to 200ml of sterile double strength modified Bennett's agar to give a final volume of 400 ml. pH of the media were tested using pH paper and adjusted with sterile 1 N NaOH or 1 N HCl to a desired pH.

Carbon utilization medium (ISP medium 9; Shirling & Gottlieb, 1966)

Basal mineral salts agar

$(NH4)_2\ SO_4$…….	2.64 g
KH_2PO_4……..	2.38 g
$K_2HPO_4.3H_2O$……	5.65 g
$MgSO_4.7H_2O$…….....	1 g
Pridham & Gottlieb trace salts*…….	1 ml
Agar……..	15 g
Distilled water……..	1000 ml
pH…….	6.8-7.0

* *Pridham & Gottlieb trace salts solution*:

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

Carbohydrate solutions were prepared separately, tyndallised and mixed thoroughly with molten basal medium agar to give an appropriate final concentration.

Chitin degradation (Hsu & Lockwood, 1975)

Colloidal chitin…….....	4 g
K_2HPO_4……..	0.7 g
KH_2PO_4……..	0.3 g
$MgSO_4.5H_2O$…….	0.5 g
$FeSO_4.7H_2O$……..	0.01 g
$ZnSO_4$……	0.001 g
$MnSO_4$……	0.001 g
Agar……	20 g
Distilled water…….....	1000 ml
pH…….	8.0

DNase test agar for DNA degradation (Difco)

Tryptose……	20 g
DNA……....	2 g
NaCl……	5 g
Agar…….....	12 g
Distilled water…….....	1000 ml
pH……	7.2

Nitrate reduction medium (Gordon & Mihm, 1962)

KNO_3……..	1 g
Lab LEMCO……..	2.4 g
Distilled water……	1000 ml
pH……..	7.0

Autoclaved at 121°C for 20 minutes. Three ml amounts dispensed into bijoux bottles.

RNA degradation (Goodfellow *et al.*, 1979)

Tryptone …….	20 g
NaCl……..……....….	5 g
Distilled water…….	1000 ml
Agar…….....	12 g
pH……	7.2

Autoclaved at 121°C for 15 minutes. RNA (3 g) was tyndallised and added to the melted basal medium prior to dispensing into plates to give a final concentration (0.3%, w/v).

Sierra's medium for degradation of Tweens (Sierra, 1957)

Basal medium

Bacto-peptone ……..…….	10 g
NaCl……	5 g
$CaCl_2.H_2O$…….....	0.1 g
Agar……	15 g
Distilled water……	1000 ml
pH……	7.4

Ten ml of each of the Tweens was tyndallised in distilled water then added to melted basal medium to give a final concentration of 1.0% (v/v).

Trybutyrin agar

Trybutyrin agar base (Sigma T3688)…….....	23 g
Trybutyrin (Sigma T8626)…….....	10 ml

Distilled water……. 990 ml

Trybutyrin agar base was dissolved in 990ml distilled water by heating and stirring on a heated magnetic stirrer. Tributyrin was added into the medium while it was being stirred. The medium was sterilized by autoclaving at 121°C for 20 minutes, cooled in a 50°C water bath and mixed by gentle swirling while pouring in order to maintain uniform turbidity in the agar medium.

Urea degradation

Basal medium

KH_2PO_4…… 9.1 g

Na_2HPO_4……..... 9.5 g

Yeast extract…… 0.1 g

Phenol red…… 0.01 g

Distilled water……. 1000 ml

pH…… 6.8

Urea solution in distilled water was filter-sterilized using cellulose acetate membrane filters (pore size 0.45 μm) then added to the basal medium to give a final concentration (2.0%, w/v). Three ml amounts were aseptically dispensed into bijoux bottles.

Appendix B. Reagents and buffers

Acidic mercuric chloride for gelatin degradation

Mercuric chloride ……..	12 g
Distilled water…….....	80 ml
Concentrated hydrochloric acid ……	16 ml

EDTA buffer (0.5 M, pH 8.0)

Disodium ethylenediaminetetraacetic acid (EDTA).....	18.612 g
Distilled water…….……..	80 ml

The solution was adjusted to pH 8.0 with approximately 2.2 g NaOH pellets and distilled water added to make a final volume of 100 ml.

Lugol's iodine for starch degradation test

Iodine…….	5 g
Potassium iodide……	10 g
Distilled water……	100 ml

Iodine and potassium iodide was dissolved in 10 ml of distilled water and make up to 100 ml. The reagent was diluted 1in 5 with distilled water.

Nitrate reduction reagents

Reagent A:

Sulphanilic acid…….	0.8 g
Acetic acid (5N)……	100 ml

Reagents B:

α-Napthylamine…….	0.8 g
Acetic acid (5N) ……	100 ml

20X SSC Buffer

NaCl (3M)……..	17.53 g
Sodium citrate (0.3M)…….	7.73 g

The solution of was adjusted to pH 7.0 with 5 N NaOH and distilled water was added to make a final volume of 100ml. The working solution was prepared by diluting the stock solution to give 0.1X SSC buffer.

Tris-Borate-EDTA buffer (TBE; 10X stock solution, pH 8.0)

Trizma base ……..	108 g
Boric acid ……..…..	55 g
EDTA ……..	7.44 g
NaOH…….....	1.0 g

Distilled water up to…….. 1000 ml

Autoclaved at 121°C for 20 minutes and stored at room temperature. The working solution was prepared by diluting the stock solution 20 times to give 0.5 X TBE buffer.

Tris-EDTA buffer, pH 8.0 (TE; 10 mM Tris, 1 mM EDTA, pH 8.0)

0.5 M EDTA, pH 8.0 ……. 2 ml

1 M Tris-HCl, pH 8.0 …….. 10 ml

Milli-Q water up to …….. 1000 ml

Autoclaved at 121°C for 20 minutes and stored at room temperature.