Novel Actinobacterial Diversity in Arid Atacama Desert Soils as a Source of New Drug Leads

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Dedicated to my family-Busarakam and beloved country-Thailand

"Bacteria dwell in virtually every spot that can sustain any form of life. And we have underestimated their global number because we, as members of a kingdom far more restricted in potential habitation, never appreciated the full range of places that might be searched."- Planet of the Bacteria. *Stephen Jay Gould*

Abstract

The search for new specialised metabolites, notably antibiotics, that can be developed for healthcare has steadily shifted towards the isolation and screening of rare and novel actinobacteria from extreme habitats on the premise that such habitats give rise to unique biodiversity that is the basis of novel chemistry. To this end, a taxonomic approach to bioprospecting for bioactive compounds was used to selectively isolate, dereplicate and classify actinobacteria from hyper-arid and extreme hyper-arid areas of the Atacama Desert in northwest Chile, namely from the Salar de Atacama and Yungay regions, respectively. Sample pretreatment and selective isolation strategies enabled the recovery of actinobacteria from each of these habitats and while population sizes were small, taxonomic diversity was high.

Relatively large numbers of *Amycolatopsis* and *Streptomyces* strains were isolated from the hyper-arid Salar de Atacama soil, as were smaller numbers of *Actinomadura*, *Kribbella*, *Lechevalieria*, *Nonomuraea* and *Saccharothrix* strains. In contrast, *Modestobacter* and *Streptomyces* isolates predominated in the extreme hyper-arid Yungay soil, the latter also contained smaller numbers of *Blastococcus*, *Couchioplanes*, *Geodermatophilus* and *Pseudonocardia* strains. With few exceptions representatives of these genera formed distinct phyletic lines in 16S rRNA gene trees. Polyphasic studies carried out on strains of ecological and biotechnological interest showed that isolates assigned to the genera *Modestobacter* and *Streptomyces* belonged to putative new species, as exemplified by the proposal for *Streptomyces leeuwenhoekii* sp. nov. for strains that formed a distinct branch in the *Streptomyces* 16S rRNA gene tree. In contrast, representatives of the genus *Amycolatopsis* were assigned to known species, albeit ones classified in a rare taxon, the *Amycolatopsis* 16S rRNA gene clade. Most of the representative isolates examined in standard plug assays inhibited the growth of one or more of a panel of five wild type microorganisms. In addition, some of the representative streptomycetes from the hyper-arid Salar de Atacama soil were found to inhibit cell envelope, cell wall, fatty acid and RNA synthesis in assays based on the use of *Bacillus subtilis* reporter genes.

The results of this project demonstrate for the first time that hyper-arid and extreme hyper-arid Atacama Desert soils are rich reservoirs of cultivable rare and novel actinobacteria with the capacity to produce a broad range of bioactive compounds that can be developed as drug leads for medicine. Indeed, microorganisms, unlike plants and animals, have overcome the prevailing harsh conditions of the Desert. Life abounds in the Atacama Desert, but most of it is microbial!

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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 and Presentations

Publications:

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Chapter 1. General Introduction

1.1. Background

The discovery of penicillin in 1929 marked the beginning of the antibiotic era and the realisation that microorganisms were a source of clinically significant natural products (Betina, 1983). The discovery and medicinal use of antibiotics over the past sixty years has without question conferred one of the greatest benefits to humankind. At present, over a thousand microbial natural products are in use as antibiotics, agrochemicals and antitumour agents (Bérdy, 2005, 2012).

It is now common knowledge that new drugs, especially antibiotics, are needed to control the spread of multi-drug resistant microbial pathogens and to treat patients with life-threatening diseases such as cancer (Payne *et al*., 2007; Fischbach, & Walsh, 2009; Genilloud, 2014). Problems caused by drug-resistant microbial pathogens are especially serious, as illustrated by infections caused by vancomycin-resistant *Enterococcus faecium*, fluoroquinoline-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* strains and even more alarmingly by panantibiotic resistant infections attributed to *Acinetobacter* and carbapenem-resistant *Klebsiella* species (Donadio *et al*., 2010a). Somewhat perversely as the prospect of a return to the pre-antibiotic days of medicine looms the number of approved therapeutic leads is in sharp decline (Donadio *et al*., 2010b; Butler & Cooper, 2011; Genilloud, 2014).

Microbial natural products are still considered to be the most promising source of new drugs (Bull & Stach, 2007; Cragg & Newman, 2013). This is partly because alternative strategies, such as combinational chemistry and fragment-based drug design, have been found to be disappointing (Newman, 2008) and partly because culture-independent molecular procedures have shown that natural habitats contain an astonishing diversity of prokaryotes, an unknown majority that constitutes an enormous genetic resource for exploitable biology (Whitman *et al.*, 1998; Bull, 2004). Indeed, it is now widely recognized that < 1.0% of microorganisms in natural ecosystems have been cultivated (Bull *et al.*, 2000; Kennedy *et al.*, 2010).

The ability of members of the microbial world to synthesize bioactive secondary metabolites is discontinuously distributed. Amongst prokaryotes, organisms classified in the phylum *Actinobacteria*, notably in the genus *Streptomyces*, remain the most abundant source of natural products, including clinically significant antibiotics, antimetabolites and antitumour agents (Bérdy, 2005; Olano *et al*., 2009; Newman & Cragg, 2013). The number of bioactive compounds synthesised by filamentous actinobacteria assigned to the class *Actinobacteria* account for nearly half of microbial secondary metabolites with around 8,000 of them derived from streptomycetes (Bérdy, 2005). Despites this amazing-actinobacterial productivity it has been estimated that only about 10% of the natural products that can be produced by streptomycetes have been discovered (Watve *et al*., 2001). Members of other actinobacteria taxa, such as the genera *Actinomadura, Amycolatopsis, Nonomuraea, Saccharothrix* and *Streptosporangium*, have featured in drug discovery programmes (Lazzaruni *et al*., 2000; Genilloud *et al*., 2011; Tiwari & Gupta, 2012a); such taxa are often referred to as "rare genera" (Tiwari & Gupta, 2012a).

The resurgence of interest in actinobacteria as a source of novel specialised (secondary) metabolites comes from the application of genomic technologies (Goodfellow & Feidler, 2010; Genilloud, 2014). Whole-genome sequencing studies have shown that the genomes of filamentous actinobacteria, unlike those of almost all other prokaryotes, are rich in biosynthetic gene clusters that code for known or predicted specialised metabolites (Goodfellow & Fiedler, 2010) while culture-independent survey have shown that large numbers of novel actinobacterial taxa are present in natural ecosystems (Stach *et al*., 2003a, b; Das *et al*., 2007; Sun *et al*., 2010). Even so, it is becoming increasingly difficult to find new chemical entities from common actinobacteria isolated from well studied habitats as screening such organisms leads to the costly rediscovery of known bioactive compounds (Busti *et al*., 2006; Lam, 2007; Williams, 2008). Consequently, new approaches are being developed for the selective isolation, dereplication and recognition of novel actinobacteria from neglected and unexplored ecosystems, as illustrated by the bioprospecting strategy recommended by Goodfellow and Fiedler (2010) and outlined in Figure 1.1. This taxonomic approach to bioprospecting has been used to isolate actinobacteria from extreme biomes, notably marine habitats, on the premise that harsh environmental conditions give rise to unique taxa which are likely to have a novel chemistry (Bull & Stach, 2007; Bull, 2011).

Novel actinobacteria from deep sea sediments have been found to be a prolific source of specialised metabolites, as exemplified by the discovery of a new family of

polycyclic polyketides, the abyssomicins, from *Verrucosispora maris* (Bister *et al*., 2004; Riedlinger *et al*., 2004; Keller *et al*., 2007; Goodfellow *et al*., 2012b); the anticancer drug, salinosporamide, from *Salinispora tropica* (Jensen *et al*., 2007; Fenical *et al*., 2009) and the demacozines from *Dermacoccus abysii*, a piezotolerant strain isolated from the Challenger Deep of the Mariana Trench (Pathom-aree *et al*., 2006; Abdel-Mageeb *et al*., 2010). Such studies have sparked a flurry of interest in marine actinobacteria as a source of natural products (Imhoff *et al*., 2011; Blunt *et al*., 2012; Zotchev, 2012; Manivasagan *et al*., 2013).

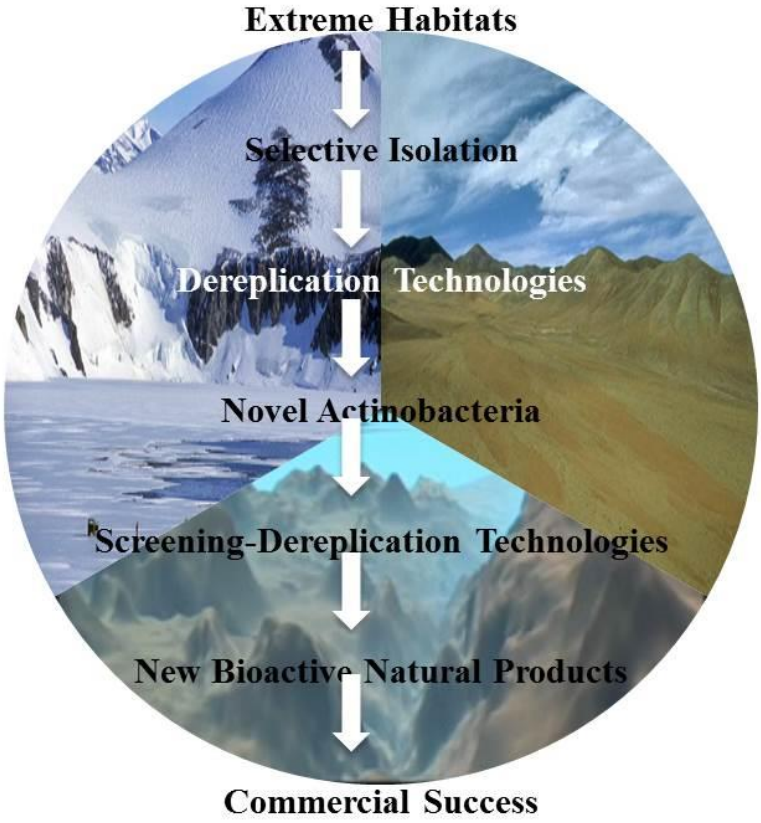


Figure 1.1. Culture-dependent bioprospecting strategy (taken from Goodfellow, 2013).

Taxonomically diverse actinobacteria, notably streptomycetes, have been isolated from marine environments using the strategy shown in Figure 1.1, and representatives of dereplicated groups found to be a good source of novel antibiotics with unique modes of action (Bull *et al*., 2005; Fiedler *et al*., 2005; Goodfellow & Fiedler, 2010). Particular strong support for culture-dependent approaches to bioprospecting comes from extensive surveys of obligate marine actinobacteria classified in the genus *Salinispora* (Jensen *et al*., 2005; Jensen, 2010; Freel *et al*., 2012;

Ahmed *et al*., 2013), notably those which show strong evidence of a coupling between taxonomic and chemical diversity.

Another neglected ecosystem that has recently attracted the attention of microbiologists is the temperate Atacama Desert in northern Chile (Azura-Bustos *et al*., 2012; Bull & Asenjo, 2013). Most Atacama Desert soils are either hyper-arid, that is, the ratio of mean annual rainfall to mean annual evaporation is < 0.005 or extreme hyper-arid where the corresponding value is < 0.002. The harshness of these conditions is compounded by very low concentrations of organic carbon, high UV radiation, the presence of inorganic oxidants and in some areas by high salinity. Despite these unfavourable conditions for microbial life both culture-dependent and culture-independent studies have revealed the presence of small populations of taxonomically diverse bacteria, including actinobacteria, in Atacama Desert soils (Drees *et al.*, 2006; Demergasso *et al.*, 2008; Fletcher *et al*., 2012; Bull & Asenjo, 2013).

Okoro and her colleagues (2009) isolated small numbers of filamentous actinobacteria from heat-pretreated samples of hyper-arid and extreme hyper-arid Atacama Desert soils using a range of selective isolation media. Most of the isolates were assigned to putatively novel *Streptomyces* species, the remainder formed new centres of taxonomic variation within two "rare taxa", the genera *Amycolatopsis* and *Lechevalieria*. In subsequent studies, the *Lechevalieria* strains were classified into three new species (Okoro *et al.*, 2010) while four of the *Streptomyces* isolates have been given species status (Santhanam *et al.*, 2012a, b, 2013; Busarakam *et al*., 2014). In addition, a further two *Streptomyces* isolates were found to produce new ansamycin and 22-membered macrolactones that showed a range of antibacterial and antitumour properties (Nachtigall *et al.*, 2011; Rateb *et al*., 2011a, b). Another putatively novel *Streptomyces* strain isolated from a high altitude Atacama Desert soil produces novel aminobenzoquinones, the abenquines, which show inhibiting activity against bacteria and dematophytic fungi (Schulz *et al.*, 2011). These initial studies show that Atacama Desert soils contain novel filamentous actinobacteria with the ability to synthesise new natural products.

1.2. Aims and content of thesis

The present study was designed to build upon and extend the pioneering investigations of Atacama Desert actinobacteria by generating a high quality library of taxonomically diverse strains of biotechnological and ecological interest. To this end, the taxonomic

approach to bioprospecting for novel bioactive compounds was used to selectively isolate, dereplicate and classify representative actinobacteria from two contrasting Atacama Desert environments, namely hyper-arid and extreme hyper-arid soils from the Chaxa de Laguna of the Salar de Atacama and the Yungay regions, respectively. Strains found to be of biotechnological or ecological interest were the focus of polyphasic studies in order to provide genotypic and phenotypic data for the formal description of new taxa. Representative strains, notably streptomycetes, were screened for bioactivity against panels of wild type organisms and *Bacillus subtilis* reporter strains in plug assays and extracts of interest sent to Professor Marcel Jaspars (University of Aberdeen) to be screened for novel chemistry. The thesis includes the following chapters.

Chapter 1. General introduction

- Background
- Aims and content of thesis
- Prokaryotic systematics
- Actinobacterial diversity and bioprospecting
- Selective isolation and recognition of novel taxa
- Extreme habitats as a source of novel actinobacteria
- Screening for bioactive compounds

Chapter 2. Materials and methods

- Sampling sites
- Physico-chemical properties of environmental samples
- Selection, maintenance and presumptive classification of actinobacteria isolated from the Salar de Atacama and Yungay environmental samples
- Comparative 16S rRNA gene sequencing studies
- Detection of additional chemical markers
- DNA-DNA relatedness assays
- Detection of phenotypic properties
- Morphology
- Screening for bioactivity
- Preliminary characterisation of bioactive compounds

Chapter 3. Biosystematic studies and screening of representative strains isolated from hyper-arid and extreme hyper-arid Atacama Desert soils

- Sampling sites

- Physico-chemical properties of environmental samples
- Selection, maintenance and presumptive classification of actinobacteria isolated from the Salar de Atacama and Yungay environmental samples
- Classification of representative actinobacteria from the Salar de Atacama and Yungay environmental samples
- Screening for bioactivity
- Preliminary characterisation of some bioactive compounds

Chapter 4. Classification of thermophilic *Amycolatopsis* strains isolated from arid desert soils

- Source, selective isolation and enumeration
- Polyphasic taxonomy of representative strains
- Formal naming of novel species and emended descriptions of *Amycolatopsis ruanii* and *Amycolatopsis thermalba*

Chapter 5. Biosystematic studies on *Modestobacter* strains isolated from extreme hyper-arid desert soil and from historic buildings

- Source, selective isolation and enumeration
- Polyphasic taxonomy of representative strains
- Formal description of novel species
- Screening of selected strains

Chapter 6. Polyphasic studies on presumptive *Streptomyces* strains isolated from hyper-arid and extreme hyper-arid Atacama Desert soils

- Source and selective isolation
- Polyphasic taxonomy of antibiotic-producing strains
- Formal naming of novel species

Chapter 7. General discussion and prospectives for future work

1.3. Prokaryotic systematics

The power of the taxonomic approach to drug discovery with particular reference to actinobacteria is based upon concepts and practices that underpin prokaryotic systematics (Goodfellow *et al.*, 1997; Schleifer, 2009; Oren & Garrity, 2014).

Prokaryotic systematics, the scientific study of the kinds and diversity of *Achaea* and *Bacteria*, is a scientific discipline which encompasses *Classification, Nomenclature* and *Identification*. The first step, classification, is the practice of assigning prokaryotes to taxonomic groups based on similarities and differences. The outcome of this process is an orderly arrangement or system designed to show natural relationships between taxa and serves 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 a prerequisite for stable nomenclature and reliable identification.

Taxonomies based on genotypic and phenotypic properties are called phenetic classifications. These classifications are built upon phenotypic data (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 ancestry. In contrast, phylogenetic classifications express inferred evolutionary relatedness between organisms and thereby reflect the extent of change over time. In practice, phenetic and phylogenetic classifications are usually found to be congruent. Current approaches to prokaryotic classification based on 16S rRNA gene sequences are usually considered to be phylogenetic, but many are in fact phenetic as they are based on similarities and differences between homologous nucleotide sequences.

The second step, nomenclature, deals with the terms used to depict ranks in the taxonomic hierarchy (e.g. species, genera, families), notably with the practice of giving correct, internationally accepted names to taxonomic groups according to rules and recommendations given in successive editions of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1975, 1992). In 1975, Lapage and his colleagues introduced two changes to the "Bacteriological Code" which had a far reaching impact on the nomenclature of prokaryotes. Thus, a definitive document and starting date was introduced for the recognition of names 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 of Bacterial Names* lost their standing in nomenclature, a development that cleared away thousands of meaningless names based on poorly described taxa. Secondly, it was decided that new taxa had to be validly published in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM; formerly the *International 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 to microbiologists as they need to know which organisms they are studying before they transmit information about them within and outside the scientific community (Tindall *et al*., 2006). In essence, an organism's name is a vehicle of communication and a key to its literature, an entry to what is known about it.

The final step, identification, is sometime seem as the most important element of prokaryotic systematics given the importance of accurately identifying unknown organisms, especially with respect to pathogenic bacteria (Priest & Williams, 1993). It is both the act and the result of determining whether unknown organisms belong to published taxa (Krieg, 2005). It involves determining the key characteristics of unknown organisms and matching them against databases containing corresponding information on validly published taxa (Priest, 2004). Prokaryotes that do not fit into established groups in the taxonomic hierarchy should be classified as new taxa.

Classifications of prokaryotes are data dependent and hence are in a continuous state of development as high quality information becomes available from the application of new and improved techniques (Tindall *et al*., 2010; ORainey & Oren, 2011; Kim *et al*., 2014; Oren & Garrity, 2014). Such taxonomies are essentially empirical as they are driven by technological advances not by theoretical considerations, as exemplified by the biological species concept (Goodfellow *et al*., 1997; Schleifer, 2009). Current approaches to the classification of prokaryotes are based on the integrated use of genotypic and phenotypic features derived from the application of chemotaxonomic, molecular systematic and 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, and still does, provide a sound basis for stable nomenclature and reliable identification, essential factors for practical or utilitarian taxonomy designed to serve different end users (Vandamme *et al*., 1996; Goodfellow *et al*., 1997; Gillis *et al*., 2005; Krieg & Padgett, 2011).

The application of polyphasic taxonomy led to significant improvements in the classification of prokaryotes, especially in groups like the *Actinobacteria* and *Cyanobacteria* where traditional approaches based on form and function were found to be unreliable (Goodfellow & Maldonado, 2006; Kroppenstedt & Goodfellow, 2006; Gupta, 2009). However, it is not possible at present to recommend a set of methods to be used in all polyphasic studies as the scope of such studies is influenced by the biological properties and rank of the taxa under study and by the equipment available to

investigators. Nevertheless, sequencing highly conserved macromolecules, notably 16S rRNA genes and conserved proteins, has provided valuable data for generating phylogenies at and above the genus level (Ludwig & Klenk, 2005; Ludwig *et al*., 2012). In contrast, DNA-DNA relatedness, molecular fingerprinting and phenotypic procedures are methods of choice for delineating taxa at and below the rank of species (Rosselló-Mora & Amann, 2001; Rosselló-Mora *et al*., 2011).

The basic taxonomic unit in prokaryotic systematics is the species though this remains an ill-defined concept (Rosselló-Mora & Amann, 2001; Staley, 2006; Schleifer, 2009). In the absence of a universally accepted definition of species (Ward, 1998; Stackebrandt *et al*., 2002), an operational or utilitarian species concept has been proposed for cultivable bacteria (Vandamme *et al*., 1996; Goodfellow *et al*., 1997; Schleifer 2009). In particular, extensive taxonomic studies led to the recommendation that genomic species should include strains with approximately 70% or more DNA-DNA relatedness with a difference of 5^{o}C or less in thermal stability (ΔTm; Wayne *et al*., 1987). In practice, polyphasic studies draw upon information acquired from chemotaxonomic, numerical phenetic and molecular systematic studies and are of considerable practical value in applied microbiology (Priest & Goodfellow, 2000; Bull, 2004; De Vos *et al*., 2009).

Chemotaxonomy. This is the study of the discontinuous distribution of chemical macromolecules (e.g. amino acids, fatty acids, polar lipids, polysaccharides, proteins and isoprenoid quinones) across different taxa and the use of such information for classification and identification (Kroppenstedt, 1985; Goodfellow & O'Donnell, 1994; Schleifer, 2009; Da Costa *et al*., 2011a, b, c). Chemotaxonomic analyses of amino acids, lipids (e.g., fatty acids, mycolic acids and polar lipids), and polysaccharides and related polymers (e.g. sugars and teichoic acids) has provide valuable data for classification of prokaryotes, notably actinobacteria at various ranks in the taxonomic hierarchy (Goodfellow, 2000). The determination of amino acid and cell wall sugar composition and peptidoglycan structure, in particular, led to marked improvements in the classification of actinobacteria (Williams *et al*., 1989; Goodfellow *et al*., 2010). The introduction of new technologies, such as analyses of proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Lanoot *et al*., 2002) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF; Dridi & Drancourt, 2011) have provided valuable information for the classification and identification of diverse bacteria, including actinobacteria (Lotz *et al*., 2010; Saleeb *et*

al., 2011). In general, good congruence has been found between the discontinuous distribution of chemical markers and phylogenetic classifications of actinobacteria (Chun & Goodfellow, 1995; Ward & Goodfellow, 2004; Goodfellow *et al.*, 2010).

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).

The conceptual basic and operation procedures of numerical taxonomy have been the subject of several comprehensive reviews (Sokal, 1985; Goodfellow *et al*., 1997) and hence will not be considered here. Indeed, conventional numerical taxonomic studies have tended to go out of fashion as they are seem to be time-consuming and laborious even though new high-throughput methods, such as commercially available 96 well phenotypic array plates, have been introduced to mitigate these problems (Bochner, 2003; Clemons, 2004; Bochner *et al.*, 2008).

The application of numerical taxonomic procedures in their heyday led to significant improvements in actinobacterial systematics. Numerical taxonomic studies were used to circumscribe taxospecies, including those in taxonomically complex genera such as *Actinomadura* (Trujillo & Goodfellow, 2003), *Actinoplanes* (Goodfellow *et al.*, 1990), *Gordonia* (Goodfellow *et al.*, 1991), *Nocardia* (Goodfellow *et al.*, 1982; Goodfellow, 1992), *Mycobacterium* (Wayne *et al.*, 1996), *Rhodococcus* (Goodfellow *et al*., 1998), *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 detailed taxonomic studies based on chemotaxonomic and molecular systematic procedures (Lanoot *et al.*, 2002; 2005; Girard *et al*., 2014a).

Molecular systematics. The most significant development in prokaryotic systematics in recent times was the realisation that bacterial genomes (and proteins) contain records of changes that have occurred since prokaryotes diverged from a common ancestor around 3.5 billion years ago (Zuckerkandl & Pauling, 1965; Woese, 1987). Molecular-sequence based approaches to systematics have a significant advantage over chemotaxonomic and numerical taxonomic methods as the acquisition of sequence data is independent of cultivation conditions. Molecular-based methods are currently the driving force in prokaryotic systematics, partly as a consequence of rapid technological advances, but also because the end product of this approach reflect natural relationships between prokaryotes as encoded in DNA and protein sequences (Head *et al.*, 1998; Woese, 1998; Gevers *et al.*, 2006; Schleifer, 2009; Alam *et al.*, 2010; Jensen, 2010; Girard *et al*., 2013, 2014).

Comparative analyses of nucleic acid and conserved protein sequences together with the identification of taxon-specific molecular signatures have had a profound impact on prokaryotic systematics (Stackbrandt & Goodfellow, 1991; Stackebrandt *et al*., 1997; Gupta, 1998, 2000; Zhi *et al*., 2009; Goodfellow *et al*., 2012a; Gao & Gupta, 2012). Comparison of almost complete 16S rRNA gene sequences has proved to be an especially effect way of establishing suprageneric relationships between prokaryotes (Woese, 1987; Ludwig & Klenk, 2001; Ludwig *et al*., 2011a; Yarza *et al*., 2012). In contrast, DNA-DNA hybridisation and molecular fingerprinting procedures together with complementary phenotypic data are invaluable in circumscribing taxa at species and infrasubspecific levels (Wayne *et al*., 1996; Rosselló-Mora & Amann, 2001; Tindall *et al*., 2010; Rosselló-Mora *et al*., 2011).

16S rRNA gene sequencing. Data derived from sequencing 16S rRNA genes have been used extensively for the classification of cultivated (Yarza *et al.*, 2012) and uncultivated actinobacteria (Stach *et al.*, 2003a, b; Kumar *et al.*, 2007) and for the design of oligonucleotide probes and primers for the identification of specific taxa (Shen & Young, 2005; Zhi *et al.*, 2009). However, 16S rRNA sequencing studies do not always allow delineation between closely related bacterial 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). In such cases molecular systematic methods that give higher resolution should be employed, as

illustrated by the delineation of *Salinispora* species based on partial *gyrB* sequences (Ahmed *et al.*, 2013).

Comparative 16S rRNA gene sequence analyses are seen to be a rapid and reliable way of classifying unknown prokaryotes. 16S rRNA gene sequence data held in the DNA Data Base of Japan (DDBJ; Kaminuma *et al*., 2011; http://www.ddbj.nig.ac.jp), the European Molecular Biology Laboratories Database (EMBL; Cochrane *et al*., 2008; http://www.ebi.ac.uk) and in special rRNA databases such as SIVA (Pruesse *et al*., 2007; http://www.arb-silva.de), RDP (Cole *et al*., 2009; http://www.rdp.cme.msu.edu) and Greengenes (De Santis *et al*., 2006; http://greengenes.lbl.gov) are retrievable for comparative taxonomic studies. In general, good, congruence has been found between phylogenetic trees derived from 16S rRNA gene sequence data and corresponding trees generated from studies based on other conserved molecules, such as elongation factors, protein-translocating ATPase subunits and RNA polymerase (Ludwig & Klenk, 2001).

***Analysis of sequence data and phylogenetic reconstruction*.** The various methods used to align and analyse 16S rRNA gene sequence data and construct and interpret phylogenetic trees have been considered in detail by Rosselló-Mora *et al*. (2011). The initial step, the alignment of rRNA gene sequences is critical for inferring phylogenetic relationships. The presence of insertions and deletions (indel sequences) may need to be addressed, especially when homology values are low; the use of secondary structural information is essential to localise 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 experience and judgment of the investigator.

Numerous tree-making methods are available to infer ancestry once nucleotide sequences have been aligned. In general, treeing approaches can be divided into two groups: cluster methods (algorithms) deal with distance data while discrete character methods use optionality criteria. 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 in 16S rRNA sequences, 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 often achieved by using the neighbour-joining (Saitou & Nei, 1987) and weighted least-squares (Fitch &

Margoliash, 1967) algorithms. 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 ultrametric and that all lineages have equally diverged. In contrast to cluster analysis, the neighbour-joining method keeps track of nodes on the tree rather than taxa or clusters of taxa. The least-squares method fits a given set of pairwise evolutionary distance estimates to an additive tree.

Widely used discrete character methods that employ optimality criteria include maximum-likelihood (Fitch, 1971) and the maximum-parsimony (Felsenstein, 1991) methods. Maximum-likelihood methods seek the tree that is most likely to generate the observed sequences. These methods examine sequences on a site-by-site basis but also incorporate on explicit model of sequence evolution to compensate for superimposed substitutions. Maximum-likelihood methods are 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 of achieving this. 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, the most parsimonious one, is taken as the one which infers evolution most closely (Felsenstein, 1981). This also examine sequences on a site-by-site basis and is successful at reconstructing inferred molecular histories, especially when the extent of change is small.

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 macromolecules is their capacity for reassociation or hybridisation. 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 amount of the 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 hybridisation (DDH) techniques which are used to make

comparisons of whole genomes between closely related prokaryotes in order to estimate their overall genomic similarities (Rosselló-Mora *et al.*, 2011).

The importance of DDH studies in circumscribing species was underlined by recommendations arising from an *ad hoc* committee on prokaryotic systematics (Wayne *et al*., 1987). 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 with 5℃ or less thermal stability (ΔTm). It is important to interpret DDH values with care as they may not reflect the actual degree of sequence similarity (Goodfellow *et al.*, 1997; Rosselló-Mora & Amann, 2001) and because results can be distorted by differences in genome size and genomic rearrangements (Kang *et al.*, 2007). It is well known that DDH data are prone to experimental error and cannot be used to generate cumulative databases as they are based on pairwise comparisons between tested strains (Goodfellow *et al.*, 1997; Stackebrandt *et al.*, 2002 Schleifer, 2009; Kang *et al.*, 2007; Rosselló-Mora *et al.*, 2011).

The strengths and weaknesses of the different DDH techniques that have been developed over the past fifty years have been reviewed by Rosselló-Mora (2006). Two experimental approaches are commonly used to measure the degree of DNA relatedness or similarity between prokaryotes. These approaches are based on assessing the degree of binding by hybridisation (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, usually 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 that of homologous DNA. The temperature at which 50% of the initial double-stranded molecules denatures into single-stranded DNA is the melting temperature or thermal denaturation midpoint (*Tm*).

A parameter commonly used to estimate DNA-DNA relatedness, Δ*Tm*, is the difference between the *Tm* of a reference strain and that of corresponding 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}); T_{or} can be estimated from the mol% G+C of the DNA of the 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, as it is rapid and inexpensive, and allows high-throughput comparisons. Comparative studies show that results derived from estimating binding percentages and ΔTm values are generally in good agreement (Roselló-Mora & Amann, 2001; Gonzalez & Saiz-Jimenez, 2005; Goodfellow *et al*., 2007).

Despite their limitations, DDH studies are still widely used for the delineation of closely related prokaryotic species, including actinobacteria (Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997; Rosselló-Mora & Amann, 2001; Kumar & Goodfellow, 2008; Rosselló-Mora *et al*., 2011). Such studies give greater resolution between strains than corresponding 16S rRNA gene sequencing studies, as is apparent from Figure 1.2. Organisms with almost identical 16S rRNA sequence similarities can be distinguished using corresponding DDH data.

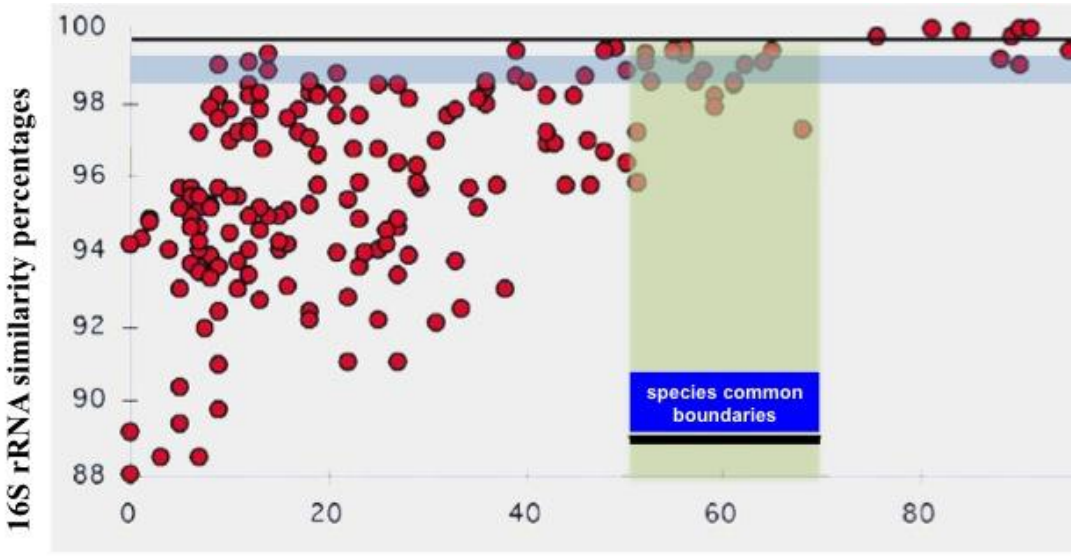


Figure 1.2. Comparison of DNA-DNA and 16S rRNA gene similarities of *Proteobacteria, Cytophaga-Flavobacterium-Bacteroides* and Gram-positive bacteria of high GC phyla. The vertical shaded zone indicates the range of cut-off values for DNA-DNA relatedness used for the delineation of genomic species while the horizontal shade zone indicates cut-off values for 16S rRNA gene sequence similarity (99 %) (adapted from Rosselló-Mora & Amann, 2001).

It is tedious and time-consuming to establish DDH similarities between pairs of closely related strains hence such experiments are to be avoided if there are good

reasons for doing so. Stackebrandt and Goebel (1994) recommended that if two strains shared 16S rRNA gene similarities at or below the 97% threshold it was unnecessary to undertake DDH determinations to prove that they belonged to the same species. This threshold was raised to 98.7-99.0%, based, as before, on an empirical dataset taken from the taxonomic literature (Stackebrandt & Ebers, 2006). Using real world 16S rRNA gene sequence and corresponding DDH data, Meier- Kolthoff *et al*. (2013) concluded that a threshold at or below 99.0% was a realistic cut-off point for recognising related pairs of actinobacterial strains belonged to different genomic species. They noted that approximately half of recent DDH experiments could safely been omitted without significant missclassification of tested strains.

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.*, 2002). They also considered that multilocus sequence analyses (MLSA) might be used as an alternative to DNA-DNA relatedness studies in defining genomic species. MLSA 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; De Vos, 2011; Cody *et al.*, 2014). The choice of genes needs to be based on their loci; selected genes should be spread across the genome (Maiden *et al.*, 1998; De Vos, 2011). MLSA 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, MLSA 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, including actinobacteria, as shown by studies on groups of closely related *Streptomyces* species (Guo *et al.*, 2008; Rong & Huang, 2010, 2012; Rong *et al.*, 2009, 2010; Adékambi *et al.*, 2011).

Embracing the genome. It has already pointed out that polyphasic taxonomic studies are being increasingly driven by advances in molecular biology, as shown by the impact that 16S rRNA gene sequence and DNA relatedness data have had on the circumscription of prokaryotic taxa, notably at the rank of species (Rosselló-Mora & Amann, 2001; Sutcliffe *et al.*, 2012). The application of polyphasic taxonomy over the

past forty years has culminated in spectacular improvements in actinobacterial systematics (Goodfellow *et al*., 2012a). However, serious problems remain, especially with the delineation of species within genera such as *Amycolatopsis* and *Streptomyces* where 16S rRNA gene sequence and associated phenotypic data are not always sufficient for the recognition of species (Kämpfer, 2012; Tan & Goodfellow, 2012). Improved methods are also needed to unambiguously establish the taxonomic status of closely related genera within morphologically complex taxa, such as the families *Pseudonocardiaceae* and *Streptomycetaceae* (Labeda *et al*., 2010; 2011). There is evidence that such problems can be addressed by the judicious use of whole genome sequence data (Girard *et al.*, 2013, 2014).

The next generation sequencing revolution has seen the number of genome sequences released into the public domain accelerate at an ever increasing rate (Shendure & Lieberman-Aiden, 2012). Currently, sequences of over 8000 bacterial genomes are publically available, including those of strains classified in the phylum *Actinobacteria*. Indeed, following the publication of the genome sequence of "*Streptomyces coelicolor*" A(3)2 (Bentley *et al*., 2002) over 100 *Streptomyces* genomes and a score of other actinobacterial genomes

have been released (http://www.genomesonline.org).

Comparative genome sequence data are being extensively used to infer genome-based phylogenics (Wu *et al*., 2009; Klenk & Göker, 2010; Gao & Gupta, 2010) and to illuminate the evolution and mechanisms of actinobacterial complexity (Girard *et al*., 2013, 2014; Chandra & Chater, 2014). In contrast, there has been a surprising reluctance amongst prokaryotic systematists to use genome sequence data to support the circumscription of novel taxa or to help unravel relationships between poorly delineated genera, despite the fact that the use of such data are a logical extension of the polyphasic taxonomic concept (Kämpfer & Glaeser, 2012). Indeed, the use of such data for descriptive purposes should provide valuable insights into the ecology, metabolism, physiology and biotechnological potential of individual novel taxa and thereby help to re-establish prokaryotic systematics as a fundamental scientific discipline. However, it is critically important that the increasing use of genome data for taxonomic purposes builds upon sound taxonomic concepts painstakingly developed over the last fifty years, not least the nomenclatural type concept (Goodfellow & Fiedler, 2010; Jensen, 2010; Whitman, 2011, Kämpfer & Glaesen, 2012; Oren & Garrity, 2014). The advantages and limitations of using whole-genome sequences in prokaryotic systematics has been summarised by Sentausa & Fournier (2013).

1.4. Actinobacterial diversity and bioprospecting

The term biodiversity or more precisely biological diversity encompasses *genetic diversity*, the distribution or variation of genes and genomes within a species (intraspecific diversity); *species or organism diversity*, the number of species in a microbial community or the variety of organisms within a region (species richness), and *ecological or ecosystem diversity*, the number of communities in an ecosystem or ecosystem diversity within a region (Harper & Hawksworth, 1994; Bull & Stach, 2004). The focus in the present study is on species diversity of actinobacteria in Atacama Desert soils, notably as a prospective source of novel specialised metabolites.

It has been estimated that a single gram of fertile soil may contain up to 8.3 million bacterial species (Schloss & Handelsman, 2006) and that the number of prokaryotic cells present in natural habitats is 4-6 $x10^{30}$ (Whitman *et al*., 1998). It is astonishing given such estimates that only about 13,000 prokaryotic species have been validly named (http://www.bacterio.net/-number.html#sinceAL). Indeed, it has been estimated less than 0.1% of prokaryotes in marine ecosystems have been cultured (Bull *et al*., 1992, 2000), a similar situation has been reported for actinobacteria (Stach *et al*., 2003a, b). It is clear from such estimates that remarkably few actinobacterial taxa have been isolated from natural ecosystems and screened for natural products.

Although only a small fraction of actinobacterial diversity in natural habitats has been isolated and included in pharmacological screening programmes these organisms have proved to be a prolific source of novel bioactive compounds, including antibiotics of therapeutic value (Lazzarini *et al*., 2000; Strohl, 2004; Genilloud *et al*., 2011; Genilloud, 2014). It seems likely that this trend will continue as novel actinobacteria are isolated and screened, not least because full genome sequences of taxonomically diverse filamentous actinobacteria have been found to contain over 20 natural product biosynthetic gene clusters for the production of specialised metabolites, as shown by studies on *Amycolatopsis mediterranei* U32^T (Zhao *et al.*, 2002), *Blastococcus saxobsidens* DD2 (Chouaia *et al*., 2012), *Geodermatophilus obscurus* G20^T (Ivanova *et al*., 2010), *Kribbella flavida* IFO 14399^T (Pukall *et al*., 2012), *Modestobacter marinus* BC 501(Normand *et al*., 2012), *Saccharopolyspora erythaea* NRRL 4338^T (Oliynyk *et al*., 2007), *Salinispora tropica* CNB 440^T (Udwary *et al*., 2007), *Saccharothrix espannaensis* DSM 44229^T(Strobel *et al*., 2012), *Streptomyces avermitilis* MA-4680^T (Ikeda *et al*., 2003) and "*Streptomyces coelicolor*"A3(2) (Bentley *et al*., 2002).

There is evidence that taxonomic diversity amongst filamentous actinobacteria can be used as a surrogate for chemical diversity, especially at the species level (Goodfellow *et al*., 2007; Jensen *et al*., 2007; Tan *et al*., 2007). It can, therefore, be anticipated that taxa which occupy gaps in actinobacterial taxospace may prove to be a rich source of new bioactive compounds (Ward & Goodfellow, 2004; Jensen, 2010). Consequently, it makes good sense to devise new strategies for the selective isolation and recognition of novel actinobacteria, including streptomycetes and members of "rare genera", as exemplified by the taxonomic approaches to drug discovery (Goodfellow & Fiedler, 2010; Genilloud *et al*., 2011; Wang *et al*., 2011; Tiwari & Gupta, 2012a, b).

Table 1.1. Antibiotics isolated from actinobacterial 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	■	■	■	■	■	■	■	■	■	■	■
Rare actinobacteria											
Actinomadura	■	■					■			■	
Actinoplanes	■	■			■	■	■		■	■	■
Actinosynnema				■							
Amycolatopsis			■		■	■	■				■
Dactylosporangium	■	■					■		■	■	
Kibdelosporangium		■				■					
Kitatospora		■			■						
Microbispora	■				■						■
Micromonospora	■	■	■		■		■		■	■	■
Microtetraspora		■			■						
Norcardia	■	■	■	■	■	■	■	■	■		■
Nocardiopsis	■	■									
Nonomuraea						■					
Pseudonocardia					■	■					■
Rhodococcus					■						■
Saccharomonospora						■					
Saccharopolyspora	■	■			■						
Saccharothrix	■	■			■	■					■
Streptoalloteichus						■					
Streptosporangium			■		■	■		■			
Thermomonospora					■						

* AG, aminoglycoside; ML, macrolide; AML, ansamacrolide; BLA, β-lactam; PEP, peptide; GP, glycopeptide; ANC, antracycline; TC, tetracycline; NUC, nucleotide; POL, polyene; QN, quinine. ■ , production (modified from Okami & Hotta, 1998).

Indeed, members of rare actinobacterial genera are a source of novel antibiotics used extensively in medicine, such as erythromycin produced by *Saccharopolyspora erythraea* (Oliynyk *et al*., 2007), gentamicin by *Micromonospora 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). Clearly, it is time to re-energise the quest for the next generation of antibiotics for healthcare from members of novel actinobacterial taxa, including the genus *Streptomyces*.

Another pertinent point is that actinobacterial antibiotics show an extensive range of chemical diversity, as shown in Table 1.1 Streptomycetes in particular synthesise a broad range of chemical entities, structural types of antibiotics commonly produced by these organisms include ansalactam rings, monocyclic lactins and products with polyether and cyclopeptide skeletons (Bérdy 2005).

1.5. Selective isolation, dereplication and recognition of novel taxa

Innumerable filamentous actinobacteria have been isolated and screened since the discovery that a *Streptomyces griseus* strain produced streptomycin (Schatz *et al*., 1944), a momentous development that stimulated the search for additional novel bioactive compounds of therapeutic value. Early search and discovery programmes were focused on streptomycetes as these organisms were easy to isolate, grow and recognise on isolation media. Intensive screening of 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 has already been pointed out that it is important to selectively isolate, dereplicate and recognise novel actinobacteria from environmental samples in order to secure high quality biological material for pharmaceutical screening programmes (Bull *et al.*, 2000; Goodfellow & Fiedler, 2010). The application of such procedures show that actinobacteria once considered to be rare in natural habitats are widely distributed and numerous, as shown by studies on acidiphilic (Kim *et al.*, 2003; Busti *et al*., 2006; Golinska *et al*., 2013a, b, c), endophytic (Janso & Carter, 2010), halophilic (Meklat *et al*., 2011), motile (Hayakawa *et al.*, 2000; Suzuki *et al.*, 2001) and marine actinobacteria (Gontang *et al*., 2007; Becerril-Espinosa *et al*., 2013) and by studies on individual taxa, such as the genera *Amcolatopsis* (Tan *et al*., 2006), *Dactylosporangium* (Kim *et al*., 2011). *Micromonospora* (Goodfellow & Haynes, 1984; Maldonado *et al*., 2008) and *Rhodococcus* (Colquhoun *et al*., 1998), *Sphaerisporangium* (Janso & Carter, 2010) and *Streptacidiphilus* (Cho *et al*., 2006).

Most actinobacteria have a saprophytic mode of life 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 many different kinds of actinobacteria present in environmental samples due to their diverse growth and incubation requirements. Consequently, numerous approaches have been recommended for the isolation of specific actinobacterial taxa (Nolan & Cross, 1988; Labeda & Shearer, 1990; Goodfellow, 2010; Tiwari & Gupta, 2012a) based on their biological properties (Cross, 1982; Wellington & Cross, 1983; Goodfellow & O'Donnell, 1989; Goodfellow, 2010). Most selective isolation procedures involve the extraction of propagules (hyphae and spores) from selected environmental samples, pretreatment(s) of samples, use of selective media and appropriate incubation conditions followed by dereplication, and recognition of target colonies.

Extraction of actinobacterial 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 always effective (Hopkins *et al.*, 1991). It is particularly important to thoroughly break up soil-sediment particles as many microorganisms, notably those showing mycelial growth, may be bound within them. 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 (Ramsay, 1984); all of these procedures address the problem of quantitative and representative sampling to varying degrees. The dispersion and differential centrifugation technique, a multistage procedure introduced by Hopkins *et al*. (1991), combines several physico-chemical treatments which have been found to be effective in increasing the number and taxonomic diversity of actinobacteria isolated from natural habitats (MacNaughton & O'Donnell, 1994; Atalan *et al*., 2000; Sembiring *et al.*, 2000; Maldonado *et al*., 2005).

Pre-treatment of environmental samples. Several pre-treatment procedures are used to select different fractions of actinobacterial communities present in environmental samples. In general, pre-treatment regimes select for target taxa by inhibiting or eliminating the growth of unwanted microorganisms. Actinobacterial spores are more

resistant to desiccation than other bacterial cells hence air-drying soil/sediment samples at room temperature helps eliminate unwanted Gram-negative bacteria which might otherwise overrun isolation plates (Williams *et al.*, 1984a; Labeda & Shearer, 1990; Sembiring *et al.*, 2000). A pre-treatment regime based on alternate drying and wetting of soil has been used to enrich for sporangia (spore vesicles)–forming genera (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 actinobacteial propagules to desiccation is usually accompanied by some degree of resistance to heat. The basis of this resistance is not clear but it is apparent that many actinobacterial spores (e.g., *Micromonospora* and *Microtetraspora*), spore vesicles (eg. *Dactylosporangium* and *Streptosporangium*) and hyphal fragments (e.g., *Rhodococcus*) are more resistant to heat than vegetative cells of other prokaryotes. Heat pretreatment procedures have been shown to be effective in the selective isolation of several actinobacterial taxa, as exemplified by the novel species of *Actinomadura, Microbispora, Microteteraspora* and *Themomonospora* on selective media inoculated with suspensions of air-dried soil that had been heated at either 100^{o}C or 120^{o}C before plating onto selective media (Nonomura & Ohara, 1969; Hayakawa *et al*., 1996). However, actinobacterial propagules are more sensitive to wet than dry heat hence much lower temperatures are used to isolate these organisms from suspensions of environmental samples. Rowbotham & Cross (1977) isolated *Dactyosporangium* and *Thermomonospora* strains from water and soil suspensions heated at either 55^{o}C for 6 minutes or at 44^{o}C for 80 minutes prior to plating onto isolation media. Similarly, Orchard & Goodfellow (1974) recommended the pre-treatment of 10^{-1} dilutions of soil at 55^{o}C for 6 minutes as an improved way of isolating nocardiae. This heat pre-treatment regime has been used to isolate what proved to be novel *Streptomyces* species from hay meadow soils (Atalan *et al.*, 2000; Manfio *et al*., 2003). Heat-pretreatment procedures usually lead to a decrease in the ratio of bacteria to actinobacteria on selective isolation plates though counts of the latter may also be reduced (Williams *et al*., 1984).

Chemical pretreatments of mixed inocula are used to isolate specific actinobacterial taxa, notably members of genera classified in the family *Streptosporangiaceae* (Hayakawa *et al.*, 1988, 1991a, 1995; Yamamura *et al.*, 2003; Goodfellow, 2010). The selective chemical procedures introduced by Hayakawa and his colleagues are based on the differential ability of actinobacterial spores to withstand

treatment with chemical germicides, such as benzethonium chloride, chlorhexidine gluconate and phenol. Treatment with these agents for 30 minutes at 30^{o}C kill 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 have been used to increase the populations of specific fractions of actinobacterial communities in environmental samples to facilitate their isolation on selective isolation media. Amendments of soil with substrates such as chitin and keratin was first used by Jensen (1930) to boost the numbers of streptomycetes. The addition of these substrates led to an increase in the number of streptomycetes through amendment with chitin also led to an increase in the number of unwanted bacteria. The practise of amending environmental samples with substrates has fallen into neglect in recent times, but this approach does provide an effective way of enhancing specific components of actinobacterial communities in soil (Williams *et al.*, 1971; Nonomura &Takagi, 1977).

Selective isolation media. Numerous media have been recommended for the isolation of a diverse actinobacterial taxa from natural habitats and more specifically for selected families, genera and species (Kurtböke, 2003; Goodfellow, 2010; Tiwari & Gupta, 2012a). 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 (Lingappa & Luckwood, 1962; Hsu & Lockwood, 1975) and starch-casein nitrate (Küster & Williams, 1964) agars tend to select for a relatively narrow range of *Streptomyces* species nor do they support the growth of actinobacteria that have different nutritional requirements (Williams *et al.*, 1984a).

Selective isolation media can be formulated in an objective way by drawing upon 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 nocardiae from soil and water samples (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 an antibiotic sensitivity database (Goodfellow *et al.*, 1989). An 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).

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 actinobacteria from competing soil inhabiting bacteria (Nonomura & Ohara, 1960; Williams & Davies, 1965), as have nalidixic acid and trimethoprim (Hayakawa *et al.*, 1996). Antibacterial antibiotics are also widely used for the selective isolation of specific actinobacterial taxa (Goodfellow, 2010).

Incubation. Incubation conditions, including gaseous regimes, incubation times and temperature, contribute to selectivity. Incubation at 25 to 30°C favours mesophilic actinobacteria whereas their thermophilic counterparts require higher temperatures. Thermophilic species, such as *Pseudonocardia thermophila* and *Saccharopolyspora rectivirgula*, only require incubation for 2-3 days at 45 to 50°C. 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.

Colony selection and dereplication. The selection of actinobacterial colonies on selective isolation plates is a time-consuming and fairly subjective stage in isolation procedures. 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 assigned to genera 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 actinobacterial 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 a rapid grouping of isolates based on colonial characteristics and molecular fingerprinting patterns (Tan *et al.*, 2006; Antony-Babu *et al.*, 2008).

A practical and reliable way of dereplicating streptomycetes was introduced by Williams *et al.* (1969) who assigned large numbers of soil streptomycetes to groups based on aerial spore mass, substrate mycelial and diffusible pigment colours produced on oatmeal agar, and on their ability to form melanin pigments on yeast extract-malt extract-iron agar. Colour-groups were subsequently shown to reflect the extent of the taxonomic diversity of streptomycetes in rhizosphere and non-rhizosphere soils (Williams & Vickers, 1988; Atalan *et al*., 2000; Sembiring *et al*., 2000), as representatives of such groups were found to belong to either validly named or novel *Streptomyces* species or species-groups based on computer-assisted identification (Williams & Vickers, 1988; Atalan *et al*., 2000) and polyphasic taxonomy (Manfio *et al*., 2003; Goodfellow *et al*., 2007).

The classification of streptomycetes into colour-groups has been used to gain an insight into the taxonomic diversity of these micro-organisms in a beach and sand dune system (Antony-Babu & Goodfellow, 2008), in marine sediments (Goodfellow & Haynes, 1984; Pathom-aree *et al*., 2006) and arid desert soils (Okoro *et al*., 2009), and thereby to the selection of representative isolates for screening assays (Goodfellow & Fiedler, 2010). A reasonable linear correlation has been found between streptomycete colour-groups and corresponding *rep*-PCR data (Antony-Babu *et al.*, 2010).

Recognition of novel taxa. The classification of representatives of dereplicated groups of actinobacteria can be achieved using a two stage procedure. Reliable methods are used to assign representative strains to higher taxonomic ranks (eg. genera, families and orders) prior to the choice of diagnostic tests for the recognition of new or validly named species. The first objective can be achieved by comparing the almost complete 16S rRNA gene sequences of the representative isolates with those of their nearest phylogenetic neighbours using appropriate databases and software (Felis *et al*., 2010; Kim *et al.*, 2012; Ludwig *et al*., 2011, 2012) and chemotaxonomic and morphological data (The Society of Actinomycetes Japan, 2001; Goodfellow *et al*., 2012b). The second stage, the classification of representatives to validly named new species can be done by using combinations of phenotypic properties with or without associated DDH assays, as exemplified by the circumscription of novel *Amycolatopsis* (Zucchi *et al*., 2012; Camas

et al., 2013), *Modestobacter* (Xiao *et al*., 2011; Qin *et al*., 2013) and *Streptomyces* species (Promnuan *et al*., 2013; Ray *et al*., 2013).

1.6. Extreme habitats as a source of novel actinobacteria

Pioneering ecological studies undertaken by Williams and his colledgues showed that the presence, distribution, numbers and kinds of actinobacteria in natural habitats were influenced by key environmental factors, such as aeration, pH, temperature and availability of organic matter and water (Williams *et al*., 1971, 1972; Goodfellow & Williams, 1988). These observations meant that the chance of isolating novel actinobacteria is dependent on the nature of environmental samples, as shown by the selective isolation of acidiphilic and acidotolerent actinobacteria from acid forest soils (Khan & Williams, 1975; Kim *et al*., 2003; ; Golinska *et al*., 2013a, b, c) and alkaliphilic streptomycetes from beach and sand dune soils (Antony-Babu *et al*., 2008). The realisation that novel actinobacteria were present in such neglected habitats induced a shift towards the isolation, classification and screening of actinobacteria from extreme habitats on the premise that harsh environmental parameters would give rise to a unique actinobacterial diversity that would be the basis of a novel chemistry (Ward & Goodfellow, 2004; Bull, 2011). Strong support for this proposition has come from bioprospecting studies on filamentous actinobacteria isolated from marine habitats (Fiedler *et al*., 2005; Bull & Stach, 2005; Jensen, 2010; Zotchev 2012; Manivasagan *et al*., 2013).

Actinobacteria from desert soils. It is surprising that so little attention has been paid to the actinobacterial composition of desert soils as deserts account for about a quarter of the earth's landmass. Most deserts lie in two areas parallel to the equator, at 25^{o}-35^{o} latitude in the northern and southern hemispheres. The desert biome can be defined on the basis of climate, as the sum of all the arid and hyper-arid areas on the planet, biologically as ecoregions that contain organisms adapted for survival in arid environments, and physically as regions with large areas of bare soil, regolith or rock and low vegetation cover. Desert landscapes are diverse, some are found on ancient crystalline rock hardened over millions of years to give flat deserts of rock and sand, as in the Sahara desert, while others are the folded product of more recent tectonic movements and have evolved into crumpled rocky mountains emerging from lowland sedimentary plains, as in Central Asia, North and South America. Life is continuously

under stress by abiotic factors in the desert biome, amongst them desiccation, temperature fluctuations, lack of organic matter and intense solar radiation. Low moisture content and water activity, in particular, limit microbial growth, abundance and diversity in hyper-arid deserts (Skujins, 1984; Kieft, 2002).

Actinobacteria have been isolated from desert soils, including from the Amargosa desert in Nevada (Luedemann, 1968), the Mojave desert in southwest United States (Garrity *et al*., 1996), the Mongolian desert (Jadambaa, 2000; Kurapova *et al*., 2012), the Namibian desert (Wink *et al.*, 2003), the Sahara desert (Hozzein *et al*., 2004; Zitouni *et al*., 2005; Meklat *et al*., 2011), the Taklamakan desert in Xinjiang province, China (Luo *et al*., 2012), the Thar desert in Rajastan, India (Harwani, 2013) and the desert ecosystem in the northeast of the Qinghai-Tibet plateau (Ding *et al*., 2012). In general, very few actinobacteria have been isolated from such desert soils but most of these that have being assigned to rare genera such as *Actinomadura, Actinoplanes, Dactylosporangium, Geodermatophilus, Microbispora, Micromonospora, Nocardiopsis, Saccharothrix* and *Saccharopolyspora*, as well as to the genus *Streptomyces*. Some desert isolates have been validly named as new genera, such as *Geodermatophilus* (Luedemann, 1968) and *Yuhushiella* (Mao *et al*., 2010) and others as new species, as exemplified by *Corynebacterium deserti* (Zhou *et al.,* 2012), *Geodermatophilus normandii* (Montero-Calasanz *et al*., 2013) and *Mycetocola manganoxydans* (Luo *et al*., 2012). In contrast, relatively large numbers of *Amycolatopsis* strains have been isolated from arid Australian soils (Tan, *et al*., 2006), including some classified to new species, such as *Amycolatopsis granulosa*, *Amycolaopsis ruanii* and *Amycolatopsis thermalba* (Zucchi *et al*., 2012).

The Atacama Desert. This temperate desert in northern Chile stretches for ~1000 kilometers along the coast of Chile in Regions II (Región de Antofagasta) and III (Región de Atacama) but is only between 100 and 200 kilometers wide. The core zone of hyper-aridity extends from 15° to 30°S at elevations from sea level to 3500 m (Houston & Hartley, 2003). The Atacama Desert is considered to be the oldest and driest desert on the planet having evolved over 100 million years of aridity and 15 million years of hyper-aridity (Hartley *et al*., 2005; Gomez-Silva *et al*., 2008).

Most Atacama Desert soils and regoliths are hyper-arid, that is, as stated earlier the ratio of mean annual rainfall to mean annual evaporation is 0.05%, the corresponding ratio for extreme hyper-arid soils is 0.002%. The availability of liquid water and high UV radiation are considered to be the key environmental factors

governing microbial colonisation in Atacama Desert soils (Bhatnagar & Bhatnagar, 2005; Paulino-Lima *et al*., 2013). The harshness of this environment is compounded by very low concentrations of organic carbon, presence of inorganic oxidants and in some areas by high salinity. The harsh conditions found in the Atacama Desert are considered by astrobiologists to provide on accurate analogue of the prevailing conditions on Mars (McKay *et al*., 2003; Navarro-González *et al*., 2003; Azura-Bustos *et al*., 2013).

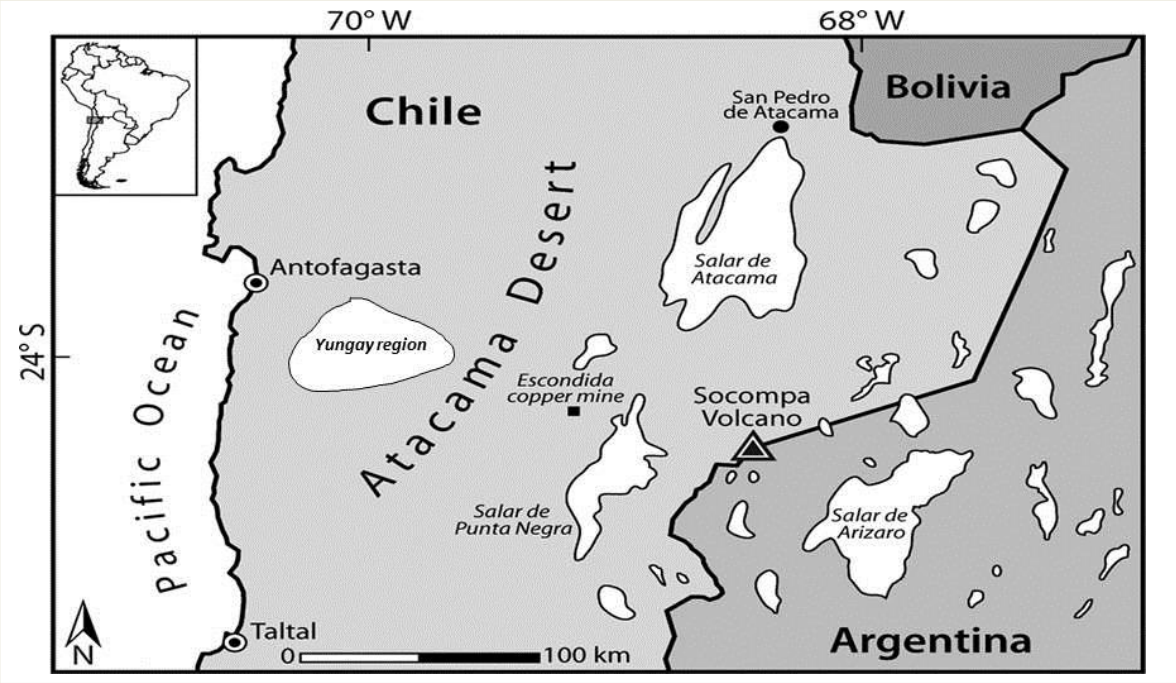


Figure 1.3. Map of Atacama Desert showing the location of the Salar de Atacama and Yungay regions (adapted from Costello *et al*., 2009).

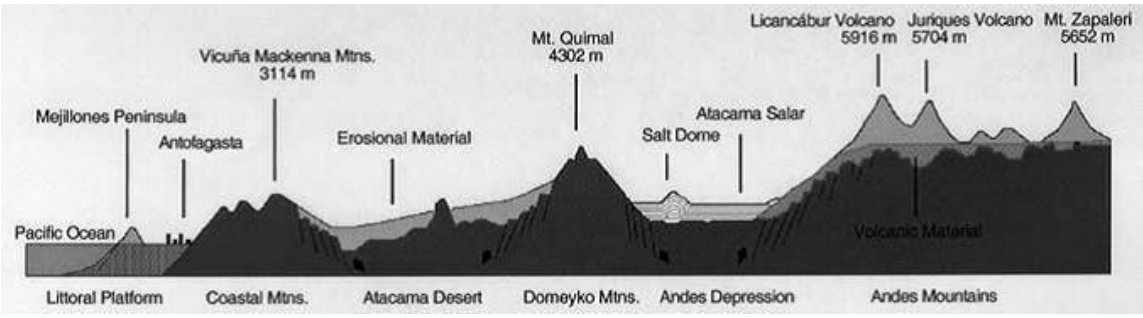


Figure 1.4. Cross-section of Atacama Desert region, near Antofagasta, Chile (adapted from Gómez-Silva *et al.*, 2008).

Extreme hyper-arid soils in the Atacama Desert occur from approximately 22^{o}S to 26^{o}S between the Coastal Mountains to the west (~1000-3000m) and the western

Andes Cordillera including the Domeyko Mountains to the east (~4000 m high), and are prominent in the Yungay region (Figure 1.3). This region receives negligible rainfall, below 1 mm $year^{-1}$ in some areas (McKay *et al*., 2003). Moisture is provided principally by intermittent fogs entering the Desert from the coast. The Yungay region, which lies within this area, is considered to be the driest part of the desert thereby providing a promising setting to determine the presence of actinobacteria in conditions of extreme hyper-aridity. In contrast, arid and hyper-arid soils are found around the Salar de Atacama (Figure 1.3); this is largest salt flat in Chile (ca. 3000 km^2) is bordered by the Cordellera de Domekyo to the west and the Andes to the east (Figure 1.4). The salar is fed by small inflowing rivers from the Andes, but the only outlet for the area is through very high evaporation rates. The Laguna de Chaxa, a product of underground rivers, lies within the Salar de Atacama. Other interesting habitats in the Atacama Desert have been considered by Bull and Asenjo (2013).

Culture-dependent and culture-independent surveys of Atacama Desert soils reveal the presence of small, but taxonomically varied microbial communities (Lester *et al*., 2007; Gómez-Silva *et al*., 2008; Bull & Asenjo, 2013; Paulino-Lima *et al*., 2013) thereby indicating that micro-organisms can withstand the extremes of a aridity that prevent the growth of plants. Drees *et al.* (2006) sampled subsurface soils across an east-west transect of the Atacama Desert and grew bacteria from all but one of the samples. Molecular profiling using denaturing gradient gel electrophoresis (DGGE) highlighted an unique microbial community in hyper-arid soils dominated by the phyla *Gammatimonadetes* and *Planctomycetes*. However, other culture-independent studies of hyper-arid soils from in and around the Yungay region were found to be dominated by actinobacteria (Demergasso *et al*., 2007; Connon *et al*., 2007; Lester *et al*., 2007). Demergasso and her coworkers also have explored microbial communities in the subsurface. A drilling project near the Salar de Grande (Region I, Región de Tarapacá, north of Antofagasta) revealed a microbial habitat at a depth of 2 m that was characterised by abundant hygroscopic salts that allowed deliquescence to occur at low relative humidities and hence microbial colonisation. Actinobacteria were among the bacteria detected in this habitat by means of a multi-signal molecular chip (Parro *et al*., 2010). In sharp contrast, culture-independent studies of high altitude wetlands from the Chilean Altiplano based on 16S rRNA phylotypes showed that the bacterial communities of these systems were dominated by *Bacteroidetes* and *Proteobacteria* (Dorador *et al*., 2009). DGGE analyses of hypersaline environments were found to be

dominated by archaea, cyanobacteria and heterotrophic bacteria (De los Ríos *et al*., 2010).

Okoro *et al*. (2009) were the first to study the diversity of culturable actinobacteria in Atacama Desert soils. The use of standard selective isolation procedures allowed them to detect small populations of filamentous actinobacteria in arid, hyper-arid and extreme hyper-arid soil samples; in some cases actinobacteria were the only bacteria to be isolated, a result in good agreement with some of the culture-independent studies (Lester *et al*., 2007; Neilson *et al*., 2012). Phylogenetic analyses and associated phenotypic tests showed that the isolates belonged to putatively novel *Amycolatopsis*, *Lechevalieria* and *Streptomyces* species. Subsequent polyphasic taxonomic studies showed that the *Lechevalieria* isolates belonged to three new species (Okoro *et al*., 2010), as did four of the *Streptomyces* isolates (Santhanam *et al*., 2012a, b; 2013; Busarakam *et al*., 2014). The results of these and an associated study (Schulz *et al*., 2011) support the view that Atacama Desert soils are a largely unexplored source of novel actinobacteria, some of which have been shown to synthesis novel bioactive compounds (Nachtigall *et al*., 2011; Rateb *et al*., 2011a, b; Schulz *et al*., 2011).

1.7. Screening for bioactive compounds

The initial steps in the culture-dependent bioprospecting strategy described by Goodfellow and Fiedler (2010) are based on the selective isolation of novel actinobacteria from neglected habitats and the selection of representative dereplicated isolates for screening. It has been shown that these steps are highly dependent on developments in actinobacterial systematics and are based partly on the hypothesis that taxonomic diversity remains a surrogate for chemical diversity (Ward & Goodfellow, 2004; Bull & Stach, 2007; Jensen, 2010) and partly on the concept that novel species may synthesis unique bioactive compounds as the evolution of specialised metabolites may be a driver of speciation (Czaran *et al*., 2002; Jensen, 2010). The remaining steps in the taxonomic approach to drug discovery involve the expression and detection of bioactive compounds from representatives of dereplicated groups grown on appropriated production media, the detection of chemical novelty using appropriate analytical chemical procedures and structural analyses of interesting leads. Numerous assays have been developed for the detection of antimicrobial, antiparasitic and antitumour metabolites (Fiedler, 2004; Singh *et al*., 2007; Donadio & Sosio, 2010).

Production media. It is good practice to grow dereplicated isolates on a range of production media (Fielder, 1994; Theobald *et al*., 2000), not least because such media can have an enormous impact on the expression of actinobacterial biosynthetic gene clusters. Goodfellow and Fiedler (2010) recommended several media formulations which they considered suitable for the production of specialised metabolites from taxonomically different actinobacteria (see Table 1.2). Media 410, which has a high concentration of carbon and nitrogen, supports the growth of most actinobacteria, notably strains classified in the order *Corynebacteriales*. This medium is not, however, suitable for the production of specialised metabolites from streptomycetes or streptosporangiae even though they grow well on it. In contrast, media 19, 400, OM and SGG induce the synthesis of novel drug candidates from *Streptomyces* strains; members of the family *Micromonosporaceae* are best grown on media 333, MMM and SGG. These observations, while somewhat empirical, do help press the point that the success of screening campaigns for specialised metabolites are highly dependent on accurate classification of novel dereplication isolates, as exemplified the production of novel antibiotics from *Streptomyces* strain C34^{T} which was isolated from the Salar de Atacama region of the Atacama Desert (Rateb *et al*., 2011b).

Table 1.2. Media suitable for submerged cultivation of actinobacteria (g/l) (taken from Goodfellow & Fiedler, 2010).

Medium	19	333	400	410	MMM	OM	SGG
Glucose	-	5	10	10	10	-	10
Glycerol	-	-	-	10	10	-	10
Mannitol	20	-	-	15	-	-	-
Casamino acid	-	-	-	15	-	-	-
Casitone	-	-	-	-	-	5	-
Cornsteep powder	-	-	-	-	-	-	2.5
Meat extract	-	-	3	-	-	-	-
Oatmeal	-	-	-	5	-	20	-
Peptone	20	3	3	10	-	-	5
Soluble starch	-	10	20	-	20	-	10
Yeast extract	-	3	5	5	5	-	2
$CaCO_3$	-	2	3	1	1	-	3

NaCl	-	-	-	-	-	-	1
NH_4NO_3	-	3	-	-	-	-	-
pH	7.5	7.2	7.0	7.0	7.6	7.3	7.3
C content (g/l)	13.8	10.5	12.7	13.4	12.5	5.2	14.0
C:N ratio	6.3:1	6.05:1	11.3:1	3.5:1	12.5:1	15:1	15.4

Primary antibiotic screening of actinomycetes is usually achieved against a panel of Gram-negative and Gram-positive bacteria (Baltz, 2007; Urban *et al*., 2007) and yeasts. Genetically engineered *Escherichia coli* K-12 strains can be used to exclude the rediscovery of the most broad-spectrum antibiotics as they contain genes that confer resistance against antibiotics such as ampicillin, chloramphenicol, streptomycin and tetracycline (Baltz, 2007). Agar plug assays are widely used to establish whether dereplicated isolates show bioactivity against such strains (Fiedler, 2004). There are several variants to this approach, one of which is to take plugs of actinobacterial strains grown on production media and place them on the surface of agar plate supporting the growth of the panel of wild type organisms then recording zones of inhibition after overnight incubation. Alternatively, culture filtrates or organic extracts of actinobacterial mycelia may be spotted onto plates supporting the growth of the microorganisms and zones of inhibition recorded following incubation.

Dereplicated isolates showing activity in the primary screens can be selected for secondary screens designed to detect the target sites of the agents of bioactivity (Fiedler *et al*., 2004). This approach can be based on mutant strains that carry reporters, such as β-galactosidase or luciferase genes, fixed to promoters that specifically respond to certain types of antibiotics which trigger the promoter resulting in the expression of the reporter gene. The product of β-galactosidase, for instance, leads to X-gal being cleaved to galactose and 5-bromo-4-chloro-3-hydroxyindole (Figure 1.5); the production of an insoluble blue pigment (5-bromo-4-chloro-3-hydroxyindole) shows that the antibiotic is repressing the target pathway. Hütter *et al*. (2004) generated several *Bacillus subtilis* reporter strains and used them to detect inhibitors of cell walls, DNA, fatty acids and protein biosynthesis. Similarly, Fiedler *et al.* (2004) used fatty acid biosynthesis pathway (FAS)-specific *B. subtilis* reporter strains against several antibiotics and identified two known FAS inhibitors, cerulenin and triclosan.

Another strategy that is increasingly being used to increase the sensitivily of screening assays is based upon antisense technology (Genilloud, 2014). This approach

involves a reduction in the level of the desired bacterial target by inducing the overexpression of the cognate antisense mRNA leading to the generation of strains that are hypersensitive to compounds which inhibit that target hence the probability of finding the inhibitor of that target as an antibacterial compound is significantly increased (Singh *et al*., 2007). This approach has led to the discovery of several potent antibiotics, including platensimycin (Wang *et al*., 2006) and platencin (Wang *et al*., 2007) from *Streptomyces platensis*.

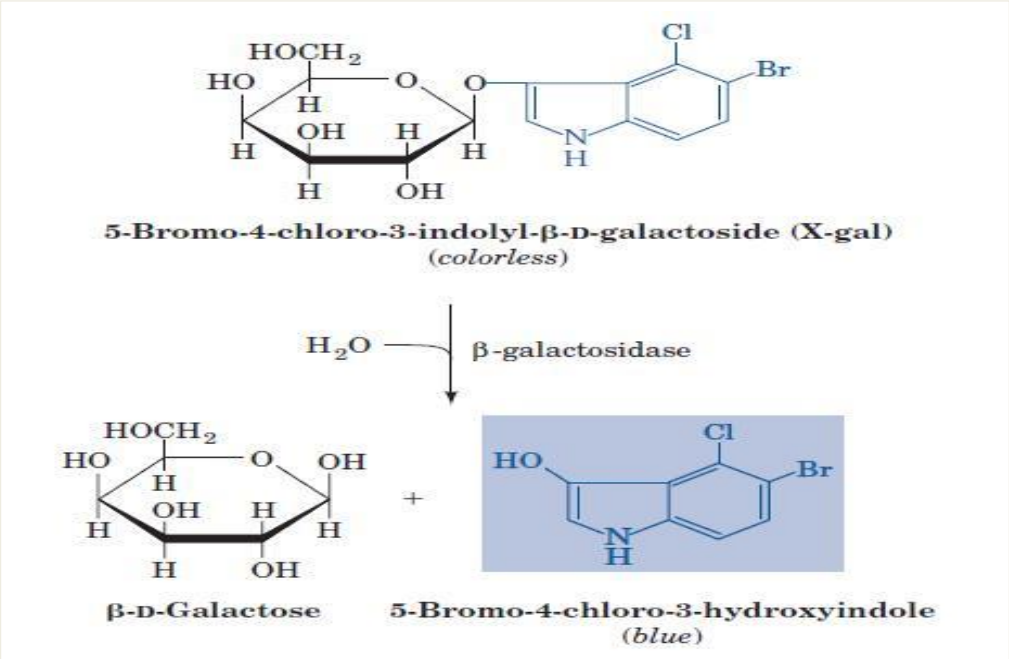


Figure 1.5. Cleavage of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to β-D-galactose and 5-bromo-4-chloro-3-hydroxyindole (Blue compound). Adapted from Voet & Voet (2011).

Chapter 2. Materials and Methods

2.1. Sampling sites

Source of environment samples. Environmental samples were collected from two locations in the Atacama Desert by Professor A.T. Bull (School of Biosciences, University of Kent, Canterbury, UK.), namely the Laguna de Chaxa, Salar de Atacama, near Tocanao and the Yungay region, quite near Antofagasta (Table 2.1).

Table 2.1. Environmental conditions at sampling sites.

Location	Latitude	Longitude	Elevation (m asl)	Date	Biome	MAR/MAE*
Laguna de Chaxa, Salar de Atacama	23°17'S	68°10'W	2,300	4-10-2004	Salt flat hyper-arid	0.009
Yungay	24°06'18.6"	70°01'55.6"	1033	13-11-2010	Extreme hyper-arid soil	0.002

*MAR, mean average rainfall; MAE, mean average evaporation. Ratio estimated from data provided by Houston (2006).

2.2. Physico-chemical properties of environmental samples

pH. The pH of the environmental samples were determined using the procedure described by Reed and Cummings (1945). Deionised water was added to two grams of each environmental sample until a thin layer of water was visible on the surface of the samples, the latter were mixed thoroughly and left for two hours at room temperature. The pH of each sample was determined, in triplicate, using a glass electrode pH meter (Model 320 Mettler-Toledo AG, CH.8603, Schwerzenbach, Switzerland).

Moisture and organic matter content. The percentage moisture content of the environmental samples were determined, in triplicate, by drying known amounts of the

samples 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 any 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.

2.3. Selective isolation, enumeration and presumptive classification of actinobacteria isolated from Atacama Desert environmental samples

Selective isolation and enumeration. Suspensions from the Salar de Atacama and Yungay environmental samples were prepared in 1/4 strength Ringer's solution (Oxoid, UK) to give $10^{-½}$ dilutions which were shaken on a tumble shaker (TMI Tumbler, Luckham Ltd., Sussex, UK) for 30 minutes, heat-pretreated at 55^{o}C for 6 minutes and 10^{-1} and 10^{-2} dilutions prepared in ¼ strength Ringer's solution. Aliquots (100 µl) of the various dilutions were spread over plates of several selective isolation media (Table 3.1) which had been dried for 15 minutes at room temperature prior to inoculation (Vickers & Williams, 1987). Three replicate plates were prepared for each dilution and for each of the isolation media. After incubation at 28^{o}C for 3 weeks, the number of actinobacteria growing on the isolation plates were counted and the results expressed as the number of colony forming units (cfu) per gram dry weight of environmental sample.

Table 2.2. Media used for the selective isolation of actinobacteria from the Atacama Desert environmental samples.

Media	Selective agents (µg ml^{-1})	Target organism(s)
Gause's No.1 agar (Zakharova *et al.*, 2003)	Nalidixic acid (10)	Rare or uncommon actinobacteria
Geodermatophilus obscurus agar (Uchida &Seino, 1997)	Nystatin (25)	*Geodermatophilus* spp.
Glucose-yeast extract agar (Athalye *et al.*, 1981)	Rifampicin (20)	*Actinomadura* spp.
HV agar (Hayakawa & Nonomura, 1987)	Humic acid (1g L^{-1})	*Streptosporangiaceae* spp.
Luedemann's agar (Luedemann, 1971)	Nystatin (25)	*Modestobacter* spp.

Microlunatus agar (Nakamura *et al*., 1995)	Nystatin (25)	*Modestobacter* spp.
Oligotrophic agar (Senechkin *et al.*, 2010)	Low carbon and nitrogen content	Rare and uncommon actinobacteria
R2A (Reasoner & Geldreich, 1985)	Nystatin (25)	*Modestobacter* spp.
Starch-casein agar (Küster & Williams, 1964)	Nystatin (25)	*Streptomyces* spp.
SM1 (Tan *et al.*, 2006)	Neomycin (1) and nystatin (25)	*Amycolatopsis* spp.

All of the media were supplemented with cycloheximide (25 µg ml^{-1}).

2.4. Selection, maintenance and presumptive classification of actinobacteria isolated from the Salar de Atacama and Yungay environmental samples

Four hundred and twenty strains randomly chosen from the selective isolation plates were subcultured onto glucose-yeast extract-malt extract agar plates (ISP2, Shirling & Gottlieb, 1966) and incubated at 28^{o}C for 14 days. Three hundred and fifteen of the strains were taken from isolation plates inoculated with suspensions of the Salar de Atacama environmental sample and 105 from the isolation plates seeded with the suspensions of the environmental sample taken from the Yungay region. The isolates considered to belong to the genus *Streptomyces* were recognised by their ability to produce leathery colonies covered by an abundant aerial spore mass while *Geodermatophilaceae* colonies were recognised by their characteristic shiny black colony. In contrast, isolates producing leathery colonies covered by little or no aerial hyphae were considered to belong to other filamentous actinobacterial groups.

Maintenance of strains. All of the isolates were grown on oatmeal (Shirling & Gottlieb, 1966) and modified Bennett's agar plates (Jones, 1949) at 28°C for 3 weeks. Suspensions of spores and mycelial fragments from each of the incubated plates were suspended in 1 ml aliquots of 20%, v/v glycerol (2 vials per strain per medium) in cryotubes, one culture prepared from each medium was kept at -80^{o}C for long-term preservation, the others, the working cultures, were kept at -20^{o}C.

Assignment to colour-groups. The presumptive streptomycete and non-streptomycete strains isolated from the Salar de Atacama and Yungay environmental samples were subcultured onto oatmeal agar (ISP medium 3, Shirling & Gottlieb, 1996) and peptone-yeast extract agar plates (PYEIA, ISP medium 6, Shirling & Gottlieb, 1966) and incubated at 28^{o}C for 14 days and 4 days, respectively. The isolates were assigned to colour-groups based on aerial spore mass, substrate mycelial and diffusible pigment colours produced on the oatmeal agar plates, using National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1958), and their ability to produce melanin pigments on the peptone-yeast extract-iron agar plates. The strains isolated from the Salar de Atacama environmental sample were assigned to 49 multi-membered and 23 single-membered colour-groups; the corresponding numbers for the strains isolated from the Yungay environmental sample were 20 and 13, respectively (Appendix 1). The codes assigned to the strains isolated from the Salar de Atacama and Yungay environmental samples include reference to the media they were isolated on and the colour-groups to which they were assigned (Tables 2.3 and 2.4).

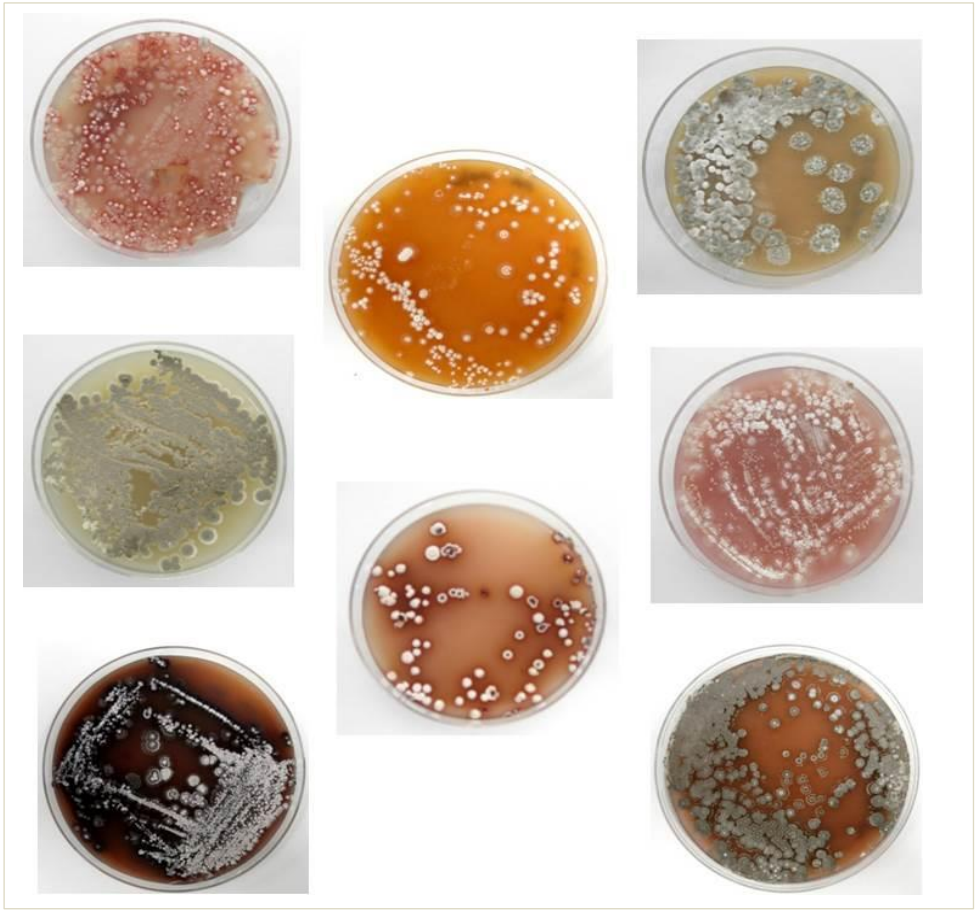


Figure 2.1. Colour-grouping of actinobacteria on oatmeal agar (ISP3 medium).

Table 2.3. Selected actinobacteria isolated from the hyper-arid environmental sample from Chaxa de Laguna, Salar de Atacama of the Atacama Desert and assigned to single- and multi-membered colour-groups.

Actinobacteria	Codes
Presumptive streptomycetes	
Multi-membered colour-groups	C34, C38, C58, C59, C79, KNN1-1a*, KNN1-2c, KNN1-3b, KNN1-5f; KNN2-1b*, KNN2-2c, KNN2-3c, KNN2-4c, KNN2-5c, KNN2-6c, KNN2-7d, KNN2-8b, KNN2-9d, KNN2-10d, KNN2-11d, KNN2-12d, KNN2-13a, KNN2-14c, KNN2-15a, KNN2-16c, KNN2-17d, KNN2-18c, KNN3-1b*, KNN3-2b, KNN3-3c, KNN3-4b, KNN3-5c, KNN3-6c, KNN3-7c, KNN3-8c, KNN3-9c, KNN3-10e, KNN3-14d, KNN3-15d, KNN3-16d, KNN3-17d, KNN3-18d, KNN3-19b, KNN4-1b*, KNN4-2e, KNN4-3b, KNN4-4a; KNN5-1a*, KNN5-2a, KNN5-3c, KNN5-4c, KNN5-5a, KNN5-6a, KNN5-7a, KNN5-8b, KNN5-9b, KNN5-10b, KNN5-13b, KNN5-14b, KNN5-15b, KNN5-16d, KNN5-17d, KNN5-18d, KNN5-19d, KNN5-20d, KNN5-25b, KNN5-28c, KNN5-29b, KNN5-30d, KNN5-31e; KNN6-1a*, KNN6-2a, KNN6-3a, KNN6-4a, KNN6-5a, KNN6-6b, KNN6-7d, KNN6-8d, KNN6-9a, KNN6-10b, KNN6-11a; KNN8-1b*, KNN8-2b, KNN8-3a, KNN8-4b, KNN8-5d, KNN8-6b, KNN8-7a, KNN8-8a, KNN8-9a, KNN8-10c, KNN8-11e, KNN9-1a*, KNN9-2e, KNN9-3a, KNN10-1a*, KNN10-2b, KNN10-3b, KNN10-4d, KNN10-5a, KNN11-1a, KNN11-2a, KNN11-3c, KNN11-4b, KNN11-5a, KNN11-6b, KNN14-1f*, KNN14-2c, KNN14-3e, KNN16-1*, KNN16-2, KNN17-1c*, KNN17-2b; KNN18-1b*, KNN18-2c, KNN18-3d, KNN24-1b*, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-5c, KNN24-6a, KNN24-7c, KNN24-8e, KNN29-1a*, KNN32-1a*, KNN32-2b, KNN35-1b*, KNN35-2b; KNN36-1*c, KNN36-2c, KNN36-3c; KNN37-1e*, KNN37-2a KNN37-3a, KNN37-4a, KNN38-1b*, KNN38-2d, KNN38-3d, KNN38-4a, KNN38-5b, KNN41-1b*, KNN41-2a, KNN48-1c*, KNN48-2a, KNN48-3e, KNN48-4e, KNN48-5e, KNN52-1c*, KNN52-2b, KNN52-3b, KNN54-1b*, KNN54-2b, KNN58-1b*, KNN58-2c, KNN60-1c*, KNN60-2d, KNN63-1b*, KNN63-2b, KNN63-3b, KNN63-4b, KNN63-5a, KNN63-6b, KNN63-7b, KNN63-8d, KNN63-9d, KNN63-10d, KNN63-11b, KNN63-12b, KNN63-13b, KNN63-14b, KNN63-15b, KNN63-16b, KNN63-17b, KNN63-18b, KNN63-19e, KNN64-1a*, KNN64-2a, KNN64-3a, KNN64-4b, KNN64-5b, KNN64-6b; KNN65-1f*, KNN65-2a, KNN65-3c, KNN65-4c, KNN65-5d, KNN67-1b*, KNN67-2b, KNN67-3b, KNN67-4b, KNN68-1b*, KNN68-2b, KNN68-3b, KNN68-4b, KNN69-1e*, KNN69-2a, KNN69-3a, KNN71-1a*, KNN71-2a, KNN73-1a*, KNN73-2a, KNN73-3d, KNN74-1c*, KNN74-2c, KNN75-1c*, KNN75-2b, KNN75-3b, KNN75-4b, KNN78-1e*, KNN78-2e, KNN79-1b*, KNN79-2b, KNN79-3d; KNN80-1c*, KNN80-2d, KNN81-1c*, KNN81-2b, KNN81-3d, KNN82-1a*, KNN82-2c, KNN85-1f*, KNN85-2b, KNN85-3b, KNN85-4c, KNN85-5c, KNN85-6c, KNN85-7c, KNN85-8a
Single-membered colour groups	KNN25c*, KNN26b* KNN27a*, KNN28a*, KNN30a*, KNN31d*, KNN33a*, KNN39c*, KNN43b*, KNN51b*, KNN56a*, KNN59e*, KNN62b*, KNN66*, KNN70b*, KNN72a*, KNN76b*, KNN83e*, KNN84c*, KNN87b*, KNN94e*
Presumptive non-streptomycetes	
Multi-membered	GY024, GY142, KNN49-1f*, KNN49-2e, KNN49-3e, KNN49-4e, KNN49-5e, KNN49-

colour-groups	6a, KNN49-7c, KNN49-8c, KNN49-9c, KNN49-10b, KNN49-11c, KNN49-12b, KNN49-13b, KNN49-14b, KNN49-15b, KNN49-16c, KNN49-17b, KNN49-18b, KNN49-19a, KNN49-20b, KNN49-21c, KNN49-22d, KNN49-23d, KNN49-24d, KNN49-25d, KNN49-26a, KNN49-27a, KNN49-28a, KNN49-29a, KNN49-30b, KNN49-31b, KNN49-32b, KNN50-1a*, KNN50-2e, KNN50-3c, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-16d, KNN50-17d KNN50-8d, KNN53-1a*, KNN53-2a, KNN53-3a, KNN54-1a*, KNN54-2b, KNN57-1b*, KNN57-2b, KNN61-1e*, KNN61-2a, KNN61-3e, KNN61-4e, KNN61-5e, KNN61-6b
Single-membered colour-groups	KNN34a*, KNN77b*

Code exemplified by strain KNN2-1b, this organism was isolated by Kanungnid Busarakam (KNN), assigned to colour-group 2 as the first member of group and was isolated on a humic acid-vitamin agar isolation plate. C-coded strains were studied by Okoro et al who isolated them from hyper-arid soil from the Salar de Atacama region of the Atacama Desert. The GY-coded strains were isolated from an arid Australian composite soil by Tan *et al*. (2006).

Codes for isolation media : (a), Gause No.1 agar; (b), HVA: Humic acid-vitamin agar; (c), Oligotrophic agar; (d), minimal medium agar; (e), SM1 agar; (f) and starch-casein agar; (g) *Geodermatophilus obscurus* agar.

*Representative of colour-groups used to detect isomers of diaminopimelic acid.

Table 2.4. Selected actinobacteria isolated from the extreme hyper-arid environmental sample from Yungay region and assigned to single- and multi-membered colour-groups.

Actinobacteria	Codes
Presumptive streptomycetes	
Multi-membered colour-groups	KNN12-1a*, KNN12-2a, KNN15-1a*, KNN15-2a, KNN16-2c, KNN19-1b*, KNN19-2c, KNN23-1a*, KNN23-2a, KNN29-2a, KNN29-3a, KNN48-7d, KNN48-8d, KNN48-9d, KNN48-10d, KNN48-11b, KNN48-12d, KNN48-13b, KNN48-14a, KNN48-15a, KNN48-16c, KNN48-17c, KNN48-18c, KNN48-19c, KNN48-20c, KNN77-1a*, KNN77-2a, KNN77-3a, KNN77-4a, KNN77-5a, KNN77-6a, KNN86-1b*, KNN86-2b, KNN86-3b, KNN86-4b, KNN86-5c, KNN86-6a, KNN88-1a*, KNN88-2b, KNN88-3b, KNN88-4a, KNN91-1a*, KNN91-2b, KNN91-3b, KNN91-4a, KNN91-5a, KNN91-6a, KNN92-1b*, KNN92-2b, KNN92-3b, KNN92-4a, KNN92-5a, KNN92-6a, KNN92-7a, KNN93-1a*, KNN93-2a, KNN93-4a, KNN 93-5a, KNN95-1f*, KNN95-2f, KNN95-3f, KNN95-4f, KNN95-5f, KNN95-6f, KNN95-7f
Single-membered colour-groups	KNN13a*; KNN20c*, KNN21a*; KNN22a*; KNN40a*; KNN42a*, KNN51b*; KNN89b*, KNN90b*; KNN96b*; KNN97b*; KNN98b*
Presumptive non-streptomycetes	
Multi-membered colour-groups	KNN7-1b*, KNN7-2b, KNN7-3b, KNN7-4b, KNN23-1a*, KNN23-2a, KNN44-1b*, KNN44-2a, KNN44-3a, KNN44-4c, KNN45-1b, KNN45-2a, KNN45-3b*, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b*, KNN46-4b, KNN46-5a, KNN46-6a, KNN46-7g,

	KNN46-8c, KNN46-9, KNN46-10, KNN46-11, KNN55-1b*, KNN55-2b
Single-membered colour-group	KNN47*

Codes as for Table 2.3.

***Detection of diaminopimelic acid isomers*.** Ninety eight strains representing each of the colour-groups (Tables 3.2 and 3.3) were examined for diaminopimelic acid (A_2pm) isomers using the procedure described by Hasegawa *et al.* (1983). One to two loopfuls of biomass scraped from strains grown on ISP2 agar plates (Shirling & Gottlieb, 1960) at 28^{o}C for 14 days were transferred to cryotubes containing 6N HCl, the preparations autoclaved at 121^{o}C for 20 minutes, left to cool at room temperature, centrifuged at 6000 rpm for 4 minutes and then stored at 4^{o}C until required. The A_2pm isomers were separated by one-dimensional thin-layer-chromatography (TLC) on aluminium-backed sheets (20 x 20 cm diameter; No.5716, Merck, Darmstadt, Germany). A pencil line was drawn 1 cm above the bottom of the TLC sheets and 3 µl of each of the hydrolysates spotted, 1 µl at a time, onto the sheets followed by drying using a hair drier to prevent the spots spreading. A 1 µl preparation, which contained a mixture of 0.01 M *LL*- and *meso*-A_2pm (Sigma), was used as a standard on each of the prepared sheets. The latter were developed in glass tanks containing the solvent system (32 methanol: 10.4 sterilised distilled water: 1.6 6N HCl : 4 pyridine, v/v) for 4-5 hours, that is, until the solvent front was 1 cm from the top of the sheets. The chromatograms were air-dried, sprayed with 2% (w/v) ninhydrin in acetone and heated at 100^{o}C for 5 minutes. The isomers of A_2pm appeared as dark purple to brown spots that had a lower retention factor (R_f) than other amino acids, the latter gave 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 and *LL*-A_2pm (from lowest to highest).

2.5. Comparative 16S rRNA gene sequencing studies

Ninety eight isolates were taken to represent multi- and single-membered colour-groups were the subject of 16S rRNA gene sequencing studies to determine whether they belonged to known or putatively novel taxa. The 42 isolates found to contain *LL*-A_2pm in whole-organism hydrolysates were considered to belong to the genus *Streptomyces*,

the remainder, which contained *meso*-A_2pm, were considered to belong to other actinobacterial genera (Table 2.5).

Extraction of genomic DNA: Bead beating method. Total genomic DNA was extracted from the 98 representative isolates. Three grams of acid washed sterile glass beads (0.1 mm in diameter, Sigma) were added to one to two loopfuls of biomass of each of the isolates grown on yeast extract-malt extract agar plates (ISP medium 2, Shirling & Gottlieb, 1966) and the resultant preparations treated for 30 seconds at 5.5 m/s in a FastPrep® (Thermo Lab Systems, Waltham, USA), a process that was repeated twice prior to the mixtures being centrifuged at 13000 rpm for 2 minutes. DNA was precipitated from the preparations by adding 400 µl of cold 100% ethanol suspended in 100 µl of 1x TE buffer (pH8) and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).The supernatants containing the extracted DNA were transferred to new tubes and kept at -20^{o}C until required.

Table 2.5. Actinobacteria taken to represent multi-membered and single-membered colour-groups examined in the comparative 16S rRNA gene sequence studies.

A. Representative isolates from the Salar de Atacama environmental sample
Multi-membered colour-groups:
C34^{O}, C38^{O}, C58^{O}, C59^{O}, C79^{O}, GY024^{T}, GY142^{T}, KNN2-4c, KNN2-6c, KNN4-1b, KNN4-4a, KNN6-6b, KNN6-9a, KNN6-10b, KNN6-11a, KNN9-1a, KNN9-2c, KNN10-4a, KNN10-5a, KNN11-1a, KNN11-5a, KNN24-1b, KNN32-1a, KNN35-1b, KNN35-2b, KNN38-1b, KNN41-1b, KNN48-1e, KNN48-3e, KNN48-6d, KNN49-1h, KNN49-3e, KNN49-5e, KNN49-6a, KNN49-11c, KNN49-12b, KNN49-26a, KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14a, KNN50-15a, KNN50-16d, KNN50-17d, KNN50-18d, KNN53-1a, KNN53-3a, KNN54-1a, KNN57-1b, KNN57-2b, KNN61-1a, KNN64-5b, KNN82-2c, KNN83e, KNN88-1c
Single-membered colour groups:
KNN25c, KNN26b, KNN34c, KNN56a, KNN70b, KNN87b, KNN94e
B. Representative isolates from the Yungay environmental sample
Multi-membered colour-groups:
KNN7-2b, KNN23-1b, KNN44-1b, KNN44-3b, KNN44-4b, KNN45-1a, KNN45-2b, KNN45-3b, KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5b, KNN46-6a, KNN46-7a, KNN46-8a, KNN46-9c, KNN46-10g, KNN46-11f, KNN55-1b, KNN55-2b
Single-membered colour-groups:
KNN13a, KNN22a, KNN42f, KNN47b, KNN51b, KNN70b, KNN89a, KNN90a

^{o}Strains isolated by Okoro *et al*. (2009) and ^{T}strains isolated by Tan *et al*. (2006).

Quality of extracted DNA. The quality of the DNA products was checked by agarose gel electrophoresis (1%, w/v agarose in 0.5xTBE, 40 minutes at 110 V). The gels were stained with ethidium bromide (0.5 µg/ml) and loaded with 4 µl of crude DNA mixed with 2µl of loading dye (0.5 µg/µl, Sigma) (Sambrook, 1989). The sizes of the DNA fragments were compared with 100 bp molecular size markers (Gene Ruler™ MBI Fermentas, Vilnius, Lithuania). After electrophoresis, the gels were visualised and photographs taken using a BioRad™ Multimager UV transilluminator.

PCR amplification of 16S rRNA gene preparations. One to two µl of each crude DNA product was used as DNA template in a 25 µl polymerase chain reaction (PCR) which contained 1x buffer (10x buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl [pH 8.8 at 25^oC] , 0.1% Tween-20), a mixture of dNTP's containing 0.125 mM of each of the four dNTP's, and 200 µM of each forward and reverse primer (27f: AGAGTTTGATCCTGGCTCAG and 1527r: AAGGAGGTGATCCAGCC, respectively; Sigma-Aldrich, Hertfordshire, UK), 1.5 µM of $MgCl_2$ and 1,25 *Taq* polymerase. Positive and negative controls were included, the negative control was sterilised distilled water and the positive control a known DNA sample. The PCR reactions were carried out as follows: initial denaturation at 95^oC for a minute, 30 cycles of 95^oC for a minute, 55^oC for a minute and 72^oC for a minute, and finally 72^oC for 5 minutes. The PCR products were checked for quality as mentioned above. The preparations were kept at -20^oC prior to use.

Purification of PCR products. Prior to sequencing, the PCR products were purified by using ExoSAP-IT* kits (USB, Corporation, Ohio, USA), according to the manufacturer's protocol. Each PCR product (5µl) was mixed with 2µl of ExoSAP-IT*, the reaction mixtures incubated at 37^oC for 15 minutes to degrade the remaining primers and nucleotides, and the preparations incubated at 80^oC for 15 minutes to inactivate the ExoSAP-IT*. The purified products were stored at -20^oC until required. The sequences of the almost complete 16S rRNA genes were determined by the Newcastle University company GENEIUS.

Phylogenetic analyses. The chromatogram files in ABI were read in Finch TV and converted to FASTA format files. The nearest match of each sequence was determined by using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI; Bethesda, USA) and pairwise sequence similarities

calculated by using the global alignment algorithm implemented using the EzTaxon e web server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.,* 2012). The aligned sequences were used to construct phylogenetic trees generated using either the neighbour-joining algorithm (Saitou & Nei, 1987) from the MEGA5 software program or the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1967) algorithms 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 analyses. 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.

The Salar de Atacama and Yungay strains included in the comparative 16S rRNA gene analyses were assigned to seven and six genera, respectively as shown in Table 2.6. It can also be seen from this Table that strains assigned to the same colour-groups as tested strains were deemed to belong to the same genus as the strains included in the comparative 16S rRNA gene analyses.

Table 2.6. Assignment of strains assigned to colour-groups containing isolates from the Salar de Atcama and Yungay samples to genera based on 16S rRNA sequencing data. In total, 420 isolates were assigned to 12 genera.

Genera	Tested strains	Additional strains in colour-groups
Actinomadura	KNN34c, KNN53-1a, KNN53-3a	- KNN53-2a
Amycolatopsis	KNN49-1f, KNN49-3e, KNN49-5e, KNN49-6q, KNN49-11c, KNN49-12b, KNN49-26a, KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN550-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-16d, KNN50-17d, KNN50-18d, KNN61-1e	KNN49-2e, KNN49-4e, KNN49-7c, KNN49-8c, KNN49-9c, KNN49-10b, KNN49-13b, KNN49-14b, KNN49-15b, KNN49-16c, KNN49-47b, KNN49-18b, KNN49-19a, KNN49-20b, KNN49-21c, KNN49-22d, KNN49-23d, KNN49-24d, KNN49-25d, KNN49-27a, KNN49-28a, KNN49-29a, KNN49-30b, KNN49-31b, KNN50-3c, KNN61-2a, KNN61-3e, KNN61-4e, KNN61-5b, KNN61-6c
Blastococcus	KNN47b	-
Couchioplanes	KNN7-2b	KNN7-1b, KNN7-3b, KNN7-4b
Geodermatophilus	KNN44-1b, KNN44-3b, KNN44-4b	KNN44-2a
Kribbella	KNN56a	

Lechevalieria	KNN94e	-
Modestobacter	KNN45-1b, KNN45-2b, KNN45-3b, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5a, KNN46-6a, KNN46-7a, KNN46-8c, KNN46-9c, KNN46-10c, KNN46-11a	KNN46-12a
Nonomuraea	KNN57-1b, KNN57-2b	-
Pseudonocardia	KNN55-1b	KNN55-2b
Saccharothrix	KNN10-4d, KNN54-1a	KNN10-1a, KNN10-2b, KNN10-3b, KNN10-5a, KNN54-2b
Streptomyces	C34, C38, C58, C59, C79, KNN2-4c, KNN2-6c, KNN4-1b, KNN4-4a, KNN6-6b, KNN6-9a, KNN6-10b, KNN6-11a, KNN9-3a, KNN10-5a, KNN11-1a, KNN11-5a, KNN13a, KNN22a, KNN23-1b, KNN24-1b, KNN25c, KNN26b, KNN32-1a, KNN35-1b, KNN35-2b, KNN38-1b, KNN41-1b, KNN42, KNN48-1c, KNN48-3e, KNN48-6e, KNN51b, KNN64-5b, KNN82-2c, KNN83e, KNN87b, KNN88-1a, KNN89a, KNN90a	KNN1-1a, KNN1-2c, KNN1-3b, KNN1-5f; KNN2-1b, KNN2-2c, KNN2-3c, KNN2-5c, KNN2-7d, KNN2-8b, KNN2-9d, KNN2-10d, KNN2-11d, KNN2-12d, KNN2-13a, KNN2-14c, KNN2-15a, KNN2-16c, KNN2-17d, KNN2-18c, KNN3-1b, KNN3-2b, KNN3-3c, KNN3-4b, KNN3-5c, KNN3-6c, KNN3-7c, KNN3-8c, KNN3-9c, KNN3-10e, KNN3-14d, KNN3-15d, KNN3-16d, KNN3-17d, KNN3-18d, KNN3-19b, KNN4-2e, KNN4-3b, KNN5-1a, KNN5-2a, KNN5-3c, KNN5-4c, KNN5-5a, KNN5-6a, KNN5-7a, KNN5-8b, KNN5-9b, KNN5-10b, KNN5-13b, KNN5-14b, KNN5-15b, KNN5-16d, KNN5-17d, KNN5-18d, KNN5-19d, KNN5-20d, KNN5-25b, KNN5-28c, KNN5-29b, KNN5-30d, KNN5-31e; KNN6-1a, KNN6-2a, KNN6-3a, KNN6-4a, KNN6-5a, KNN6-7d, KNN6-8d, KNN8-1b, KNN8-2b, KNN8-3a, KNN8-4b, KNN8-5d, KNN8-6b, KNN8-7a, KNN8-8a, KNN8-9a, KNN8-10c, KNN8-11e, KNN9-1a, KNN9-2e, KNN10-1a, KNN10-2b, KNN10-3b, KNN10-4d, KNN11-2a, KNN11-3c, KNN11-4b, KNN11-6b, KNN12-1a, KNN12-2a, KNN14-1f, KNN14-2c, KNN14-3e, KNN15-1a, KNN15-2a, KNN16-1c, KNN16-2c, KNN17-1c, KNN17-2b KNN18-1b, KNN18-2c, KNN18-3d, KNN19-1b, KNN19-2c, KNN23-2a, KNN23-2a, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-5c, KNN24-6a, KNN24-7c, KNN24-8e, KNN27a, KNN28a, , KNN29-1a, KNN29-2a, KNN29-3a, KNN30a, KNN31d, KNN32-2b, KNN33a, KNN36-1c, KNN36-2c, KNN36-3c; KNN37-1e, KNN37-2a KNN37-3a, KNN37-4a, KNN38-1b, KNN38-2d, KNN38-3d, KNN38-4a, KNN38-5b, KNN39, KNN41-2a, KNN43b, KNN48-2a, KNN48-4e, KNN48-5e, KNN48-7d, KNN48-8d, KNN48-9d, KNN48-10d, KNN48-11b, KNN48-12d, KNN48-13b, KNN48-14a, KNN48-15a, KNN48-16c, KNN48-17c, KNN48-18c, KNN48-19c, KNN48-20c, KNN51b, KNN52-1c, KNN52-2b, KNN52-3b, KNN54-1b, KNN54-2b, KNN56a, KNN58-1b, KNN58-2c, KNN59e, KNN60-1c, KNN60-2d, KNN62b, KNN63-1b, KNN63-2b, KNN63-3b, KNN63-4b, KNN63-5a, KNN63-6b, KNN63-7b, KNN63-8d, KNN63-9d, KNN63-10d, KNN63-11b, KNN63-12b,

	KNN63-13b, KNN63-14b, KNN63-15b, KNN63-16b, KNN63-17b, KNN63-18b, KNN63-19e, KNN64-1a, KNN64-2a, KNN64-3a, KNN64-4b, KNN64-6b; KNN65-1f, KNN65-2a, KNN65-3c, KNN65-4c, KNN65-5d, KNN66b, KNN67-1b, KNN67-2b, KNN67-3b, KNN67-4b, KNN68-1b, KNN68-2b, KNN68-3b, KNN68-4b, KNN69-1e, KNN69-2a, KNN69-3a, KNN70b, KNN71-1a, KNN71-2a, KNN72a, KNN73-1a, KNN73-2a, KNN73-3d, KNN74-1c, KNN74-2c, KNN75-1c, KNN75-2b, KNN75-3b, KNN75-4b, KNN77-1a, KNN77-2a, KNN77-3a, KNN77-4a, KNN77-5a, KNN77-6a, KNN78-1e, KNN78-2e, KNN79-1b, KNN79-2b, KNN79-3d, KNN80-1c, KNN80-2d, KNN81-1c, KNN81-2 b, KNN81-3d, KNN82-1a, KNN82-2c, KNN83e, KNN84c, KNN85-1f, KNN85-2b, KNN85-3b, KNN85-4c, KNN85-5c, KNN85-6c, KNN85-7c, KNN85-8a, KNN86-1b, KNN86-2b, KNN86-3b, KNN86-4b, KNN86-5c, KNN86-6a, KNN88-2b, KNN88-3b, KNN88-4a, KNN87b, KNN91-1a, KNN91-2a, KNN91-3a, KNN91-4a, , KNN91-5a, KNN91-6a, KNN92-1b, KNN92-2b KNN92-3b, KNN92-4a, KNN92-5a, KNN92-6a, KNN92-7a, KNN94e, KNN97b, KNN98b

2.6. Detection of additional chemical markers

Actinobacteria taken to represent each of the genera highlighted in the comparative 16S rRNA gene sequencing studies were examined for key chemical markers known to be of value in actinobacterial systematics (Table 2.7).

Table 2.7. Representative actinobacteria included in the chemotaxonomic analyses.

Codes	Source	Genera
KNN53-1a, KNN53-2a	Salar de Atacama	*Actinomadura*
KNN49-5e, KNN49-26a, KNN50-8b, KNN50-11c, KNN50-16d	Salar de Atacama	*Amycolatopsis*
KNN47b	Yungay	*Blastococcus*
KNN7-2b	Yungay	*Cochioplanes*
KNN44-1b	Yungay	*Geodermatophilus*
KNN56a	Salar de Atacama	*Kribbella*
KNN94e	Salar de Atacama	*Lechevalieria*
KNN45-2b, KNN46-4b	Yungay	*Modestobacter*
KNN57-1b	Salar de Atacama	*Nonomuraea*
KNN55-1b	Yungay	*Pseudonocardia*
KNN10-4d	Salar de Atacama	*Saccharothrix*
C34, C38, C58, C59, C79	Salar de Atacama	*Streptomyces*

Whole-cell sugar analyses. All of the representative strains (Table 2.7) assigned to genera based on 16S rRNA gene sequences were examined for the presence of diagnostic whole-cell sugars using the procedure described by Hasegawa *et al.* (1983).

One to two loopfuls of each strain were transferred to cryotubes containing 0.25 N HCl, the preparations autoclaved at 121^{o}C for 15 minutes and 3 µl of each of the resultant hydrolysates and 1 µl of a standard solution of sugars (each at 1%, w/v) applied to 10 x10 cm cellulose TLC plates (10 x 20 cm; No.5552, Merck). The standard sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose (10 mg of each in 1 ml of pyridine). The TLC sheets were developed for 2 hours in a glass tank containing the solvent system, namely 10 n-butanol : 6 sterile distilled water : 6 pyridine : 1 toluene (v/v). The chromatograms were air-dried, developed for 2 hours, air-dried again, sprayed with aniline phthalate reagent and heated at 100^{o}C for 4 minutes when the hexose and pentose sugars gave brown-red and grey-green spots, respectively. The sugar patterns of the strains were identified by comparison against the standard sugars.

Menaquinone analyses. Menaquinones were extracted from the 21 representative strains (Table 2.7), by using the small-scale integrated procedure of Minnikin *et al.* (1984). Freeze-dried biomass (100 mg) 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 layers transferred to individual clean vials. The lower layers were treated with a further 1ml of petroleum ether, mixed for 30 minutes, and the upper layers added to the corresponding initial preparations. The combined extracts were transferred to small glass vials, evaporated to dryness using a nitrogen stream and stored at - 20°C until required.

The extracted menaquinones were resuspended in 200 µl of isopropanol and analysed using a high performance liquid chromatograph (LC-10AS, Shimadzu Co., New Jersey, USA) fitted with a reverse-phase C18 column (250 × 4.0 mm, 5 µm particle size; RP-18-Lichrosorb column; Capital Analytical, Leeds, UK). Methanol/isopropanol (2:1, v/v) was used as the isocratic mobile phase with a flow rate of 1 ml/minute at ambient temperature; aliquots of each sample (10 µl) were injected into the chromatograph. 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 analyses. Polar lipids were extracted from the biomass preparations

remaining after the extraction of the menaquinones (Minnikin *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%, w/v aqueous NaCl (9:10:3, v/v/v) added and the resultant preparations mixed on a tube rotator for an hour. Each supernatant was transferred to a clean test tube and 0.75 ml of chloroform/methanol/0.3%, w/v aqueous NaCl (5:10:4, v/v/v) added prior to vortexing for 30 minutes and centrifuging at 2000 rpm for 5 munutes, the supernatants were then transfered to a clean tube; repeat this step was repeated twice. Each of the resultant preparations was mixed thoroughly after the addition of 1.3 ml of chloroform and 1.3 ml of 0.3% NaCl, then centrifugation and the lower layers transferred to clean vials, evaporated to dryness in a nitrogen stream and stored at -20^{o}C. The lipid extracts were dissolved in 50 µl of chloroform/methanol (2:1, v/v) and 10 µl of the resultant samples applied to the corners of five 10 x 10 cm silica-gel TLC plates (No. 1.05721, Merck). 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 until the solvent front was 1cm below the top of the plates. The resultant spots were characterised using differential stains after Komagata and Suzuki (1987):

(a) The first plate was sprayed with 10% (w/v) phosphomolybdic acid in ethanol (Sigma P-1518) and charred at 120^{o}C for 10 minutes to detect all lipids.

(b) The second plate was sprayed with ninhydrin 0.2% (w/v) in ethanol and heated at 100^{o}C for 5 minutes to detect lipids with free amino groups. Phosphatidylethanolamine (PE) and phosphatidyl-N-methylethanolamine (PME) appear as dark pink spots on a white background. The same sheet was sprayed with Dittmer's reagent (molybdenum blue; Sigma M-3389) to detect phosphorus containing lipids which appear as blue spots on a white background.

(c) The third plate was sprayed with aqueous sodium metaperiodate (1%, v/v) and left for 10 minutes before decoloring with SO_2, followed by spraying with Schiff's reagent (Sigma) and a further decoloring with SO_2. Phosphatidylglycerol gives a bright purple spot immediately and phosphatidylinositol a brown coloured spot on a white background.

(d) The fourth plate was sprayed with α-naphthosulfuric acid (10.5 ml of 15% α-naphthol (Sigma N2480) in 95% ethanol 6.5 ml of concentrated H_2PO_4), 40.5 ml of 95%

EtOH and 4 ml of water) and heated at 100^{o}C for 10 minutes. Phosphatidylinositol dimannosides (PIDM) and glycolipids appear as brown spots on a white background.
(e) The final plate was sprayed with Dragendorff reagent (0.11 M potassium iodide and 0.6 mM bismuth nitrate in 3.5 M acetic acid) to detect phosphatidylcholine (PC), PE and PME which appear as orange spots on a yellow background.
The lipids on each of the TLC plates were identified by comparing their motilities with those of authentic lipids (Sigma PH-9).

2.7. DNA-DNA relatedness assays

Isolates found to be closely related to type strains of known species based on 16S rRNA gene sequence data were subjected of DNA:DNA relatedness studies. High purity genomic DNA, was prepared using the CTAB method (Kieser *et al*., 2000) from isolates KNN49-5e, KNN49-26a, KNN50-2e, KNN50-8b, KNN50-11c, KNN50-16d, KNN45-2 and KNN46-4.

Determination of optimal temperature for renaturation. DNA-DNA relatedness values were determined between isolate KNN49-5e and *A. ruanii* NGM112^{T}, isolate KNN49-26a and *A. ruanii* NGM112^{T}, isolate KNN50-2e and *A. thermalba* SF45^{T}, isolate KNN50-8b and *A. ruanii* NGM112^{T}, isolate KNN50-11c and *A. thermalba* SF45^{T}, isolate KNN50-16d and *A. thermalba* SF45^{T}, isolate KNN45-2b and *M. marinus* 42H12-1^{T} using the procedure described by Gonzalez & Saiz-Jimenez (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). The G+C contents of the strains were determined fluorimetrically. To this end, 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. Ten μl of 1x SYBR Green I (Molecular Probes) was added to each of the preparations and a thermal ramp run from 25 to 100^{o}C at a 1^{o}C rise per minute in a 185-5201 CFX connected (Bio Rad, Hertfordshire, UK). Double-stranded DNA was bound with SYBR Green I; when the studied-double strand DNA dissociated

into single-stranded DNA, the fluorescence was released and measured as described below. 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 reference strains and hybrids for estimation of ΔT_m values between the pairs of test strains was carried out by adding genomic DNA (250 ng/µl in 0.1xSSC, Appendix 2) samples from both the reference and the test strains to a 0.5 ml centrifuge tube. The homologous DNA control and hybrid DNA samples were denatured and rehybridised in a *T*-gradient Thermocycler® (Whatman-Biometra) using the following conditions: 99℃ for 10 minutes, T_{or} for 8 hours followed by progressive steps in which the temperature was dropped by 10℃ then held for an hour until the temperature reached 25℃. The tubes were then held at 4℃, 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℃ for 15 minutes, and a thermal ramp from 25 to 100℃ at 0.2℃ per second; fluorescence measurements were taken at each step of the ramp. The T_m of the homologous and hybrid DNA preparations were 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℃ or more are considered to show that tested strains belong to different genomic species (Wayne *et al.*, 1987; Rosselló-Mora & Amann, 2001).

2.8. Detection of phenotypic properties

2. 8. 1. *Test strains and data acquisition*

Phenotypic tests were carried out on representative strains of the genera *Amycolatopsis* (Table 2.8), *Modestobacter* (Table 2.9) and *Streptomyces* (Table 2.10) together with

appropriate marker type strains. The tests were performed using standard microbiological methods and by using API ZYM kits , 20 NE API kits (BioMerieux® Co.) and GEN III MicroPlates (BiOLOG).

Table 2.8. *Amycolatopsis* strains isolated from the Salar de Atacama environmental sample and examined for phenotypic properties together with the type strains of their nearest phylogenetic neighbours.

Strains	**Source/strain histories**
Isolates	KNN49-1h, KNN49-3e, KNN49-5e, KNN49-6a, KNN49-7c, KNN49-10b, KNN49-11c, KNN49-12b, KNN49-26a, KNN49-32e, KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-16d, KNN50-17d, KNN50-18d*, GY024^{A} and GY142^{A}
A. eurytherma Kim *et al.* (2002)	NT202^{T} (=DSM 44348^{T}=NCIMB 13795^{Tc}), isolated from arid scrubland soil
A. granulosa Zucchi *et al.* (2012b)	GY307^{T} (= NCIMB 14709^{T}= NRRL B-24844^{T}), isolated from an arid soil sample from Marla, Australia
A. methanolica Boer *et al.* (1990)	NCIB 11946^{T}; isolated from a New Guinea soil
A. thermoflava Chun *et al.* (1999)	N1165^{T} (= CIP106795^{T} = DSM 44574^{T} = NBRC 14333^{T} = JCM 10669^{T} = NRRL B-24140^{T}), isolated from a soil sample from Hainan Island, China
A. thermalba Zucchi *et al.* (2012a)	SF45^{T} (=NCIMB14705^{T}=NRRL B-24845^{T}), isolated from an arid soil sample from Marla, Australia
A. ruanii Zucchi *et al.* (2012a)	NMG112^{T}(= NCIB14711^{T} = NRRL B-24848^{T}), isolated from an arid soil sample from Marla, Australia
A. thermophila Zucchi *et al.* (2012b)	GY088^{T} (= NCIMB 14699^{T} = NRRL B-24836^{T}) isolated from a composite Australian soil sample
A. tucumanensis Albarracín *et al.* 2010	Professor Martha Trujillo, University of Salamanca, Spain, strain ABO^{T} (= DSM45259^{T} = JCM17017^{T} = LMG 24814^{T}), isolated from a sediment sample polluted with copper and collected in Tucumán, Argentina.
A. viridis Zucchi *et al.* (2012b)	GY115^{T} (= NCIMB 14700^{T} = NRRL B-24837^{T}), isolated from a composite Australian soil sample

^{T} Type strain.

Table 2.9. *Modestobacter* strains isolated from the Yungay environmental sample and examined for phenotypic properties together with the type strains of their nearest phylogenetic neighbours.

Strains	Source/strain histories
Isolates	KNN45-1a, KNN45-2b, KNN45-3b, KNN45-4b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-6a, KNN46-7a, KNN46-8d, KNN46-9c and KNN46-10g
M. marinus Xiao *et al.* (2011)	Professor Martha Trujillo, University of Salamanca, Spain, strain 42H12-1^T (= CGMCC 4.5581^T=DSM 45201^T), isolated from deep-sea sediment from the Atlantic Ocean
M. multiseptatus Mevs *et al.* (2000) emend. Reddy *et al.* (2007).	Professor Martha Trujillo, strain AA-826^T (= CIP 106529^T=DSM 44406^T=JCM 12207^T), isolated from Antarctic surface soil from Linnaeus Terrace (1600 m) of Asgard Range Transantarctic Mountains
M. roseus Qin *et al.* 2013	DSM 45764^T (= KCTC 19887^T = KLBMP 1279^T = NBRC 108673^T), isolated from the coastal halophyte *Salicornia europaea* Linn.
M. versicolor Reddy *et al.* 2007	Professor Martha Trujillo, CP153-2^T (= ATCC BAA-1040^T=DSM 16678^T =JCM 15578^T) isolated from a soil crust

^{T} Type strain.

Table 2.10. *Streptomyces* strains isolated from either the Salar de Atacama or Yungay environmental samples and examined for phenotypic properties together with the type strain of *Streptomyces fimbriatus*.

Strains	Source/strain histories
Isolates	C34^{*,1}, C38^{*,1}, C58^{*,1}, C59^1, C79^{*,1} KNN2-4^1, KNN2-6^1, KNN5-48^1, KNN6-6^1, KNN6-9^1, KNN10-4^1, KNN10-5^1, KNN11-1^1, KNN11-5^1, KNN13^2, KNN23-1^2, KNN24-1^1, KNN25^1, KNN26^1, KNN32-1^1, KNN35-1^1, KNN35-2^1, KNN38-1^1, KNN42^2, KNN48-1^1, KNN51^2, KNN64-5^1, KNN70^1, KNN88-1^2 and KNN90^2
Streptomyces fimbriatus (Millard & Burr 1926) Waksman and Lechevalier 1953	NRRL B-3175^T (=AS 4.1598^T = ATCC 15051^T = CBS 453.65^T = DSM 40942^T = NBRC 15411^T = JCM 5080^T = NCIB 13039^T = VKM Ac-761^T) isolated from a case of common potato scab

Strains isolated from ^{1}Salar de Atacama and ^{2}Yungay environmental samples.

^{T} Type strain.

Inoculation of test media. Most of the tests were carried out in Replidishes (Sterilin Ltd., Staffordshire, UK) at 28°C unless stated otherwise, and the 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. Inocula were prepared by scraping spores/hyphal fragments from yeast extract-malt extract agar plates (ISP medium 2, Shirling & Gottlieb, 1966), 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 solution to give a turbidity of 5 on the McFarland scale (Murray *et al*., 1999). Each inoculum (1 ml) was pipetted into a sterile compartment of a Replidish 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 medium. The phenotypic tests carried out in either Bijoux tubes or Replidishes are shown in Table 2.11. All of the tests were carried out in duplicate. Details of the composition of the test media are shown in Appendix 3.

Table 2.11. Phenotypic tests carried out in bijou tubes or Replidishes.

Type of test	Tests
A. Biochemical tests (w/v):	Aesculin hydrolysis (0.1%)[A,M,S], Allantoin hydrolysis (0.1%)[A,M,S], Arbutin hydrolysis (0.1%) [A,M,S], Catalase production*; [A,M,S], Hydrogen sulphide production*;[A,M,S], Nitrate reduction*; [A,M,S], Urease production*; [A,M,S]
B. Degradation tests (%, w/v):	Adenine (0.4) [A,M,S], Casein (1) [A,M,S], Cellulose (1.0) [A,M,S], Chitin (0.2) [A,M,S], DNA (0.2) [A,M,S], Elastin (0.3) [A,M,S], Gelatin (0.4) [A,M,S], Guanine (0.05) [A,M,S], Hypoxanthine (0.4) [A,M,S], RNA (0.3) [A,M,S], Tributyrin (0.1 % v/v) [A,M,S], L-tyrosine (0.5) [A,M,S], Starch (0.1) [A,M,S], Tween 20 (1% v/v) [A,M,S], Tween 40 (1% v/v) [A,M,S], Tween 60 (1% v/v) [A,M,S], Tween 80 (1% v/v) [A,M,S], Uric acid (0.5) [A,M,S], Xanthine (0.4) [A,M,S], Xylan (0.4%) [A,M,S],
C. Nutritional tests Growth on sole carbon sources: 1. Monosaccharides (at 1%, w/v)	Hexoses: D-fructose[A], D-galactose[A], D-glucose[A], D-mannose[A], L-sorbose[A], D-xylose[A] Pentoses: Amygdalin[A], L-arabinose[A], L-fucose[A], D-turanose[A], L-rhamnose[A] , D-ribose[A]
2. Disaccharides (at 1%, w/v)	D-trehalose[A], D-cellobiose[A]
3. Tri & tetrasaccharides (at 1%, w/v)	D(+) melezitose[A] , D(+) raffinose[A], α-lactose[A], D-maltose[A], D-melibiose[A], D-sucrose[A]
4. Polysaccharides (at 1%, w/v)	D-cellubiose[A] , Dextrin[A], Glycogen[A], Inulin[A], Pectin[A]
5. Sugar alcohols (at 1%, w/v)	Adonitol[A], Dulcitol[A], *meso*-inositol[A], D-mannitol[A], D-sorbitol[A],

6. Glycosides (at 1%, w/v)	Aesculin [A], Arbutin [A], Salicin [A]
7. Alcohols (1%,v/v)	Butan-1-ol [A], Ergosterol [A], Ethanol [A], Ethylamine [A], Methanol [A], Methylamine [A], Propan-1-ol [A], Propan-2-ol [A], Propylene glycol [A]
8. Sodium salts (0.1% w/v)	Acetate [A], Adipate [A], Alginate [A], Azelate [A], Benzoate [A], Butyrate [A], Citrate[A], Fumarate [A], Hippurate [A], Malate [A], Malonate [A], Oxalate [A], Pimelate [A], Propionate [A], Pyruvate [A], Succinate [A], Tartrate [A], Urea [A], Uric acid [A]
D. Tolerance tests[1]:	
1. pH	2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0, 10.0, 11.0 and 12.0 [A,M,S]
2. Temperature	4, 10, 20, 30, 40, 50, 55, 58 and 60^{o}C [A,M,S]
3. Growth in presence of (%w/v)	3.1 Sodium chloride [A,M,S] : 2.5, 5, 7.5 and 10.0 3.2 Lysozyme (0.05) 3.3 Resistance to antibiotics (µg/ml)[1; A,M]: Gentamicin sulphate (8)' Neomycin sulphate (4, 8) , Novobiocin sulphate (8), Penicillin G sulphate (16), Rifampicin (16)' Streptomycin sulphate(4) , Tetracycline sulphate (8)' Vancomycin sulphate (2)

Tests carried out on (A) *Amycolatopsis*, (M) *Modestobacter* and (S) *Streptomyces* strains.

2. 8. 2. Biochemical tests

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 showing 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 actinobacteria 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) 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 in 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.

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 iron in the medium to form a black-brown melanin-like polymer. Blackening 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 and Williams (1964). Tubes of nitrate broth 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; blackening of lead acetate strips after incubation for 7 days was scored as a positive result.

Nitrate reduction. This test is used 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 3) were added. The sulfanilic acid in reagent A reacts with nitrite to yield 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.

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

2. 8. 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 Tyndallisation 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 as positive.

DNA and RNA. The degradation of DNA was detected using Bacto DNase test agar (Difco; Appendix 3) which contains DNA (0.2%, w/v). Ribonucleic acid breakdown was examined in tryptose agar supplemented with RNA (0.3%, w/v, Goodfellow *et al.*, 1979). 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 3), 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 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 performed 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 3); 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 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 tributyrin agar (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 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+}) 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) were inoculated, incubated and examined for the characteristic precipitates which indicated a positive response.

2. 8. 4. Nutritional tests

The ability of some test strains to metabolise carbon compounds as sole sources of carbon for energy and growth was examined using carbon utilisation agar (ISP medium 9; 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 (negative control) and onto the basal medium supplemented with 1%, glucose (positive control). A positive result was recorded when growth on the test medium was equal or greater to 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. 8. 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 45°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 values (Table 2.8) 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) (Appendix 2). 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. Some test strains were examined for their ability to grow in the presence of different antibiotics at various concentrations. 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.

2.9. Data acquired using test kits

API ZYM kits. Suspensions of the *Amycolatopsis, Modestobacter* and *Streptomyces* strains were prepared from cultures grown on yeast extract-malt extract agar (ISP medium 2; Shirling & Gottlieb, 1966) plates for 7 days at 28^{o}C. Biomass was transferred to sterile disposal bijoux bottles containing 2 ml of sterile ¼ strength Ringer's solution and emulsified by vigorous vortexing to provide turbidity readings equivalent to McFarland scale 5.0 (Murray *et al*., 1999). Aliqouts (50µl) of the suspensions were incubated at 28^{o}C overnight prior to the addition of single drops of reagent ZYM A (Tris-HCl and sodium lauryl sulphate in water) and one drop of reagent ZYM B (diazonium salt Fast Blue BB in 2-methoxyethanol) to each cupule. The strips were then developed in strong light for 5 minutes to eliminate any yellow colour which may appear in the cupules due to any excess of Fast Blue BB which had not reacted. Enzymetic activity was revealed by the colour changes shown in Table 2.12, reactions were scored from 0 (no enzyme activity) to 5 (very strong enzyme activity), according to the intensity of the colour reaction in each cupule. Scores of 0 to 2 and 3 to 5 were classified as negative (0) and positive (1), respectively.

GEN III MicroPlate™ BiOLOG tests. Suspensions of the *Amycolatopsis, Modestobacter* and *Streptomyces* strains were prepared as mentioned for the API ZYM tests. Test strains were examined to establish their carbon utilisation and chemical sensitivity patterns based on 71 and 23 assays, respectively (Table 2.13). Inocula were

prepared in Inocula Fluid (IF, BiOLOG) and vigorous by vortexed to give a turbidity reading equivalent to McFarland scale 5. Aliqouts (100 µl) of the inocula were added to the wells in the Microplates which were then covered with a lid and incubated at 28^{o}C for 3 to 36 hours. Colour densities in the wells were detected using a spectrophotometer (model Multiskan Ascent, Thermo Labsystems) using software version 1.3.1. Positive results were read when colourimetric readings for both the carbon utilisation and chemical tests were greater than the control and negative results when the readings were less or equal to those of the negative control.

2.10. Morphology

Selected *Actinomadura, Nonomuraea, Pseudonocardia, Saccharothrix* and *Streptomyces* strains (Table 2.14) were grown on oatmeal agar at 28^{o}C for 21 days prior to detecting spore chain arrangement and spore surface ornamentation which were observed by examining gold-coated dehydrated specimens under a scanning electron microscope (Cambridge Stereoscan 240 instrument), as described by O'Donnell *et al.* (1993).

Table 2.12. API ZYM colourimetric enzyme assays.

Test no.	Enzyme detected	Substrate	pH	Positive result	Negative result
1	Control	-	-	No colour	No colour
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	
3	Esterase (C4)	2-napthyl butyrate	6.5	Violet	
4	Esterase lipase (C8)	2-napthyl caprylate	7.5	Violet	
5	Lipase	2-napthyl myristate	7.5	Violet	
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange	
7	Valine arylamidase	L-valyl-2-naphthylamide	7.5	Orange	
8	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange	
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	AS control
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11	Acid phosphatase	2-naphthyl phosphatase	5.4	Violet	
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue	
13	α-Galactosidase	6-Br-2-naphthyl-αD-	5.4	Violet	

		galactopyranosidase			
14	β-Galactosidase	2-naphthyl-αD-galactopyranosidase	5.4	Violet	
15	β-Glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue	
16	α-Glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet	
17	β-Glucosidase	6-Br-2-naphthyl-αD-glucopyranoside	5.4	Violet	
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosamine	5.4	Brown	
19	α-Mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet	
20	α-Fucosidase	2-naphthyl-αL-fucopyranoside	5.4	Violet	

Table 2.13. Colourimetric carbon utilization and tolerance testing using GEN III Microplates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 negative control	A2 Dextrin	A3 D-Maltose	A4 D Trehalose	A5 D-cellobiose	A6 Gentiobose	A7 N-acetyl-β-D-mannosamine	A8 D-turanose	A9 Stachyose	A10 Positive control	A11 pH6	A12 pH5
B	B1 D-raffinose	B2 α-D-lactose	B3 D-melibiose	B4 β-methyl-D-glucoside	B5 D-salicin	B6 N-acetyl-D-glucosamine	B7 N-acetyl-β—D-mannosamine	B8 D-acetyl-galactosam-ine	B9 N-acetyl-Neuraminic acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C	C1 α-D-glucose	C2 D-mannose	C3 D-fructose	C4 D-galactose	C5 3-methyl-glucose	C6 D-fucose	C7 L-fucose	C8 L-rhamnose	C9 inosine	C10 1% sodium lactate	C11 Fusidic acid	C12 D-serine
D	D1 D-sorbitol	D2 D-mannose	D3 D-arabitol	D4 myo-inositol	D5 glycerol	D6 D-glucose-6-PO_4	D7 D-fructose-6-PO_4	D8 L-rhamnose	D9 D-serine	D10 Troleandomy-cin	D11 rifamycin SV	D12 minocycline
E	E1 gelatin	E2 glycyl-L-proline	E3 L-alanine	E4 L-arginine	E5 L-aspartic acid	E6 L-glutaminc acid	E7 L-histidine	E8 L-pyroglutamic acid	E9 L-serine	E10 lincomycin	E11 guanidine HCl	E12 Niaproof4
F	F1 pectin	F2 D-galacturonic acid	F3 L-galacturon-ic acid lactone	F4 D-gluconic acid	F5 D-glucuronic acid	F6 glucoronamide	F7 mucic acid	F8 quinic acid	F9 D-saccharic acid	F10 vancomycin	F11 tetrazolium violet	F12 tettrazolium blue
G	G1 ρ-hydroxy phenylacetic acid	G2 methyl pyruvate	G3 D-lactic acid methyl ether	G4 L-lactic acid	G5 citric acid	G6 α-keto-glutaric acid	G7 D-malic acid	G8 L-malic acid	G9 bromo-succinic acid	G10 nalidixic acid	G11 lithium chloride	G12 potassium tellurite
H	H1 tween30	H2 γ-amino-butyric acid	H3 α-hydroxy butyrc acid	H4 β-hydroxy-D,L-butyric acid	H5 α-keto-butyric acid	H6 acetoacetic acid	H7 propionic acid	H8 acetic acid	H9 formic acid	H10 azetreonam	H11 sodiumbuty-rate	H12 sodium bromide

Table 2.14. *Actinomadura, Nonomuraea, Pseudonocardia, Saccharothrix* and *Streptomyces* strains examined for spore chain arrangement and spore ornamentation using scanning electron microscopy.

Genera	Code
Actinomadura	KNN34c, KNN53-1a
Nonomuraea	KNN57-1b, KNN57-2b
Pseudonocardia	KNN51-1b
Saccharothrix	KNN54-1a
Streptomyces	C34, C38, C58, C59, C79, KNN2-6c, KNN13a, KNN17-2c, KNN21a, KNN23-1b, KNN24-1b, KNN26b, KNN35-1b, KNN35-2b, KNN38-1b, KNN42f, KNN48-1c, KNN51-1b, KNN66b, KNN83e, KNN87b and KNN90a

2.11. Screening for bioactivity

Plug assays. One hundred and thirty four isolates, 106 from the Salar de Atacama and 28 from the Yungay environmental sample (Table2.15) were taken to represent multi-membered and single-membered colour-groups and screened for antimicrobial activity against a panel of wild type microorganisms using a standard agar plug assay (Fiedler, 2004),
The isolates were grown on ISP medium 2 (Shirling & Gottlieb, 1966), prepared with 0.8% agar, and incubated for 14 days at 30^{o}C.

- Five plugs were taken from each plate using a sterile cork borer and transferred to Petri dishes labelled *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae* and *Staphylococcus aureus.*

- Lysogeny broth (50 ml) was mixed with 50 ml of nutrient agar in four separate sterile bottles and 100 µl of the *B. subtilis, E. coli, P. fluorescens*, and *S. aureus* wild type strains added to the appropiate bottle.

- 50 ml of yeast-potato dextrose (YPD) agar was added to 50 ml of YPD broth and 200 µl of the *S. cerevisiae* wild type strain.

The resultant preparations were carefully poured into the corresponding Petri dishes containing the agar plugs until the bottom of the dishes were covered. The media were

allowed to solidify and the plates incubated overnight at 30^{o}C and then examined for the presence and extent of zones of inhibition around the inoculated agar plugs.

Reporter strains. The 92 isolates which showed activity in the plug assays were examined against five *B. subtilis* reporter strains designed to detect specific modes of action on the target cells (Table 2.16).

- Five plugs were taken from each plate, as explained above, and transferred to Petri dishes labelled with the names of the five *B. subtilis* reporter strains.
- 25 ml Luria broth (LB) 100 µl of erythromycin, 100 µl of X-gal and 100 µl of each reporter strain were added to 75 ml of nutrient agar; for the *phi*105^{CH} reporter strain 100 µl of chloramphenicol was used instead of erythromycin.
- The resultant media were carefully poured into Perti dishes until all of the plugs were covered.

Table 2.15. Representative strains isolated from the Salar de Atacama and Yungay environmental samples and examined in the plug assays against a panel of pathogenic microorganisms.

Salar de Atacama	Yungay
Multi-membered colour-groups: KNN1-2c, KNN1-5f, KNN2-2c, KNN2-5c, KNN2-6c, KNN2-10d, KNN2-11d, KNN3-1b, KNN3-2b, KNN3-11d, KNN3-17d, KNN4-1b, KNN4-3b, KNN4-4a, KNN5-24b, KNN5-25d, KNN6-2a, KNN6-5a, KNN6-6b, KNN6-10b, KNN6-11a, KNN8-3a, KNN8-5b, KNN8-8a, KNN8-9c, KNN8-10e, KNN9-1a, KNN9-2c, KNN10-3b, KNN10-4d, KNN10-5a, KNN11-2a, KNN11-4b, KNN11-5a, KNN11-6a, KNN14-3e, KNN17-1c, KNN17-2b, KNN18-3d, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-7e, KNN24-8e, KNN24-9e, KNN32-1a, KNN35-1b, KNN35-2b, KNN36-1c, KNN36-3c, KNN37-1e, KNN37-5a, KNN38-1b, KNN38-5b, KNN48-3e, KNN48-6d, KNN49-5e, KNN49-26a, KNN50-8b, KNN50-11c, KNN52-2b, KNN53-1a, KNN53-2a, KNN53-3a, KNN54-1a, KNN57-1b, KNN58-1b, KNN61-1a, KNN63-2b, KNN63-15b, KNN64-3a, KNN64-5b, KNN65-1f, KNN65-5d, KNN67-4b, KNN68-2b, KNN68-4b, KNN69-1a, KNN69-2, KNN69-3a, KNN71-2a, KNN73-2a, KNN74-2c, KNN75-4b, KNN81-2b, KNN82-1a, KNN85-1f Single-membered colour groups: KNN25c, KNN26b, KNN27a, KNN28a, KNN29a, KNN30a, KNN31d, KNN33a, KNN34c, KNN39c, KNN43b, KNN56a, KNN66b, KNN72a, KNN76b, KNN83e, KNN87b, KNN94es	Multi-membered colour groups: KNN7-2b, KNN13a, KNN21a, KNN23-1b, KNN44-2a, KNN44-4b, KNN45-1a, KNN45-2b, KNN45-3b, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5b, KNN46-6a, KNN46-7a, KNN46-8a, KNN55-1b, KNN86-1b, KNN88-1a, KNN91-1a Single-membered colour groups: KNN47b, KNN51b, KNN89a, KNN90a, KNN96a, KNN97a

Positive controls (10 µl) were placed on Whatman filter paper discs (6 mm diameter) and applied to the corresponding plates which were incubated overnight at 30^{o}C and examined for blue rings around Whatman filter paper (Brunstein, 2010), The reporter genes are induced by bioactive substances and causing the cleavage X-gal to galactose and 5-bromo-4-chloro-3-hydroxyindole. The presence of a blue halo around inhibition zones which is due to this latter compound, indicates the mode of action of the bioactive compounds.

Positive results in the plug assays based on the *B. subtilis* reporter strains were recorded when a blue halo was formed round zones of inhibition.

Table 2.16. *Bacillus subtilis* strains containing reporter genes and positive controls used in the plug assays designed to determine the mode of action of unknown antimicrobial compounds.

Reporter genes	Positive controls	Targets
yvqI^{ER}	Bacitracin	Cell wall synthesis
yvgS^{ER}	Rifampicin	RNA synthesis
ypuA^{ER}	Cefoxitin	Cell envelope synthesis
*Phi*105^{CH}	Nalidixic acid	DNA synthesis
yjaX^{ER}	Triclosan	Fatty acid synthesis

ER: erythromycin resistant, CH: chloramphenicol resistant.

Disk diffusion assays. **.** The mycelial extracts of the 92 representative isolates tested in the plug assays were examined against the *E. coli* and *S. cerevisiae* strains and against the 5 *B. subtilis* reporter strains. Each isolate was grown on ISP medium 2 (Shirling & Gottlieb, 1966), prepared with 0.8% agar, and incubated for 7 days at 30^{o}C. Cultures growing on the ISP medium were crushed using a sterile syringe, kept overnight at -20^{o}C, the supernatants (extracts) collected using wide bore pipette tips and transferred to 10 ml Falcon tubes. The media used for the disk diffusion assays were prepared as for the plug assays. The resultant media were poured into the corresponding Petri dishes and allowed to solidify. Twenty sterile Whatman filter paper disks (6 mm diameter) impregnated with 10 µl of extracts were placed on the agar surface of each Petri dish, the plates incubated overnight at 30^{o}C and the extent of zones of inhibition measured.

2.12. Preliminary characterisation of bioactive compounds

Strains. Six strains belonging to the genera *Amycolatopsis* (isolates KNN50-8b and KNN50-16d) and *Streptomyces* (isolates KNN26b, KNN38-1b, KNN64-5b and KNN90a) were chosen because they produced extensive inhibition zones in the plug assays and were distinct from their closest phylogenetic neighbours in the corresponding *Amycolatopsis* and *Streptomyces* 16S rRNA gene trees.

Production media and cultivation conditions. Four media were employed for submerged cultivation of the selected strains, namely yeast-extract malt-extract medium (ISP medium 2, Shirling and Gottleb, 1966), medium 19, medium 410 and starch-casein agar (Goodfellow & Fiedler, 2010) containing 1 gram Amberlite per 50 ml of production medium for secondary metabolites adsorption from cultivated medium (Sigma-Aldrich, Gillingham Dorset, UK). The inoculated media were shaken at 180 rpm for 14 days.

Preparation of extracts. The Amberlite beads were separated from production media biomass by centrifugation at 2,000 rpm for 5 minutes then washed 4-5 times with sterile distilled water. The Amberlite bead preparations were then shaken in 50 ml of methanol overnight prior to centrifugation at 3000 rpm for 10 minutes and the Amberlite discarded. The resultant supernatants were dried under a nitrogen gas stream and the dried preparations sent to Professor Marcel Jaspars at Aberdeen University for preliminary chemical analyses.

Chemical analyses. Each preparation was dissolved in methanol to give a final concentration of 0.5mg/mL, filtered and submitted for liquid chromatographic/mass spectrometric (LCMS) analysis. High resolution mass spectral data were obtained from a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump using the following conditions : capillary voltage 45 V, capillary temperature 260°C, auxilliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV. mass range 100–2000 amu (maximum resolution 30000). An Agilent Poroshell 120, EC-C18, 2.1x100 mm, 2.7um UPLC column was used for LC/MS with a mobile phase of 0-100% MeCN over 30 minutes at 0.5 ml/min flow rate. The results were presented as mass spectrophotometric (MS), liquid spectrophotometric (LC) and ultraviolet (UV) traces.

Chapter 3. Biosystematic Studies and Screening of Representative Strains Isolated from Hyper-arid and Extreme hyper-arid Atacama Desert Soils

3.1. Abstract

Atacama Desert soils are a largely untapped source of actinobacteria with a potential to produce biologically active specialised metabolites for the control of drug resistant pathogens. This study was designed to recover and screen taxonomically diverse actinobacteria from two habitats in the Atacama Desert, a hyper-arid soil from the Salar de Atacama region and an extreme hyper-arid soil from the Yungay region. The hyper-arid soil yielded relatively large numbers of *Amycolatopsis* and *Streptomyces* strains and small numbers of *Actinomadura*, *Kribbella*, *Lechevalieria*, *Nonomuraea* and *Saccharothrix* strains. *Modestobacter* and *Streptomyces* were predominant in the extreme hyper-arid soil through small numbers of *Blastococcus*, *Couchioplanes* and *Geodermatophilus* strains were isolated. With few exceptions representatives of the genera formed distinct phyletic lines in 16S rRNA gene trees. Most of the representative isolates examined in standard plug assays inhibited the growth of one or more of a panel of five wild type micro-organisms while some of the streptomycetes from the hyper-arid Salar de Atacama soil were shown to inhibit cell envelope, cell wall, fatty acid and RNA synthesis in assays based on *Bacillus subtilis* reporter strains. In contrast, few of the non-streptomycetes produced bioactive compounds through this may reflect the use of an inappropriate production media. Nevertheless, these results confirm that novel actinobacteria, notably streptomycetes, from hyper- and extreme hyper-arid Atacama Dsert soils are a rich source of diverse bioactive compounds that may be developed as resources for healthcare.

3.2. Introduction

Previously unknown filamentous actinobacteria, notably streptomycetes, are proving to be a rich source of new natural products, especially antibiotics, that can be developed as, resources for healthcare. Streptomycetes account for around 40% of known specialised metabolites and have genomes that typically contain over twenty biosynthetic gene

clusters that encode for known or predicted secondary metabolites (Bérdy, 2012). Although an underused resource, it is difficult to find new specialised metabolites from known actinobacteria as screening them tends to lead to the rediscovery of compounds of known provenance (Busti *et al*., 2000; Lam, 2007; Williams, 2008). Consequently, new strategies are being developed to selectively isolate, dereplicate and recognise novel actinobacteria for pharmaceutical screening programmes, as exemplified by the taxonomic approach to drug discovery recommended by Goodfellow & Fiedler (2010). This approach to bioprospecting has been used to isolate actinobacteria from extreme biomes, especially marine habitats, on the premise that harsh environmental conditions give rise to unique actinobacteria presenting novel chemistry (Bull & Stach, 2007; Bull 2011).

Novel actinobacteria isolated from deep-sea sediments have been shown to be an especially prolific source of new antibiotics, as illustrated by the discovery of caboxamycin, a benzoxazole antibiotic produced by a *Streptomyces* strain isolated from the Canary Basin in the Atlantic Ocean (Hohmann *et al*., 2009) and the abyssomicins, a family of polycyclic polyketides isolated from a *Verrucosispora mari*s strain derived from a sediment sample collected from the Sea of Japan (Riedlinger *et al*., 2004; Keller *et al*., 2007; Goodfellow *et al*., 2012b). *Salinispora*, an obligate marine genus which has a pan-tropical distribution in near shore marine sediments, is proving to be a particularly rich source of new antibiotics (Mincer *et al*., 2002; Fenical & Jensen, 2006; Jensen *et al*., 2007; Jensen, 2010). *Salinispora arenicola* and *Salinospora tropica*, the founder members of the genus have a large fraction of their genomes encoding for the biosynthesis of structurally unique specialised metabolites which are produced in species-specific patterns, *Salinispora arenicola* synthesises metabolites in the rifampicin and staurosporine classes and *S. tropica* compounds in the salinosporamide and spiralide clades, including salinisporamide A, an anticancer agent. Such studies helped promote an explosion of interest in marine actinobacteria as a source of new specialised metabolites (Blunt *et al*., 2012; Zotchev, 2012; Manivasagan *et al*., 2013).

The taxonomic approach to drug discovery has been used to isolate and screen novel filamentous actinobacteria from Atacama Desert soils in Northern Chile (Bull & Asenjo, 2013). The Atacama Desert is the oldest and driest desert on the planet having evolved over several million years of aridity and hyper-aridity (Gómez-Silva *et al*., 2008). Environment conditions in the desert have been considered too extreme to support any form of life due to a dearth of liquid water, virtual absence of organic matter, presence of inorganic oxidants and high levels of UV radiation. Nevertheless,

small numbers of phylogenetically novel filamentous actinobacteria, notably streptomycetes, have been isolated from hyper-arid and extreme hyper-arid Atacama Desert soils (Okoro *et al*., 2009) and shown to synthesise novel specialised metabolites (Bull & Asenjo, 2013). Three of the putatively novel *Streptomyces* isolates have been validly named as *Streptomyces atacamensis*, *Streptomyces bullii* and *Streptomyces deserti* and another two found to syntheses new antibiotics and anticancer agents, the atacamycins, chaxalactins and chaxamycins (Nachtigall *et al*., 2011; Rateb *et al*., 2011a, b; Bull & Asenjo, 2013). Another putatively novel *Streptomyces* strain from high altitude Atacama altiplano soil produced new aminobenzoquinones, the abenquines, which show inhibitory activity against bacteria and dematophilic fungi (Schulz *et al*., 2011). In addition, three isolates assigned to the rare genus *Lechevalieria* (Okoro *et al*., 2009) have been validly named as *Lechevalieria atacamensis*, *Lechevalieria deserti* and *Lechevalieria roselyniae* (Okoro *et al*., 2010).

The present study was designed to build upon and extend the pioneering studies of Okoro *et al*. (2009, 2010). To this end, large numbers of actinobacteria were isolated from two contrasting locations in the Atacama Desert using a broad range of selective isolation media, dereplicated, assigned to genera and screened for their ability to inhibit the growth of a panel of micro-organisms and *Bacillus subtilis* reporter strains, the latter designed to detected modes of action of bioactive compounds.

3.3. Materials and Methods

3. 3. 1. Sampling sites

Environmental samples were collected from two locations in the Atacama Desert by Professor A.T. Bull (School of Biosciences, University of Kent, Canterbury, UK.), namely the Laguna de Chaxa, Salar de Atacama, near Tocanao (latitude $23^{o}17'S$, longitude $68^{o}10'W$), and the Yungay region (latitude 24°06'18.6" and longitude 70°01'55.6") (see Chapter 2).

3. 3. 2. Physico-chemical properties of environmental samples

The pH of the environmental samples were determined using the procedure described by Reed and Cummings (1945). The pH of each sample was determined, in triplicate,

using a glass electrode pH meter (Model 320 Mettler-Toledo AG, CH.8603, Schwerzenbach, Switzerland). The percentage moisture content of the environmental samples were determined, in triplicate, by drying known amounts of the samples 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 any organic matter. After cooling overnight in a desiccator, the average loss in weight for each set of samples was recorded as the total organic matter content.

3. 3. 3. Selective isolation and enumeration of actinobacteria isolated from the environmental samples

Soil suspensions from the Salar de Atacama and Yungay environmental samples were prepared in 1/4 strength Ringer's solution (Oxoid, UK) to give $10^{-½}$ dilutions which were shaken on a tumble shaker (TMI Tumbler, Luckham Ltd., Sussex, UK) for 30 minutes, heat-pretreated at 55^{o}C for 6 minutes, as described by Okoro *et al.* (2009). Aliquots (100 µl) of the various dilutions were spread over plates of several selective isolation media (Table 3.1) which had been dried for 15 minutes at room temperature prior to inoculation (Vickers & Williams, 1987). Three replicate plates were prepared for each dilution and for each of the isolation media. After incubation at 28^{o}C for 3 weeks, the number of actinobacteria growing on the isolation plates were counted and the results expressed as the number of colony forming units (cfu) per gram dry weight of environmental sample.

Table 3.1. Media used for the selective isolation of actinobacteria from the Atacama Desert environmental samples.

Media	Selective agents (µg ml^{-1})	Target organism(s)
Gause's No.1 agar (Gause *et al*., 1957; Zakharova *et al.*, 2003)	Nalidixic acid (10)	Rare or uncommon actinobacteria
Geodermatophilus obscurus agar (Uchida &Seino, 1997)	Nystatin (25)	*Geodermatophilus* spp.
Glucose-yeast extract agar (Athalye *et al.*, 1981)	Rifampicin (20)	*Actinomadura* spp.
HV agar (Hayakawa & Nonomura, 1987)	Humic acid (1g L^{-1})	*Streptosporangiaceae* spp.

Luedemann's agar (Luedemann, 1971)	Nystatin (25)	*Modestobacter* spp.
Microlunatus agar (Nakamura *et al*., 1995)	Nystatin (25)	*Modestobacter* spp.
Minimal medium agar (Johnson *et al.*, 1981)	Nystatin (25)	Rare or uncommon actinobacteria
Oligotrophic agar (Senechkin *et al.*, 2010)	Low carbon and nitrogen content	Rare and uncommon actinobacteria
R2A (Reasoner & Geldreich, 1985)	Nystatin (25)	*Modestobacter* spp.
Starch-casein agar (Küster &Williams, 1964)	Nystatin (25)	*Streptomyces* spp.
SM1 (Tan *et al.*, 2006)	Neomycin (1) and nystatin (25)	*Amycolatopsis* spp.

All of the media were supplemented with cycloheximide (25 µg ml^{-1}).

3. 3. 4. Selection, maintenance and presumptive classification of actinobacteria isolated from the Salar de Atacama and Yungay environmental samples

Four hundred and twenty strains chosen to represent the various colony types growing on the selective isolation plates were subcultured onto yeast extract-malt extract agar plates (International *Streptomyces* Project [ISP2], Shirling & Gottlieb, 1966) and incubated at 28^{o}C for 14 days. Three hundred and fifteen of the strains were taken from the isolation plates inoculated with suspensions of the Salar de Atacama environmental sample and 105 from those seeded with suspensions of the environmental sample taken from the Yungay region. Colonies that produced a leathery substrate mycelium covered by an abundant aerial spore mass were considered to belong to the genus *Streptomyces*, and those which formed shiny black, mucoid colonies were assigned to the family *Geodermatophilaceae*. In contrast, isolates producing leathery colonies covered by little or no aerial hyphae were assumed to belong to a range of filamentous actinobacterial taxa.

Maintenance of strains. All of the isolates were grown on oatmeal (Shirling & Gottlieb, 1966) and modified Bennett's agar plates (Jones, 1949) at 28°C for 3 weeks. Suspensions of spores and mycelial fragments from each of the inoculated plates were suspended in 1 ml aliquots of 20%, v/v glycerol (2 vials per strain per medium) in

cryotubes, one culture prepared from each medium was kept at -80^{o}C for long-term preservation, the others, the working cultures, were kept at -20^{o}C.

Assignment to colour-groups. The presumptive streptomycete and non-streptomycete strains isolated from the Salar de Atacama and Yungay environmental samples were subcultured onto oatmeal agar (ISP medium 3, Shirling & Gottlieb, 1996) and peptone-yeast extract-iron agar (PYEIA, ISP medium 6, Shirling & Gottlieb, 1966) and incubated at 28^{o}C for 14 days and 4 days, respectively. The isolates were assigned to colour-groups based on aerial spore mass, substrate mycelial and diffusible pigment colours produced on the oatmeal agar plates, using National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1958) and by their ability to produce melanin pigments on the PYEIA plates (Tables 3.2 and 3.3).

Table 3.2. Actinobacteria isolated from the hyper-arid Salar de Atacama environmental sample and assigned to single- and multi-membered colour-groups.

Actinobacteria	Codes
Presumptive streptomycetes:	
Multi-membered colour-groups	C34, C38, C58, C59, C79, KNN1-1a*, KNN1-2c, KNN1-3b, KNN1-5f; KNN2-1b*, KNN2-2c, KNN2-3c, KNN2-4c, KNN2-5c, KNN2-6c, KNN2-7d, KNN2-8b, KNN2-9d, KNN2-10d, KNN2-11d, KNN2-12d, KNN2-13a, KNN2-14c, KNN2-15a, KNN2-16c, KNN2-17d, KNN2-18c, KNN3-1b*, KNN3-2b, KNN3-3c, KNN3-4b, KNN3-5c, KNN3-6c, KNN3-7c, KNN3-8c, KNN3-9c, KNN3-10e, KNN3-14d, KNN3-15d, KNN3-16d, KNN3-17d, KNN3-18d, KNN3-19b, KNN4-1b*, KNN4-2e, KNN4-3b, KNN4-4a; KNN5-1a*, KNN5-2a, KNN5-3c, KNN5-4c, KNN5-5a, KNN5-6a, KNN5-7a, KNN5-8b, KNN5-9b, KNN5-10b, KNN5-13b, KNN5-14b, KNN5-15b, KNN5-16d, KNN5-17d, KNN5-18d, KNN5-19d, KNN5-20d, KNN5-25b, KNN5-28c, KNN5-29b, KNN5-30d, KNN5-31e; KNN6-1a*, KNN6-2a, KNN6-3a, KNN6-4a, KNN6-5a, KNN6-6b, KNN6-7d, KNN6-8d, KNN6-9a, KNN6-10b, KNN6-11a; KNN8-1b*, KNN8-2b, KNN8-3a, KNN8-4b, KNN8-5d, KNN8-6b, KNN8-7a, KNN8-8a, KNN8-9a, KNN8-10c, KNN8-11e, KNN9-1a*, KNN9-2e, KNN9-3a, KNN10-1a*, KNN10-2b, KNN10-3b, KNN10-4d, KNN10-5a, KNN11-1a, KNN11-2a, KNN11-3c, KNN11-4b, KNN11-5a, KNN11-6b, KNN14-1f*, KNN14-2c, KNN14-3e, KNN16-1c*, KNN16-2c, KNN17-1c*, KNN17-2b; KNN18-1b*, KNN18-2c, KNN18-3d, KNN24-1b*, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-5c, KNN24-6a, KNN24-7c, KNN24-8e, KNN29-1a*, KNN32-1a*, KNN32-2b, KNN35-1b*, KNN35-2b; KNN36-1*c, KNN36-2c, KNN36-3c; KNN37-1e*, KNN37-2a KNN37-3a, KNN37-4a, KNN37-5a*, KNN38-1b*, KNN38-2d, KNN38-3d, KNN38-4a, KNN38-5b, KNN41-1b*, KNN41-2a, KNN48-1c*, KNN48-2a, KNN48-3e, KNN48-4e, KNN48-5e, KNN52-1c*, KNN52-2b, KNN52-3b, KNN54-1b*, KNN54-2b, KNN58-1b*, KNN58-2c, KNN60-1c*, KNN60-2d, KNN63-1b*, KNN63-

	2b, KNN63-3b, KNN63-4b, KNN63-5a, KNN63-6b, KNN63-7b, KNN63-8d, KNN63-9d, KNN63-10d, KNN63-11b, KNN63-12b, KNN63-13b, KNN63-14b, KNN63-15b, KNN63-16b, KNN63-17b, KNN63-18b, KNN63-19e, KNN64-1a*, KNN64-2a, KNN64-3a, KNN64-4b, KNN64-5b, KNN64-6b; KNN65-1f*, KNN65-2a, KNN65-3c, KNN65-4c, KNN65-5d, KNN67-1b*, KNN67-2b, KNN67-3b, KNN67-4b, KNN68-1b*, KNN68-2b, KNN68-3b, KNN68-4b, KNN69-1e*, KNN69-2a, KNN69-3a, KNN71-1a*, KNN71-2a, KNN73-1a*, KNN73-2a, KNN73-3d, KNN74-1c*, KNN74-2c, KNN75-1c*, KNN75-2b, KNN75-3b, KNN75-4b, KNN78-1e*, KNN78-2e, KNN79-1b*, KNN79-2b, KNN79-3d; KNN80-1c*, KNN80-2d, KNN81-1c*, KNN81-2b, KNN81-3d, KNN82-1a*, KNN82-2c, KNN85-1f*, KNN85-2b, KNN85-3b, KNN85-4c, KNN85-5c, KNN85-6c, KNN85-7c, KNN85-8a
Single-membered colour groups	KNN25c*, KNN26b* KNN27a*, KNN28a*, KNN30a*, KNN31d*, KNN33a*, KNN39c*, KNN43b*, KNN51b*, KNN56a*, KNN59e*, KNN62b*, KNN66*, KNN70b*, KNN72a*, KNN76b*, KNN83e*, KNN84c*, KNN87b*, KNN94e*
Presumptive non-streptomycetes:	
Multi-membered colour-groups	KNN49-1f*, KNN49-2e, KNN49-3e, KNN49-4e, KNN49-5e, KNN49-6a, KNN49-7c, KNN49-8c, KNN49-9c, KNN49-10b, KNN49-11c, KNN49-12b, KNN49-13b, KNN49-14b, KNN49-15b, KNN49-16c, KNN49-17b, KNN49-18b, KNN49-19a, KNN49-20b, KNN49-21c, KNN49-22d, KNN49-23d, KNN49-24d, KNN49-25d, KNN49-26a, KNN49-27a, KNN49-28a, KNN49-29a, KNN49-30b, KNN49-31b, KNN49-32b, KNN50-1a*, KNN50-2e, KNN50-3c, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-16d, KNN50-17d KNN50-8d, KNN53-1a*, KNN53-2a, KNN53-3a, KNN54-1a*, KNN54-2b, KNN57-1b*, KNN57-2b, KNN61-1e*, KNN61-2a, KNN61-3e, KNN61-4e, KNN61-5e, KNN61-6b
Single-membered colour-groups	KNN34a*, KNN77b*

Codes exemplified by strain KNN2-1b, this organism was isolated by Kanungnid Busarakam (KNN), assigned to colour-group 2 as the first member of group and was isolated on a humic acid-vitamin agar isolation plate. The C-coded isolates represent a colour-group delineated by Okoro *et al*. (2009).

Codes for selective isolation media : (a), Gause No.1 agar; (b), HVA, humic acid-vitamin agar; (c), Oligotrophic agar; (d), minimal medium agar; (e), SM1 agar; (h), SCAV agar

*Representatives of colour-groups used to detect isomers of diaminopimelic acid in whole-organism hydrolysates.

The C-coded strains are known to contain *LL*-A_2pm in whole-organism hydrolysates (Okoro *et al*., 2009).

Table 3.3. Actinobacteria isolated from the extreme hyper-arid Yungay environmental sample and assigned to single- and multi-membered colour-groups.

Actinobacteria	Codes
Presumptive streptomycetes:	
Multi-membered colour-groups	KNN12-1a*, KNN12-2a, KNN15-1a*, KNN15-2a, KNN16-2c, KNN19-1b*, KNN19-2c, KNN23-1b*, KNN23-2a, KNN29-2a, KNN29-3a, KNN48-7d, KNN48-8d, KNN48-9d, KNN48-10d, KNN48-11b, KNN48-12d, KNN48-13b, KNN48-14a, KNN48-15a, KNN48-16c, KNN48-17c, KNN48-18c, KNN48-19c, KNN48-20c, KNN77-1a*, KNN77-2a, KNN77-3a, KNN77-4a, KNN77-5a, KNN77-6a, KNN86-1b*, KNN86-2b, KNN86-3b, KNN86-4b, KNN86-5c, KNN86-6a, KNN88-1a*, KNN88-2b, KNN88-3b, KNN88-4a, KNN91-1a*, KNN91-2b, KNN91-3b, KNN91-4a, KNN91-5a, KNN91-6a, KNN92-1b*, KNN92-2b, KNN92-3b, KNN92-4a, KNN92-5a, KNN92-6a, KNN92-7a, KNN93-1a*, KNN93-2a, KNN93-4a, KNN 93-5a, KNN95-1f*, KNN95-2f, KNN95-3f, KNN95-4f, KNN95-5f, KNN95-6f, KNN95-7f
Single-membered colour-groups	KNN13a*; KNN20c*, KNN21a*; KNN22a*; KNN40a*; KNN42a*, KNN51b*; KNN89b*, KNN90b*; KNN96b*; KNN97b*; KNN98b*
Presumptive non-streptomycetes:	
Multi-membered colour-groups	
Geodermatophilaceae	KNN44-1b*, KNN44-2a, KNN44-3a, KNN44-4c, KNN45-1b, KNN45-2a, KNN45-3b*, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b*, KNN46-4b, KNN46-5a, KNN46-6a, KNN46-7g, KNN46-8c, KNN46-9, KNN46-10, KNN46-11
Others	KNN7-1b*, KNN7-2b, KNN7-3b, KNN7-4b, KNN23-1a*, KNN23-2a, KNN55-1b*, KNN55-2b
Single-membered colour-group	KNN47*

KNN and media codes as for Table 3.2.; Additional codes (f) *Geodermatophilus obscurus* agar; (g) Luedemann's agar.

Detection of diaminopimelic acid isomers. Ninety eight strains representing each of the colour-groups (Tables 3.2 and 3.3) were examined for diaminopimelic acid (A_2pm) isomers using the procedure described by Hasegawa *et al.* (1983) and biomass scraped from strains grown on ISP2 agar plates (Shirling & Gottlieb, 1960) incubated at 28^{o}C for 14 days.

3. 3. 5. Classification of representative actinobacteria from the Salar de Atacama and Yungay environmental samples to generic rank

Phylogenetic analyses. Ninety eight isolates were taken to represent 64 multi- and 34 single-membered colour-groups (Table 3.4). Genomic DNA of the selected isolates was extracted from biomass scrapped from yeast-extract malt-extract plates (ISP2 medium, Shirling & Gottlieb, 1966) that had been incubated for 14 days at 28^{o}C. PCR amplification of the 16S rRNA purified gene products of the isolates was carried out, as described by Kim & Goodfellow (2002). The resultant almost complete 16S rRNA gene sequences (~1300 to 1450 nucleotide [nt]) were submitted to the EzTaxon server (http://eztaxon-e.ezbiocloud.net/; Kim *et al*. (2012) and aligned with corresponding 16S rRNA gene sequences of closely related type strains of the appropicate genus (Table 3.5) using CLUSTAL W version 1.8 software (Thompson *et al.,* 1994). Individual phylogenetic trees were generated from the aligned sequences using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the MEGA and PHYML software packages (Guindon & Gascuel, 2003; Tamura *et al.,* 2011; evolutionary matrices for the neighbour-joining analyses were prepared using the Jukes and Cantor (1969) model. The topology of the inferred evolutionary trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining datasets using MEGA 5 software.

Table 3.4. Representative actinobacteria taken to represent multi-membered and single-membered colour-groups examined in the comparative 16S rRNA gene sequence studies.

A. Representative isolates from the Salar de Atacama environmental sample
Multi-membered colour-groups:
C34*, C38*, C58*, C59*, C79*, KNN2-4c, KNN2-6c, KNN4-1b, KNN4-4a, KNN6-6b, KNN6-9a, KNN6-10b, KNN6-11a, KNN9-1a, KNN9-2c, KNN10-4a, KNN10-5a, KNN11-1a, KNN11-5a, KNN24-1b, KNN32-1a, KNN35-1b, KNN35-2b, KNN38-1b, KNN41-1b, KNN48-1e, KNN48-3e, KNN48-6d, KNN49-1h, KNN49-3e, KNN49-5e, KNN49-6a, KNN49-11c, KNN49-12b, KNN49-26a, KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14a, KNN50-15a, KNN50-16d, KNN50-17d, KNN50-18d, KNN53-1a, KNN53-3a, KNN54-1a, KNN57-1b, KNN57-2b, KNN61-1a, KNN64-5b, KNN82-2c,

KNN83e, KNN88-1c

Single-membered colour groups:

KNN25c, KNN26b, KNN34c, KNN56a, KNN70b, KNN87b, KNN94e

B. Representative isolates from the Yungay environmental sample

Multi-membered colour-groups:

KNN7-2b, KNN23-1b, KNN44-1b, KNN44-3b, KNN44-4b, KNN45-1a, KNN45-2b, KNN45-3b, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5b, KNN46-6a, KNN46-7a, KNN46-8a, KNN46-9c, KNN46-10g, KNN46-11f, KNN55-1b, KNN55-2b

Single-membered colour-groups:

KNN13a, KNN22a, KNN42f, KNN47b, KNN51b, KNN70b, KNN89a, KNN90a

*Strains isolated by Okoro *et al*. (2009).

***Chemotaxonomy*.** Wet biomass of 21 representative isolates was prepared by harvesting from shake flasks of yeast extract malt-extract broth (ISP medium 2, Shirling & Gottlieb, 1966) after 14 days at 28^{o}C. The resultant preparations, which represented the 12 genera detected in the 16S rRNA gene sequencing studies (Table 3.5). Standard procedures used to establish the predominant menaquinones (Minnikin *et al*., 1984) and polar lipids (Minnikin *et al*., 1984). Similarly, standard procedures were examined for the presence of diagnostic sugars using procedure described by Hasegawa *et al.* (1983), the sugar paterns of the strains were identified by comparison against on in house standard solution. (Minnikin *et al*., 1984)

Table 3.5. Representative actinobacteria included in the chemotaxonomic analyses.

Codes	Source	Genera
KNN53-1a, KNN53-2a	Salar de Atacama	*Actinomadura*
KNN49-5e, KNN49-26a, KNN50-8b, KNN50-11c	Salar de Atacama	*Amycolatopsis*
KNN47b	Yungay	*Blastococcus*
KNN7-2b	Yungay	*Cochioplanes*
KNN44-1b	Yungay	*Geodermatophilus*

KNN56a	Salar de Atacama	*Kribbella*
KNN94e	Salar de Atacama	*Lechevalieria*
KNN45-2b, KNN46-3b	Yungay	*Modestobacter*
KNN57-1b	Salar de Atacama	*Nonomuraea*
KNN55-1b	Yungay	*Pseudonocardia*
KNN10-4d	Salar de Atacama	*Saccharothrix*
C34, C38, C58, C59, C79	Salar de Atacama	*Streptomyces*

Morphology. Selected *Actinomadura, Nonomuraea, Pseudonocardia, Saccharothrix* and *Streptomyces* isolates (Table 3.6) were grown on oatmeal agar at 28^{o}C for 21 days prior to detecting spore chain arrangement and spore surface ornamentation using gold-coated dehydrated specimens under a scanning electron microscope (Cambridge Stereoscan 240 instrument), as described by O'Donnell *et al.* (1993).

Table 3.6. *Actinomadura, Nonomuraea, Pseudonocardia, Saccharothrix* and *Streptomyces* strains examined for spore chain arrangement and spore surface ornamentation using scanning electron microscopy.

Genera	Codes
Actinomadura	KNN34c, KNN53-1a
Nonomuraea	KNN57-1b, KNN57-2b
Pseudonocardia	KNN55-1b
Saccharothrix	KNN10-4d, KNN54-1a
Streptomyces	C34, C38, C58, C59, C79, KNN2-6c, KNN13a, KNN17-2b, KNN21a, KNN23-1b, KNN24-1b, KNN26b, KNN35-1b, KNN35-2b, KNN38-1b, KNN42f, KNN48-1c, KNN51b, KNN66b, KNN83e, KNN87b and KNN90a

3. 3. 6. Screening for bioactivity

Plug assays. One hundred and thirty four isolates, 106 from the Salar de Atacama and 28 from the Yungay environmental sample (Table 3.7) taken to represent multi-membered and single-membered colour-groups were screened for antimicrobial activity against a panel of wild type microorganisms using a standard agar plug assay (Fiedler, 2004), as detailed on page 61 in the Materials and Methods section.

Table 3.7. Representative strains isolated from the Salar de Atacama and Yungay environmental samples examined against the panel of wild type microorganisms in plug assays.

Salar de Atacama	Yungay
Multi-membered colour-groups: KNN1-2c, KNN1-5f, KNN2-2c, KNN2-5c, KNN2-6c, KNN2-10d, KNN2-11d, KNN3-1b, KNN3-2b, KNN3-11d, KNN3-17d, KNN4-1b, KNN4-3b, KNN4-4a, KNN5-24b, KNN5-25d, KNN6-2a, KNN6-5a, KNN6-6b, KNN6-10b, KNN6-11a, KNN8-3a, KNN8-5b, KNN8-8a, KNN8-9c, KNN8-10e, KNN9-1a, KNN9-2c, KNN10-3b, KNN10-4d, KNN10-5a, KNN11-2a, KNN11-4b, KNN11-5a, KNN11-6a, KNN14-3e, KNN17-1c, KNN17-2b, KNN18-3d, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-7e, KNN24-8e, KNN24-9e, KNN32-1a, KNN35-1b, KNN35-2b, KNN36-1c, KNN36-3c, KNN37-1e, KNN37-5a, KNN38-1b, KNN38-5b, KNN48-3e, KNN48-6d, KNN49-5e, KNN49-26a, KNN50-8b, KNN50-11c, KNN52-2b, KNN53-1a, KNN53-2a, KNN53-3a, KNN54-1a, KNN57-1b, KNN58-1b, KNN61-1a, KNN63-2b, KNN63-15b, KNN64-3a, KNN64-5b, KNN65-1f, KNN65-5d, KNN67-4b, KNN68-2b, KNN68-4b, KNN69-1a, KNN69-2, KNN69-3a, KNN71-2a, KNN73-2a, KNN74-2c, KNN75-4b, KNN81-2b, KNN82-1a, KNN85-1f Single-membered colour groups: KNN25c, KNN26b, KNN27a, KNN28a, KNN29a, KNN30a, KNN31d, KNN33a, KNN34c, KNN39c, KNN43b, KNN56a, KNN66b, KNN72a, KNN76b, KNN83e, KNN87b, KNN94es	Multi-membered colour groups: KNN7-2b, KNN13a, KNN21a, KNN23-1b, KNN44-2a, KNN44-4b, KNN45-1a, KNN45-2b, KNN45-3b, KNN45-4b, KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5b, KNN46-6a, KNN46-7a, KNN46-8a, KNN55-1b, KNN86-1b, KNN88-1a, KNN91-1a Single-membered colour groups: KNN47b, KNN51b, KNN89a, KNN90a, KNN96a, KNN97a

Reporter strains. The 92 isolates which showed activity in the plug assays were examined against five *B. subtilis* reporter strains designed to detect specific modes of action on the target cells (Table 3.8), as details on pages 61 and 63 in the Materials and Methods section.

Disk diffusion assays. The mycelial extracts of the 92 representative isolates tested in the plug assays were examined against the *E. coli* and *S. cerevisiae* strains and against the 5 *B. subtilis* reporter strains. Each isolate was grown on ISP medium 2 (Shirling & Gottlieb, 1966), prepared with 0.8% agar, and incubated for 7 days at 30^{o}C. Cultures growing on the ISP medium were crushed using a sterile syringe, kept overnight at -20^{o}C, the supernatants (extracts) collected using wide bore pipette tips and transferred to 10 ml Falcon tubes. The media used for the disk diffusion assays were prepared as for the plug assays. The resultant media were poured into the corresponding Petri dishes and allowed to solidify. Twenty sterile Whatman filter paper disks (6 mm diameter) impregnated with 10 µl of extracts were placed on the agar surface of each Petri dish,

the plates incubated overnight at 30^{o}C and the extent of zones of inhibition measured.

3. 3. 7. Preliminary characterisation of bioactive compounds

Selection of strains. Six strains belonging to the genera *Amycolatopsis* (isolates KNN50-8b and KNN50-16d) and *Streptomyces* (isolates KNN26b, KNN38-1b, KNN64-5b and KNN90a) were chosen because they produced extensive inhibition zones in the plug assays and were distinct from their closest phylogenetic neighbours in the corresponding *Amycolatopsis* and *Streptomyces* 16S rRNA gene trees.

Production media and growth conditions. Each of the selected strains was shaken at 180 rpm at 28^{o}C for 14 days in 50 ml of each of the production media, namely yeast-extract malt-extract broth (ISP medium 2, Shirling & Gottlieb, 1966), medium 19 and medium 410 broths (Goodfellow & Fiedler, 2010); each formulation contained 1 gram Amberlite (Sigma-Aldrich, Gillingham, Dorset, UK) per 50 ml of production medium for secondary metabolite adsorption. The inoculated media were shaken at 180 rpm for 14 days at 28^{o}C.

Preparation of extracts. The Amberlite beads were separated from production media biomass by centrifugation at 2,000 rpm for 5 minutes then washed 4-5 times with sterile distilled water. The Amberlite bead preparations were then shaken in 50 ml of methanol overnight prior to centrifugation at 3000 rpm for 10 minutes and the Amberlite discarded. The resultant supernatants were dried under a nitrogen gas stream and the dried preparations sent to Professor Marcel Jaspars at Aberdeen University for chemical analyses.

Chemical analyses. Each preparation was dissolved in methanol to give a final concentration of 0.5mg/mL, filtered and submitted for liquid chromatographic/mass spectrometric (LCMS) analysis. High resolution mass spectral data were obtained from a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump using the following conditions: capillary voltage 45 V, capillary temperature 260°C, auxilliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV. mass range 100–2000 amu (maximum resolution 30000). An Agilent Poroshell 120, EC-C18, 2.1x100 mm, 2.7um UPLC

column was used for LC/MS with a mobile phase of 0-100% MeCN over 30 minutes at 0.5 ml/min flow rate. The results were presented as mass spectrophotometric (MS), and ultraviolet (UV) traces.

3.4. Results

3. 4. 1. Physico-chemical properties of environmental samples

The pH, moisture and organic matter contents of the Salar de Atacama and Yungay environmental samples are shown in Table 3.9. The pH values recorded for the two samples were similar, but the Salar de Atacama environmental sample contained a little more moisture and organic matter when compared with that from the Yungay sample.

Table 3.8. Physico-chemical properties of the environmental samples.

Environmental sample	pH	Moisture content (%)	Organic matter content (%)
Salar de Atacama	7.7±0.2*	0.007	0.03
Yungay region	7.5±0.2*	0.004	0.01

*Standard deviation.

3. 4. 2. Enumuration, detection of isomers of diaminopimelic acid and colour-grouping

Number of actinobacteria isolated from the Salar de Atacama and Yungay environmental samples. Small numbers of actinobacteria, but very few unwanted bacteria, were detected on the isolation media inoculated with suspensions of the Salar de Atacama and Yungay environmental samples (Table 3.10). The highest counts were recorded on the humic acid vitamin agar plates for both the Salar de Atacama and Yungay environmental samples, namely $1.3x10^4$ and 5×10^3 cfu/gram weight of environmental sample, respectively.

Assignment to colour-groups. The 420 strains isolated from the two environmental samples were assigned to 63 multi- and 35 single-membered colour-groups based on aerial spore mass, substrate mycelial and diffusible pigment colours after growth on the oatmeal agar at 28^{o}C for 14 days, (melanin pigments were only produced by members

of colour-groups KNN16, KNN29 and KNN44; see Appendix 1). Eighty three of the 98 representatives of the colour-groups, namely 92 *Streptomyces* and a single *Kribbella* strain, containied *LL*-A_2pm in whole-cell hydrolysates and hence were considered belong to the genus *Streptomyces*. The remaining isolates contained *meso*-A_2pm in whole-organism hydrolysates and hence were considered to be non-streptomycetes. The balance of isolates in the multi-membered colour-groups were assigned to the same taxa as the corresponding representative strain (see Table 3.24).

Table 3.9. Number of actinobacteria (cfu/g dry weight environmental sample) growing on selective media inoculated with suspensions of the Salar de Atacama and Yungay environmental samples and incubated at 28^{o}C for 3 weeks.

Sample	Media										
	Gause's No.1	*Geodermatophilus obscurus* agar	Glucose-yeast extract agar	Humic acid vitamin agar	Luedemann's agar	*Microlunatus* agar	Minimal medium agar	Oligotrophic agar	R 2A agar	Starch-casein agar	SM1 agar
Salar de Atacama	$1.1x10^4$	None	None	$1.3x10^4$	None	None	$1.3x10^2$	$0.3x10^2$	None	$0.1x10^2$	$0.3x10^2$
Yungay	$3x10^2$	$2.3x10^2$	$2.4x10^2$	$5x10^3$	$1x10^2$	$3x10^2$	$0.2\ x10^2$	$1.3x10^2$	$2.4x10^2$	$2.3x10^2$	$4.7x10^2$

The 315 strains isolated from the Salar de Atacama environmental sample were assigned to 49 multi- and 23 single-membered colour-groups (Table 3.2; Appendix 1); these strains were comprised of 250 streptomycetes and 65 non-streptomycetes, that is 79% and 21% of the total number of isolates, respectively. The streptomycetes were assigned to 43 multi-membered colour-groups and the latter to the remaining 6 colour-groups. Similarly, 13 out of the 23 single-membered colour-groups were composed of streptomycetes, the remaining ones were considered to be non-streptomycetes.

Over half of the streptomycetes were recovered in colour-groups KNN2, KNN5 and KNN63. The largest of these taxa, colour-group KNN5, contained 25 isolates (7.9% of the total number of isolates); these strains produced a medium gray aerial spore mass, a gray greenish yellow substrate mycelium and a pale gray greenish yellow diffusible pigment. The next largest taxon, colour-group KNN2, included 23 strains (7.3% of the total number of isolates); these strains formed a medium gray aerial spore mass, an

olivaceous black substrate mycelium and a light olivaceous gray diffusible pigment. Similarly, colour-group KNN63, encompassed 19 strains (6% of the total number of isolates); these strains formed a yellowish white aerial spore mass, a yellowish white substrate mycelium but did not produce a diffusible pigment.

The 65 non-streptomycete strains isolated from the Salar de Atacama environmental sample were assigned to 6 multi-membered (63 isolates) and 2 single-membered colour-groups. The largest multi-membered colour-group, KNN49, contained 32 strains (10.2% of the total number of isolates); these organisms produced a white aerial spore mass, a yellowish substrate mycelium but did not form a diffusible pigment. The next largest taxon, colour-group KNN 50, encompassed 18 isolates (5.7% of the total number of isolates); these organisms produced a yellowish white aerial spore mass, a yellowish substrate mycelium but no diffusible pigment.

The 105 strains isolated from the Yungay environmental sample were assigned to 20 multi-membered and 13 single-membered colour groups (Table 3.3; Appendix 1). Seventy-seven of the isolates were assigned to the genus *Streptomyces* (73% of the total), the remaining 28 to non-streptomycete taxa (27%). The largest taxon, colour–group KNN48, included 14 isolates (13.3% of total number of isolates); these strains produced a yellowish white aerial spore mass, a yellowish substrate mycelium but did not produce diffusible pigments. Colour-groups KNN92 and KNN95 each contained 7 isolates (6.7% of the total number of isolates). Members of colour-group KNN92 formed a dark pale gray aerial spore mass, a blackish purple and a light reddish brown diffusible pigment and those assigned to colour-group KNN95 produced a yellowish white aerial spore mass, a middle orange yellow substrate mycelium and a gray yellow diffusible pigment.

The isolates assigned to the family *Geodermatophilaceae* fell into 3 colour-groups, all of which encompassed isolates that formed black shiny colonies. The largest of these taxa, colour-group KNN46, contained 12 isolates (11.4% of the total number of isolates) which exhibited shiny black colonies, did not form diffusible pigments or an aerial spore mass. Similarly, colour-group KNN 45 contained 4 isolates (3.8%) which formed shiny orange-black colonies. The final colour-group, KNN44 contained 4 isolates (3.8%) which produced shiny black colonies and a light yellowish brown diffusible pigment.

Forty three of the multi-membered colour-groups were comprised of streptomycetes isolated from the Salar de Atacama environmental sample, and a further 14 streptomycetes isolated from the Yungay environmental sample. In contrast, colour-groups KNN16, KNN29 and KNN48 encompassed streptomycetes isolated from each of the environmental samples. Colour-group KNN16 contained 2 strains, a single isolate from each of the environmental samples; these organisms produced a pale yellow aerial spore mass, a yellowish white substrate mycelium, but did not form a diffusible pigment. Similarly, colour-group KNN29 contained 3 isolates, one from the Salar de Atacama environmental sample and two from the Yungay environmental sample; these isolates formed a yellowish white aerial spore mass, a yellowish white substrate mycelium, and a yellowish white diffusible pigment. The final colour-group, KNN48, contained 5 isolates from the Salar de Atacama environmental sample and 15 from the corresponding Yungay environmental sample; these organisms formed a yellowish white aerial spore mass, a yellowish white substrate mycelium and a light orange yellow diffusible pigment.

3. 4. 3. Classification of representative strains isolated from the Salar de Atacama and Yungay environmental samples based on chemotaxonomic, morphological and phylogenetic data

Phylogenetic analyses. Eighty three out of the 98 representatives of the multi-and single-membered colour-groups contained *LL*-A_2pm in whole-organism hydrolysates, formed streptomycetes-like colonies and were recovered within the evolutionary radiation encompassed by the genus *Streptomyces* based on 16S rRNA gene sequence data. The remaining 15 isolates, which contained *meso*-A_2pm in whole-organism hydrolysates, were found to belong to 10 taxa based on 16S rRNA gene sequence data, namely the genera A*ctinomadura, Amycolatopsis, Blastococcus, Couchioplanes, Geodermatophilus, Lechevalieria, Modestobacter, Nonomuraea, Pseudonocardia* and *Saccharothrix*.

The genus *Actinomadura*. Three isolates from the Salar de Atacama environmental sample were recovered within the *Actinomadura* 16S rRNA gene tree (Figure 3.1). Isolates KNN53-1a and KNN53-3a, representatives of a colour-group which containing three isolates, were found to share a 16S rRNA gene sequence similarity of 99.9%, a value shown to correspond to 2 nucleotide (nt) differences at 1332 sites (Table 1;

Appendix 4). These isolates formed a distinct branch in the *Actinomadura* 16S rRNA gene tree together with the type strains of *Actinomadura apis* and *Actinomadura rifamycini*; this taxon was supported by all of the tree-making algorithms and by a 96% bootstrap value. Strains KNN53-1a and KNN53-3a were most closely related to *A. apis* IMI 17.1^{T} sharing a 99.7% 16S rRNA gene similarity with the latter, a value that corresponded to 6 and 4 nt differences, respectively at 1351 locations. Similarly, isolate KNN34c, the representative of a single membered colour-group, formed a well delineated branch in the *Actinomadura* 16S rRNA gene sequence tree together with the type strains of *Actinomadura flavalba*, *Actinomadura rugatobispora* and *Actinomadura vinacea*; the taxonomic integrity of this subclade was supported by all of the tree-making algorithms and by a 65% bootstrap value. Isolate KNN34c was found to be most closely related to *A. rugatobispora* NBRC 14382^{T}, the two strains shared a 16S rRNA gene sequence similarity of 98.8%, a value found to be equivalent to 16 nt differences at 1336 locations (Appendix 4, Table 1). This isolate was also loosely related to *A. vinacea* ICM 3325^{T}, these strains shared a 16S rRNA gene sequence similarity of 98.5%, a value corresponding to 20 nt differences at 1338 locations.

The genus *Amycolatopsis*. Twenty six strains isolated from the Salar de Atacama environmental sample were recovered within the *Amycolatopsis methanolica* 16S rRNA subclade (Figure 3.2). Thirteen of the isolates had identical or almost identical 16S rRNA gene sequences to that of the type strain *Amycolatopsis ruanii*, as association that was supported by all of the tree-making algorithms and by a 99% bootstrap value. Similarly, 13 isolates had identical or almost identical 16S rRNA gene sequences to that of the type strain of *Amycolatopsis thermalba*, a relationship that was underpinned by all of the tree-making algorithms and by a 99% bootstrap value. In contrast, strains GY024 and GY142, which were isolated from an arid Australian environmental sample formed a distinct branch in the *A. methanolica* 16S rRNA gene subclade. These isolates shared a 16S rRNA gene similarity of 99.7%, a value shown to correspond to 4 nt differences at 1366 sites (Appendix 4, Table 2), the taxonomic integrity of this subclade was supported by all of the tree-making algorithms and by a 100% bootstrap values. Isolates GY024 and GY142 were found to be most closely related to *A. ruanii* NGM112^{T} sharing 16S rRNA gene sequence similarities with the latter of 98.9 and 98.7%, respectively, values corresponding to 15 and 14 nt differences at 1395 and 1410 locations (Table 2, Appendix 4). The results obtained for the *Amycolatopsis* strains will be considered in detail in Chapter 4.

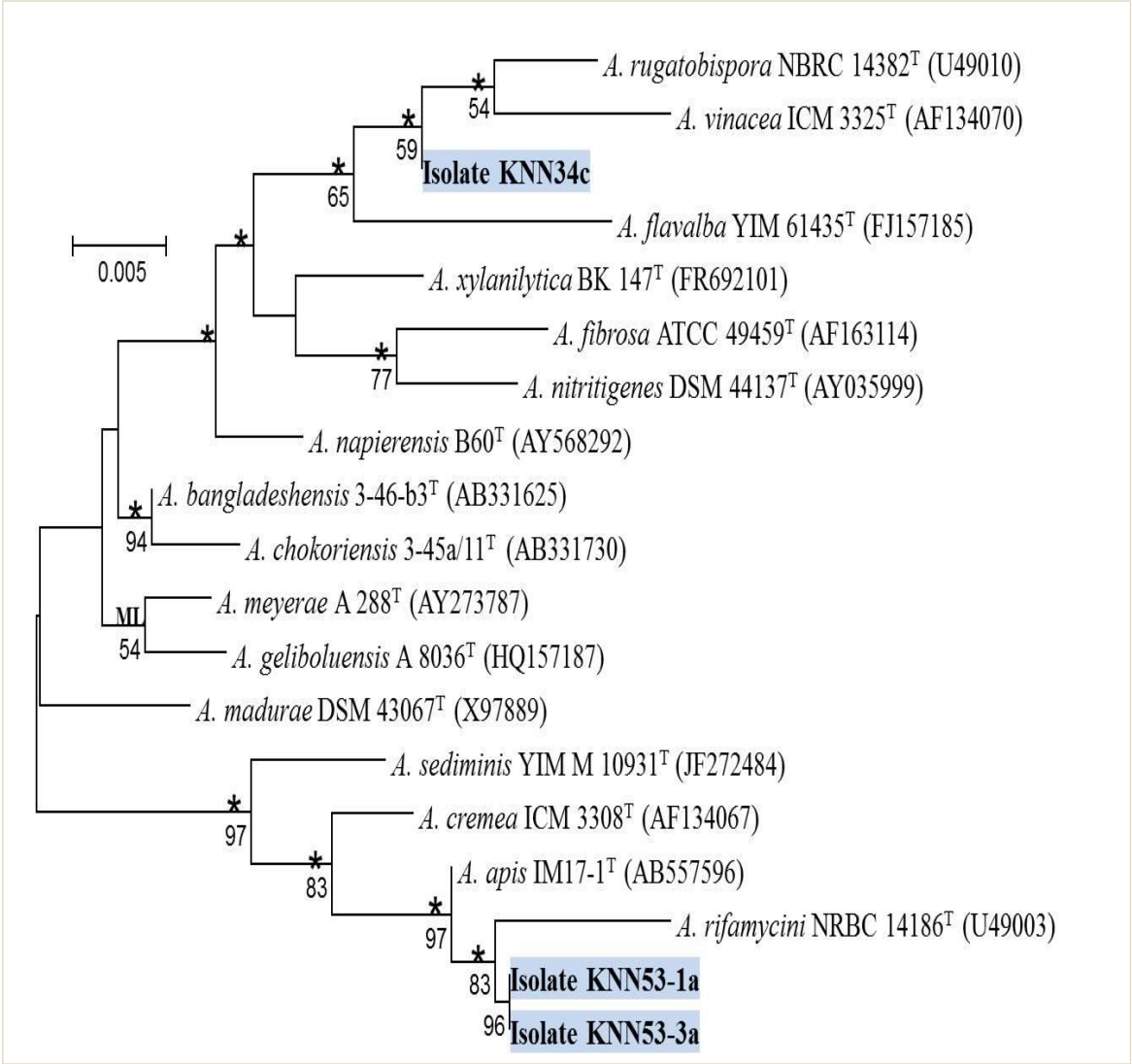


Figure 3.1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the hyper-arid Salar de Atacama environmental sample and relationships between them and type strains of the most closely related *Actinomadura* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates a branch of the tree that was supported by the maximum-likelihood tree-making method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

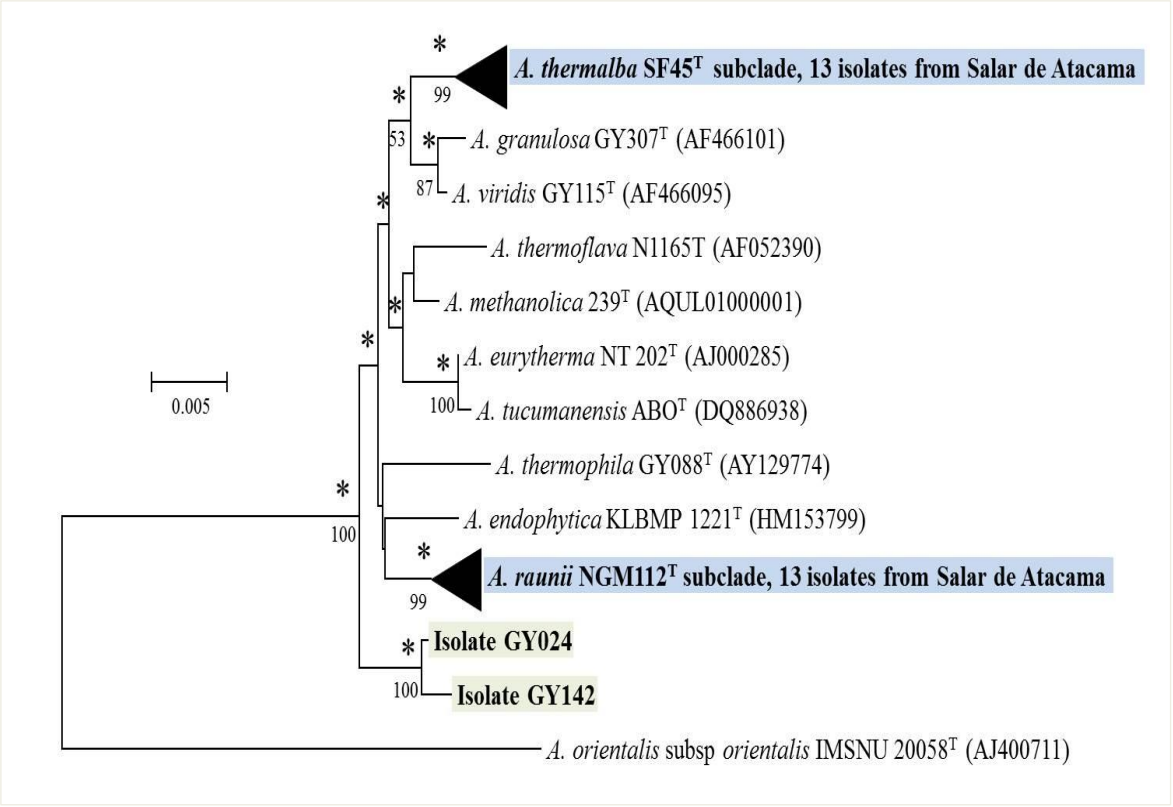


Figure 3.2. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the hyper-arid Salar de Atacama environmental sample and relationships between them and the type strains of the most closely related *Amycolatopsis* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position. Blue and green coloured subclades signified strains isolated from the hyper-arid Salar de Atacama soil and from the composite Australian soil, respectively.

The family *Geodermatophilaceae*. Sixteen isolates from the Yungay environmental sample were assigned to the *Geodermatophilaceae* 16S rRNA gene tree (Figure 3.3). The single strain assigned to the genus *Blastococcus*, isolate KNN47b, the representative of a single-membered group, formed a well delineated phyletic line with *Blastococcus saxobidens* BC448^T, this taxon was supported by all of the tree-making algorithms and by 99% bootstrap value. The two strains shared a 16S rRNA gene sequence similarity of 99.0%, a value shown to correspond to 14 nt differences at 1422 sites. Isolate KNN47b was also closely related to *Blastococcus endophytica* YIM68236^T, these organisms shared a 16S rRNA gene sequence similarity of 98.2 %, a

value equivalent to 15 nt differences at 1422 sites (Appendix 4, Table 3).

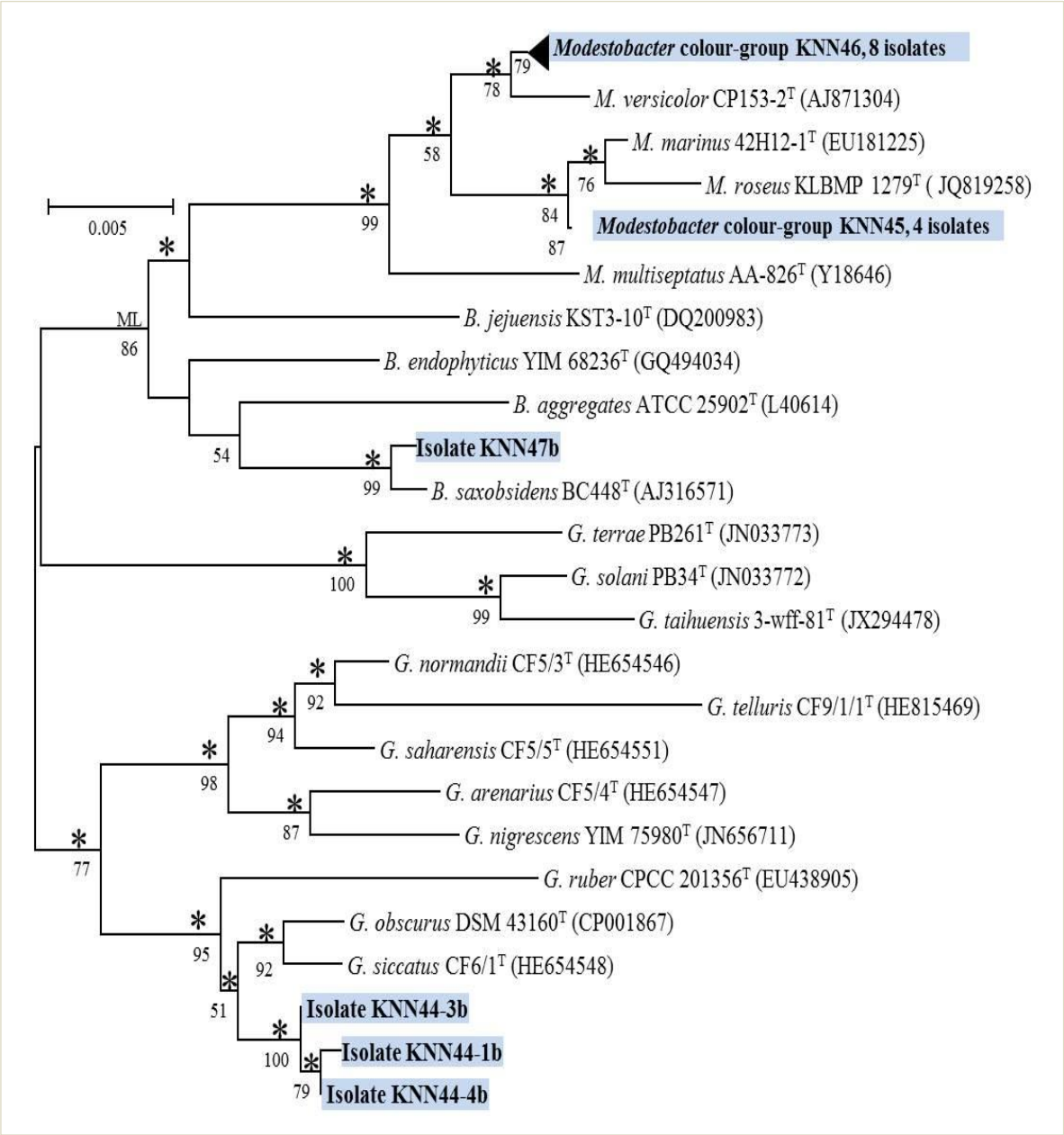


Figure 3.3. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the Yungay environmental sample and relationships between them and the type strains of the most closely related *Blastococcus*, *Geodermatophilus* and *Modestobacter* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates a branch of the tree that was supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets;

only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Three strains , isolates KNN44-1b, KNN44-3b and KNN44-4b, representatives of a colour-group that contained 4 isolates, formed a well delineated branch in the *Geodermatophilus* 16S rRNA gene tree together with the type strains of *Geodermatophilus obscurus, Geodermatophilus ruber* and *Geodermatophilus siccatus*; the taxonomic status of this subclade was supported by all of the tree-making algorithms and by a 95% bootstrap value. Isolate KNN44-3b shared a 16S rRNA gene sequence similarity to isolate KNN44-4b of 99.9%, a value equivalent to a single nt difference at 1324 sites. Isolates KNN44-1b and KNN44-3b shared a 16S rRNA gene sequence of 99.7%, a value corresponding to 4 nt differences at 1300 sites. Isolate KNN44-1b was also found to share a 16S rRNA gene sequence similarity to isolate KNN44-4 of 99.9%, a value that corresponded to a single nt difference at 1288 sites. Isolates KNN44-1b, KNN44-3b and KNN44-4b were most closely related to *Geodermatophillus obscurus* DSM 43160^T sharing 16S rRNA gene sequence similarities with the latter of 99.9, 98.6 and 99.9%, respectively, values equivalent to 13, 20 and 13 nt differences at 1300, 1428 and 1324 locations. These isolates were also closely related to *Geodermatophilus siccatus* CF6^T sharing 16S rRNA sequences similarities with the latter within the range of 98.5 to 98.9%.

The 12 *Modestobacter* isolates were assigned to 2 subclades in the *Modestobacter* 16S rRNA gene tree that were related to the type strains of *Modestobacter marinus* and *Modestobacter versicolor*, respectively (Figure 3.5). The 4 isolates that were most closely related to *M. marinus* 42H12-1^T were from colour-group KNN45, the remaining 8 isolates that were most closely related to *M. versicolor* CP153-2^T, all of these isolates belonged to colour-group KNN46. The results obtained for the *Modestobacter* isolates will be considered in detail in Chapter 5.

The genus *Couchioplanes*. Isolate KNN7-2b, a representative of a colour-group KNN7 containing 4 isolates, formed a distinct phyletic branch in the *Couchioplanes* 16S rRNA gene tree (Figure 3.4). This isolate was most closely related to the type strain of *Couchioplanes caeruleus* subsp. *azureus*, a relationship that was supported by all of the tree-making algorithms, but not by a high bootstrap value. The two strains shared a 16S

rRNA gene sequence similarity of 98.6%, a value shown to correspond to 20 nt differences at 1386 locations (Table 3.11). Isolate KNN7-2b was also closely related to *Pseudosporangium ferrugineum* 3-4-a-19^T, these strains shared a 16S rRNA gene sequence similarity of 98.5%, a value equivalated to 25 nt differences at 1381 locations (Table 3.11).

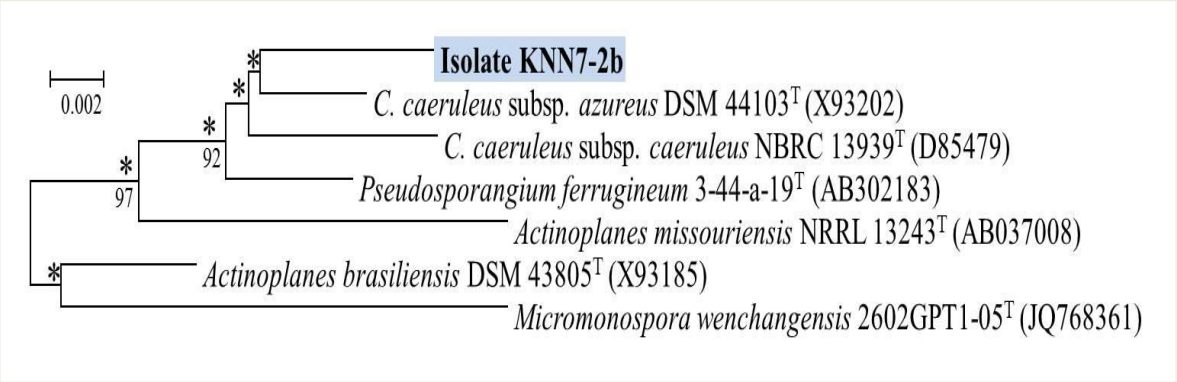


Figure 3.4. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN7-2b isolated from the Yungay environmental sample and closely related members of the genera *Couchioplanes* and *Pseudosporangium*. Asterisks indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parisimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Table 3.10. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strain KNN7-2b isolated from the extreme hyper-arid Yungay environmental sample and its nearest phylogenetic neighbours.

Isolate	**1**	2	3	4	5	6	7
1. Isolate KNN7-2b	**---**	**20/1386**	**25/1381**	**21/1385**	**41/1375**	**30/1378**	**50/1382**
2. *C. caeruleus* subsp. *azureus*	**98.6**	---	15/1445	15/1449	31/1436	28/1442	40/1446
3. *C. caeruleus* subsp. *caeruleus*	**98.2**	99.0	---	17/1451	33/1440	29/1438	47/1447
4. *Pseudosporangium ferrugineum*	**98.5**	99.0	98.8	---	31/1442	26/1442	43/1469
5. *Actinoplanes missouriensis*	**97.0**	97.8	97.7	97.9	---	34/1430	50/1441
6. *Actinoplanes braziliensis*	**97.8**	98.1	98.0	98.2	97.6	---	30/1438
7. *Micromonospora wenchangensis*	**96.4**	97.2	96.8	97.1	96.5	97.9	---

Strain codes, as given in Figure 3.4.

The genus *Kribbella*. Strain KNN56a, which was isolated from the Salar de Atacama environmental sample, represented a single-membered colour-group. It formed a well delineated branch in the *Kribbella* 16S rRNA gene tree (Figure 3.5) together with the type strain of *Kribbella antibiotica*, the taxonomic status of which was supported by all of the tree-making algorithms and by a 56% bootstrap value. The two strains shared a 16S rRNA gene sequence similarity of 99.3%, a value found to correspond to 10 nt differences at 1417 sites. Isolate KNN56a was closely related to *Kribbella albertanoniae* BC640^T, these strains shared a 16S rRNA gene sequence similarity of 99.2%, a value corresponding to 11 nt differences at 1421 sites (Table 3.12).

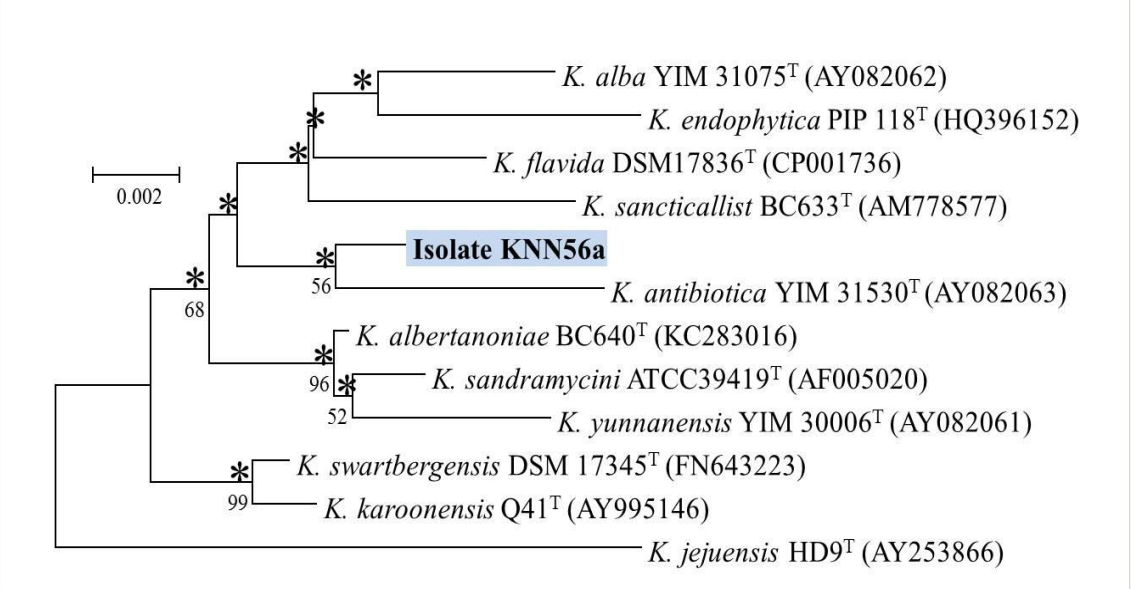


Figure 3.5. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN56a isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Kribbella* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Table 3.11. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between strain KNN56a isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Kribbella* species.

Isolate	**1**	2	3	4	5	6	7	8	9	10	11	12
1. KNN56 a	**---**	**10/**	**11/**	**11/**	**12/**	**13/**	**14/**	**15/**	**17/**	**17/**	**17/**	**29/**

		1417	1421	1344	1379	1408	1419	1374	1420	1419	1382	1419
2. *K. antibiotica*	**99.3**	---	13/ 1417	19/ 1342	20/ 1377	17/ 1406	21/ 1417	20/ 1372	19/ 1416	23/ 1417	16/ 1380	35/ 1417
3. *K. albertanoniae*	**99.2**	99.1	---	11/ 1344	12/ 1379	4/ 1408	12/ 1419	17/ 1374	6/ 1420	17/ 1419	19/ 1382	27/ 1419
4. *K. swartbergensis*	**99.2**	98.6	99.2	---	3/ 1304	13/ 1333	13/ 1344	18/ 1299	17/ 1343	16/ 1344	22/ 1307	26 /1344
5. *K. karoonensis*	**99.1**	98.6	99.1	99.8	---	12/ 1376	14/ 1379	19/ 1372	18/1 378	17/ 1379	23/ 1379	27/ 1379
6. *K. sandramycini*	**99.1**	98.8	99.7	99.0	99.1	---	14/ 1408	19/ 1371	8/ 1407	19/ 1408	21/ 1379	29/ 1408
7. *K. flavida*	**99.0**	98.5	99.2	99.0	99.0	99.0	---	13/ 1374	18/ 1418	13/ 1419	18/ 1382	30/ 1419
8. *K. sancticallist*	**98.9**	98.5	98.8	98.6	98.6	98.6	99.1	---	23/ 1373	18/ 1374	19/ 1374	35/ 1374
9. *K. yunnanensis*	**98.8**	98.7	99.6	98.7	98.7	99.4	98.7	98.3	---	23/ 1418	25/ 1381	33/ 1418
10. *K. alba*	**98.8**	98.4	98.8	98.8	98.8	98.7	99.1	98.7	98.4	---	15/ 1382	32/ 1419
11. *K. endophytica*	**98.8**	98.8	98.6	98.3	98.3	98.5	98.7	98.6	98.2	98.9	---	39/ 1382
12. *K. jejuensis*	**98.0**	97.5	98.1	98.1	98.0	97.9	97.9	97.5	97.7	97.7	97.2	---

Strain codes, as given in Figure 3.5.

The genus *Lechevalieria.* Strain KNN94e, the representative of a single-membered colour-group, was isolated from the Salar de Atacama environmental sample. This isolate formed a well delineated branch in the *Lechevalieria* 16S rRNA gene tree together with the type strains of *Lechevalieria atacamensis*, *Lechervalieria deserti* and *Lechevalieria roselyniae*; the taxonomic status of this subclade was supported by all of the tree-making algorithms and by a 100% bootstrap value (Figure 3.6). The isolate shared a 100% 16S rRNA gene sequence with *L. atacamensis* C61^T. It was also closely related to *L. roselyniae* C81^T; these strains shared a 16S rRNA gene sequence similarity of 99.8%, a value that corresponed to 2 nt differences at 1375 locations (Table 3.13).

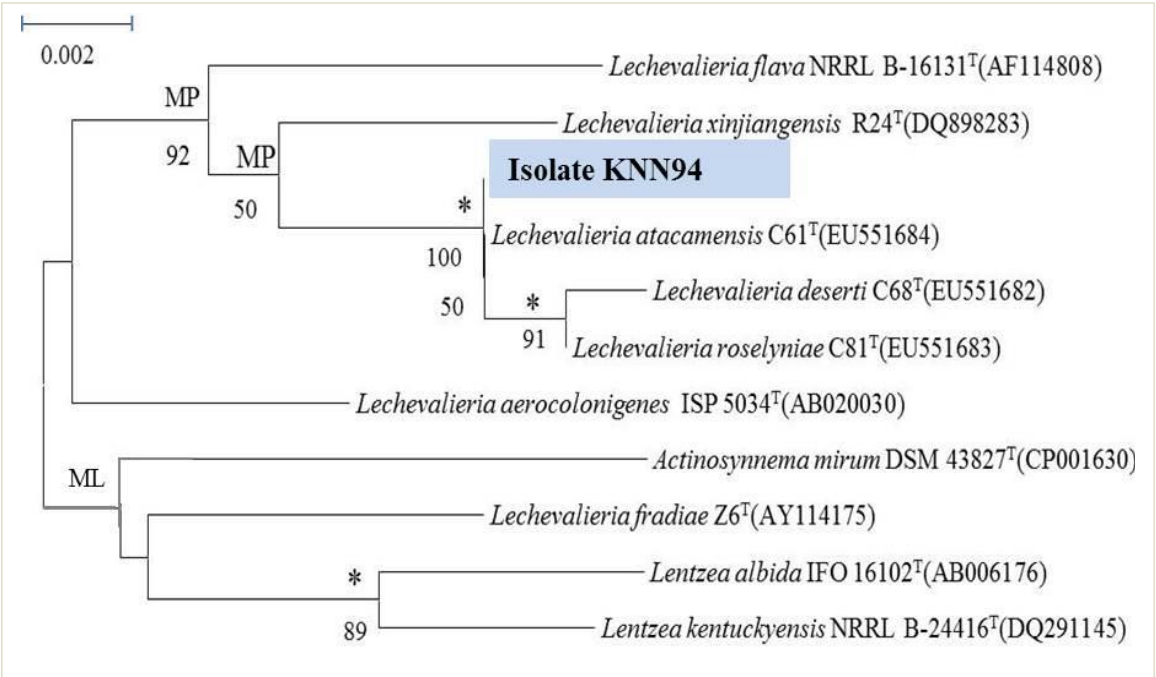


Figure 3.6. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN94e isolated from the Salar de Atacama environmental sample and closely related type strains of *Lechevalieria* and *Lentzea* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms. ML and MP indicates branches of the tree that were supported by the maximum-likelihood and maximum-parsimony methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Table 3.12. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate KNN94e from the hyper-arid Salar de Atacama environmental sample and the type strains of *Lechevalieria* and closely related *Lentzea* species.

Isolate	**1**	2	3	4	5	6	7	8	9	10
1. Isolate KNN94e	---	**20/ 1375**	**14/ 1375**	**0/ 1375**	**4/ 1375**	**2/ 1375**	**21/ 1375**	**19/ 1375**	**16/ 1373**	**19/ 1373**
2. *L. flava*	**98.5**	---	17/ 1377	20/ 1377	25/ 1377	23/ 1377	20/ 1377	24/ 1377	32/ 1375	25/ 1375
3. *L. xinjiangensis*	**99**	98.8	---	14/ 1377	19/ 1377	17/ 1377	17/ 1377	29/ 1377	30/ 1375	25/ 1375
4. *L. atacamensis*	**100**	98.5	99	---	5/ 1377	3/ 1377	21/ 1377	19/ 1377	16/ 1375	19/ 1375
5. *L. deserti*	**99.7**	98.2	98.6	99.6	---	2/ 1377	26/ 1377	24/ 1377	21/ 1375	24/ 1375
6. *L. roselyniae*	**99.8**	98.3	98.8	99.8	99.8	---	24/ 1377	22/ 1377	19/ 1375	22/ 1375
7. *L. aerocolonigenes*	**98.5**	98.5	98.8	98.5	98.1	98.3	---	17/ 1377	25/ 1375	17/ 1375
8. *L. fradiae*	**98.6**	98.3	97.9	98.6	98.3	98.4	98.8	---	21/ 1375	19/ 1375
9. *Lentzea albida*	**98.8**	97.7	97.8	98.8	98.5	98.6	98.2	98.5	---	12/ 1373
10. *Lentzea kentuckyensis*	**98.6**	98.2	98.2	98.6	98.2	98.4	98.8	98.6	99.1	---

Strain codes, as given in Figure 3.6.

The genus *Nonomuraea*. Strain KNN57-2b, a representative of a colour-group KNN57 which contained 2 isolates, was isolated from the Salar de Atacama environmental sample and shown to belong to the *Nonomuraea* 16S rRNA gene tree (Figure 3.7). It formed a distinct branch in the tree and was most closely related to *Nonomuraea candida* HMC10^T; these strains shared a 16S rRNA gene sequence similarity of 99.8%, a value shown to correspond to 7 nt differences at 1368 locations. The isolate was also closely related to *Nonomuraea jabiensis* A4036^T, these strains shared a 16S rRNA gene sequence similarity of 99.0%, a value shown to correspond to 14 nt differences at 1403

locations (Table 3.14).

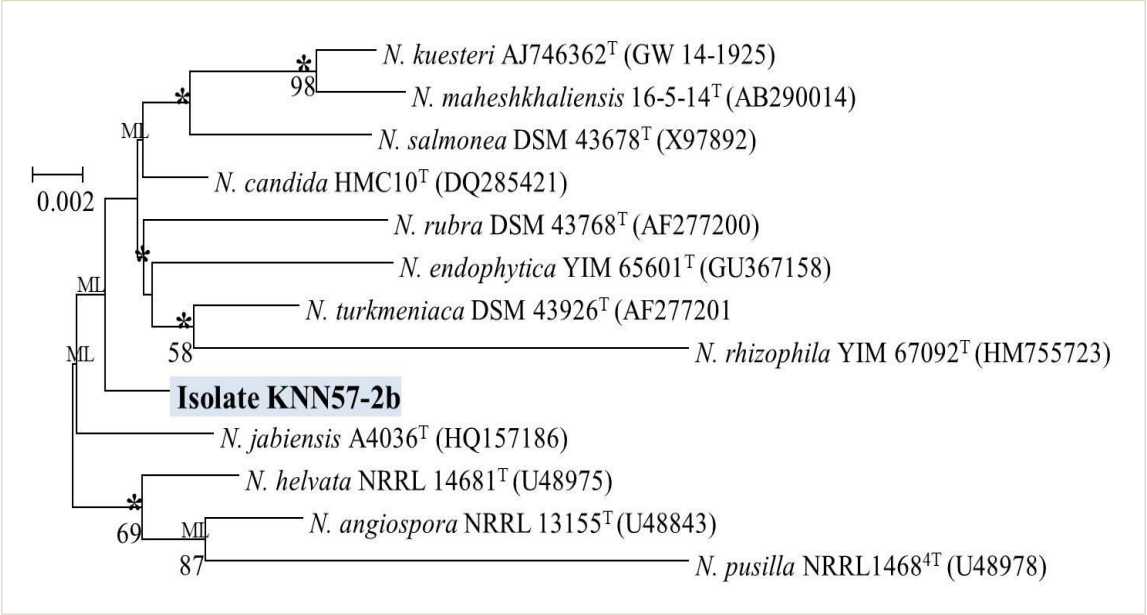


Figure 3.7. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN57-2b isolated from the Salar de Atacama environmental sample and the type strains of closely related *Nonomuraea* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide nucleotide position.

Table 3.13. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strain KNN57-2b from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Nonomuraea* species.

Isolate	**1**	2	3	4	5	6	7	8	9	10	11	12	13
1. KNN57-2b	---	**7/ 1368**	**14/ 1403**	**19/ 1405**	**17/ 1399**	**17/ 1405**	**19/ 1399**	**19/ 1405**	**19/ 1404**	**22/ 1404**	**23/ 1397**	**38/ 1405**	**41/ 1401**
2. *N. candida*	**99.8**	---	17/ 1370	20/ 1370	24/ 1364	14/ 1370	21/ 1366	18/ 1370	11/ 1369	23/ 1370	18/ 1363	35/ 1370	46/ 1368
3. *N. jabiensis*	**99.0**	98.8	---	27/ 1406	14/ 1399	28/ 1405	25/ 1401	22/ 1405	23/ 1404	23/ 1404	25/ 1398	36/ 1406	41/ 1403
4. *N. endophytica*	**98.7**	98.5	98.1	---	28/ 1401	23/ 1407	29/ 1402	31/ 1407	22/ 1408	32/ 1406	30/ 1400	43/ 1408	52/ 1405
5. *N. agiospora*	**98.8**	98.2	99.0	98.0	---	31/ 1401	16/ 1398	29/ 1401	27/ 1401	26/ 1400	31/ 1393	46/ 1401	32/ 1399
6. *N. salmonea*	**98.8**	99.0	98.0	98.4	97.8	---	31/ 1401	22/ 1407	23/ 1406	23/ 1406	28/ 1398	46/ 1407	55/ 1403
7. *N. helvata*	**98.6**	98.5	99.2	97.9	98.9	97.8	---	26/ 1401	27/ 1401	27/ 1400	27/ 1396	43/ 1402	40/ 1400
8. *N. kuesteri*	**98.7**	98.7	98.4	97.8	97.9	98.4	98.1	---	21/ 1406	8/ 1407	29/ 1398	41/ 1408	53/ 1403

9. *N. turkmeniaca*	**98.7**	99.2	98.4	98.4	98.1	98.4	98.1	98.5	---	26 /1405	24/ 1399	35/ 1406	50/ 1405
10. *N. maheshkhaliensis*	**98.4**	98.3	98.4	97.7	98.1	98.4	98.1	99.4	98.2	---	34/ 1397	44/ 1407	48/ 1402
11. *N. rubra*	**98.4**	98.7	98.2	97.9	97.8	98	98.1	97.9	98.3	97.6	---	45/ 1399	56/ 1397
12. *N. rhizophila*	**97.3**	97.5	97.4	97.0	96.7	96.7	96.9	97.1	97.5	96.9	96.8	---	71/ 1403

Strain codes, as given in Figure 3.7.

The genus *Pseudonocardia.* Two isolates, strains KNN55-1b and KNN55-2b, from the Yungay environmental sample were recovered in the *Pseudonocardia* 16S rRNA gene tree (Figure 3.8). The two strains, which formed colour-group KNN55, were found to share a 16S rRNA gene sequence similarity of 96.9%, a value shown to correspond to 41 nucleotide (nt) differences at 1318 sites (Table 3.15). The strains formed distinct branches in the *Pseudonocardia* 16S rRNA gene tree together with the type strains of *Pseudonocardia petroleophila* and *Pseudonocardia seraimata*; a taxon that was supported by all of the tree-making algorithms, but not by high bootstrap values. Strain KNN55-1b was most closely related to *P. petroleophila* ATCC 15777^T sharing a 99.0% 16S rRNA gene sequence similarity with the latter, a value corresponding to 14 nt differences at 1363. It was also loosely associated with *P. serranimata* YIM 63233^T; these strains shared a 96.3% 16S rRNA gene sequence similarity, a value equivalent to 14 nt differences at 1310 sites. In turn, isolate KNN55-2b was most closely related to *P. petroleophila* ATCC 15777^T; these strains shared a 99.0% 16S rRNA gene sequence similarity, a value corresponding to 14 nt differences at 1370 locations. It was also closely related to *P. serranimata* YIM 63233^T; these organisms shared a 96.1% 16S rRNA gene sequence similarity, a value corresponding to 51 nt differences 1317 sites.

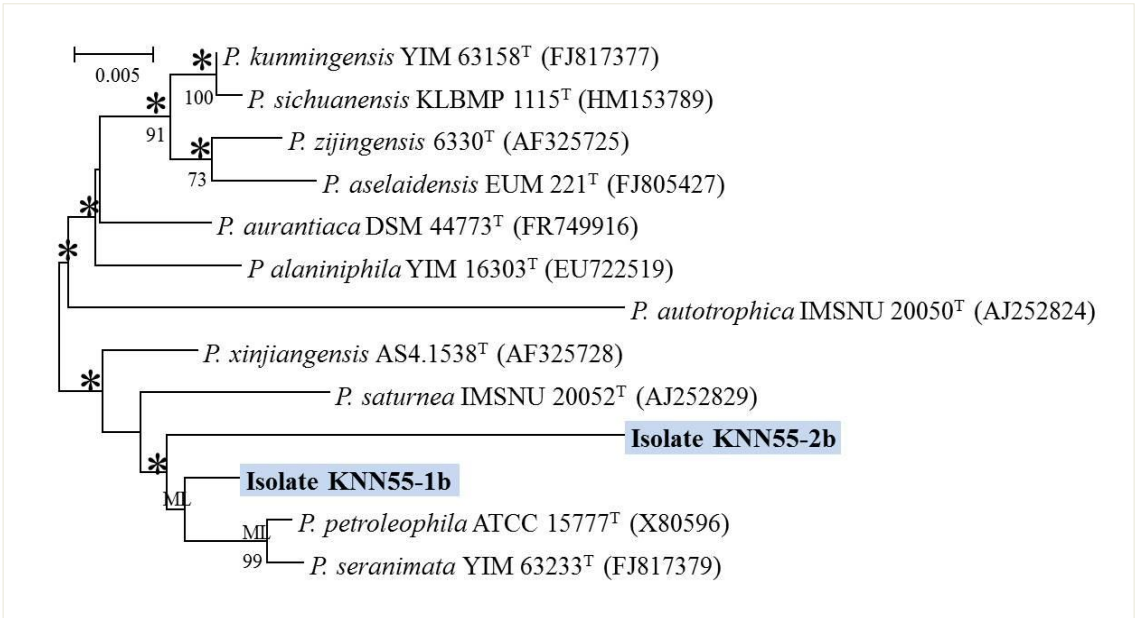


Figure 3.8. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains KNN55-1b and KNN55-2b isolated from the Yungay environmental sample and relationships between them and the type strains of the most closely related *Pseudonocardia* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were also supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Table 3.14. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the extreme hyper-arid Yungay environmental sample and between them and the type strains of closely related *Pseudonocardia* species.

Isolate	**1**	**2**	3	4	5	6	7	8	9	10	11	12	13
1. KNN55-2b	---	**41/ 1318**	**48/ 1310**	**51/ 1317**	**50/ 1303**	**62/ 1314**	**54/ 1318**	**64/ 1314**	**65/ 1308**	**67/ 1316**	**60/ 1315**	**69/ 1316**	**84/ 1316**
2. KNN55-1 b	**96.9**	---	**14/ 1363**	**14/ 1370**	**19/ 1325**	**24/ 1365**	**25/ 1371**	**26/ 1367**	**28/13 61**	**29/ 1369**	**31/ 1368**	**33/ 1369**	**70/ 1369**
3. *P. petroleophila*	**96.3**	**99.0**	---	5/ 1364	26/ 1317	22/ 1357	34/ 1365	24/ 1360	28/ 1354	27/ 1362	29/ 1361	30/ 1362	76/ 1362
4. *P. seranimata*	**96.1**	**99.0**	99.6	---	29/ 1324	25/ 1364	31/ 1372	27/ 1367	27/ 1361	30/ 1369	30/ 1368	33/ 1369	75/ 1369
5. *P. xinjiangensis*	**96.2**	**98.6**	98.0	97.8	---	25/ 1322	26/ 1325	27/ 1322	18/ 1316	26/ 1323	27/ 1323	34/ 1323	60/ 1323
6. *P. kunmingensis*	**95.3**	**98.2**	98.4	98.2	98.1	---	33/ 1365	2/ 1364	22/ 1358	17/ 1365	23/ 1365	13/ 1365	67/ 1365
7. *P. saturnea*	**95.9**	**98.2**	97.5	97.7	98.0	97.6	---	35/ 1368	36/ 1362	42/13 70	43/13 69	40/ 1370	63/ 1370
8. *P. sichuanensis*	**95.1**	**98.1**	98.2	98.0	98.0	99.9	97.4	---	24/ 1361	19/ 1368	25/ 1368	15/ 1368	69/ 1368
9. *P. aurantiaca*	**95.0**	**97.9**	97.9	98.0	98.6	98.4	97.4	98.2	---	21/ 1362	21/ 1362	31/ 1362	61/ 1362
10. P zijingensis	**94.9**	**97.9**	98.0	97.8	98.0	98.8	96.9	98.6	98.5	---	28/ 1369	16/ 1370	63/ 1370
11. *P. alaniniphila*	**95.4**	**97.7**	97.9	97.8	98.0	98.3	96.9	98.2	98.5	98.0	---	34/13 69	60/ 1369
12. *P. aselaidensis*	**94.8**	**97.6**	97.8	97.6	97.4	99.1	97.1	98.9	97.7	98.8	97.5	---	72/ 1370
13. *P. autotrophica*	**93.6**	**94.9**	94.4	94.5	95.5	95.1	95.4	95.0	95.5	95.4	95.6	94.7	---

Strain codes, as given in Figure 3.8.

The genus *Saccharothrix*. Two isolates, KNN10-4d and KNN54-1a, which represented colour-groups containing 5 and 2 strains, respectively, were isolated from the Salar de Atacama environmental sample and recovered in the *Saccharothrix* 16S rRNA gene sequence tree (Figure 3.9). The isolates shared a 16S rRNA gene similarity of 99.1%, a value shown to correspond to 12 nt differences at 1346 sites (Table 3.16). Isolate KNN10-4d formed a well delineated branch in the *Saccharothrix* 16S rRNA gene tree together with the type strains of *Saccharothrix espanaensis*, *Saccharothrix texaensis* and *Saccharothrix variisporea*; the taxonomic integrity of this subclade was supported

by all of the tree-making algorithm but not by a high bootstrap value. The isolate was found to be most closely related to *S. texasensis* NRRL B-16107^T sharing a 98.9% 16S rRNA gene sequence similarity with the latter, a value corresponding to 15 nt differences at 1346 locations. Isolate KNN10-4d was also closely related to *S. espanaensis* DSM 44229^T; these strains shared a 98.8% 16S rRNA gene sequence similarity, a value equivalent to 15 nt differences at 1346 locations. Similarly, isolate KNN54-1a formed a well delineated branch in the *Saccharothrix* 16S rRNA gene tree together with the type strains of *Saccharothrix hoggarensis, Saccharothrix longispora* and *Saccharothrix saharensis*, the taxonomic status of this subclade was supported by all of the tree-making algorithms and by a low bootstrap value of 51%. The isolate was most closely related to *S. saharensis* SA152^T ; these organisms shared a 99.6% 16S rRNA gene sequence similarity, a value corresponding to 12 nt differences at 1346 locations. Isolate KNN54-1a was also closely related to *Saccharothrix xinjiangensis* NBRC 101911^T; these strains shared a 99.3% 16S rRNA gene sequence similarity, a value equivalent to 6 nt differences at 1346 locations.

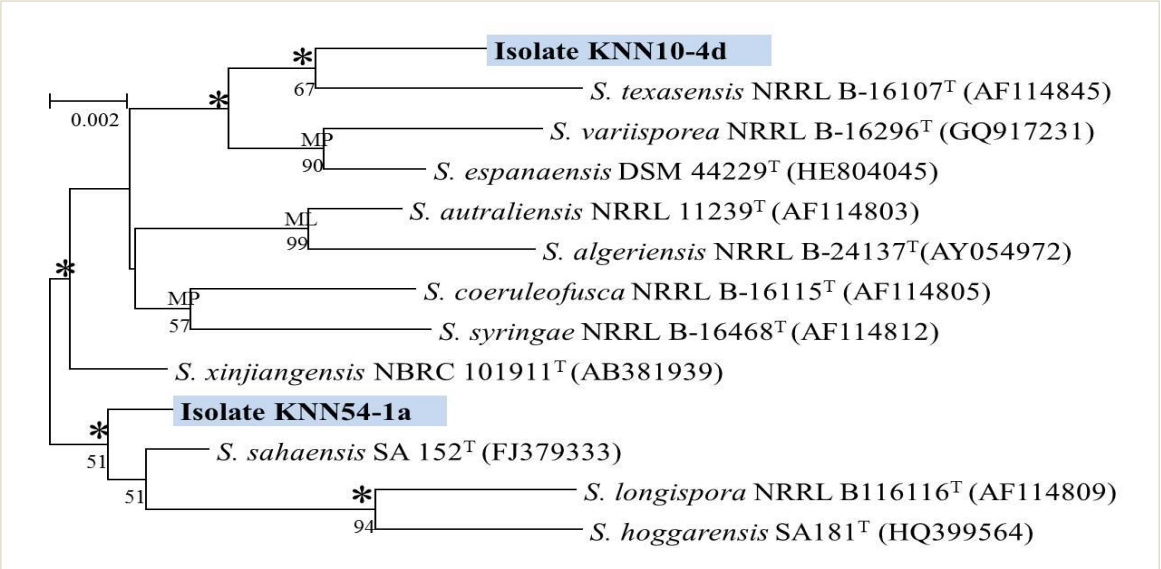


Figure 3.9. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains KNN10-4d and KNN54-1a isolated from the Salar de Atacama environmental sample and relationships between them and the type strains of closely related *Saccharothix* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML and MP indicate branches of the tree that were supported by the maximum-likelihood and maximum-parsimony methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of

1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Table 3.15. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the hyper-arid Salar de Atacama environmental sample and between them and the type strains of closely related *Saccharothix* species.

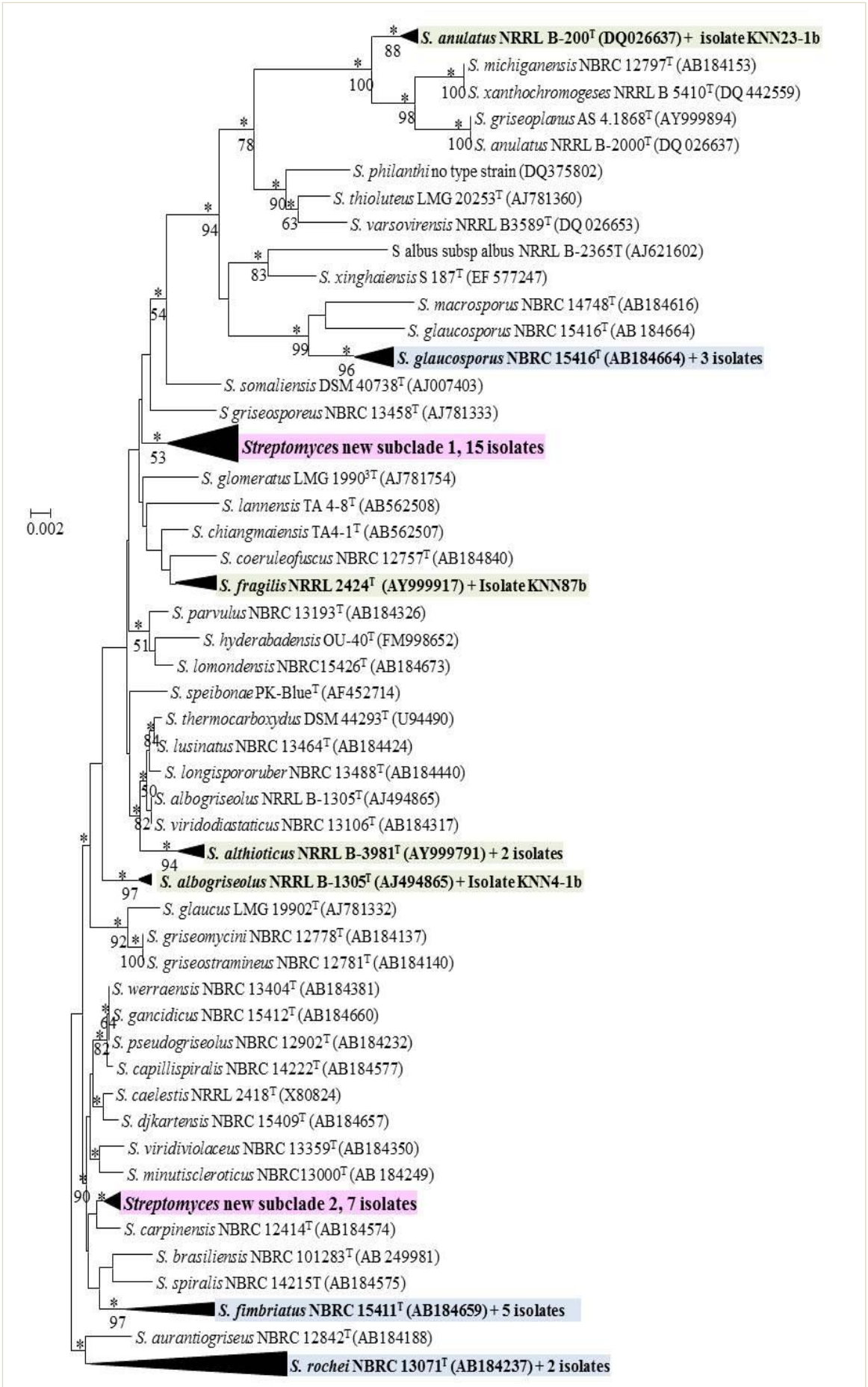
Isolate	**1**	**2**	3	4	5	6	7	8	9	10	11	12	13
1. KNN54-1a	---	**12/ 1346**	**6/ 1346**	**10/ 1346**	**16/ 1346**	**15/ 1346**	**20/ 1346**	**22/ 1346**	**18/ 1346**	**17/ 1346**	**19/ 1346**	**19/ 1346**	**21/ 1346**
2. KNN10-4d	**99.1**	---	**17/ 1346**	**21/ 1346**	**24/ 1346**	**18/ 1346**	**15/ 1346**	**20/ 1346**	**15/ 1346**	**29/ 1346**	**30/ 1346**	**23/ 1346**	**29/ 1346**
3. *S. saharensis*	**99.6**	**98.7**	---	6/ 1346	19/ 1346	20/ 1346	15/ 1346	25/ 1346	22/ 1346	19/ 1346	15/ 1346	19/ 1346	23/ 1346
4. *S. xinjiangensis*	99.3	98.4	99.6	---	15/ 1346	16/ 1346	21/ 1346	21/ 1346	18/ 1346	23/ 1346	21/ 1346	13/ 1346	19/ 1346
5. *S. autraliensis*	**98.8**	**98.2**	98.6	98.9	---	17/ 1346	30/ 1346	21/ 1346	15/ 1346	28/ 1346	32/ 1346	19/ 1346	11/ 1346
6. *S. coeruleofusca*	**98.9**	**98.7**	98.5	98.8	98.7	---	27/ 1346	25/ 1346	21/ 1346	32/ 1346	33/ 1346	16/ 1346	24/ 1346
7. *S. texasensis*	**98.5**	**98.9**	98.9	98.4	97.8	98.0	---	24/ 1346	18/ 1346	33/ 1346	22/ 1346	28/ 1346	32/ 1346
8. *S. variisporea*	**98.4**	**98.5**	98.1	98.4	98.4	98.1	98.2	---	11/ 1346	33/ 1346	38/ 1346	22/ 1346	26/ 1346
9. *S. espanaensis*	**98.7**	**98.9**	98.4	98.7	98.9	98.4	98.7	99.2	---	31/ 1346	35/ 1346	21/ 1346	18/ 1346
10. *S. longispora*	**98.7**	**97.9**	98.6	98.3	97.9	97.6	97.6	97.6	97.7	---	14/ 1347	33/ 1346	32/ 1346
11. *S. hoggarensis*	**98.6**	**97.8**	98.9	98.4	97.6	97.6	98.4	97.2	97.4	99.0	---	31/ 1346	36/ 1346
12. *S. xinjiangensis*	**98.6**	**98.3**	98.6	99.0	98.6	98.8	97.9	98.4	98.4	97.6	97.7	---	25/ 1346
13. *S. syringae*	**98.4**	**97.9**	98.3	98.6	99.2	98.2	97.6	98.1	98.7	97.6	97.3	98.1	---

Codes, as given in Figure 3.9

The genus *Streptomyces.* The 37 representative *Streptomyces* strains isolated from the Salar de Atacama and Yungay environmental samples (Table 3.4) were recovered within the *Streptomyces* 16S rRNA gene tree (Figure 3.10). Five of the 16S rRNA subclades contained one or more type strains of *Streptomyces* species, the remaining two, labelled *Streptomyces* new subclades 1 and 2, encompassed strains isolated from the Salar de Atacama and the Yungay environmental samples, respectively (Table 3.4). The taxonomic status of the 15 isolates in *Streptomyces* new subclade 1 was supported by all of the tree-making methods through the bootstrap value was low at 53%. Seven isolates were assigned to *Streptomyces* new subclade 2, the taxonomic status of which was underpinned by all of the tree-making algorithms, but not by a high bootstrap value. The detailed taxonomic relationships between the isolates assigned to these taxa and between them and their closest phylogenetic neighbours will be considered in detail in Chapter 6, as will relationships between the 5 isolates assigned to the *Streptomyces fimbriatus* 16S rRNA gene clade. Members of these taxa are of interest as they contain

strains known or likely to produce novel natural products. The 9 remaining *Streptomyces* were assigned to the subclades six of *Streptomyces* subclade, namely *Streptomyces albogriseolus* (1 isolate), *Streptomyces althioticus* (2 isolates), *Streptomyces anulatus* (1 isolate), *Streptomyces fragilis* (1 isolate), *Streptomyces glaucosporus* (3 isolates) and *Streptomyces rochei* (2 isolates) all which were named after the earliest described type strains, as shown in Figure 3.10.

Figure 3.10. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the assignment of *Streptomyces* strains isolated from the hyper-arid Salar de Atacama and the extreme hyper-arid Yungay environmental samples to subclades containing one or more the type strains of closely related *Streptomyces* species. The number of isolates in each subclade is shown; subclades containing one or more *Streptomyces* type strains were named after the earliest validly named species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML and MP indicate branches of the tree that were supported by the maximum-likelihood and maximum-parsimony methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.


S. anulatus NRRL B-200T (DQ026637) + isolate KNN23-1b
S. michiganensis NBRC 12797T (AB184153)
S. xanthochromogeses NRRL B 5410T (DQ 442559)
S. griseoplanus AS 4.1868T (AY999894)
S. anulatus NRRL B-2000T (DQ 026637)
S. philanthi no type strain (DQ375802)
S. thioluteus LMG 20253T (AJ781360)
S. varsovirensis NRRL B3589T (DQ 026653)
S albus subsp albus NRRL B-2365T (AJ621602)
S. xinghaiensis S 187T (EF 577247)
S. macrosporus NBRC 14748T (AB184616)
S. glaucosporus NBRC 15416T (AB 184664)
S. glaucosporus NBRC 15416T (AB184664) + 3 isolates
S. somaliensis DSM 40738T (AJ007403)
S griseosporeus NBRC 13458T (AJ781333)
Streptomyces new subclade 1, 15 isolates
S. glomeratus LMG 1990 3T (AJ781754)
S. lannensis TA 4-8T (AB562508)
S. chiangmaiensis TA4-1T (AB562507)
S. coeruleofuscus NBRC 12757T (AB184840)
S. fragilis NRRL 2424T (AY999917) + Isolate KNN87b
S. parvulus NBRC 13193T (AB184326)
S. hyderabadensis OU-40T (FM998652)
S. lomondensis NBRC15426T (AB184673)
S. speibonae PK-BlueT (AF452714)
S. thermocarboxydus DSM 44293T (U94490)
S. lusinatus NBRC 13464T (AB184424)
S. longispororuber NBRC 13488T (AB184440)
S. albogriseolus NRRL B-1305T (AJ494865)
S. viridodiastaticus NBRC 13106T (AB184317)
S. althioticus NRRL B-3981T (AY999791) + 2 isolates
S. albogriseolus NRRL B-1305T (AJ494865) + Isolate KNN4-1b
S. glaucus LMG 19902T (AJ781332)
S. griseomycini NBRC 12778T (AB184137)
S. griseostramineus NBRC 12781T (AB184140)
S. werraensis NBRC 13404T (AB184381)
S. gancidicus NBRC 15412T (AB184660)
S. pseudogriseolus NBRC 12902T (AB184232)
S. capillispiralis NBRC 14222T (AB184577)
S. caelestis NRRL 2418T (X80824)
S. djkartensis NBRC 15409T (AB184657)
S. viridiviolaceus NBRC 13359T (AB184350)
S. minutiscleroticus NBRC13000T (AB 184249)
Streptomyces new subclade 2, 7 isolates
S. carpinensis NBRC 12414T (AB184574)
S. brasiliensis NBRC 101283T (AB 249981)
S. spiralis NBRC 14215T (AB184575)
S. fimbriatus NBRC 15411T (AB184659) + 5 isolates
S. aurantiogriseus NBRC 12842T (AB184188)
S. rochei NBRC 13071T (AB184237) + 2 isolates
0.002

***Streptomyces albogriseolus* sublade.** Strain KNN4-1b, a representative of the four isolates assigned to colour-group KNN4, was isolated from the Salar de Atacama environmental sample and recovered in the *S. albogriseolus* 16S rRNA subclade (Figure 3.11). The isolate formed a well delineated phyletic line together with the type strain of *Streptomyces arovirens*; the taxonomic status of this subclade was supported by all of the tree-making algorithms and by a 96% bootstrap value. Isolate KNN4-1b was most closely related to *Streptomyces atrovirens* NRRL B-16357^T and *Streptomyces albogriseolus* NRRL B-1305^T, the isolate shared a 16S rRNA gene sequence similarity with these strains of 99.9% and 99.4%, respectively, values corresponding to a single and 9 nt differences at 1397 locations (Table 3.17).

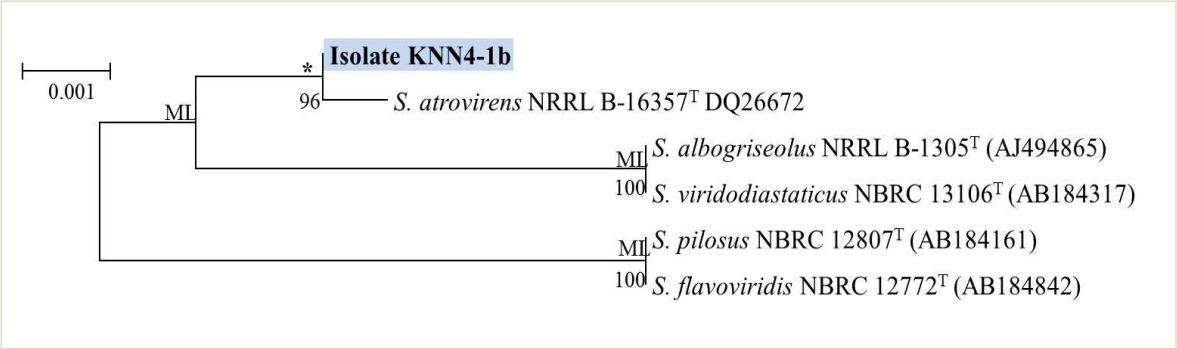


Figure 3.11. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between *Streptomyces* strain KNN4-1b isolated from the hyper-arid Salar de Atacama environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.001 substitutions per nucleotide position.

Table 3.16. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolate KNN4-1b from the hyper-arid Salar de Atacama environmental sample and between it and the type strains of closely related *Streptomyces* species.

Isolate	**1**	2	3	4	5	6
1. Isolate KNN4-1b	**---**	**1/1397**	**9/1397**	**9/1389**	**12/1380**	**12/1388**
2. *S. atrovirens*	**99.9**	---	11/1484	10/1463	13/1454	13/1462

3. *S. albogriseolus*	**99.4**	99.3	---	0/1463	17/1454	17/1462
4. *S. viridodiastaticus*	**99.4**	99.3	100	---	17/1454	17/1462
5. *S. pilosus*	**99.1**	99.1	98.8	98.8	---	0/1454
6. *S. flavoviridis*	**99.1**	99.1	98.8	98.8	100	---

Strain codes, as given in Figure 3.11.

***Streptomyces althioticus* subclade.** Strains KNN2-4c and KNN5-25d, representatives of the twenty three and twenty five isolates assigned to colour-groups KNN2 and KNN5, respectively, were isolated from the Salar de Atacama environmental sample and recovered in *S. althioticus* 16S rRNA gene tree (Figure 3.12). These two strains shared a 16S rRNA gene sequence similarity of 98.2%, a value corresponding to 23 nt differences at 1305 locations. Isolate KNN2-4c formed a well delineated phyletic line together with the type strains of *Streptomyces griseoincarnatus*, *Streptomyces labedae* and *Streptomyces variabilis*; the taxonomic status of this subclade was supported by all of the tree-making algorithms and by a 67% bootstrap value. The isolate shared a 99.3% a 16S rRNA gene similarity with these strains, a value corresponding to 10 nt differences at 1355 locations. In turn, isolate KNN5-25c formed a distinct phyletic line in the *S. althioticus* 16S rRNA subclade. It was most closely related to *S. althioticus* NRRL B-3981^T and *Streptomyces matensis* NBRC 12889^T, sharing 16S rRNA gene sequence similarities with the later equivalent to 99.1%, a value corresponding to in each case to 12 nt differences at 1283 locations (Table 3.18).

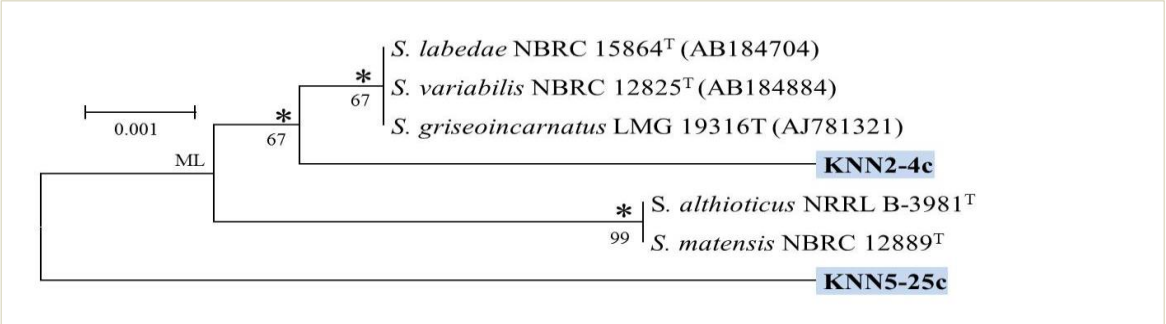


Figure 3.12. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains KNN2-4c and KNN5-25d isolated from the hyper-arid Salar de Atacama environmental sample and between them and the type strains of the most closely related *Streptomyces* species. The asterisk indicates a branch of the tree that was also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values

above 50% are shown. The scale bar indicates 0.001 substitutions per nucleotide position.

Table 3.17. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains KNN2-4c and KNN5-25d isolated from the hyper-arid Salar de Atacama environmental sample and between them and type strains of closely related *Streptomyces* species.

Isolate	1	2	3	4	5	6	7
1.Isolate KNN2-4c	---	**23/1305**	**12/1346**	**13/1346**	**10/1355**	**10/1355**	**10/1355**
2. Isolate KNN5-25c	**98.2**	---	**17/1300**	**17/1300**	**20/1309**	**20/1309**	**20/1309**
3. *S. althioticus*	**99.1**	**98.7**	---	0/1350	7/1351	7/1351	7/1351
4. *S. matensis*	**99.0**	**98.7**	100.0	---	8/1351	8/1351	8/1351
5. *S. griseocarnatus*	**99.3**	**98.5**	99.5	99.4	---	0/1360	0/1360
6. *S. labedae*	**99.3**	**98.5**	99.5	99.4	100.0	---	0/1360
7. *S. variabilis*	**99.3**	**98.5**	99.5	99.4	100.0	100.0	---

Strain codes, as given in Figure 3.12.

***Streptomyces anulatus* subclade.** Isolate KNN23-1b, a representative of two isolates assigned to colour-group KNN23, was isolated from the Yungay environmental sample and assigned to the *S. anulatus* 16S rRNA subclade (Figure 3.13). It formed a well delineated phyletic line at the periphery of a subclade which contained the type strains of *Streptomyces albidochromogenes*, *Streptomyces chryseus*, *Streptomyces flavidovirens* and *Streptomyces helvaticus*; the taxonomic integrity of this subclade was supported by the maximum-likelihood tree-making algorithm and by a 63% bootstrap value. The isolate was most closely related to *S. albidochromogenes* NBRC 101003^T and *S. flavidovirens* NBRC 13039^T; sharing 16S rRNA gene sequence similarities with these organisms of 99.5%, a value corresponding in each case to 7 nt differences at 1378 locations (Table 3.19).

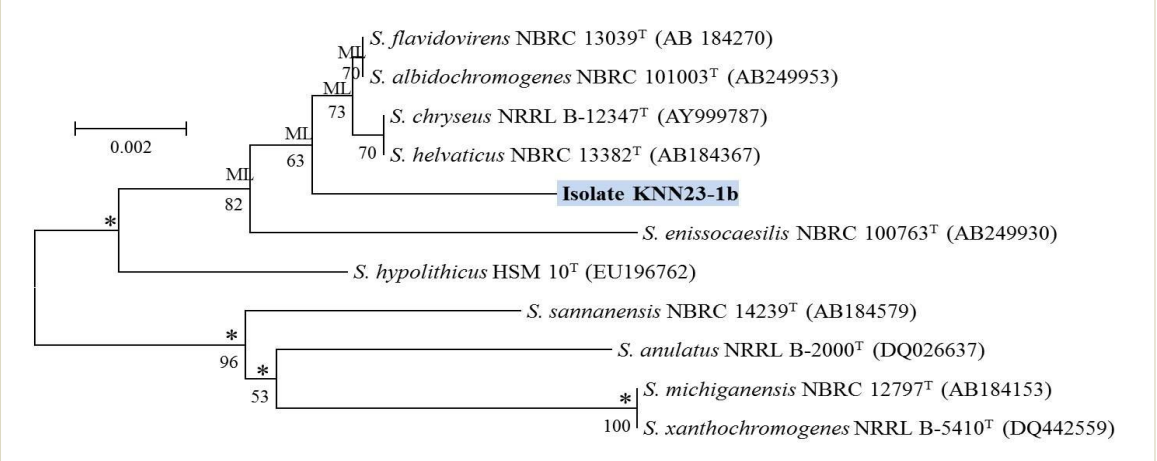


Figure 3.13. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN23-1b isolated from the extreme hyper-arid Yungay environmental sample and the type strains of closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Table 3.18. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strain KNN23-1b isolated from the extreme hyper-arid environmental sample and the type strains of closely related *Streptomyces* species.

Isolate	1	2	3	4	5	6	7	8	9	10	11
1. Isolate KNN23-1b	---	**7/ 1378**	**7/ 1378**	**8/ 1378**	**8/ 1378**	**17/ 1378**	**23/ 1391**	**27/ 1378**	**27/ 1378**	**27/ 1378**	**27/ 1378**
2. *S. flavidovirens*	**99.5**	---	0/ 1379	1/ 1379	1/ 1379	12/ 1379	12/ 1378	23/ 1379	21/ 1379	24/ 1379	24/ 1379
3. *S. albidochromogenes*	**99.5**	100	---	1/ 1379	1/ 1379	12/ 1379	12/ 1378	23/ 1379	21/ 1379	24/ 1379	24/ 1379
4. *S. chryseus*	**99.4**	99.9	99.9	---	0/ 1379	13/ 1379	11/ 1378	22/ 1379	20/ 1379	23/ 1379	23/ 1379
5. *S. helvaticus*	**99.4**	99.9	99.9	100	---	13/ 1379	11/ 1378	22/ 1379	20/ 1379	23/ 1379	23/ 1379
6. *S. enssocaesilis*	**98.8**	99.1	99.1	99.1	99.1	---	21/ 1378	29/ 1379	21/ 1379	29/ 1379	29/ 1379
7. *S. hypolithicus*	**98.4**	99.1	99.1	99.2	99.2	98.5	---	20/ 1378	21/ 1378	21/ 1378	21/ 1378
8. *S. anulatus*	**98.0**	98.3	98.3	98.4	98.4	97.9	98.6	---	16/ 1379	17/ 1379	17/ 1379
9. *S. sannanensis*	**98.0**	98.5	98.5	98.6	98.6	98.5	98.5	98.8	---	16/ 1379	16/ 1379
10. *S. michiganensis*	**98.0**	98.3	98.3	98.3	98.3	97.9	98.5	98.8	98.8	---	0/ 1379
11. *S. xanthochromogenes*	**98.0**	98.3	98.3	98.3	98.3	97.9	98.5	98.8	98.8	100	---

Strain codes, as given in Figure 3.13.

***Streptomyces fragilis* subclade.** Strain KNN87b, the representative of a single-membered colour-group, was isolated from the Salar de Atacama environmental sample and recovered in the *S. fragilis* 16S rRNA subclade (Figure 3.14). It shared an identical 16S rRNA gene sequence with the type strain of *Streptomyces bullii* (Table 3.20), these strains were most closely related to *Streptomyces chromofuscus* NBRC 12851^T sharing a 99.4% 16S rRNA gene similarity with the latter, a value corresponding 12 nt differences at 1388 sites (Table 3.20).

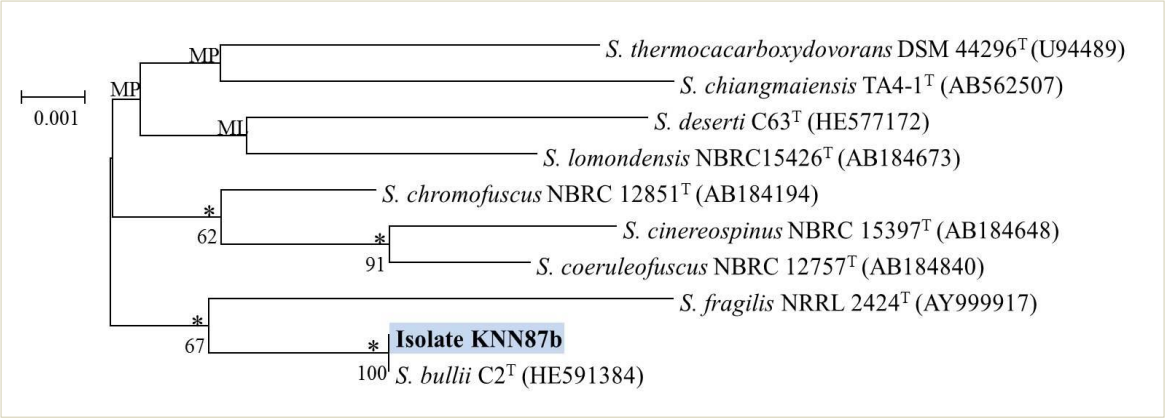


Figure 3.14. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain KNN87b isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML and MP indicate branches of the tree that were supported by the maximum-likelihood and maximum-parsimony methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.001 substitutions per nucleotide position.

Table 3.19. Nucleotide similarities (%)and differences based on almost complete 16S rRNA gene sequences showing relationships between strain KNN87b isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Streptomyces* species.

Isolate	**1**	2	3	4	5	6	7	8	9	10
1. Isolate KNN87b	---	**0/ 1388**	**12/ 1388**	**15/ 1388**	**16/ 1388**	**17/ 1388**	**17/ 1388**	**17/ 1388**	**17/ 1387**	**19/ 1386**
2.*S. bullii*	**100**	---	12/ 1390	15/ 1390	16/ 1390	17/ 1390	17/ 1390	17/ 1390	17/ 1389	19/ 1388
3. *S. chromofuscus*	**99.1**	99.1	---	21/ 1390	19/ 1390	13/ 1390	10/ 1390	15/ 1390	18/ 1389	15/ 1388
4. *S. fragilis*	**98.9**	98.9	98.5	---	24/ 1390	23/ 1390	20/ 1390	24/ 1390	21/ 1389	26/ 1388
5. *S. thermocarboxydovorans*	**98.9**	98.9	98.6	98.3	---	22/ 1390	24/ 1390	19/ 1391	17/ 1389	19/ 1388
6. *S. cinereospinus*	**98.8**	98.8	99.1	98.4	98.4	---	9/ 1390	19/ 1390	24/ 1389	24/ 1388
7. *S. coeruleofuscus*	**98.8**	98.9	99.3	98.6	98.3	99.4	---	20/ 1390	20/ 1389	19/ 1388
8. *S. deserti*	**98.8**	98.8	98.9	98.3	98.6	98.6	98.6	---	15/ 1389	29/ 1388
9. *S. lomodensis*	**98.8**	98.8	98.7	98.5	98.8	98.3	98.6	98.9	---	18/ 1387
10. *S. chiangmaiensis*	**98.6**	98.6	98.9	98.1	98.6	98.3	98.6	97.9	98.7	---

Strain codes, as given in Figure 3.14.

***Streptomyces radiopugnans* subclade.** Isolates KNN51b and KNN89a, representatives of single-membered colour-groups, encompassed strains isolated from the Salar de Atacama environmental sample and isolate KNN70b, a representative of a single-membered colour-group from the Yungay environmental sample, were recovered in the *S. radiopugnans* 16S rRNA subclade (Figure 3.15). Isolates KNN51b and KNN70b were shown to have a 98.6% 16S rRNA gene sequence similarity, a value corresponding to 20 nt differences at 1394 locations (Table 3.21). Isolate KNN51b shared a 98.1% 16S rRNA gene sequence similarity to isolate KNN89a, a value corresponding to 26 nt differences at 1383 locations. Similarly, isolates KNN70b and KNN89a shared a 97.0% 16S rRNA gene sequence similarity, a value equivalent to 42 nt differences at 1385 locations.

Isolates KNN51b and KNN70b formed a well circumscribed branch in the 16S rRNA gene tree together with *Streptomyces atacamensis* C60^T, a taxon that was supported by all of the tree-making algorithms and by a bootstrap value of 96%. Strain KNN51 shared a 99.2% 16S rRNA gene sequence similarity with the *S. atacamensis* strain, a value equivalent to 12 nt differences at 1406 locations; the corresponding value between isolate KNN70b and *S. atacamensis* C60^T was 98.6%, a value corresponding to 19 nt differences at 1396 locations. Strain KNN51b was also closely related to *Streptomyces radiopugnans* R97^T; these strains shared a 16S rRNA gene sequence similarity of 98.7%, a value corresponding to 19 nt differences at 1403 locations. Isolate KNN70b was also closely reated to *S. radiopugnans* R97^T ; the two strains shared a 16S rRNA gene sequence similarity of a 98.1%, a value corresponding to 26 nt differences at 1396 locations.

Isolate KNN89a was recovered at the periphery of a taxon that contained isolates KNN51b and KNN70b and the type strains of *S. atacamensis, Streptomyces fenghuangensis, Streptomyces nanheiensis* and *S. radiopugnans*, together with isolate KNN89a; this taxon was supported by all of the tree-making algorithms and by a bootstrap value 69%. Isolate KNN89a shared a 16S rRNA gene sequence similarity to isolates KNN51 and KNN70b of 98.1% and 97.0 %, respectively, values corresponding to 26 and 42 nt differences at 1383 and 1385 locations. Isolate KNN89a was most closely related to *S. radiopugnans* R97^T; these strains shared a 98.4% 16S rRNA gene sequence similarity, a value equivalent to 22 nt differences at 1383 locations. It was also closely related *S. nanhaiensis* SCSIO01248^T; these organisms shared a 98.3% 16S rRNA gene similarity, a value corresponding to 23 nt differences at 1386 locations.

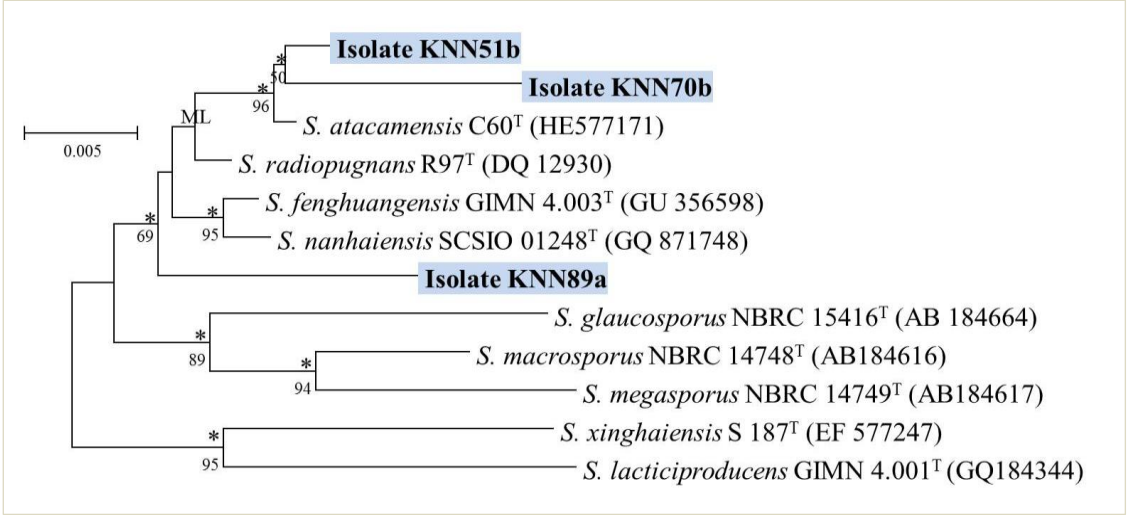


Figure 3.15. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains KNN51b, KNN70b and KNN89a isolated from the Salar de Atacama and Yungay environmental samples and between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates a branch of the tree that was supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Table 3.20. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolates KNN51b, KNN70b and KNN89a and between them and closely related type strains of the *Streptomyces* species.

Isolate	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12
1. Isolate KNN51b	---	**20/ 1394**	**26/ 1383**	**12/ 1406**	**19/ 1403**	**26/ 1403**	**26/ 1404**	**45/ 1404**	**43/ 1405**	**57/ 1402**	**52/ 1401**	**47/ 1405**
2. Isolate KNN70b	**98.6**	---	**42/ 1385**	**19/ 1396**	**26/ 1396**	**35/ 1397**	**35/ 1398**	**53/ 1397**	**52/ 1398**	**64/ 1396**	**61/ 1395**	**58/ 1397**
3. Isolate KNN89a	**98.1**	**97.0**	---	**28/ 1384**	**22/ 1383**	**25/ 1385**	**23/ 1386**	**50/ 1385**	**41/ 1386**	**48/ 1383**	**53/ 1383**	**51/ 1387**
4. *S. atacamensis*	**99.2**	**98.6**	**98.0**	---	9/ 1409	17/ 1409	18/ 1410	35/ 1410	33/ 1411	48/ 1409	45/ 1406	42/ 1411
5. *S. radioplugnans*	**98.7**	**98.1**	**98.4**	99.4	---	12/ 1408	13/ 1409	39/ 1408	31/ 1409	42/ 1408	41/ 1406	41/ 1408
6. *S. fenghuangensis*	**98.2**	**97.5**	**98.2**	98.8	99.2	---	10/ 1411	43/ 1410	32/ 1411	45/ 1408	42/ 1408	38/ 1410
7. *S. nanhaiensis*	**98.2**	**97.5**	**98.3**	98.7	99.1	99.3	---	43/ 1411	35/ 1412	47/ 1409	48/ 1408	38/ 1411
8. *S. glaucosporus*	**96.8**	**96.2**	**96.4**	97.5	97.2	97.0	97.0	---	39/ 1412	51/ 1408	70/ 1407	49/ 1413
9. *S. macrosporus*	**96.9**	**96.3**	**97.0**	97.7	97.8	97.7	97.5	97.2	---	50/ 1409	57/ 1408	30/ 1412
10. *S.*	**95.9**	**95.4**	**96.5**	96.6	97.0	96.8	96.7	96.4	96.5	---	43/	64/

xinghaiensis											1406	1408
11. *S. lactiproducens*	**96.3**	**95.6**	**96.2**	96.8	97.1	97.0	96.6	95.0	96.0	96.9	---	70/ 1408
12. *S. megaspores*	**96.7**	**95.9**	**96.3**	97.0	97.1	97.3	97.3	96.5	97.9	95.5	95.0	---

Strain codes, as given in Figure 3.15.

***Streptomyces rochei* subclade.** Isolate KNN22a, a representative of a single-membered colour-group, was isolated from the Yungay environmental sample and isolate KNN32-1a, a representative of colour-group KNN32 which contianed two strains, was isolated from the Salar de Atacama environmental sample. Both isolates formed distinct phyletic lines in the *S. rochei* 16S rRNA gene subclade (Figure 3.16). These isolates shared a 16S rRNA gene sequence similarity of a 99.6%, a value corresponding to 5 nt differences at 1331 locations. They formed a distinct branch in the *S. rochei* subclade with *Streptomyces mutabilis*; a taxon supported by all the tree-making algorithms but not by a high bootstrap value. Strain KNN22a was most closely related to *Streptomyces ghanaensis* KCTC9882^T, these strains shared a 16S rRNA gene sequence similarity of 99.3% , a value equivalent to 10 nt differences at 1398 locations (Table 3.22). The isolate was also closely related to *S. rochei* NBRC12908^T, these strains shared a16S rRNA gene sequence similarity of 99.1%, a value equivalent to 13 nt differences at 1405 locations. In turn, isolate KNN32-1a was most closely related to *Streptomyces mutabilis* NBRC12800^T, these strains shared a 16S rRNA gene sequence similarity of 99.6%, a value corresponding to 5 nt differences at 1334 locations. Isolate KNN32-1a was also closely related to *S. rochei* NBRC12908^T, these organisms were found to have a 16S rRNA gene sequence similarity of 99.6%, a value equivalent to 5 nt differences at 1332 lacations.

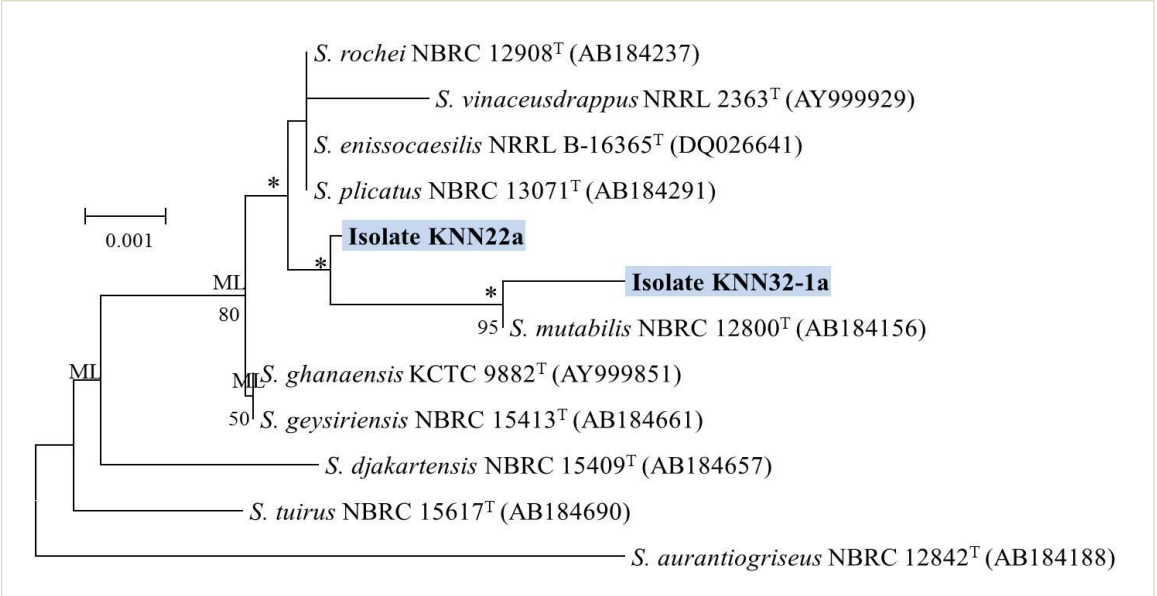


Figure 3.16. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains KNN32-1a and KNN22a isolated from the Salar de Atacama and Yungay environmental samples, respectively and between them and the type strains of closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are shown. The scale bar indicates 0.001 substitutions per nucleotide position.

Table 3.21. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains KNN22a and KNN32-1a isolated from the Atacama Desert environmental samples and between them and the type strains of closely related *Streptomyces* species.

Isolate	**1**	**2**	3	4	5	6	7	8	9	10	11	12
1.Isolate KNN22a	---	5/ 1331	13/ 1405	13/ 1405	13/ 1405	10/ 1398	15/ 1405	14/ 1405	15/ 1400	20/ 1405	21/ 1405	27/ 1404
2. Isolate KNN32-1a	**99.6**	**---**	**5/ 1332**	**5/ 1332**	**5/ 1332**	**6/ 1332**	**5/ 1334**	**6/ 1332**	**7/ 1327**	**10/ 1332**	**8/ 1332**	**17/ 1331**
3. *S. enissocaesilis*	**99.1**	**99.6**	---	0/ 1461	0/ 1463	1/ 1420	4/ 1458	1/ 1463	2/ 1468	7/ 1435	8/ 1454	14/ 1463
4. S. rochei	**99.1**	**99.6**	100	---	0/ 1461	1/ 1418	4/ 1456	1/ 1461	2/ 1456	7/ 1433	8/ 1452	14/ 1460
5. S. plicatus	**99.1**	**99.6**	100	100	---	1/ 1420	4/ 1458	1/ 1463	2/ 1458	7/ 1435	8/ 1454	14/ 1462
6. *S. ghanaensis*	**99.3**	**99.6**	99.3	99.9	99.9	---	5/ 1420	0/ 1420	3/ 1415	6/ 1420	7/ 1420	13/ 1419
7. *S. mutabilis*	**98.9**	**99.6**	99.7	99.7	99.7	99.7	---	5/ 1458	6/ 1453	9/ 1435	8/ 1454	16/ 1457
8. *S. geysiriensis*	**99.0**	**99.6**	99.9	99.9	99.9	100	99.7	---	3/ 1458	6/ 1435	7/ 1454	13/ 1462
9. *S. vinceusdrappus*	**98.9**	**99.5**	99.9	99.9	99.9	99.8	99.6	99.8	---	9/ 1430	10/ 1449	16/ 1458
10. *S. djakartensis*	**98.6**	**99.3**	99.5	99.5	99.5	99.6	99.4	99.6	99.4	---	7/ 1435	15/ 1434
11. S. tuirus	**98.5**	**99.4**	99.5	99.5	99.5	99.5	99.5	99.5	99.3	99.5	---	14/ 1453
12. *S. aurantiogriseus*	**98.1**	**98.7**	99.0	99.0	99.0	99.1	98.9	99.1	98.9	99.0	99.0	---

Strain codes, as given in Figure 3.16.

3. 4. 4. Chemotaxonomy

The chemotaxonomic properties of the representative isolates included in the phylogenetic analyses are show in Table 3.23. The *Actinomadura, Amycolatopsis, Blastococcus, Couchioplanes, Geodermatophilus, Lechevalieria, Modestobacter,*

Nonomuraea, Pseudonocardia and *Saccharothrix* strains contained *meso*-A_2pm in whole-organism hydrolysates whereas those assigned to the genera *Kribbella* and *Streptomyces* had hydrolysates rich in the corresponding *LL*-isomer. Nearly half of the isolates contained menaquinones with nine isoprene units though the number of hydrogenated units tended to differ. The representatives of the genera *Amycolatopsis, Blastococcus* and *Pseudonocardia* contained arabinose and galactose as diagnostic whole-organism sugars whereas the *Actinomadura, Couchioplanes, Geodermatophilus, Kribbella, Lechevalieria, Modestobacter, Nonomuraea* and *Streptomyces* strains showed a range of whole-cell sugars patterns. Similarly, considerable variation was found in the polar lipid patterns between the tested isolates.

Table 3.22. Chemotaxonimic properties of isolates assigned to genera circumscribed in the comparative 16S rRNA gene sequence analyses*.

Isolate code	Chemotaxonomic markers			
	Diaminopimelic acid isomer[2]	Predominant Menaquinones[3]	Major polar lipid[4]	Diagnostic whole-cell sugars[5]
Actinomadura: KNN34c	*Meso*-A_2pm	DPG, PG, PI	DPG, PG, PI	Gal, Glu, Mad, Man, Rib
KNN49-26a	*Meso*-A_2pm	MK-$9_{(H6)}$		Gal, Glu, Mad, Man, Rib
Amycolatopsis: KNN49-5e	*Meso*-A_2pm	MK-$9_{(H2)}$, MK$9_{(H4)}$, MK-$9_{(H6)}$, MK-$9_{(H8)}$	DPG, PE, PME	Ara, Gal
KNN49-26a	*Meso*-A_2pm	MK$9_{(H4)}$, MK-$9_{(H6)}$, MK-$9_{(H8)}$	DPG, PE, PME	Ara, Gal
KNN50-8b	*Meso*-A_2pm	MK$8_{(H4)}$, MK-$9_{(H4)}$, MK-$9_{(H6)}$	DPG, PE, PG, PI, PME	Ara, Gal
KNN50-11c	*Meso*-A_2pm	MK-$8_{(H4)}$, MK-$9_{(H4)}$	DPG, PE, PG, PI, PME	Ara, Gal
Blastococcus: KNN47b	*Meso*-A_2pm	MK-$9_{(H4)}$	DPG, PC, PG, PI, PE	Ara, Gal
Couchioplanes: KNN7-2	*Meso*-A_2pm	MK-$9_{(H4)}$	PG, PE, PI	Ara, Glu, Gal, Xyl
Geodermatophilus: KNN44-1b	*Meso*-A_2pm	MK-$9_{(H4)}$, MK$9_{(H0)}$	DPG, PC, PI	Rib, Glu
Kribbella: KNN56a	*LL*-A_2pm	MK-$9_{(H4)}$	DPG, PCPG, PI	Glu, Gal
Lechevalieria: KNN94e	*Meso*-A_2pm	MK-$9_{(H4)}$	DPG, PE, PG, PI	Gal, Glu, Man, Rib, Rha
Modestobacter:				

KNN45-2b KNN46-4b	*Meso*-A_2pm *Meso*-A_2pm	MK-$9_{(H4)}$ MK-$9_{(H4)}$	DPG, PG, PE, PI DPG, PG, PE, PI	Gal, Glu, Xyl Gal, Glu, Xyl
Nonomuraea: KNN57-2b	*Meso*-A_2pm	MK-$9_{(H4)}$	DPG, PE	Mad, Man
Pseudonocardia: KNN55-1b	*Meso*-A_2pm	MK-$8_{(H4)}$	PC, PE	Ara, Gal
Saccharothrix: KNN10-4d	*Meso*-A_2pm	MK-$9_{(H4)}$, MK-10 $_{(H4)}$	DPG, PE, PIMS	Gal, Rha, Man

*Data on *Streptomyces* isolates can be found in Chapter 6.

[1]ND, not determined..

[2]Diaminopimelic acid isomers: *meso*-A_2pm, *meso*-diaminopimelic acid; *LL*-A_2pm, *LL*-diaminopimelic acid.

[3]Menaquinone:exemplified by MK-$9_{(H4)}$, tetrahydrogenated menaquinones with nine isoprene units.

[4]Polar lipids: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, posphatidylglycerol; PI, phosphatidylinositol, PIMS, phosphatidylinositol manosides (PIMs) and PME, phosphatidylmethylethanolamine.

[5]Whole-cell sugars: Ara, arabinose; Gal, Galactose; Glu, glucose, Man, mannose; Mad, maduros; Rha, rhamnose; Rib, ribose and Xyl, xylose.

3. 4. 5. Morphology

The arrangement of aerial hyphae and spores of the 20 representative isolates included in the scaning electron microscopy studies are shown in Figures 3.17 and 3.18. The two *Saccharothrix* strains produced straight chains of rough ornamented spores, the *Pseudonocrdia* strain formed straight chains of smooth surfaced spores whereas the *Actinomadura*, *Nonomuraea* and *Streptomyces* strains showed variation in spore chain arrangement and spore surface ornamentation.

Figure 3.17. Scanning electron micrographs of isolates grown on glucose yeast-extract malt-extract agar for 14 days at 28^{o}C, (A) *Actinomadura* strain KNN34c showing smooth ornamented spores in straight chains; (B) *Actinomadura* strain KNN53-1a showing spiral chains of rough ornamented spores; (C) *Actinomadura* strain KNN53-3a showing a straight chain of smooth surfaced spores; (D) *Nonomuraea* strain KNN57-1b showing spiral chains of rough ornamented spores; (E) *Nonomuraea* KNN 57-2b showing straight chains of smooth surfaced spores; (F) *Pseudonocardia* strain KNN55-1b showing straight chains of smooth ornamented spores; (G, H) *Saccharothrix* strains KNN10-4d and KNN 54-1a (H) showing straight chains of smooth ornamented spores. Scale bars : 1µm.

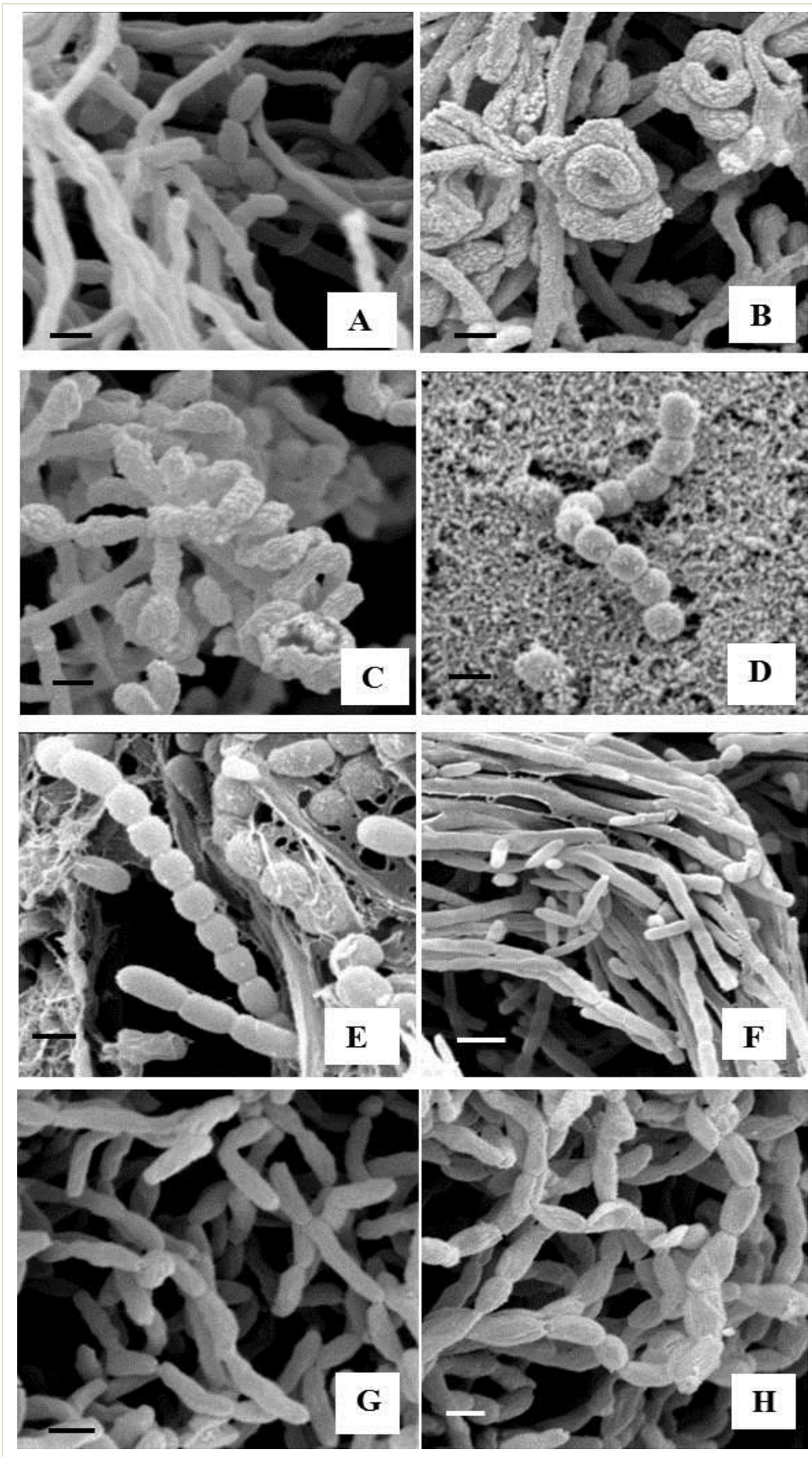
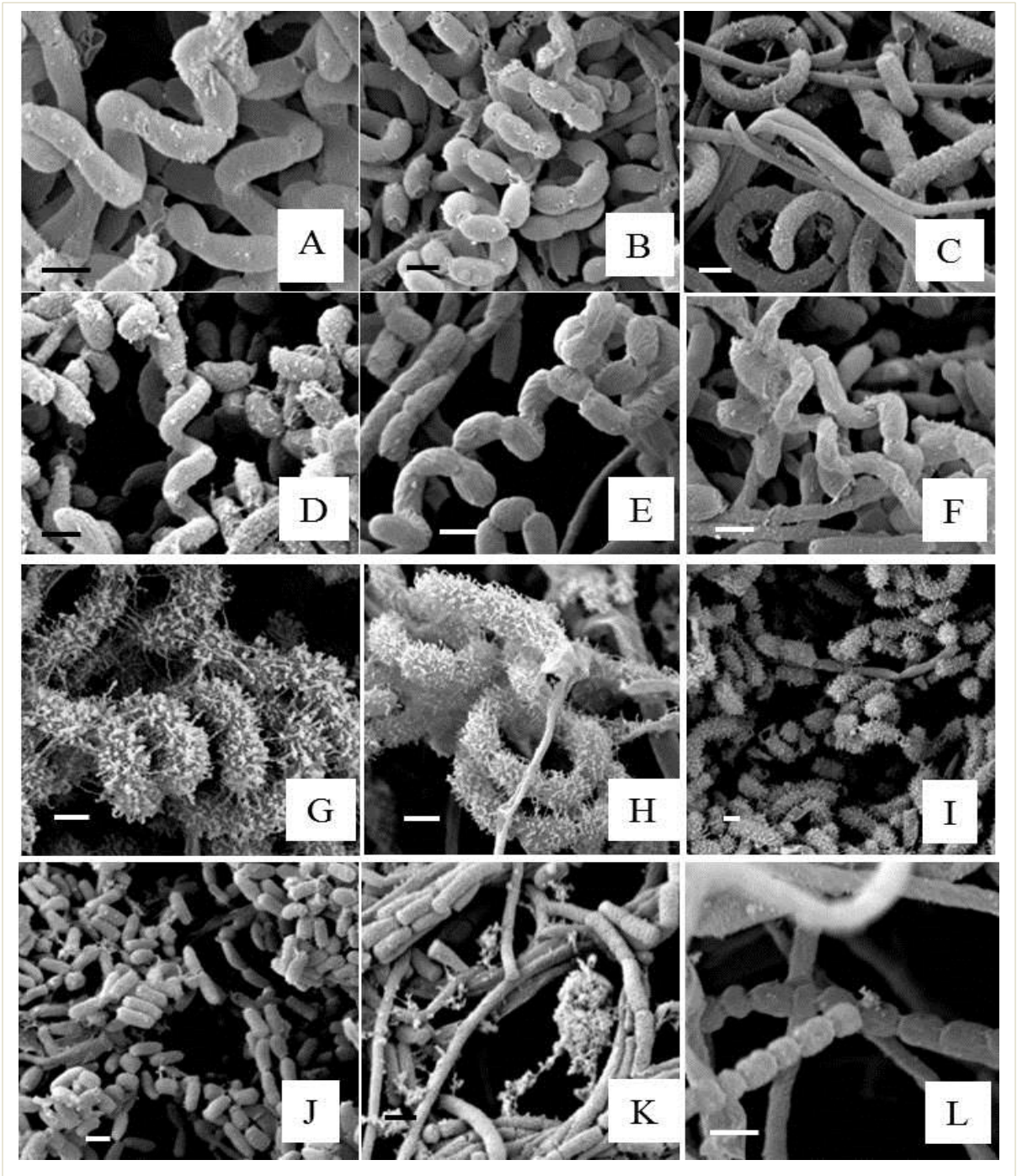

A
B
C
D
E
F
G
H

Figure 3.18. Scanning electron micrographs of streptomycetes isolates grown on oatmeal agar after 14 days at 28°C. (A), C34^{T} showing smooth ornamented spore in open spiral chains; (B), isolate C38 showing spiral chains of smooth surfaced spores; (C), isolate KNN35-2b showing loose spiral chains of smooth surfaced spores; (D, E and F) isolates KNN17-2b, KNN24-1b and KNN26b showing loose spiral chains of smooth surfaced spores; (G, H and I), isolates C59 KNN40b and KNN48-1c showing spiral chains of hairy ornamented spores; (J, K and L) isolates KNN51b, KNN89a and KNN90a showing straight chains of smooth surfaced spores. Scale bars : 1µm.



3. 4. 6. Selectivity of selection media

Most of the strains isolated from the Salar de Atacama and Yungay environmental samples were assigned to the genus *Streptomyces* (79.4% and 73.3%, respectively) as shown in Table 3.24. In contrast, relatively high numbers of *Amycolatopsis* strains were isolated from the hyper–arid Salar de Atacama environmental sample (13.1%). The corresponding Yungay environmental sample was found to contain a relatively high number of isolates (5%) belonging to the family *Geodermatophilaceae*. Isolates recovered in the same multi-membered colour-groups as the representative isolates were assumed to belong to the same genus as the studied strains (Table 3.24).

Table 3.23. Assignment of strains assigned to colour-groups containing isolates from the Salar de Atcama and Yungay environmental samples to genera based on 16S rRNA sequencing data of representative strains.

Genera	Tested strains	Additional strains in colour-groups
Actinomadura	KNN34c,	None
	KNN53-1a, KNN53-3a	KNN53-2a
Amycolatopsis	KNN49-1f, KNN49-3e, KNN49-5e, KNN49-6q, KNN49-11c, KNN49-12b, KNN49-26a,	KNN49-2e, KNN49-4e, KNN49-7c, KNN49-8c, KNN49-9c, KNN49-10b, KNN49-13b, KNN49-14b, KNN49-15b, KNN49-16c, KNN49-47b, KNN49-18b, KNN49-19a, KNN49-20b, KNN49-21c, KNN49-22d, KNN49-23d, KNN49-24d, KNN49-25d, KNN49-27a, KNN49-28a, KNN49-29a, KNN49-30b, KNN49-31b
	KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN550-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-16d, KNN50-17d, KNN50-18d,	KNN50-3c
	KNN61-1e	KNN61-2a, KNN61-3e, KNN61-4e, KNN61-5b, KNN61-6c
Blastococcus	KNN47b	None
Couchioplanes	KNN7-2b	KNN7-1b, KNN7-3b, KNN7-4b
Geodermatophilus	KNN44-1b, KNN44-3b, KNN44-4b	KNN44-2a
Kribbella	KNN56a	None

Lechevalieria	KNN94e	None
Modestobacter	KNN45-1b, KNN45-2b, KNN45-3b, KNN45-4b	None
	KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5a, KNN46-6a, KNN46-7a, KNN46-8c, KNN46-9c, KNN46-10c, KNN46-11a	KNN46-12a
Nonomuraea	KNN57-1b, KNN57-2b	None
Pseudonocardia	KNN55-1b	KNN55-2b
Saccharothrix	KNN10-4d	KNN10-1a, KNN10-2b, KNN10-3b, KNN10-5a,
	KNN54-1a	KNN54-2b
Streptomyces	KNN2-4c, KNN2-6c	KNN2-1b, KNN2-2c, KNN2-3c, KNN2-5c, KNN2-7d, KNN2-8b, KNN2-9d, KNN2-10d, KNN2-11d, KNN2-12d, KNN2-13a, KNN2-14c, KNN2-15a, KNN2-16c, KNN2-17d, KNN2-18c, C34, C38, C58, C59, C79,
	KNN4-1b, KNN4-4a,	KNN4-2e and KNN4-3b
	KNN5-4, KNN5-5	KNN5-1a, KNN5-2a, KNN5-3c, KNN5-4c, KNN5-5a, KNN5-6a, KNN5-7a, KNN5-8b, KNN5-9b, KNN5-10b, KNN5-13b, KNN5-14b, KNN5-15b, KNN5-16d, KNN5-17d, KNN5-18d, KNN5-19d, KNN5-20d, KNN5-25b
	KNN6-6b, KNN6-9a, KNN6-10b, KNN6-11a	KNN6-1a, KNN6-2a, KNN6-3a, KNN6-4a, KNN6-5a, KNN6-7d, KNN6-8d
	KNN9-3a	KNN9-1a, KNN9-2e
	KNN11-1a, KNN11-5a	KNN11-2a, KNN11-3c, KNN11-4b, KNN11-6b,
	KNN13a	None
	KNN22a	None
	KNN23-1b	KNN23-2a, KNN23-2a
	KNN24-1b	KNN24-2c, KNN24-3c, KNN24-4c, KNN24-5c, KNN24-6a, KNN24-7c, KNN24-8e
	KNN25c	None
	KNN26b	None
	KNN32-1a	KNN32-2b,
	KNN35-1b, KNN35-2b	None
	KNN38-1b	KNN38-1b, KNN38-2d, KNN38-3d, KNN38-4a, KNN38-5b

KNN41-1b	KNN41-2a
KNN42	None
KNN48-1c, KNN48-3e, KNN48-6e	KNN48-2a, KNN48-4e, KNN48-5e, KNN48-7d, KNN48-8d, KNN48-9d, KNN48-10d, KNN48-11b, KNN48-12d, KNN48-13b, KNN48-14a, KNN48-15a, KNN48-16c, KNN48-17c, KNN48-18c, KNN48-19c, KNN48-20c
KNN51b	None
KNN64-5b	KNN64-1a, KNN64-2a, KNN64-3a, KNN64-4b, KNN64-6b
KNN82-2c	KNN82-1a, KNN82-2c
KNN83e	None
KNN87b	None
KNN88-1a	KNN88-2b, KNN88-3b, KNN88-4a
KNN89a	None
KNN90a	None

Codes for isolation media : (a), Gause No.1 agar; (b), HVA, humic acid-vitamin agar; (c), Oligotrophic agar; (d), minimal medium agar; (e), SM1 agar; (f) *Geodermatophilus obscurus* agar; (g) Luedemann's agar. (h), SCAV agar.

The number of actinobacteria isolated from the Salar de Atacama environmental sample on the selective isolation media are shown in Table 3.25. Eighty of the streptomycetes were isolated from humic acid-vitamin agar plates (25.4% of the total number of isolates), 56 on Gause's No. 1 agar (17.8%), 53 on oligotrophic agar (16.8%), 33 on minimal agar (10.5%), 18 on SM 1 agar (5.7%) and 5 on starch casein-vitamin agar (1.6%) plates. In turn, 13 of the *Amycolatopsis* isolates were recovered from humic acid-vitamin agar and from minimal medium agar (4.1%), 10 from oligotrophic medium agar, 10 from SM1 agar (3.2%), 8 from Gause's No. 1 agar plates (2.5%) and a single isolate from a starch casein-vitamin agar plate (0.3%). Three of *Saccharothrix* strains were isolated from Gause's No.1 agar plates and the remaining three from a humic acid-vitamin agar plate (1%). Three out of the 4 *Actinomadura* strains were isolated on Gause's No.1 agar plates (1%) and the remaining one from a minimal medium agar plate (0.3%). The two *Nonomuraea* strains were isolated from humic acid-vitamin agar plates (0.6%), the single *Kribbella* from Gause's No.1 agar (0.3%) and the only *Lechevalieria* strain from SM1 agar (0.3%).

Table 3.24. Assignment of representative strains isolated on a range of selective media inoculated with suspensions of the Salar de Atacama environmental sample to genera based on 16S rRNA gene sequences.

Genus	Media					
	Gause's No.1	Humic acid-vitamin agar	Minimal medium agar	Oligitrophic medium agar	SM1 agar	Starch casein-vitamin agar
Actinomadura	3	-	1	-	-	-
Amycolatopsis	8	13	13	10	10	1
Kribbella	1	-	-	-	-	-
Lechevalieria	-	-	-	-	1	-
Nonomuraea	-	2	-	-	-	-
Saccharothrix	3	3	-	1	-	-
Strreptomyces	56	80	33	53	18	5
Total	71	98	47	64	29	6

The corresponding numbers of actinobacteria isolated on the selective media inoculated with suspensions of the Yungay environmental sample are shown in Table 3.26. Thirty six streptomycetes were isolated on Gause's No.1 agar (34.3%), 20 on humic acid-vitamin agar (19%), 8 on oligotrophic medium agar (7.6%), 7 on starch casein-vitamin agar (6.7%) and 6 on minimal medium agar (5.75%) plates. Similarly, 8 of the 12 *Modestobacter* isolates (11.4%) were recovered from humic acid-vitamin agar, 4 from Gause No.1 agar (3.8%), 2 from *Geodermatophilus obscurus* agar plates (1.9%) and the remaining two strains from the Luedemann agar and oligotrophic agar plates (1%). Similarly, 4 out of the 5 *Couchioplanes* isolates were recovered from humic acid-vitamin agar plates and the remaining one from a starch casein-vitamin agar plate (1%). Similarly, 4 out of the 5 *Geodermatophilus* strains were isolated from humic acid-vitamin agar plates (4.8%), the remaining one from a Gause's No.1 agar plate (1%). The 2 *Pseudonocardia* isolates were recovered from humic acid-vitamin agar plates (1.9%) and the single strain of *Blastococcus* from same isolation medium (1%).

Table 3.25. Assignment of representative strains isolated on a range of selective media inoculated with suspensions of the Yungay environmental sample to genera based on 16S rRNA gene sequences.

Genus	Media						
	Gause No.1 agar	Humic acid-vitamin agar	*Geodermatophilus obscurus* agar	Luedemann's agar	*Microlunatus* agar	Minimal medium agar	Starch casein- vitamin agar
Blastococcus	-	1	-	-	-	-	-
Couchioplanes	-	4	-	-	-	-	1
Geodermatophilus	1	4	-	-	-	-	-
Modestobacter	4	8	2	1	-	1	-
Pseudonocardia	-	2	-	-	-	-	-
Streptomyces	36	20	-	-	6	8	7
Total	41	39	2	1	5	9	8

No actinobacteria were isolated on the SM1 agar plates.

3. 4. 7. Screening for antimicrobial activity

Plug assays. The results obtained for the 136 representative isolates screened for antimicrobial activity in the agar plug assays are shown in Table 3.27. Ninety six isolates showed activity against the *Bacillus subtilis* strain (69.1%), 10 against the *Escherichia coli* strain (7.4%), 11 against the *Pseudomonas fluorescens* strain (8.1%), 75 against *Staphylococcus aureus* strain (56.0%) and 38 against the *Saccharomyces cerevisiae* strain (27.9%). Isolates KNN26b, KNN32-1a, KNN35-1b, KNN35-2b, KNN37-5a, KNN38-1b, KNN63-2b, KNN64-3a, KNN64-5b and KNN90a (7.4% of isolates) gave zones of inhibition against all five wild type strains. In contrast, 43 isolates (31.6%) did not show any activity against the panel of strains, namely 15 isolates from the Salar de Atacama soil (11.0%) and 28 from the Yungay soils (20.6%).

Table 3.26. Inhibition zones (mm) shown by representative strains isolated from the Salar de Atacama and Yungay environmental samples in the plug assays after incubation overnight at 30^{o}C.

Isolates	Panel of wild type strains				
	Bacillus subtilis	*Escherichia coli*	*Pseudomonas fluorescens*	*Staphylococcus aureus*	*Saccharomyces cerevisiae*
Salar de Atacama:					
KNN1-2c	18	-	-	30	-
KNN1-5f	17	-	-	40	-
KNN2-2c	18	-	-	35	11
KNN2-5c	16	-	-	25	-
KNN2-6c	19	-	-	30	12
KNN2-10d	18	-	-	-	-

KNN2-11d	17	-	-	25	11
KNN3-1b	19	-	-	24	-
KNN3-2b	16	-	-	20	-
KNN3-11d	17	-	-	35	-
KNN3-17d	18	-	-	35	-
KNN4-3b	18	-	-	20	-
KNN5-24b	15	-	-	-	-
KNN5-25d	16	-	-	-	-
KNN6-2a	18	-	-	40	-
KNN6-5a	19	-	-	30	-
KNN6-6b	18	-	-	-	20
KNN6-10b	17	-	-	25	-
KNN6-11a	17	-	-	22	-
KNN8-3a	18	-	-	10	-
KNN8-5b	19	-	-	30	-
KNN8-8a	16	-	-	-	12
KNN8-9c	16	-	-	-	-
KNN8-10e	17	-	-	-	-
KNN9-1a	18	-	-	20	11
KNN9-2c	18	-	-	30	11
KNN10-3b	18	-	-	24	-
KNN10-4d	19	-	-	30	-
KNN10-5a	18	-	-	20	-
KNN11-2a	18	-	-	20	18
KNN11-4b	18	-	-	28	-
KNN11-5a	18	-	-	20	35
KNN11-6a	18	-	-	15	-
KNN14-3e	18	-	-	20	-
KNN17-1c	18	-	-	30	-
KNN17-2b	18	-	-	35	-
KNN18-3d	18	-	-	28	20
KNN24-2c	18	-	-	25	-
KNN24-3c	18	-	-	15	-
KNN24-4c	18	-	-	13	-
KNN24-7c	18	-	-	35	18
KNN24-8e	18	-	-	Weak	-
KNN24-9e	18	-	-	25	-
KNN25c	18	-	-	14	18
KNN26b	18	17	22	24	22
KNN27a	18	-	-	25	-
KNN28a	18	-	-	25	18
KNN29a	18	-	-	-	-

KNN30a	18	-	-	28	-
KNN31d	18	-	-	30	-
KNN32-1a	18	Weak	13	22	18
KNN33a	18	-	-	-	-
KNN35-1b	24	20	20	24	18
KNN35-2b	24	13	18	18	25
KNN36-1c	18	-	-	-	-
KNN36-3c	18	-	-	-	-
KNN37-1e	18	-	-	-	40
KNN37-5a	18	21	20	20	16
KNN38-1b	18	15	20	30	13
KNN38-5b	18	-	-	25	14
KNN43b	18	-	-	35	-
KNN48-3e	18	-	-	18	-
KNN48-6e	18	-	-	22	-
KNN52-2b	18	-	-	-	-
KNN58-1b	19	-	-	30	15
KNN63-2b	18	12	14	10	13
KNN63-15b	16	-	-	15	-
KNN64-3a	18	17	20	20	14
KNN64-5b	20	16	21	30	18
KNN65-1f	18	-	-	20	18
KNN65-5d	19	-	-	22	16
KNN66b	18	-	-	35	18
KNN67-4b	18	-	-	-	-
KNN68-2b	17	-	-	-	45
KNN68-4b	16	-	-	-	15
KNN69-1e	18	-	-	30	18
KNN69-2a	19	-	-	25	18
KNN69-3a	19	-	-	16	-
KNN71-2a	18	-	-	35	18
KNN72a	18	-	-	17	-
KNN73-2a	17	-	-	35	-
KNN74-2c	19	-	-	35	18

KNN75-4b	18	-	-	23	18
KNN76b	-	-	-	Weak	-
KNN81-2b	18	-	-	-	35
KNN82-1a	16	-	-	30	-
KNN83e	19	-	-	22	-
KNN85-1f	18	-	-	25	18
KNN87b	18	-	-	30	-
KNN94e	16	-	-	-	-
Yungay:					
KNN13a	13	-	-	-	-
KNN21a	19	-	-	12	-
KNN23-1b	13	-	21	-	-
KNN90a	16	16	22	24	24

+: positive, -: negative results.

Strains negative against all five wild type strains: A) isolates from the Salar de Atacama environmental sample: 1) streptomycetes including KNN4-1b, KNN4-4a, KNN39c, KNN50-11c, , KNN61-1a and 2) non-streptomycetes including KNN34c (*Actinomadura*), KNN49-5e (*Amycolatopsis*) , KNN49-26a (*Amycolatopsis*), KNN50-8b* (*Amycolatopsis*), KNN53-1a (*Actinomadura*), KNN53-2a (*Actinomadura*), KNN53-3a (*Actinomadura*), KNN54-1a (*Saccharothrix*), KNN56a (*Kribbella*), and KNN57-1b (*Nonomuraea*); and B) isolates from the Yungay environmental sample: 1) streptomycetes including KNN61-1e, KNN67-4b, KNN86-1b, KNN88-1a, KNN88-2a, KNN89a, KNN91-1a, KNN96a KNN97a and KNN88a ; and 2) non-streptomycetes KNN7-2b (*Couchioplanes*), KNN44-2b (*Geodermatophilus*), KNN44-4a (*Geodermatophilus*), KNN45-1a (*Modestobacter*), KNN45-2b (*Modestobacter*), KNN45-3b (*Modestobacter*), KNN45-4b (*Modestobacter*), KNN46-1b (*Modestobacter*), KNN46-2b (*Modestobacter*), KNN46-3b (*Modestobacter*), KNN46-4b (*Modestobacter*), KNN46-5b (*Modestobacter*), KNN46-6a (*Modestobacter*), KNN46-7a (*Modestobacter*), KNN46-8a (*Modestobacter*), KNN47b (*Blastococcus*), KNN51b (*Pseudonocardia*) and KNN55-1b (*Pseudonocardia*).

*Isolate KNN50-8b did not shown any activity against the five wild type strains but was included in the preliminary study of bioactive compounds, as a non-creative strain.

Plug assays with Bacillus subtilis reporter strains. The results obtained for the 94 isolates included in the plug assays based on the *B. subtilis* reporter strains are shown in Table 3.28. Twenty two strains (23.4%) gave blue halos against the *yvq*I^{ER} , *yja*X^{ER}, *ypuA*^{ER} and *yvg*S^{ER} reporter strains, six (6.4%) against the *yja*X^{ER}, *ypuA*^{ER} and *yvg*S^{ER} reporter strains, and three (3.2%) against the *yvq*I^{ER} , *ypuA*^{ER} and *yvg*S^{ER} reporter strains. Three strains (3.2%) were shown to inhibit DNA synthesis, twenty eight (29.8%) cell envelope synthesis, thirty four (36.2%) fatty acid synthesis, thirty two (34.0%) cell wall synthesis and a further thirty two (34%) against RNA synthesis, as shown in Table 3.28, and as exemplified in Figure 3.19. In contrast, 40 isolates (42.6%) showed bioactivity but did not form blue halos.

Table 3.27. Diameter of zones of inhibition (mm) produced by strains isolated from the Salar de Atacama environmental sample against the *Bacillus subtilis* reporter strains after incubating overnight at 30^{o}C. Isolates found to give blue halos are shown in blue.

Isolates	Reporter genes				
	*phi*105^{CH}	*yvq*I^{ER}	*yja*X^{ER}	*ypuA*^{ER}	*yvg*S^{ER}
Multi-membered colour-groups:					
KNN1-2c	23	20	23	22	22
KNN1-5f	20	18	15	16	17
KNN2-2c	15	17	15	15	13
KNN2-5c	16	14	15	15	16
KNN2-6c	17	18	18	18	20
KNN2-10d	25	17	25	22	21
KNN2-11d	15	15	20	15	16
KNN3-1b	20	20	28	23	23
KNN3-2b	22	18	23	22	23
KNN3-11d	13	12	10	11	14
KNN3-17d	24	21	23	22	23
KNN4-1b	-	-	-	-	-
KNN4-3b	20	20	22	23	22
KNN4-4 a	-	-	-	-	-
KNN5-25d	21	17	19	18	19
KNN6-2a	20	19	20	20	20
KNN6-5a	14	15	14	13	15
KNN6-6b	Weak	Weak	13	13	13
KNN6-10b	24	16	25	23	23
KNN6-11a	22	22	25	24	23
KNN8-3a	20	16	20	18	20
KNN8-5b	25	25	25	25	25
KNN8-8a	Weak	Weak	Weak	Weak	Weak
KNN8-9a	23	17	25	20	20
KNN8-10a	Weak	Weak	Weak	Weak	Weak
KNN9-1a	Weak	13	Weak	Weak	Weak
KNN9-2c	22	19	22	20	22

*KNN10-3b	23	18	20	20	20
*KNN10-4d	18	18	20	17	21
*KNN10-5a	20	16	20	20	19
KNN11-2a	13	12	10	13	14
KNN11-4b	Weak	13	13	12	10
KNN11-5a	Weak	Weak	14	10	19
KNN11-6a	24	12	15	18	19
KNN14-3e	15	14	18	15	15
KNN17-1c	30	19	35	28	35
KNN17-2b	23	30	28	28	30
KNN18-3d	16	15	20	18	17
KNN24-2c	21	20	24	21	22
KNN24-3c	15	19	17	13	15
KNN24-4c	-	-	-	-	-
KNN24-7c	24	25	25	20	25
KNN24-8e	15	13	14	15	14
KNN24-9e	18	18	19	17	16
KNN32-1a	16	21	18	19	18
KNN35-2b	20	21	20	21	21
KNN36-1c	Weak	Weak	Weak	10	10
KNN36-3c	12	14	Weak	11	12
KNN37-1e	20	20	20	20	17
KNN37-5a	20	19	18	16	18
KNN38-1b	27	18	18	22	15
KNN38-5b	20	18	22	20	22
KNN48-3e	20	19	22	22	22
KNN48-6d	21	25	27	24	24
KNN52-2b	15	Weak	12	16	15
KNN57-1b	-	-	-	-	-
KNN58-1b	24	16	25	23	22
KNN63-2b	15	16	18	18	18
KNN63-5b	Weak	20	Weak	Weak	Weak
KNN64-3b	28	17	21	21	23
KNN64-5b	27	20	22	20	21
KNN65-1f	23	18	25	25	24
KNN65-5d	25	19	22	25	25
KNN67-4b	11	Weak	15	17	16
KNN68-2 b	Weak	Weak	10	Weak	13
KNN68-4 b	Weak	Weak	Weak	11	10
KNN69-1e	21	15	23	21	21
KNN69-2a	22	25	25	23	23

KNN69-3a	13	Weak	12	13	15
KNN71-2a	21	17	23	21	21
KNN73-2a	18	17	18	17	18
KNN74-2c	23	18	23	20	22
KNN75-4b	15	13	15	15	15
KNN81-2b	Weak	12	11	11	14
KNN82-1a	15	12	Weak	Weak	12
KNN85-1f	20	18	21	20	22
Single-membered colour-groups:					
KNN25c	15	15	15	12	15
KNN26b	25	19	19	28	25
KNN27a	25	21	20	21	20
KNN28a	12	22	14	12	14
KNN29a	Weak	Weak	12	12	12
KNN30a	13	Weak	17	16	16
KNN31d	10	Weak	13	Weak	11
KNN33a	14	12	12	11	10
KNN39c	-	-	-	-	-
KNN43b	25	18	25	23	25
KNN56a	-	-	-	-	-
KNN66a	25	18	22	22	22
KNN72a	16	15	17	16	17
KNN76b	-	-	-	-	-
KNN83e	20	19	20	20	22
KNN87b	20	23	24	27	25
KNN94e	Weak	10	Weak	Weak	Weak

- : negative result. * *Saccharothrix* strains.

Disc diffusion assays. The results obtained with the mycelial extracts of the 94 strains isolated from the Salar de Atacama environmental sample are shown in Table 3.29, and illustrated in Figure 3.20. Twenty four of the isolates (25.5%) gave strong positive results with the *B. subtilis* reporter strains, whereas 28 (29.8%) showed very weak or weak activity. In contrast, negative results were obtained against all of the *B. subtilis*

reporter strains with 31 of the extracts (33.0%). The mycelial extracts of three strains inhibited cell wall synthesis (3.2%), three inhibited fatty acid synthesis (3.2%), seventeen cell envelope synthesis (18.1%) and a further sixteen RNA synthesis (17.0%), none of the extracts showed activity against DNA synthesis. The mycelial extracts of eight strains (8.5%) showed activity against to all of *B. subtilis* reporter strains but did not produced blue halos. Seventy four strains presented strong activity against the *B. subtilis* reporter strains in the plug assays, but only thirteen of the corresponding mycelial extracts showed strong activity against to the *B. subtilis* reporter strains.

Figure 3.19. Zones of inhibition produced by representative strains isolated from the Salar de Atacama environmental sample against the *Escherichia coli* strain and against the *Bacillus subtilis* reporter strains (Table 2.16, page 63) in agar plug assays after incubating overnight at 30^{o}C.

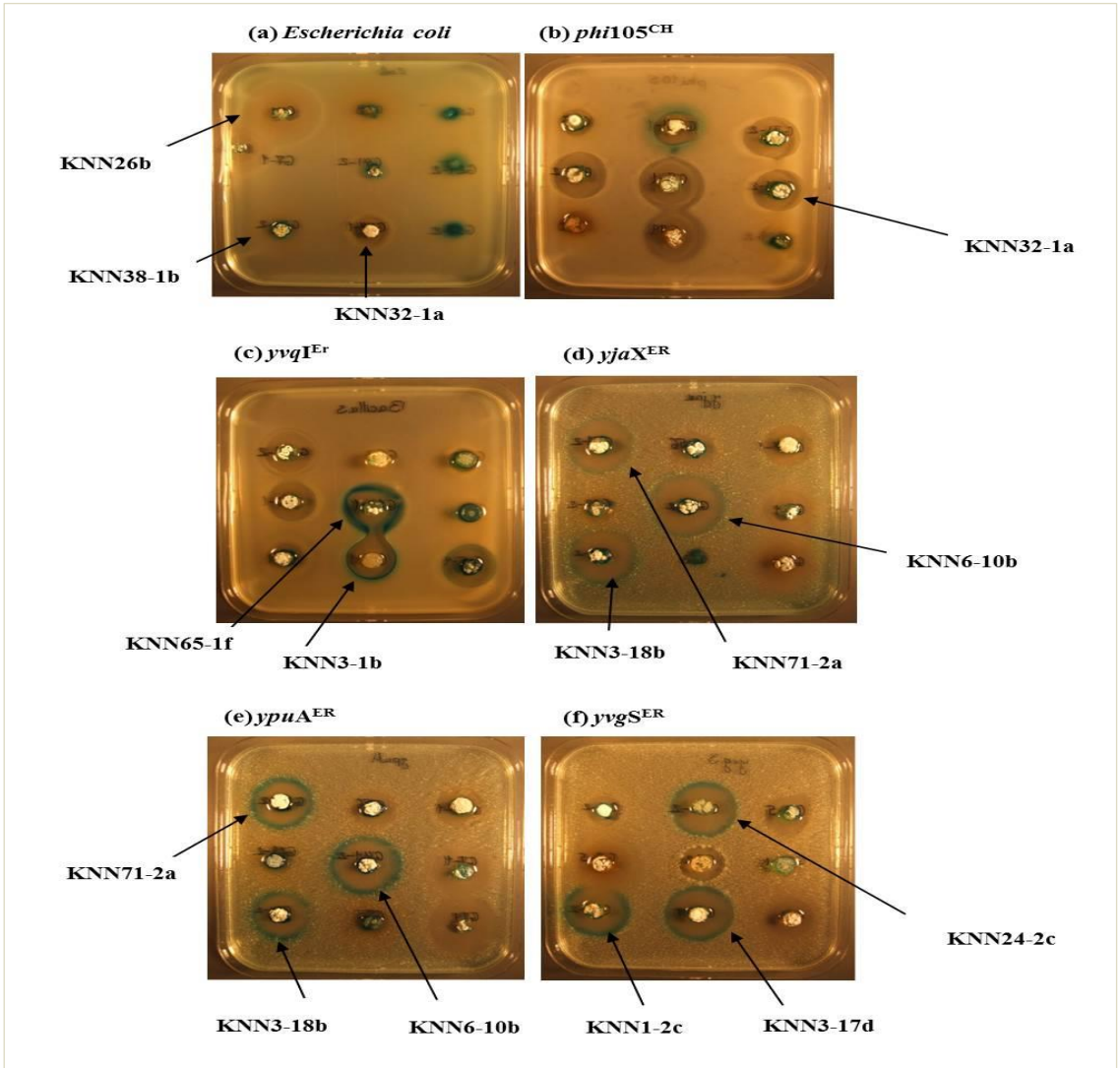


Table 3.28. Inhibition zones (mm) produced by mycelial extracts obtained from strains isolated from the Salar de Atacama environmental sample against the *Bacillus subtilis* reporter strains after incubating overnight at 30°C. Strains which gave blue halos are shown in blue.

Isolates	Reporter genes				
	*phi*105^{CH}	*yvq*I^{ER}	*yja*X^{ER}	*ypuA*^{ER}	*yvg*S^{ER}
Multi-membered colour-groups:					
Streptomycetes					
KNN1-2c	23	20	23	22	22
KNN1-5f	20	18	15	16	17
KNN2-2c	15	17	15	15	13
KNN2-5c	16	14	15	15	16
KNN2-6c	17	18	18	18	20
KNN2-10d	25	17	25	22	21
KNN2-11d	15	15	20	15	16
KNN3-1b	20	20	28	23	23
KNN3-2b	22	18	23	22	23
KNN3-11d	13	12	10	11	14
KNN3-17d	24	21	23	22	23
KNN4-1b	-	-	-	-	-
KNN4-3b	20	20	22	23	22
KNN4-4 a	-	-	-	-	-
KNN5-25d	21	17	19	18	19
KNN6-2a	20	19	20	20	20
KNN6-5a	14	15	14	13	15
KNN6-6b	Weak	Weak	13	13	13
KNN6-10b	24	16	25	23	23
KNN6-11a	22	22	25	24	23
KNN8-3a	20	16	20	18	20
KNN8-5b	25	25	25	25	25
KNN8-8a	Weak	Weak	Weak	Weak	Weak
KNN8-9a	23	17	25	20	20
KNN8-10a	Weak	Weak	Weak	Weak	Weak
KNN9-1a	Weak	13	Weak	Weak	Weak
KNN9-2c	22	19	22	20	22
KNN11-2a	13	12	10	13	14
KNN11-4b	Weak	13	13	12	10
KNN11-5a	Weak	Weak	14	10	19
KNN11-6a	24	12	15	18	19
KNN14-3e	15	14	18	15	15
KNN17-1c	30	19	35	28	35
KNN17-2b	23	30	28	28	30
KNN18-3d	16	15	20	18	17
KNN24-2c	21	20	24	21	22
KNN24-3c	15	19	17	13	15
KNN24-4c	-	-	-	-	-

KNN24-7c	24	25	25	20	25
KNN24-8e	15	13	14	15	14
KNN24-9e	18	18	19	17	16
KNN32-1a	16	21	18	19	18
KNN35-2b	20	21	20	21	21
KNN36-1c	Weak	Weak	Weak	10	10
KNN36-3c	12	14	Weak	11	12
KNN37-1e	20	20	20	20	17
KNN37-5a	20	19	18	16	18
KNN38-1b	27	18	18	22	15
KNN38-5b	20	18	22	20	22
KNN48-3e	20	19	22	22	22
KNN48-6d	21	25	27	24	24
KNN52-2b	15	Weak	12	16	15
KNN58-1b	24	16	25	23	22
KNN63-2b	15	16	18	18	18
KNN63-5b	Weak	20	Weak	Weak	Weak
KNN64-3b	28	17	21	21	23
KNN64-5b	27	20	22	20	21
KNN65-1f	23	18	25	25	24
KNN65-5d	25	19	22	25	25
KNN67-4b	11	Weak	15	17	16
KNN68-2 b	Weak	Weak	10	Weak	13
KNN68-4 b	Weak	Weak	Weak	11	10
KNN69-1e	21	15	23	21	21
KNN69-2a	22	25	25	23	23
KNN69-3a	13	Weak	12	13	15
KNN71-2a	21	17	23	21	21
KNN73-2a	18	17	18	17	18
KNN74-2c	23	18	23	20	22
KNN75-4b	15	13	15	15	15
KNN81-2b	Weak	12	11	11	14
KNN82-1a	15	12	Weak	Weak	12
KNN85-1f	20	18	21	20	22
Non-streptomycetes					
Saccharothrix					
KNN10-3b	23	18	20	20	20
KNN10-4d	18	18	20	17	21

KNN10-5a	20	16	20	20	19
KNN54-1a	-	-	-	-	-
Nonomuraea KNN57-1b	-	-	-	-	-
Single-membered colour-groups:					
KNN25c	15	15	15	12	15
KNN26b	25	19	19	28	25
KNN27a	25	21	20	21	20
KNN28a	12	22	14	12	14
KNN29a	Weak	Weak	12	12	12
KNN30a	13	Weak	17	16	16
KNN31d	10	Weak	13	Weak	11
KNN33a	14	12	12	11	10
KNN39c	-	-	-	-	-
KNN43b	25	18	25	23	25
KNN66a	25	18	22	22	22
KNN72a	16	15	17	16	17
KNN76b	-	-	-	-	-
KNN83e	20	19	20	20	22
KNN87b	20	23	24	27	25
KNN94e	Weak	10	Weak	Weak	Weak
Non-streptomycetes					
Kribbella KNN56a	-	-	-	-	-

- : negative result.

Strains negative against all of the *B. subtilis* reporter strains: KNN5-25d, KNN9-1a, KNN9-2c, KNN25c, KNN26b, KNN27a, KNN28a, KNN29a, KNN32-1a, KNN33a, KNN35-1b, KNN35-2b, KNN37-5a, KNN39c, KNN52-2b, KNN56a, KNN57-1b, KNN64-3a, KNN64-5b, KNN67-4b, KNN68-2b, KNN68-4b, KNN72a, KNN75-4b, KNN81-2b, KNN82-1a and KNN94e.

3. 4. 8. Preliminary characterisation of some bioactive compounds

Three out of the 6 tested strains, namely *Amycolatopsis* strain KNN50-8b and *Streptomyces* strains KNN26b and KNN64-5b were found to produce interesting

specialised metabolites. The results obtained for isolate KNN26b, a member of *S. fimbriatus* 16S rRNA gene clade, are shown in Figure 3.21. Extracts of this organism showed HPLC peaks between 10-15 minutes (molecular weight between 220-590), which suggested the presence of novel metabolites or peptides as these peaks that did not correspond with compounds contained in the Dictionary of Natural Products (Buckingham, 2013), the Reaxys online (http://www.reaxys.com) or the AntiBase 2012 (Laatsch, 2013) databases. Isolate KNN50-8b, a member of *A. ruanii* 16S rRNA gene subclade, produced one major and several minor compounds as shown in Figure 3.22, that did not feature in the databases mentioned above. Isolate KNN64-5b, another member of the *S. fimbriatus* 16S rRNA gene clade, produced 5-6 derivatives of prospective novel aromatic polyene compounds, as shown in Figure 3.23, that did not correspond to entries in any of the three databases. The remaining strains, *Amycolatopsis* isolate KNN50-16d and *Streptomyces* isolates KNN38-1b and KNN90a, did not yield any specialised compounds of interest.

Figure 3.20. Zones of inhibition produced by mycelial extracts of representative strains isolated from the Salar de Atacama environmental sample against the *Bacillus subtilis* reporter strains after incubating overnight at 30°C. Strains which gave blue halos are highlighted.

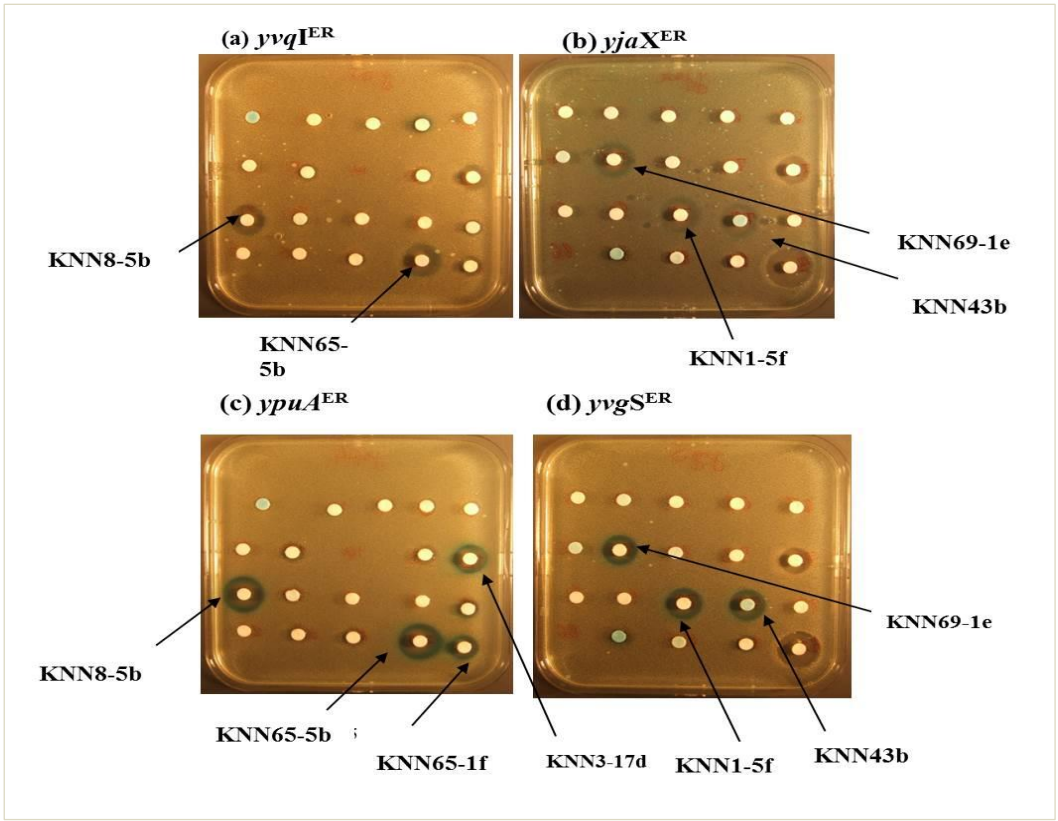


Figure 3.21. HPLC and UV traces obtained in liquid chromatographic analyses for *Streptomyces fimbriatus*-like strain KNN26b extracts prepared from medium 19. The red circle highlights the peaks of prospective novel major and minor novel specialised compounds.

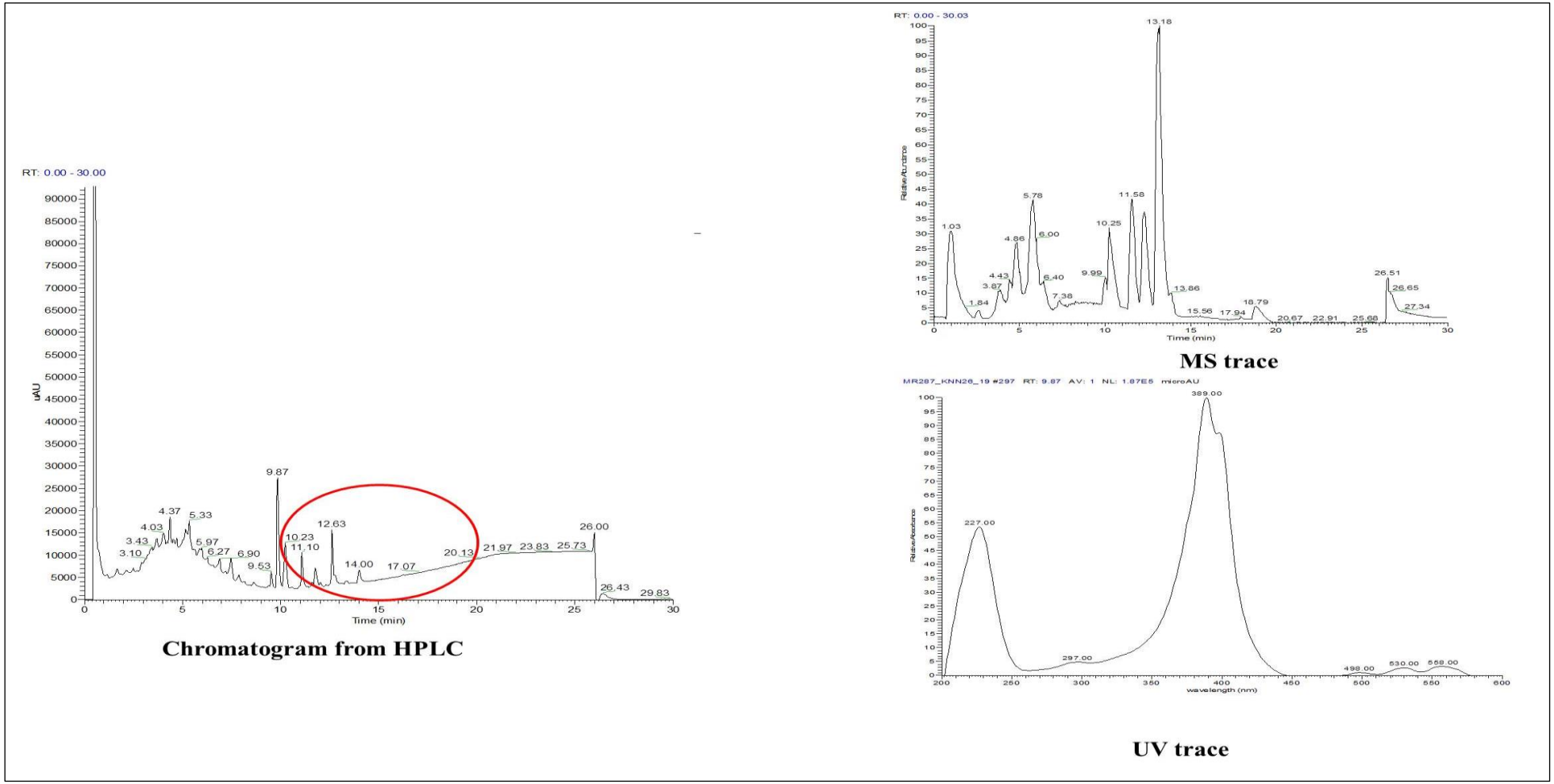


Figure 3.22. HPLC and UV traces obtained in liquid chromatographic analyses for *Amycolatopsis ruanii*-like isolate KNN50-8b extracts prepared from medium 19. The red circle highlights the peaks of major and minor specialised metabolites of interest compounds.

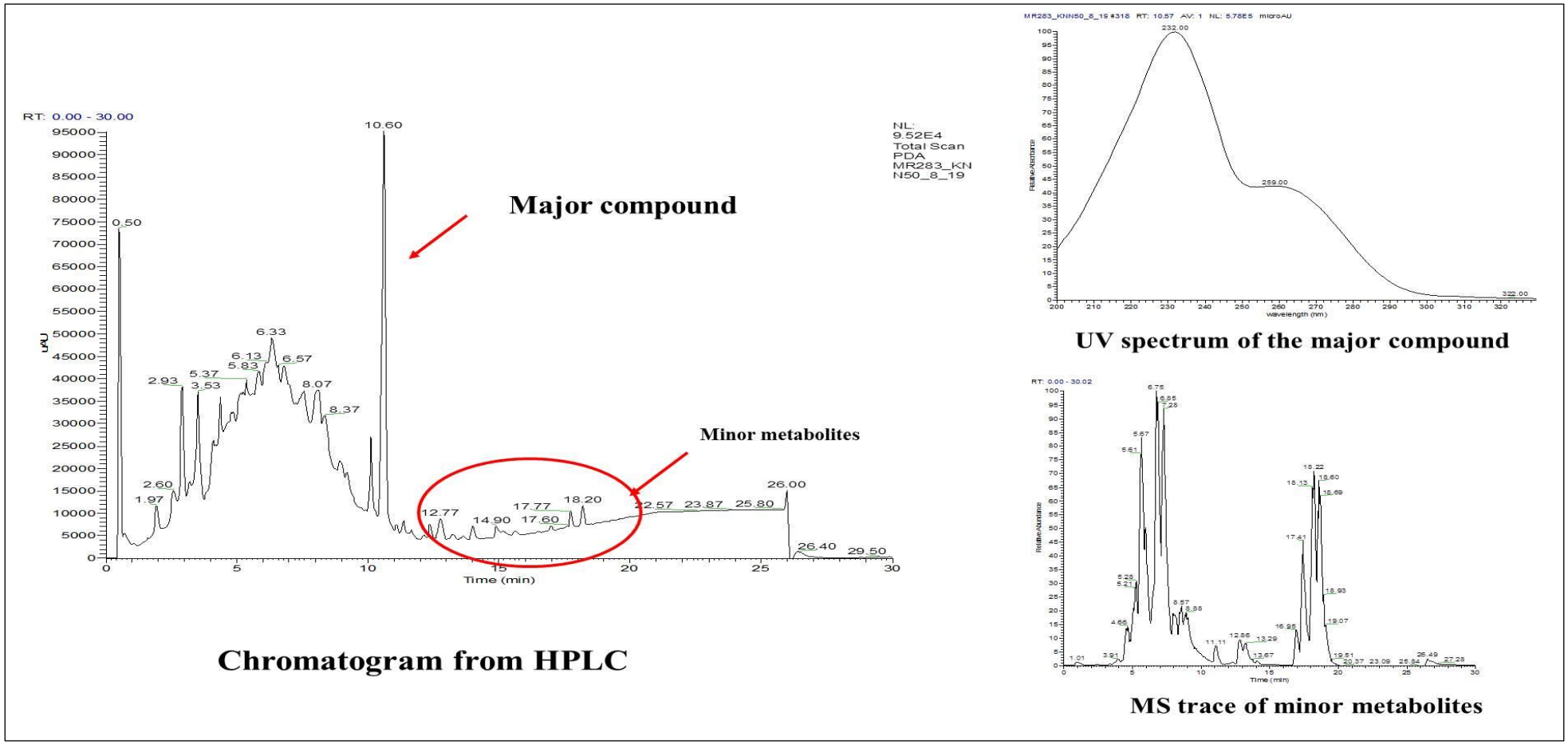
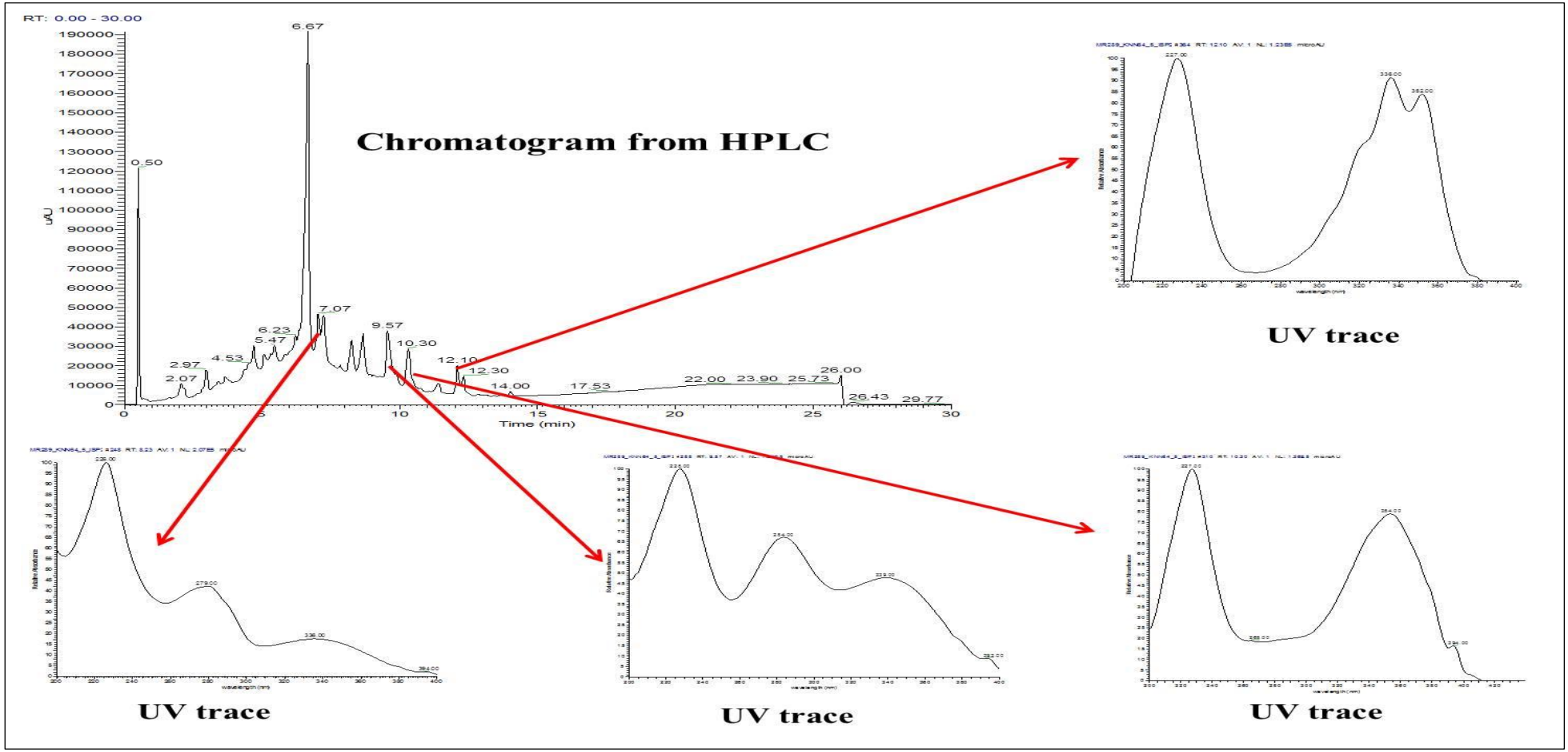


Figure 3.23. HPLC and UV traces obtained in liquid chromatographic analyses for *Streptomyces fimbriatus*-like strain KNN64-5b extracts prepared from glucose yeast-extract malt-extract broth. The individual arrows highlight peak of presumptive novel compounds.



3.5. Discussion

The outcomes of the present investigation confirm and extend those reported in the pioneering biosystematic studies on cultivable Atacama Desert actinobacteria (Okoro *et al*., 2009; Bull & Asenjo, 2012). Once again, small numbers of taxonomically diverse filamentous actinobacteria with the capacity to produce bioactive compounds were isolated from hyper- and extreme hyper-arid Atacama Desert soils. Low numbers of actinobacteria were isolated from the Salar de Atacama and Yungay environmental samples, counts ranging from $0.1x10^2$ to $1.3x10^4$ and $1.0x10^2$ to $5x10^3$ colony forming units per gram dry weight soil, respectively with the highest number in each case recorded from humic acid-vitamin agar plates. In general, these counts are similar to those recorded for comparable soils by Okoro *et al*. (2009). These results together with those from previous culture-dependent (Cameron *et al*., 1966; Opfel & Zerbal, 1967) and culture-independent studies (Wintzingerode *et al*., 1997; Navarro-Gonzalez *et al*., 2003; Drees *et al*., 2006; DiRuggiero *et al*., 2013) show that actinobacteria are present in arid Atacama Desert soils, sometimes in high abundance (Connon *et al*., 2007; Neilson *et al*., 2012).

Nearly all of the colonies growing on the selective isolation plates were identified as actinobacteria based on characteristic morphological features, notably the ability to form extensively branched substrate mycelia, and in many cases, aerial hyphae. Indeed, on this basis most of the representative isolates from the hyper-arid Salar de Atacama and extreme hyper-arid Yungay soils were assigned to the genus *Streptomyces*, namely 79.3 and 73.3%, respectively. The 250 representative streptomycetes from the Salar de Atacama soil were recovered in 43 multi- and 21 single-membered colour-groups and the corresponding 77 representative isolates from the Yungay soil in 14 multi- and 12 single-membered colour-groups. These data show that the streptomycete communities in these soils, while small, are taxonomically diverse as it is known that colour-groups based on the ability of streptomycetes to produce diagnostic pigments on oatmeal and peptone-yeast extract-iron agars are reliable indicators of species diversity (Pathom-aree *et al*., 2006; Antony-Babu & Goodfellow, 2008; Antony-Babu *et al.*, 2010; Goodfellow & Fiedler, 2010). It is evident, therefore, that taxonomically diverse streptomycetes are present in arid Atacama Desert soil, as found in the pilot study of Okoro *et al*. (2009).

Whole-organism hydrolysates of strains taken to represent the *Streptomyces* colour-groups

were rich in the diagnostic marker, *LL*-diaminopimelic acid thereby underpinning the generic status of these taxa (Kämpfer, 2012). It was surprising that only 10 out of the 327 streptomycetes formed melanin pigments on peptone-yeast extract-iron agar as melanin and melanin-like pigments have been shown to protect micro-organisms against UV radiation and oxidants (Casadevall *et al*., 2000; Langfelder *et al*., 2003; Dharmik & Gomashe, 2013). However, there is evidence that some actinobacteria only produce melanin-like pigments under oligotrophic conditions (Reddy *et al.,* 2007) while other micro-organisms, such as cyanobacteria and fungi, may produce secondary metabolites as sunscreens (Cao & Garcia-Puhel, 2011).

The phylogenetic analyses of the 62 Salar de Atacama strains taken to repesent 21 multi- and 6 single-membered colour-groups fell within the evolutionary radiation occupied by the genera *Actinomadura, Amycolatopsis, Kribbella, Lechevalieria, Nonomuraea, Saccharothrix* and *Streptomyces*. Most of the *Amycolatopsis* and *Streptomyces* isolates represented large multi-membered colour-groups, as exemplified by *Amycolatopsis* colour-groups KNN49 (31 isolates) and KNN50 (18 isolates) and *Streptomyces* colour-groups KNN2 (18 isolates), KNN 5 (21 isolates) and KNN48 (20 isolates). These data together with those reported by Okoro *et al.* (2009) indicate that *Amycolatopsis* and *Streptomyces* are the predominant culturable genera present in hyper-arid Salar de Atacama soil. In contrast, the *Actinomadura, Nonomuraea* and all but one of the *Saccharothrix* strains represented colour-groups encompassing between 2 and 5 isolates; the *Kribbella, Lechevalieria* and the remaining *Saccharothrix* strains belonged to single-membered colour-groups. All of these isolates represent genera considered to be rare in natural habitats (Tiwari & Gupta, 2012a, b); of these genera only members of the genus *Lechevalieria* had previously been isolated from Salar de Atacama soil (Okoro *et al*., 2010).

Comparative 16S rRNA gene sequence analyses showed that 25 isolates from the extreme hyper-arid Yungay soil, which represented 6 multi- and 4 single-membered clusters, belonged to the genera *Blastococcus* (KNN47b), *Couchioplanes* (KNN7-2b), *Geodermatophilus* (KNN44-1b, KNN44-3b and KNN44-4b), *Modestobacter* (KNN45-1a, KNN45-2b, KNN45-3b, KNN45-4b and KNN46-2b, KNN46-3b, KNN46-4b, KNN46-6b, KNN46-7a, KNN46-8a, KNN46-9c and KNN49-10f), *Pseudonocardia* (KNN55-1b and KNN55-2b) and *Streptomyces* (KNN22a, KNN23-1b, KNN32-1a, KNN42f, KNN47b and KNN70b); these assignments were underpinned by the results of chemotaxonomic and

morphological data from representatives of these taxa as the latter gave results typical of the constituent genera (Goodfellow *et al.*, 2012a). In this case, most of the isolates were assigned to the genera *Modestobacter* and *Streptomyces*, as illustrated by *Modestobacter* colour-group KNN46 (12 isolates) and *Streptomyces* colour-group KNN48 (15 isolates). Isolates representing single-membered colour-groups were assigned to the genera *Blastococcus* and *Streptomyces* whereas the *Couchioplanes*, *Geodermatophilus* and *Pseudonocardia* strains represented colour-groups that contained 4, 4 and 2 isolates, respectively.

While based on a relatively small number of isolates, the phylogenetic data provide evidence that hyper-arid and extreme hyper-arid Atacama Desert soils are populated by markedly different actinobacterial communities. It is especially interesting that representatives of the genera *Blastococcus, Geodermatophilus* and *Modestobacter*, which together constitute the family *Geodermatophilaceae*, were only found in the extreme hyper-arid soil, as members of these poorly studied genera are typically inhabitants of exposed surfaces, such as ancient monuments and rocks (Urzì *et al*., 2004; Reddy *et al*., 2007; Montero-Calasanz *et al*., 2013), surface soils (Luedemann, 1968) and regoliths (Mevs *et al*., 2004). In constrast, *Amycolatopsis* strains were only isolated from the hyper-arid Salar de Atacama soil; members of this taxon are known to be present in arid Australian soils (Tan *et al*., 2006). Furthermore, *Streptomyces* strains, while prevalent in both the Salar de Atacama and Yungay soils, were with very few exception, recovered in site specific colour-groups. Relatively little in known about the abundance and distribution of the remaining genera in natural habitats through *Actinomadura, Nonomuraea, Pseudonocardia* and *Saccharothrix* strains have been isolated from arid soils (Wink *et al*., 2003; Zitouni *et al*., 2004; Babulola *et al*., 2009; Lee & Lee, 2010; Zhang *et al.*, 2010; Kurapova *et al.*, 2012; Camas *et al*., 2013; Ding *et al*., 2013).

The results of the phylogenetic analyses based on 16S rRNA gene sequences of representative *Streptomyces* strains isolated from the hyper-arid Salar de Atacama soil showed that nearly all of them formed distinct phyletic lines or subclades that can be considered as putatively novel *Streptomyces* species. Similar deductions drawn by Okoro *et al.* (2009) for strains isolated from hyper-arid and extreme hyper-arid Atacama Desert soils were substantiated when three strains forming distinct lineages in the *Streptomyces* 16S rRNA gene tree were validly named as *Streptomyces atacamensis*, *Streptomyces bullii* and *Streptomyces deserti* (Santhaman *et al*., 2012a, b, 2013). In the present study, isolate KNN87, which formed a single-membered colour-group, was found to have an identical 16S rRNA gene

sequence to the type strain of *S. bullii* thereby providing good grounds for considering it to be a member of this species. However, it was especially interesting that most of the *Streptomyces* strains from the present study belonged to well delineated multi-membered subclades recognised by Okoro *et al.* (2009), notably the *S. fimbriatus* subclade and a large taxonomically distinct taxon which encompassed strains shown to synthesised specialised metabolites (Nachtigall *et al*., 2011; Rateb *et al*., 2011a, b; Bull & Asenjo, 2012). The detailed taxonomic relationships within these taxa and their closest phylogenetic neighbours will be considered in Chapter 6, as will relationships between the seven strains which formed a well delineated *Streptomyces* subclade that was most closely related to the type strain of *Streptomyces carpinenis*. Corresponding studies will be reported in Chapter 4 on isolates assigned to the *Amycolatopsis methanolica* 16S rRNA gene clade given their potential biotechnological, ecological and taxonomic significance.

Most of the remaining isolates included in the phylogenetic studies formed distinct phyletic lines within evolutionary space populated by the genera *Actinomadura, Blastococcus, Couchioplanes, Geodermatophilus, Nonomuraea, Pseudonocardia* and *Saccharothrix*, as did the balance of the *Streptomyces* strains. Some of these isolates may well form the nuclei of new species as they shared 16S rRNA gene similarities below the 99.0% cut-off point recommended by Meier- Kolthoff *et al*. (2013) to trigger the need for DNA:DNA pairing studies between potentially novel isolates and very close taxonomic neighbours. Consequently, isolates KNN7-2b, KNN10-4d, KNN34c, KNN44-3b and KNN55-2 probably represent new species in the genera *Couchioplanes, Saccharothrix, Actinomadura, Geodermatophilus* and *Pseudonocardia*, respectively. In contrast, more extensive taxonomic studies, including the application of DNA:DNA pairing or whole-genome sequence procedures, are needed to establish the taxonomic status of the remaining isolates recovered in the genera *Actinomadura*, *Geodermatophilus*, *Pseudonocardia*, *Saccharothrix* and *Streptomyces* and the strains assigned to the genera *Kribbella* and *Nonomuraea*. The relationships between the *Modestobacer* isolates and between them and associated type strains will be considered in Chapter 5.

At the generic level, more extensive actinobacterial diversity was recorded amongst isolates from the hyper-arid Atacama Desert soil when compared with the work of Okoro *et al*. (2009), for in addition to *Amycolatopsis, Lechevalieria* and *Streptomyces* strains a small number of *Actinomadura, Kribbella, Nonomuraea* and *Saccharothrix* strains were isolated.

These differences can be attributed to factors such as sampling error and to the use of different selective media. However, in the present study *Amycolatopsis* and *Streptomyces* strain grew on all of the isolation media, not just on those designed for this purpose. Indeed, the humic acid agar plates not only supported the growth of representative strains of *Amycolatopsis* and *Streptomyces*, but also that of the two *Nonomuraea* strains and three out of the six *Saccharothrix* isolates. Similar results were recorded for the extreme hyper-arid Yungay soil, as exemplified by the isolation of more representatives of the family *Geodermatophilaceae* on humic acid-vitamin agar than on the media designed to isolate members of this taxon. These apparently anomalous results can be attributed to the lack of competition between taxonomically different bacteria, including actinobacteria, on isolation plates, which in turn may reflect the small number of the bacterial propagules in the soil suspensions. Indeed, in light of these results humic acid-vitamin agar can be recommended as the medium of choice for the selective isolation of taxonomically diverse actinobacteria from hyper-arid and extreme hyper-arid Atacama Desert soils. Nevertheless, additional taxonomic surveys of arid Atacama Desert soils should be based on the use of several selective isolation media, not least ones formulated for the isolation members of the family *Conexibacteriaceae*, *Nitriliruptoraceae*, *Patulibacteriaceae*, *Rubrobacteriaceae* and *Solirubrobacteriaceae* all of which have been detected in such soils by pyrosequencing community DNA samples (Neilson *et al*., 2012).

Ninety two out of 136 representative strains (67.6%) isolated from the hyper-arid and extreme hyper-arid Atacama Desert soils showed activity against one or more of the five strains used in the standard plug assays thereby providing further evidence that the use of dereplicated strains results in high hit rates (Qin *et al*., 2009; Goodfellow & Fiedler, 2010; Yuan *et al*., 2014). Most of the isolates showed activity against the *B. subtilis* and *S. aureus* strains, a third against *S. cerevisiae*, but few inhibited the growth of the *E. coli* and *P. fluorescens* strains; these results are in line with those of previous studies on filamentous actinobacteria (Eccleston *et al*., 2008; Qin *et al*., 2009). In contrast, 15 of the isolates from the hyper-arid Salar de Atacama soil (11.0%) and 28 of those from the Yungay (20.6%) did not show any activity against the panel of wild type micro-organisms, most of these isolates belonged to non-streptomycete taxa.

Nine out of the ten strains that inhibited the growth of all of the wild type strains in the plug assays were isolated from the hyper-arid Salar de Atacama soil and the remaining one,

isolate KNN90a, from the extreme hyper-arid Yungay soil. These strains are of particular interest as they may produce one or more novel broad spectrum antibiotics (or a range of specialised metabolites) and hence are prime candidates in the search for such compounds (Fishbach & Walsh, 2009). The ten isolates, which represented eight colour-groups, were all assigned to be the genus *Streptomyces*: strains KNN26b, KNN38-1b, KNN64-3a, KNN64-5b to the S. *fimbriatus* 16S rRNA subclade, strains KNN35-1b and KNN35-2b to *Streptomyces* news ubclade 2, while strain KNN32-1a formed a distinct phyletic line in the *S. rochei* 16S rRNA subclade; the remaining three strains, isolates KNN37-5a, KNN63-2b and KNN90a, were not included in the 16S rRNA sequence analyses. It is also interesting that six of the strains were isolated on humic acid-vitamin agar plates, the remaining four on a Gause's No.1 agar plate. The potential importance of the isolates classified in the *S. fimbriatus* 16S rRNA subclade was underpinned by results from the preliminary chemical analyses which showed that strains KNN26b and KNN64-5b produced potentially novel bioactive compounds.

Twenty two of the Salar de Atacama isolates (23.4%) were found to inhibit cell envelope, cell wall, fatty acid and RNA synthesis as they produced blue halos with the *B. subtilis* reporter strains *ypuA*^{ER}, *yvq*I^{ER}, *yja*X^{ER} and *yvg*S^{ER}, respectively. These isolates belonged to 16 *Streptomyces* colour-groups and hence represent diverse taxa within this genus. Isolate KNN5-25d formed a well distinct phyletic line in the *S. althioticus* 16S rRNA subclade while KNN38-5b was member of *S. fimbriatus* 16S rRNA subclade (the remaining isolates were not included in the 16S rRNA gene sequencing studies). It was particularly interesting that five of the twenty two isolates, strains KNN6-2a, KNN6-10b, KNN9-2c, KNN10-4d and KNN24-2c, were assigned to *Streptomyces* 16S rRNA new subclade 1 which will be considered in Chapter 6 under the heading *Streptomyces leeuwenhoekii* 16S rRNA gene subclade. The 40 isolates (42.6%) which did not exhibit blue halos but did show bioactivity against one or more of the *B. subtilis* reporter strains are of interest as the nature of their bioactivity remains to be determined.

It is becoming increasingly apparent that rare actinobacteria from extreme environments are a useful source of novel bioactive compounds (Bredholt *et al*., 2007, 2008; Genilloud *et al.*, 2011; Tiwari & Gupta, 2012b). However, in the present study only three out of the thirty isolates assigned to the genera *Actinomadura, Amycolatopsis, Blastococcus, Couchioplanes, Geodermatophilus, Kribbella, Modestobacter, Nonomuraea, Pseudonocardia* and *Saccharothrix* showed activity in the plug assays. The three isolates found to be active,

strains KNN10-3b, KNN10-4d, KNN10-5d, inhibited the growth of the *B. subtilis* and *S. aureus* strains and synthesised one or more bioactive compounds that targeted cell envelope, fatty acid and RNA synthesis. It is, however, important to remember that the success or otherwise of screening programmes is not only dependent on the use of novel taxonomically diverse actinobacteria but is also influenced by the composition of media used to cultivate strains. Consequently, strains showing inactivity in screening assays need to be grown on a range of production media as this may have a dramatic effect on the expression of constituent secondary metabolite gene clusters (Goodfellow & Fiedler, 2010).

The results of the present study provide further evidence that small numbers of taxonomically diverse actinobacteria with the capacity to synthesise a broad range of bioactive compounds are present in highly arid Atacama Desert soils. Indeed, this is the first report describing the isolation of members the genera *Actinomadura, Blastococcus, Couchioplanes, Geodermatophilus, Kribbella, Modestobacter, Pseudonocardia* and *Saccharothrix* strains from such soils. However, complementary culture-independent studies of community DNA isolated from Salar de Atacama and Yungay soils are needed to determine whether the taxa isolated in the present study are representative of the actinobacterial communities present in these soils.

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Chapter 4. Classification of thermophilic *Amycolatopsis* strains isolated from arid desert soils

4.1. Abstract

The taxonomic position of twenty six filamentous actinobacteria isolated from a hyper-arid Atacama Desert soil and two from an arid Australian composite soil were established using a polyphasic approach. All of the isolates gave the diagnostic amplification product using 16S rRNA oligonucleotide primers specific for the genus *Amycolatopsis*. 16S rRNA gene analyses showed that all of the isolates belonged to the *Amycolatopsis methanolica* 16S rRNA gene subclade. Representative isolates had chemotaxonomic and morphological properties typical of members of the genus *Amycolatopsis*. The Atacama Desert isolates were assigned to one or other of two recognised species, namely *Amycolatopsis ruanii* and *Amycolatopsis thermalba* based on 16S rRNA gene sequence, DNA:DNA relatedness and phenotypic data; emended descriptions are given for these species. In contrast, the two strains from the arid Australian composite soil, isolates GY024 and GY142, formed a distinct branch at the periphery of the *A. methanolica* 16S rRNA gene subclade, a taxon that was supported by all of the tree-making algorithms and by a 100% bootstrap value. Isolate GY024 was distinguished from the type strains of all of species classified in the *A. methanoloca* 16S rRNA subclade using a broad range of phenotypic properties. The combined genotypic and phenotypic data show that strain GY024 merits recognition as a new species within the *A. methanolica* group of thermophilic strains. The name proposed for the new species is *Amycolatopsis deserti* sp. nov.; the type strain is GY024^{T} (=NCIMB……=NRRL….).

4.2. Introduction

The genus *Amycolatopsis* Lechevalier *et al.* (1986) is classified in the family *Pseudonocardiaceae* (Labeda & Goodfellow, 2012a) of the order *Pseudonocardiales* (Labeda & Goodfellow, 2012b). It can be distinguished from other genera classified in the family by using genus-specific 16S rRNA oligonucleotide primers (Tan *et al*., 2006) and by a combination of chemotaxonomic and morphological markers (Labeda *et al*.,

2011; Labeda & Goodfellow, 2012a). The genus encompasses 65 validly published species (Euzéby, 2013) most of which have been proposed using polyphasic taxonomic data, as exemplified by descriptions of *Amycolatopsis cihanbeyliensis* (Tatar *et al*., 2013), *Amycolatopsis jiangsuensis* (Xing *et al*., 2014), *Amycolatopsis magusensis* (Camas *et al*., 2013) and *Amycolatopsis umgeniensis* (Everest *et al*., 2013). Closely related *Amycolatopsis* species can be distinguished by using a combination of phenotypic features (Tan & Goodfellow, 2012; Camas *et al*., 2013; Everest *et al*., 2013). Members of genus are widely distributed in natural habitats, especially in geographically diverse soils (Saintpierre-Bonaccio *et al*., 2005; Tan *et al*., 2006; Nie *et al*., 2012; Camas *et al*., 2013; Everest *et al*., 2013), others have been isolated from clinical material (Labeda *et al*., 2003, Huang *et al*., 2004), marine sediment (Bian *et al*., 2009), a salt lake (Tang *et al*., 2010) and plants (Duangmal *et al*., 2011; Miao *et al*., 2011).

Amycolatopsis species can be assigned to several multi- and single- membered phyletic lines based on *gyrB, recN* and 16S rRNA sequence analyses (Everest & Meyers, 2009; Everest *et al*., 2011; Tan & Goodfellow 2012)., The two most populated phyletic groups, the *Amycolatopsis methanolica* and *Amycolatopsis orientalis* 16S rRNA subclades, are difficult to distinguish using phenotypic criteria though members of the former grow well at temperatures up to 45^{o}C (Tan & Goodfellow, 2012; Zucchi *et al*., 2012a, c) and hence can be considered to be thermophilic actinobacteria (Cross, 1968; Brock, 1986). The *A. methanolica* 16S rRNA subclade encompasses *Amycolatopsis methanolica* (De Boer *et al*., 1990), the earliest described species, *Amycolatopsis eurytherma* (Kim *et al*., 2002a), *Amycolatopsis granulosa* (Zucchi *et al*., 2012a), *Amycolatopsis ruanii* (Zucchi *et al*., 2012c), *Amycolatopsis thermoflava* (Chun *et al*., 1999), *Amycolatopsis thermophila* (Zucchi *et al*., 2012a), *Amycolatopsis thermalba* (Zucchi *et al*., 2012b), *Amycolatopsis tucumanensis* (Albarraćin *et al*., 2010) and *Amycolatopsis viridis* (Zucchi *et al*., 2012c). These species can be distinguished using a broad range of phenotypic properties (Zucchi *et al*., 2012b, c) and are of potential value in biotechnology, notably as vehicles for fermentation overproduction of aromatic amino acids (De Boer *et al*., 1990; Abou-Zeid *et al*., 1995) and as agents of bioremediation (Albarracín *et al*., 2008, 2010). Members of the *A. methanolica* 16S rRNA gene clade are rare actinomycetes as all but one of the constituent species, namely *A. eurytherma*, rest on descriptions of single strains that were mainly isolated from arid soil collected in Australia, China and New Guinea.

The present study was designed to establish the taxonomic provenance of strains isolated from arid Atacama Desert and Australian desert soils and assigned to the *A. methanolica* 16S rRNA gene clade based on characteristic colonial features and an ability to grow at 50^{o}C. The isolates were compared with the type strains of species classified in the *A. methanolica* 16S rRNA gene clade using a polyphasic taxonomic approach. The resultant data showed that the twenty six Atacama Desert isolates were *bona fide* members of either *A. ruanii* or *A. thermalba* whereas the two isolates from the Australian soil belong to a new *Amycolatopsis* species for which the name *Amycolatopsis taniae* sp. nov. is proposed.

4.3. Materials and Methods

4. 3. 1. Isolation of *Amycolatopsis* strains

Filamentous actinobacteria were sought from a hyper-arid soil sample collected from the Chaxa de Laguna of the Salar de Atacama in the Atacama Desert of Northern Chile (23^{o} 17'S, 68^{o} 100' W) using a range of selective isolation media (Table 4.1). The media were incubated at 28^{o}C for 3 weeks following inoculation with a 10^{-1} soil suspension that had been heated at 55^{o}C for 6 minutes, as described by Okoro *et al*. (2009). Counts of *Amycolatopsis* strains growing on each of the isolation media were expressed as the number of colony forming units per gram dry weight soil. Strains GY024 and GY142 were isolated on SM1 agar plates that had been inoculated with tenfold dilutions of an arid Australian composite soil and incubated at 28^{o}C for 3 weeks, as described by Tan *et al.* (2006).

4. 3. 2. Test strains, maintenance and cultural conditions

Twenty-six isolates from the hyper-arid Salar de Atacama soil with the typical colonial appearance of *Amycolatopsis* strains were taken from the selective isolation plates and subcultured onto modified Bennett's agar plates (Jones, 1949), incubated at 28^{o}C for 14 days and checked for purity by microscopic examination of Gram-stained smears. These Atacama strains together with isolates GY024^{T} and GY142 and the type strains of *Amycolatopsis* species classified in the *A. methanolica* 16S rRNA gene subclade were maintained on modified Bennett's agar slopes (Jones, 1949) and in 20%, v/v glycerol -20 and -80^{o}C. Biomass for the chemotaxonomic studies carried out on representative

isolates and the type strains of *A. ruanii* and *A. thermalba* was prepared in shake flasks (200 revolutions per minute) of yeast extract-malt extract broth (International Streptomyces Project [ISP] medium 2; Shirling & Gottlieb, 1966) for 14 days at 28^{o}C, washed twice in distilled water and freeze-dried. Biomass for the molecular systematic studies was prepared in the same way but was stored as washed preparations at -20^{o}C.

4. 3. 3. PCR amplification using genus-specific primers

Genomic DNA was extracted from the twenty six representatives of the Salar de Atcama soil and from the 2 isolates from the Australian composite soil and the DNA preparations examined to determine whether they produced the diagnostic amplification product with the genus-specific *Amycolatopsis* 16S rRNA oligonucleotide primers AMY2 amd ATOP, as described by Tan *et al.* (2006).

4. 3. 4. Phylogeny

Genomic DNA was extracted from all of the isolates and PCR amplification and 16S rRNA gene sequencing achieved after Kim and Goodfellow (2002). 16S rRNA gene sequences of the strains [1310-1350 nucleotides (nt)] were aligned manually, using MEGA version 5 software (Tamura *et al*., 2011), against corresponding sequences of the type strains of *Amycolatopsis* species retrieved from the GenBank database using the EzTaxon-e server (Kim *et al*., 2012) and phylogenetic trees generated using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from MEGA 5software programme. The same procedure was used to examine relationships between the isolates and closely related *Amycolatopsis*. The appropriate nucleotide substitutions for the maximum-likelihood analyses were selected by the Bayesian Information Criterion (BIC) using MEGA 5 software and found to follow the Tamura 3-parameter (T92+G+I) model (Tamura, 1992). The topologies of all of the evolutionary trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset based on 1,000 replicates using MEGA 5 software. The root position of the neighbour-joining trees were inferred by using *Actinokineospora riparia* DSM 44259^{T} (GenBank AF114802) as the outgroup.

4. 3. 5. Chemotaxonomy

All of the isolates were examined for the presence of isomers of diaminopimelic acid (A_2pm) using the procedure described by Staneck and Robert (1974). In addition, six strains from the Salar de Atacama soil (isolates KNN49-5e, KNN49-26a, KNN50-8b, KNN50-2e, KNN50-11c and KNN50-16d) and isolates GY024 and GY142 from the Australian composite soil were examined for additional chemotaxonomic markers considered to be typical of members of the genus *Amycolatopsis* (Tan & Goodfellow, 2012). Standard procedures were used to determine the predominant isoprenoid quinones (Minnikin *et al*., 1984**),** muramic acid type (Uchida *et al.*, 1999), sugars (Schaal, 1985), and polar lipids (Minnikin *et al.*, 1984), using *A. ruanii* NMG112^T and *A. thermalba* SF45^T as controls; mycolic acids were sought from these isolates using the acid methanolysis procedure described by Minnikin *et al*. (1980). Fatty acids extracted from the representative isolates and type strains of *A. ruanii* and *A. thermalba* were methylated and analysed using the standard Sherlock Microbial Identification (MIDI) system, ACTINO version 5. The G+C mol% of the DNA of the representatives from the hyper-arid Salar de Atacama soil isolates and isolates GY024^T and GY142 from the Australian composite soil were determined, in triplicate, following the procedure described by Gonzalez and Saiz-Jimenez (2002).

4. 3. 6. Phenotypic tests

All of the isolates were examined for cultural properties following growth on oatmeal and peptone-yeast extract-iron agars (ISP media 3 and 6; Shirling & Gottlieb, 1966) after incubation for 14 and 4 days, respectively. The isolates and the type strains of species classified in the *A. methanolica* 16S rRNA gene subclade were also examined for phenotypic properties known to be of value in *Amycolatopsis* systematics (De Boer *et al*., 1990; Kim *et al*., 2002; Tan & Goodfellow, 2012) following incubation at 28^{o}C for 3 weeks. Additional biochemical features were obtained by using API ZYM test strips (BioMérieux), following the manufacturer's protocol. A standard inoculum equivalent to 5.0 on the McFarland scale (Murray *et al.*, 1999) was used to inoculate the test media. All of the tests were carried out in duplicate.

4. 3. 7. DNA-DNA relatedness

DNA:DNA relatedness experiments were carried out, in triplicate, between six selected pairs of strains (Table 4.3) by measuring the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔTm) preparations using the fluorimetric method, as described by Gonzalez & Sait-Jimenez (2005). The optimal temperature for renaturation (T_m) was calculated using the equation T_{or}-0.51 (GC%) + 47.

4.4. Results

Selective isolation, enumeration and initial classification of isolates. Low numbers of strains growing on the isolation media were assigned to the genus *Amycolatopsis* (Table 4.1) as they formed characteristic flat to slightly raised, round colonies covered by a powdery white aerial mycelia. The highest count, $6.0x10^2$ cfu/g dry weight soil, was recorded from the humic acid-vitamin agar plates. The twenty six isolates taken to represent the strains isolated from the Salar de Atacama soil and the two representatives from the Australian composite soil produced the genus-specific amplification product with the AMY2 and ATOP 16S rRNA primers and formed yellowish white aerial hyphae, yellowish white substrate mycelia but no diffusible pigments on oatmeal agar; melanin pigments were not produced on peptone-yeast extract-iron agar.

Table 4.1. Numbers and representatives of *Amycolatopsis* isolates growing on the selective isolation media after incubation for 21 days at 28^{o}C.

Selective media	Target organisms	Number of *Amycolatopsis* isolates (cfu/g dry weight soil)	Reprresentative isolates
Gause's No.1 agar supplemented with nalidixic acid (10 µg/ml) (Zakharova *et al.*, 2003)	Rare or uncommon actinobateria	$4.3x10^2$	KNN49-6a, KNN49-26a, KNN50-1a
Humic acid-vitamin agar (Hayakawa & Nonomura, 1987)	*Streptosporangiaceae* spp.	$6.0x10^2$	KNN49-10b, KNN49-12b, KNN50-8b, KNN50-9b
Minimal medium agar (Johnson *et al.*, 1981)	Rare or uncommon actinobateria	$4.7x10^2$	KNN49-11, KNN50-4, KNN50-5, KNN50-11, KNN50-12, KNN50-13

Oligotrophic agar (Senechkin *et al.*, 2010)	Rare or uncommon actinobateria	$5.7x10^2$	KNN50-7, KNN50-15, KNN50-14, KNN50-16, KNN50-17, KNN50-18
Starch-casein agar (Küster & Williams, 1964)	*Streptomyces* spp.	$0.3x10^2$	KNN49-1
SM1 agar supplemented with neomycin (1 µg/ml) (Tan *et al.*, 2006)	*Amycolatopsis* spp.	$4.7x10^2$	KNN49-3e, KNN49-5e, KNN49-32e, KNN50-2e, KNN50-6e, KNN50-10e

All of the media were supplemented with cycloheximide and nystatin, each at 25 µg ml^{-1}.

Phylogeny. All of the isolates fell within the evolutionary radiation encompassed by the *A. methanolica* 16S rRNA gene subclade, which, in turn, was sharply separated from all of the remaining *Amycolatopsis* strains (Figure S1). The recovery of the isolates within the *A. methanolica* 16S rRNA gene subclade in the more detailed 16S rRNA gene sequence analyses was supported by all of the tree-making algorithms and by a 100% bootstrap value (Figure 4.1). The isolates from the hyper-arid Salar de Atacama soil were closely related either to the type strain of *A. ruanii* or to the corresponding *A. thermalba* strain, results underpinned by all of the tree-making methods and in each case by a 99% bootstrap value. Indeed, four strains (isolates KNN49-3e, KNN49-5e, KNN50-5c and KNN50-13c) had identical 16S rRNA gene similarities with *A. ruanii* NMG112^T, the remainder shared similarities with the latter within the similarity range 99.7 to 99.9 %, values corresponding to 1 to 4 nt diffences at 1313 to 1403 locations (Table S1). Similarly, four strains, isolates KNN49-6a, KNN50-1a, KNN50-2e and KNN50-12c, had identical 16S rRNA gene sequences with *A. thermalba* SF45^T, the remaining nine isolates were found to have similarities with the latter within the 16S rRNA similarity range 99.8 to 99.9%, a value equivalent to 1 to 3 nt differences at 1315 to 1361 locations.

The Australian strains, isolates GY024^T and GY142, formed a distinct branch at the periphery of the *A. methanolica* 16S rRNA gene subclade, the integrity of this taxon was supported by all of the tree-making algorithms and by a 100% bootstrap value (Figure 4.1). The two isolates shared a 16S rRNA gene similarity of 99.7%, which corresponded to 4 nt differences at 1366 locations. Strain GY024^T was most closely related to the type strain of *A. ruanii* having a 98.9% 16S rRNA gene sequence similarity with the latter, a value equivalent to 15 nt differences at 1328 sites. Similarly, isolate GY142 was marginally more closely related to *A. thermalba* SF45^T than to *A.*

ruanii NMG112^{T} sharing a 16S rRNA gene sequence similarity with the former of 98.8%, a value equivalent to 17 nt differences at 1369 sites.

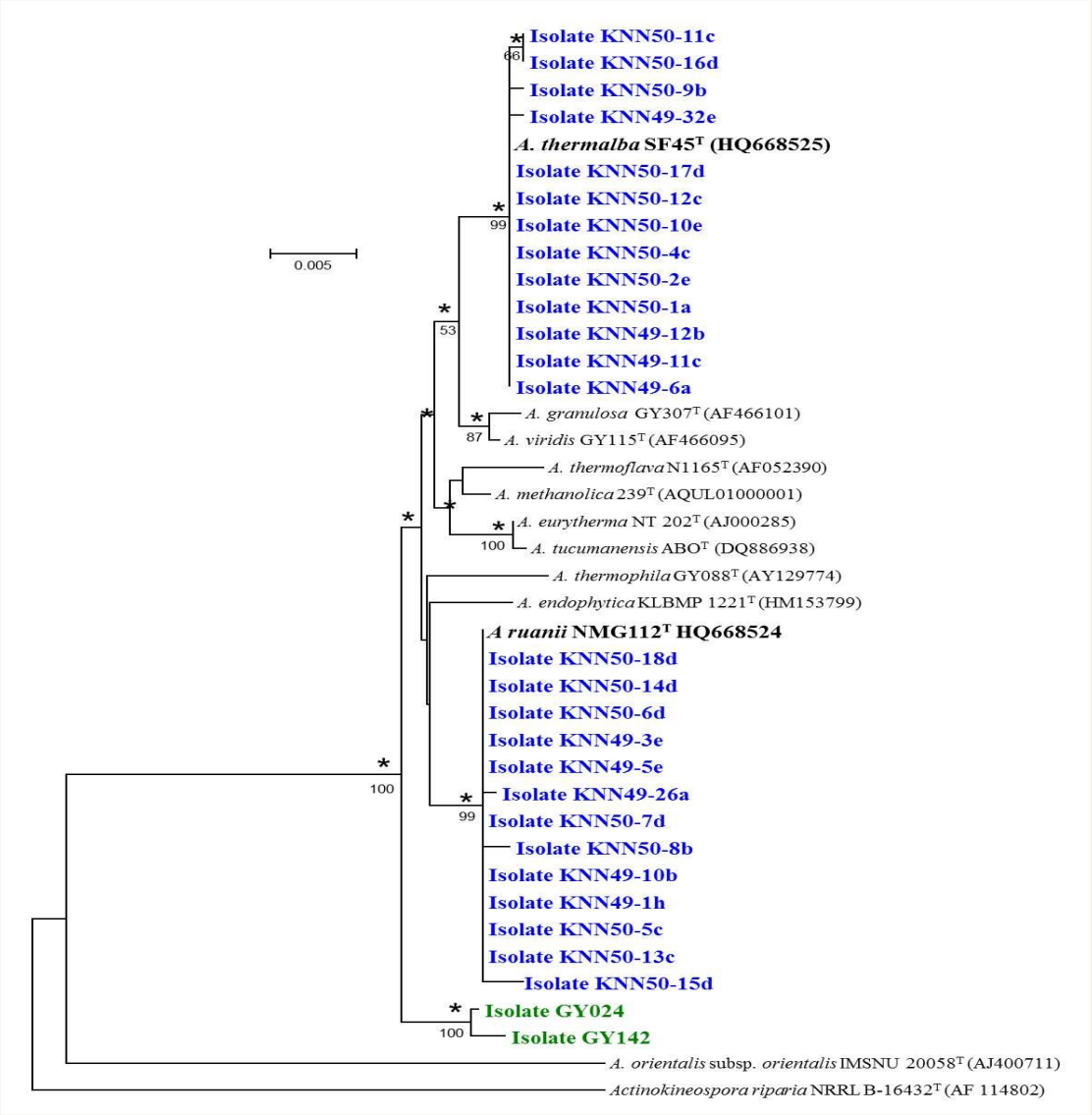


Figure 4.1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (~1350 bp) showing relationships between the isolates and and between them and the type strains of closely related *Amycolatopsis* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets;only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position. Blue and green characters indicated the strains were isolated from Salar de Atacama, Atacama Desert, Chile and the composite Australia soil, respectively.

Chemotaxonomy. Whole organism hydrolysates of all of the isolates contained *meso*-A_2pm. The six representative isolates from the Salar de Atacama soil, the two from the Australian composite soil and the type strains of *A. ruanii* and *A. thermalba* contained arabinose and galactose in whole-organism hydrolysates, N-acetylated muramic acid, tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue, and major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol and phosphatidylinositol, as exemplified in Figure 4.2, but lacked mycolic acids. All of the isolates contained major proportions (>10%) of *iso*-$C_{16:0}$ (23.5-30.6%), $C_{16:0}$ (12-15.4%) and *anteiso*-$C_{17:0}$ (12.2-13.1%), small amounts (<10%) of *iso*-$C_{15:0}$ (3.3-4.4%), $C_{16:1}\omega6c/C_{16:1}$ (2.6-6.0%), *iso*-$C_{17:0}$ (5.8-7.1%), $C_{17:1}\omega6c$ (5.6-9.7%) and $C_{18:0}$ (6.7-8.5) and trace to small amounts of several components, some of which were discontinuously distributed (Table 4.2). The DNA G+C contents of isolates KNN49-5e, KNN49-26e and KNN50-8b, representatives of the *A. ruanii* 16S rRNA gene subclade, were 76.18±1%, 75.18±1% and 77.18±1%, respectively whereas those for the representatives of the *A. thermalba* 16S rRNA gene subclade, namely isolates KNN50-2e, KNN50-11c and KNN50-16d were 78.17±1%, 79±1% and 78.28±1%, respectively and those for isolate GY024^T and GY142 were 70.1±1% and 70.4±1%, respectively.

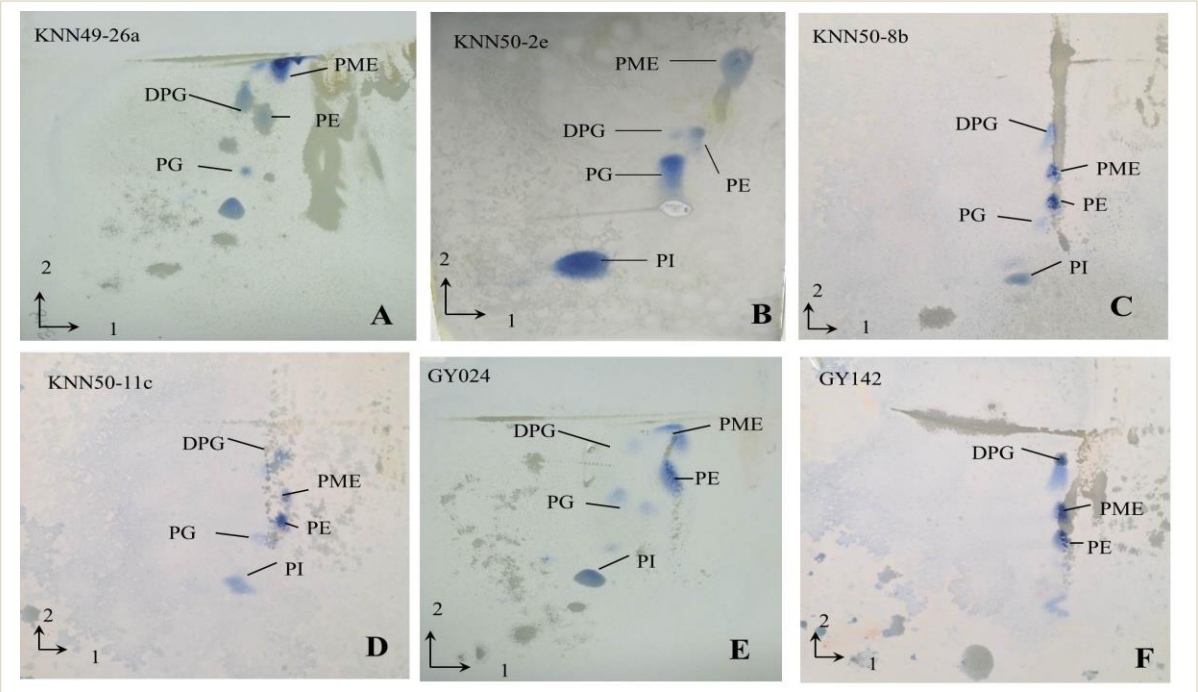


Figure 4.2. Two-dimensional thin-layer chromatography of polar lipids of isolates (A) KNN49-26a, (B) KNN50-2e, (C) KNN50-8b, (D) KNN50-11c, (E) GY024 and (F)

GY142 stained with molybdenum blue spray (Sigma). Chloroform:methanol:water (32.5:12.5:2.0, v/v) was used in the first direction, followed by chloroform : acetic acid : methanol : water (40.0 : 7.5 : 6.0 : 2.0,v/v) in the second direction. Abbrevations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidyl inositol; PME, phosphatidylmethylethanolamine.

Table 4.2 Fatty acid composition (%) of *Amycolatopsis* isolates and the type strains of their closest phylogenetic neighbours.

Fatty acids	Strains							
	KNN49-5e	KNN49-26a	KNN50-8b	KNN50-2e	KNN50-11c	KNN50-16d	*A. ruanii* NMG112^T	*A.thermalba* SF45^T
iso-$C_{11:0}$	-	-	-	-	-	-	0.1	-
iso-$C_{12:0}$	-	-	-	-	-	-	0.1	-
$C_{12:0}$	-	0.2	0.2	0.3	0.3	0.2	0.1	0.3
$C_{11:0}$ 2OH	-	-	-	-	-	-	0.1	-
iso-$C_{13:0}$	-	0.1	-	-	0.1	-	0.5	-
anteiso-$C_{13:0}$	-	-	-	-	-	-	0.1	-
$C_{13:0}$	-	-	-	-	-	-	0.1	-
iso-$C_{14:0}$	0.6	0.5	0.4	0.5	0.5	0.5	3.8	0.6
anteiso-$C_{14:0}$	-	-	-	-	-	-	0.2	-
$C_{14:0}$	0.6	0.7	0.6	0.7	0.9	0.6	0.8	0.6
iso-$C_{15:0}$	3.3	4.5	4.2	4.2	4.1	4.4	23.7	47.1
anteiso-$C_{15:0}$	1.0	1.2	1.0	1.5	1.5	1.0	6.3	1.1
$C_{15:1}$ ω6c	0.1	-	-	-	-	-	-	-
iso-$C_{16:1}$ H	0.8	0.8	0.9	0.5	0.5	0.8	-	0.9
$C_{16:0}$ N alcohol	0.1	0.2	0.2	0.1	0.1	0.2	0.3	-
iso-$C_{16:0}$	30.6	28.5	29.3	29.4	28.6	30.1	12.8	35.7
anteiso-$C_{16:0}$	0.4	0.9	1.2	0.9	1.0	0.8	0.7	0.5
$C_{16:1}$ ω6c/$C_{16:1}$ ω7c	6.0	5.5	3.2	3.3	2.6	5.4	6.7	7.2
$C_{16:0}$	15.4	12.8	14.2	13.6	14.2	12.0	14.4	9.8
iso-$C_{17:1}$ ω9c/$C_{16:0}$ 10 methyl	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.4
anteiso-$C_{17:1}$ ω9c	0.1	0.2	0.2	0.2	0.2	0.2	-	-
iso-$C_{17:0}$	5.8	6.7	7.1	6.8	6.9	6.7	3.9	8.1
anteiso-$C_{17:0}$	12.2	15.0	16.2	16.6	18.1	13.9	5.5	12.8
$C_{17:1}$ ω8c	1.4	0.8	0.7	0.6	0.4	1.2	3.5	0.7
$C_{17:1}$ ω6c	5.6	9.4	8.3	9.0	9.3	8.3	1.3	7.4
$C_{17:0}$	1.5	1.5	1.8	1.5	1.4	1.7	8.9	0.8
$C_{17:0}$ 10 methyl	0.2	0.1	-	-	-	0.1	0.3	-
$C_{16:0}$ 3OH	-	-	-	0.1	0.1	0.1	-	-

$C_{18:3}$ ω6c (6, 9, 12)	-	-	-	0.2	-	0.2	-	-
$C_{17:0}$ 10-methyl	0.2	-	0.1	-	-	-	-	-
iso-$C_{18:0}$	0.5	0.4	0.5	0.5	0.4	0.5	0.2	0.3
$C_{18:1}$ ω9c	4.7	2.1	1.7	1.5	1.0	2.8	1.5	1.5
$C_{18:1}$ ω6c/$C_{18:1}$ ω7c	0.3	-	-	-	-	-	-	-
$C_{18:0}$	8.5	6.8	6.5	7.0	6.7	7.3	3.7	4.1
$C_{17:0}$ 2OH	-	-	-	-	0.1	-	-	-
$C_{17:0}$ 3OH	-	-	-	-	-	-	0.2	-
cyclo-$C_{19:0}$ ω10c/19ω6	0.6	1.0	1.3	1.0	1.2	1.0	-	0.3

DNA:DNA relatedness. It is apparent from Table 4.3 that without exception, the DNA:DNA relatedness studies carried out by thermal denaturation of homologous DNA and associated heterologous DNA hybrid preparations between members of the *A. ruanii* and *A. thermalba* 16S rRNA gene subclades revealed Δ*T*m values below the cut-off point recommended for the recognition of genomic species (Δ*T*m >5.0^{o}C) and hence above the DNA:DNA relatedness threshold of 70% (Wayne *et al*., 1987). In contrast, the corresponding assays between the type strains of *A. ruanii* and *A. thermalba* were above these recommended thresholds, these strains shared a Δ*T*m value of 13±1^{o}C and a DNA:DNA relatedness value of 30±0.1%. The results of these experiments are exemplified in Figure 4.3.

Table 4.3. Relationships between representative isolates and between them and their most closely related type strain based on Δ*T*m (^{o}C) values together with corresponding figures expressed as percentage DNA:DNA relatedness values.

Strains	**Mean Δ*T*m* (^{o}C)**	**DNA:DNA relatedness values ± standard deviations**	**Nucleotide base differences / total number of 16S rRNA nucleotides**
***Amycolatopsis ruanii* subclade:**			
Isolate KNN49-5e versus isolate KNN49-26a	2.1±0.1	82+0.1	1/1382
Isolate KNN49-5e versus isolate KNN50-8b	4.2±0.1	72±0.1	2/1314
Isolate KNN49-26e versus isolate KNN50-8b	4.2±0.1	72±0.1	3/1316
Isolate KNN49-5e versus *A. ruanii* NMG112^{T}	3.2±0.1	75±0.1	0/1382
Isolate KNN49-26e versus *A. ruanii* NMG112^{T}	4.0±0.1	76+0.1	4/1402
Isolate KNN50-8b versus *A. ruanii* NMG112^{T}	3.9±0.1	76±0.1	4/1359

***Amycolatopsis thermalba* subclade:**			
Isolate KNN50-2e versus isolate KNN50-16d	2.7±0.1	80±0.1	1/1311
Isolate KNN50-11c versus isolate KNN50-16d	3.1±0.1	76±0.1	2/1321
Isolate KNN50-2e versus *A. thermalba* SF45^T	2.9±0.1	80±0.1	0/1357
Isolate KNN50-16d versus *A. thermalba* SF45^T	2.7±0.1	80±0.1	1/1315
A. ruanii NMG112^T versus *A. thermalba* SF45^T	13.0±0.1	30±0.1	13/1392

* Based on triplicate analyses.

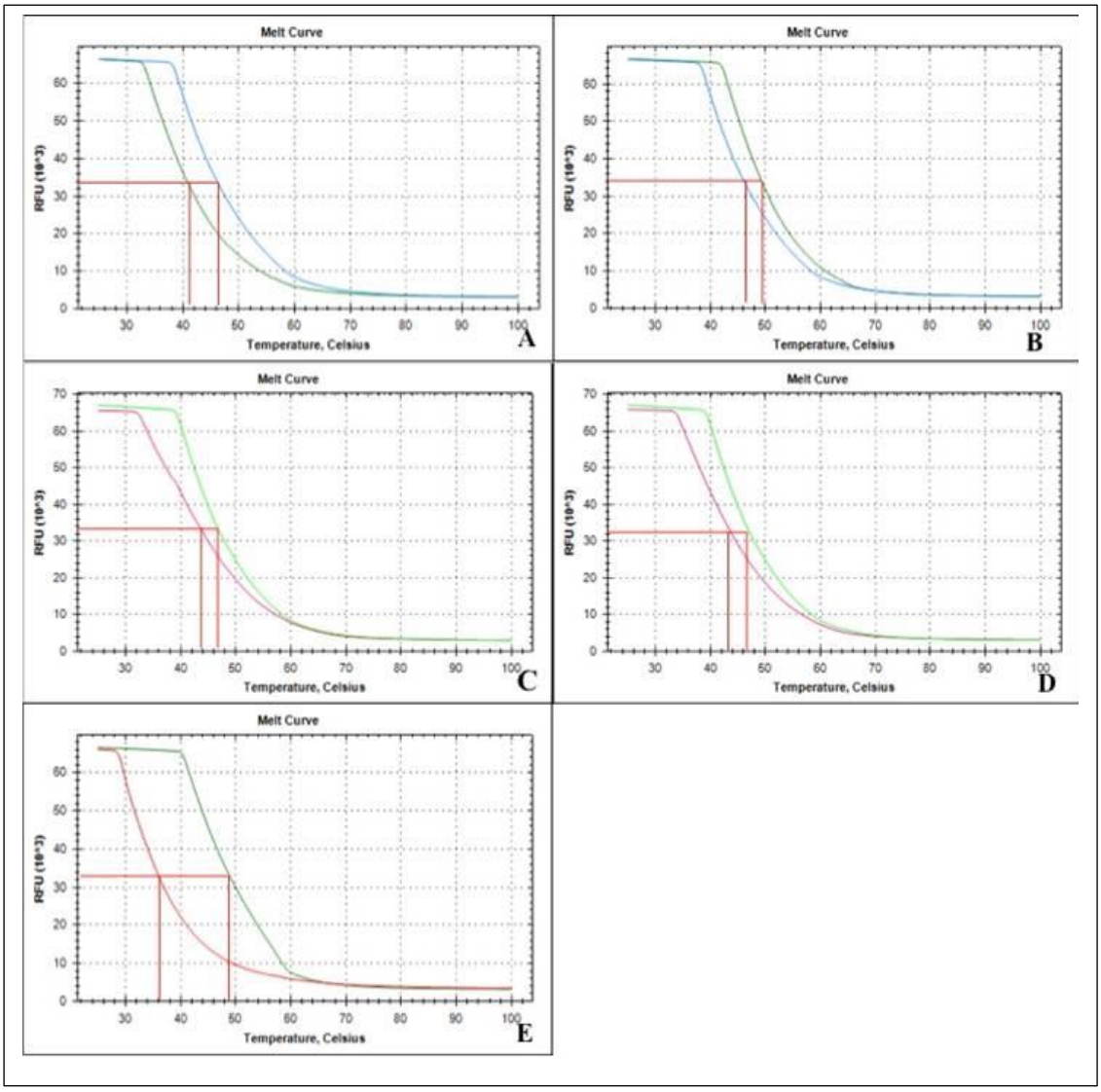


Figure 4.3. DNA:DNA relatedness studies between (A), isolate KNN49-5e gDNA and isolate KNN49-5e : KNN50-8b hybrid DNA; (B), isolate KNN50-8b gDNA and isolate KNN50-8b : *A. ruanii* NMG112^T hybrid DNA; (C), isolate KNN50-2e gDNA and isolate KNN50-2e : isolate KNN50-16d hybrid DNA; (D), isolate KNN50-2e gDNA and isolate isolate KNN50-2e: *A. thermalba* SF45^T hybrid and (E), *A. ruanii* NMG112^T gDNA and *A. ruanii* NMG112T: *A. thermalba* SF45^T hybrid DNA.

Phenotypic properties. Identical results were obtained between the duplicated cultures for all of the phenotypic tests. The isolates and the type strains of *Amycolatopsis* species classified in the *A. methanolica* 16S rRNA gene cluster grew from pH 6.0 to 10, from 30 to 50^{o}C, in the presence of 2.5% NaCl, degraded arbutin, casein, cellulose, gelatin, Tween 40 and 60, produced α-chymotrypsin, acid phosphatase and α-fucosidase (API ZYM tests) and formed acid from L-fructose and L-fucose. In contrast, the isolates and marker strains did not grow at 4^{o}C, in the presence of 10% NaCl, did not degraded chitin, guanine, Tween 20 or 80, did not produce acid from D-glucose, D-glycogen, did not grow on butanol, ergosterol, propan-1-ol; propan-2-ol, sodium hippurate or sodium malate as sole carbon sources (at 1%, w/v), did not produce α-galactosidase (API ZYM tests) and were sensitive to lysozyme (0.005%, w/v), but resistant to vancomycin hydrochloride (10 μg/ml).

It can be seen from Table 4.4 that all of the isolates assigned to the *A. ruanii* or *A. thermalba* 16S rRNA gene subclades gave identical results to those recorded for the respective type strain. The members of these taxa can be distinguished from one another and from the type strains of the remaining *Amycolatopsis* species classified in the *A. methanolica* 16S rRNA gene subclade using a combination of phenotypic properties. The *A. ruanii* strains, unlike the *A. thermalba* strains, degraded hypoxanthine, used D-arabinose and D-galactose as sole carbon sources, grew in the presence of 7.5% NaCl (w/v) and produced N-acetyl-β-glucosamidase. In contrast, the *A. thermalba* strains, unlike the *A. ruanii* counterparts, hydrolysed allantoin, produced acid from xylose (1%, w/v), and used methanol (1%, v/v) as a sole carbon source, produced α- and β-glucosidases and naphthol-AS-BI-phosphohydrolase (API ZYM tests) and grew at 20^{o}C and 55^{o}C. Similarly, isolates GY024^{T} and GY142 can be distinguished from one another and from all of the *Amycolatopsis* type strains belonging to species classified in the *A. methanolica* 16S rRNA subclade using a range of phenotypic properties (Table 4.4). Isolate GY024^{T}, unlike isolate GY142, degraded adenine and guanine and produced acid from D-adonitol, conversely only isolate GY142 formed acid from D-galactose, D-melibiose, D-melezitose, D-ribose, D-sucrose and D-turanose and grew in the presence of 7.5% NaCl (w/v).

Table 4.4. Phenotypic characteristics distinguishing between *Amycolatopsis* isolates and between them and validly named *Amycolatopsis* species classified in the *Amycolatopsis methanolica* 16S rRNA gene clade.

Phenotypic tests	***A. methanolica* 239^T**	***A. eurytherma* NT 202^T**	***A. granulosa* GY 037^T**	***A. ruanii* NGM 112^T + 13 Salar de Atacama isolates***	***A. thermoflava* NRRL1433^T**	***A. thermalba* SF 45^T + 13 Salar de Atacama isolates***	***A. thermophila* GY088^T**	***A. tucumanensis* ABO^T**	***A. viridis* GY115^T**	**Isolate GY024**	**Isolate GY142**
Acid production from (1%, w/v):											
D-adonitol	+	+	-	+	+	-	+	+	-	+	-
D-Amygdalin	+	+	-	+	+	+	-	+	-	-	+
L-arabinose	+	+	+	+	+	+	-	+	+	-	-
D-arabinose	+	+	-	+	+	-	-	-	-	-	-
D-arabitol	+	+	-	+	+	-	+	+	+	-	-
L-arabitol	+	+	-	+	+	+	-	+	-	+	+
Meso-erythritol	-	+	+	+	+	+	-	+	-	-	-
D-galactose	+	+	+	+	+	-	+	+	+	-	+
Meso-inositol	+	+	-	+	-	+	-	+	-	+	+
D-lactose	+	+	+	+	+	+	-	+	+	+	+
D-maltose	-	-	-	+	-	+	-	-	-	-	-
D-mannitol	-	+	-	+	+	+	-	+	-	+	+
D-melezitose	-	+	+	+	-	+	-	+	-	_	+
D-melibiose	-	-	-	-	+	-	-	-	-	-	+
D-ribose	+	+	-	+	+	+	-	+	-	-	+
D-sorbitol	+	+	-	+	+	+	-	+	-	+	+
D-sucrose	+	+	-	-	+	+	-	+	-	-	+
D-trehalose	+	+	+	-	+	+	-	-	+	+	+
D-turanose	+	+	-	-	-	-	-	+	+	-	+
D-xylose	+	+	+	+	+	+	-	-	+	+	+
D-xylitol	+	+	-	+	+	+	-	+	-	+	+
API ZYM tests:											
N-acetyl-β-glucosamidase	+	+	-	+	+	-	+	-	-	+	+
Alkaline phosphatase	+	+	+	+	+	+	-	-	+	+	+
Cysteine arylamidase	+	+	+	-	+	-	-	-	+	+	+
Esterase (C4)	+	+	+	+	-	+	+	+	+	-	-
Esterase (C8)	+	+	+	+	-	+	+	+	+	+	+
β-galactosidase	+	-	+	-	-	-	-	-	+	-	-
α-glucoside	+	-	-	-	-	+	-	+	-	-	-
β-glucosidase	-	-	-	-	-	+	-	+	-	-	-
β-glucuronidase	-	-	+	-	-	-	-	-	+	-	-
Leucine arylamidase	+	+	+	+	+	+	-	+	+	+	+
Lipase (C14)	+	+	+	-	-	-	-	-	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	-	+	+	+	-	+	-	-
Trypsin	+	+	+	-	+	-	-	-	-	-	-
Valine arylamidase	+	-	-	-	-	-	+	-	+	-	-
Degradation tests (%, w/v):											
Allantoin (0.1)	-	+	-	-	+	+	-	-	-	-	-
Adenine (0.4)	-	-	-	-	-	-	-	-	+	+	-
Elastin (0.3)	-	+	-	+	+	+	-	+	-	+	+
Guanine (0.4)	-	-	-	-	-	-	-	-	-	+	-
Hypoxanthine (0.4)	+	+	+	+	+	-	-	-	+	-	-

Starch (0.1)	+	+	+	+	+	+	+	-	+	+	+
Tyrosine (0.4)	+	+	+	-	-	-	+	-	+	-	+
Xanthine (0.4)	+	-	-	-	+	+	-	-	-	-	-
Xylan (0.4)	-	+	-	-	+	-	-	-	-	-	-
Growth in the presence of (%w/v):											
5% NaCl	+	+	+	+	+	-	+	+	+	+	+
7.5% NaCl	+	+	-	+	+	-	+	+	-	-	+
Growth on sole carbon sources (1%, w/v or 1%, v/v):											
Ethanol	+	+	-	+	-	+	-	+	-	+	+
Methanol	+	-	-	-	-	+	+	+	-	+	+
Sodium acetate	+	+	-	-	+	+	-	+	-	+	+
Sodium benzoate	-	-	+	-	+	+	-	+	-	+	+
Sodium butyrate	+	-	+	-	+	+	-	+	-	+	+
Sodium citrate	+	+	-	-	+	-	-	+	-	+	+
Sodium fumarate	-	-	-	-	+	-	-	+	-	+	+
Sodium malonate	+	+	-	-	+	-	-	+	-	+	+
Sodium propionate	+	+	-	-	+	-	-	+	-	+	+
Sodium pyruvate	+	+	-	-	+	-	-	+	-	+	+
Sodium succinate	+	+	-	-	+	-	-	-	-	+	+
Sodium tartrate	-	-	+	-	+	-	+	+	-	+	+
Growth at:											
pH5	+	+	+	+	+	-	+	+	+	-	+
10^{o}C	+	-	-	-	-	-	+	-	+	-	-
20^{o}C	-	-	-	-	+	+	+	+	+	-	-
50^{o}C	+	+	+	+	+	+	+	+	+	+	+
55^{o}C	-	+	+	-	+	+	-	+	-	+	+
Resistant to:											
Novobiocin (10 µg/ml)	-	+	-	-	+	+	-	-	+	-	-
Streptomycin sulphate (10 µg/ml)	-	+	+	+	-	-	-	+	-	+	+
Lysozyme resistance (0.005%, w/v)	-	-	-	-	+	-	-	+	-	-	-

*Codes for these isolates are given in Figure 4.1.

4.5. Discussion

All of the isolates gave the amplification products characteristic of the genus *Amycolatopsis* with the 16S rRNA oligonucleotide primers AMY2 and ATOP, formed complex mixtures of *iso*-branched, straight chain saturated and unsaturated fatty acids typical of the genus, and were recovered in the *A. methanolica* 16S rRNA gene subclade. The representative isolates from the *A. ruanii* and *A. thermalba* 16S rRNA subclades and isolates GY024^{T} and GY142 gave chemotaxonomic and morphological features typical of the genus *Amycolatopsis* (Tan & Goodfellow, 2012). All of the isolates formed a branched substrate mycelium that fragmented into rod-shaped elements (0.3-0.4 µm x 1.2-1.8 µm), formed colonies covered with powdery white aerial hyphae, produced whole-organism hydrolysates rich in *meso*-A_2pm, arabinose and galactose (wall chemotype IV after Lechevalier & Lechevalier, 1970), contained tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue, and major amounts of DPG, PE, PG, PI and PME (phospholipid type II

sensu Lechevalier *et al*., 1977, 1986). All of these properties are consistent with the classification of the isolates in the genus *Amycolatopsis* (Tan & Goodfellow, 2012).

The 26 strains isolated from the hyper-arid Salar de Atacama soil were assigned to either the *A. ruanii* or *A. thermalba* phyletic lines and found to have identical or almost identical 16S rRNA gene similarity values with their respective type strain. DNA:DNA gene relatedness values between representative isolates from the *A. ruanii* 16S rRNA subclade and between them and *A. ruanii* NMG112^T were within the range 72-82$\pm$0.1%, values well above the cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al*., 1987). In turn, isolates representing those belonging to the *A. thermalba* 16S rRNA subclade were shown to belong to the same genomic species as the type strain of *A. thermalba* as the corresponding DNA:DNA relatedness values were within the range 76-80%. Members of each of these species had many phenotypic features in common but can be separated from one another and from the type strains of the remaining *Amycolatopsi*s species classified in the *A. methanolica* 16S rRNA subclade using a broad range of phenotypic features. Since the original descriptions of *A. ruanii* and *A. thermalba* were based on single isolates it is proposed that the descriptions of these species be emended in light of the current data.

Isolates GY024^T and GY142 from the Australian arid composite soil formed a distinct branch towards the periphery of the *A. methanolica* 16S rRNA gene tree. They shared a high 16S rRNA gene sequence similarity but were readily distinguished using a combination of phenotypic properties. The two strains need to be the subject of DNA:DNA pairing assays to establish whether belong to the same or different novel *Amycolatopsis* species.

The results of the present study confirm and extend those from previous investigations in showing that thermophilic *Amycolatopsis* strains constitute a distinct phyletic branch within the evolutionary radiation occupied by the genus *Amycolatopsis* (Tan *et al*., 2006; Everest & Myers, 2009; Everest *et al*., 2011; Tan & Goodfellow, 2012; Zucchi *et al*., 2012a, b, c). There is evidence that this taxon is underspeciated and more distantly related to *Amycolatopsis orientalis*, the type species of the genus, than to members of some other genera classified in the family *Pseudonocardiaceae* (Tan *et al*., 2006; Ludwig *et al*., 2012; Guoping Zhou *et al*., *pers. com.*). Indeed, it seems likely that *A. methanolica* and the closely related thermophilic species should be classified in a new genus though further comparative taxonomic studies are need to confirm this.

Almost nothing is known about the distribution, numbers, kinds and roles of thermophilic *Amycolatopsis* strains in natural habitats (Tan *et al*., 2006; Tan & Goodfellow, 2012) though it would seem from the present study that *A. ruanii* and *A. thermalba* strains may be major components of the cultivable actinobacterial community in the hyper-arid Salar de Atacama soil. Similarly, *A. taniae* strains may be common in Australian arid soils as isolates GY024^T and GY142 were representatives of the largest colour-group recovered in the survey undertaken by Tan *et al*. (2006). However, additional biosystematic studies are needed to build upon these preliminary findings, not least by establishing the ecological roles that thermophilic *Amycolatopsis* strains play in arid desert soils.

Description of *Amycolatopsis deserti* sp. nov.

Amycolatopsis deserti (dè.ser.t. L. gen.gem.n. *deserti* of desert, pertaining to the socrce of the isolates.

The description is based on data from this and an ealier study (Tan *et al*., 2006).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile, catalase-positive actinobacterium that forms an extensively branched substrate mycelium that fragments into granular ornamented cylindrical elements (0.3-0.4 x 1.1-1.7 µm). Produces abundant white aerial hyphae, a pale yellow substrate mycelium and a yellow diffusible pigment on oatmeal agar. Grows from pH 6-10, from 25-50^{o}C, optimum temperature is 45^{o}C and in the presence of NaCl (5%, w/v). Additional phenotypic test results are cited either in the main text or in Table 4.3. Chemotaxonomic properties are typical of the genus.

The type strain GY024^T (=DSMZ……=NRRL…….) was isolated from an Australian arid composite soil.

Emended description of *Amycolatopsis ruanii* Zucchi *et al*. 2012b

The description is based on this and the earlier study of Zuchii *et al*. (2012b).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile, catalase-positive actinobacteria that form an extensively branched substrate mycelium that fragments into granular ornamented cylindrical elements (0.3-0.35 x 1.2-1.7 µm) on modified Bennett's agar supplemented with mannitol and soybean flour, but does not form diffusible pigment on this medium. Abundant yellowish white aerial hyphae, a yellowish white substrate mycelium, but no diffusible pigments are produced on

oatmeal agar. Grows from 20-50^{o}C, optimum temperature is 45^{o}C, from pH 5.0-10.0 and in the presence of NaCl (2.5%, w/v). Additional phenotypic properties are cited in the main text and in Table 4.3. Chemotaxonomic properties are typical of the genus.

The type strain NMG112^{T} (=NCIMB14711^{T}=NRRL B-24848^{T}) was isolated from an Australian arid composite soil and additional members of the taxon from a hyper-arid Atacama Desert soil.

Emended description of *Amycolatopsis thermalba* Zucchi *et al.* 2012b

The description is based on this and the earlier study Zucchi *et al* (2012b).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile, catalase-positive actinobacteria that form an extensively branched substrate mycelium that fragments into granular ornamented cylindrical elements (0.3-0.4 x 1.5-1.8 µm). Abundant, white aerial hyphae, a yellow substrate mycelium and a medium yellow olive brown diffusible pigment are produced on modified Bennett's agar supplemented with mannitol and soybean flour. Melanin pigments are not formed on peptone-yeast extract-iron agar. Grows from 20-55^{o}C, optimum temperature is 45^{o}C, from pH 6-10.0 and in the presence of NaCl (2.5%, w/v). Additional phenotypic properties are cited in the main text and in Table 4.3. Chemotaxonomic properties are typical of the genus. Common in a hyper-arid Salar de Atacama soil of the Atacama Desert.

The type strain, SF45^{T} (=NCIMB 14705^{T}=NRRL B-24845^{T}) was isolated from an arid Australian composite soil and the remaining members of the taxon from a hyper-arid Atacama Desert soil.

Acknowledgement

The fatty acid analyses and some of the chemotaxonomic data were generated by Dr. Tiago Zucchi (Microbiology Laboratory, EMBRAPA, Jaguariu´na, Brazil).

Supplemented Table. **N**ucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from a hyper-arid Atacama Desert soil or from an arid Australian composite and between them and the type strains of closely related *Amycolatopsis* species.

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Isoalte KNN50-8b	---	**17/ 1285**	**21/ 1315**	**16/ 1314**	**2/ 1314**	**2/ 1312**	**16/ 1315**	**2/ 1317**	**3/ 1316**	**16/ 1317**	**16/ 1317**	**17/ 1312**	**2/ 1313**	**16/ 1314**	**7/ 1314**	**19/ 1313**	**17/ 1316**	**3/ 1313**	**2/ 1314**	**16/ 1314**	**19/ 1317**	**7/ 1317**
2. Isolate GY024	**98.7**	---	**4/ 1366**	**25/ 1353**	**15 /1353**	**15/ 1354**	**26/ 1369**	**15/ 1371**	**16/ 1370**	**26/ 1370**	**26/ 1370**	**40/ 1372**	**15/ 1281**	**28/ 1374**	**18/ 1282**	**16/ 1282**	**30/ 1338**	**16/ 1370**	**15/ 1347**	**26/ 1369**	**24/ 1351**	**16/ 1371**
3. Isolate GY142	**98.4**	**99.7**	---	**27/ 1378**	**17/ 1378**	**18/ 1378**	**31/ 1395**	**21/ 1398**	**22/ 1397**	**31/ 1397**	**30/ 1396**	**39/ 1392**	**19/ 1311**	**29/ 1392**	**20/ 1314**	**18/ 1313**	**34/ 1365**	**19/ 1393**	**17/ 1372**	**28/ 1393**	**26/ 1380**	**26/ 1397**
4. Isolate KNN49-6a	**98.8**	**98.2**	**98.0**	---	**24 /1382**	**24/ 1380**	**0/ 1381**	**24/ 1382**	**25/ 1381**	**0/ 1383**	**0/ 1383**	**7/ 1381**	**14/ 1310**	**1/ 1382**	**17/ 1311**	**1/ 1311**	**14/ 1367**	**25/ 1381**	**19/ 1376**	**0/ 1383**	**1/ 1381**	**29/ 1382**
5. Isolate KNN49-5e	**99.9**	**98.9**	**98.8**	**98.3**	---	**0/ 1380**	**24/ 1380**	**0/ 1382**	**1/ 1381**	**24/ 1382**	**24/ 1382**	**23/ 1380**	**0/ 1310**	**25/ 1381**	**3/ 1311**	**15/ 1311**	**29/ 1366**	**1/ 1381**	**0/ 1376**	**24/ 1382**	**23/ 1380**	**5/ 1382**
6. Isolate KNN49-3e	**99.9**	**98.9**	**98.7**	**98.3**	**100.0**	---	**25/ 1379**	**0/ 1382**	**1/ 1381**	**25/ 1381**	**25/ 1381**	**25/ 1381**	**0/ 1308**	**26/ 1380**	**3/ 1309**	**15/ 1309**	**29/ 1364**	**0/ 1383**	**0/ 1374**	**25/ 1381**	**23/ 1378**	**4/ 1382**
7. Isolate KNN50-10e	**98.8**	**98.1**	**97.8**	**100.0**	**98.3**	**98.2**	---	**25/ 1401**	**26/ 1399**	**0/ 1402**	**0/ 1401**	**21/ 1396**	**14/ 1311**	**1/ 1397**	**20/ 1313**	**4/ 1312**	**14/ 1368**	**26/ 1395**	**19/ 1374**	**0/ 1397**	**4/ 1383**	**31/ 1400**
8. Isolate KNN50-4c	**99.9**	**98.9**	**98.5**	**98.3**	**100.0**	**100.0**	**98.2**	---	**1/ 1402**	**25/ 1403**	**25/ 1402**	**38/ 1398**	**0/ 1313**	**26/ 1398**	**6/ 1315**	**18/ 1314**	**29/ 1369**	**1/ 1398**	**0/ 1376**	**25/ 1398**	**26/ 1384**	**6/ 1403**
9. Isolate KNN49-26a	**99.8**	**98.8**	**98.4**	**98.2**	**99.9**	**99.9**	**98.1**	**99.9**	---	**26/ 1401**	**26/ 1400**	**38/ 1396**	**1/ 1312**	**27/ 1396**	**7/ 1314**	**19/ 1313**	**30/ 1369**	**2/ 1397**	**1/ 1375**	**26/ 1397**	**27/ 1383**	**7/ 1401**
10. Isolate KNN49-12b	**98.8**	**98.1**	**97.8**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	---	**0/ 1403**	**21/ 1398**	**14/ 1313**	**1/ 1399**	**20/ 1315**	**4/ 1314**	**14/ 1370**	**26/ 1397**	**19/ 1376**	**0/ 1399**	**4/1385**	**31/ 1402**
11. Isolate KNN49-11c	**98.8**	**98.1**	**97.9**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	**100.0**	---	**21/ 1398**	**14/ 1313**	**1/ 1399**	**19/ 1314**	**3/ 1313**	**14/ 1370**	**26/ 1397**	**19/ 1376**	**0/ 1399**	**3/ 1384**	**31/ 1402**
12. Isolate KNN49-32e	**98.7**	**97.1**	**97.2**	**99.5**	**98.3**	**98.2**	**98.5**	**97.3**	**97.3**	**98.5**	**98.5**	---	**15/ 1308**	**23/ 1398**	**18/ 1309**	**2/ 1309**	**15/ 1365**	**38/ 1397**	**19/ 1374**	**20/ 1397**	**6/ 1379**	**41/ 1398**
13. Isolate KNN50-18d	**99.9**	**98.8**	**98.6**	**98.9**	**100.0**	**100.0**	**98.9**	**100.0**	**99.92**	**98.9**	**98.9**	**98.9**	---	**14/ 1310**	**5/ 1312**	**17/ 1312**	**15/ 1312**	**1/ 1309**	**0/ 1310**	**14/ 1310**	**17/ 1313**	**5/ 1313**
14. Isolate KNN50-17d	**98.8**	**98.0**	**97.9**	**99.9**	**98.2**	**98.1**	**99.9**	**98.1**	**98.1**	**99.9**	**99.9**	**98.4**	**98.9**	---	**17/ 1311**	**1/ 1311**	**15/ 1366**	**27/ 1396**	**20/ 1375**	**1/ 1398**	**2/ 1380**	**32/ 1398**
15. Isolate KNN50-15d	**99.5**	**98.6**	**98.5**	**98.7**	**99.8**	**99.8**	**98.5**	**99.5**	**99.5**	**98.5**	**98.6**	**98.6**	**99.6**	**98.7**	---	**18/1314**	**20/1313**	**4/1310**	**3/1311**	**17/1311**	**18/1315**	**10/1314**
16. Isolate KNN50-16d	**98.6**	**98.8**	**98.6**	**99.9**	**98.9**	**98.9**	**99.7**	**98.6**	**98.6**	**99.7**	**99.8**	**99.9**	**98.7**	**99.9**	**98.6**	---	**2/1312**	**16/1310**	**15/1311**	**1/1311**	**2/1314**	**22/1313**
17. Isolate KNN50-11c	**98.7**	**97.8**	**97.5**	**99.0**	**97.88**	**97.9**	**99.0**	**97.9**	**97.8**	**99.0**	**99.0**	**98.9**	**98.9**	**98.9**	**98.5**	**99.9**	---	**30/1365**	**29/1366**	**14/1367**	**17/1370**	**34/1369**
18. Isolate KNN50-14d	**99.8**	**98.8**	**98.6**	**98.2**	**99.9**	**100.0**	**98.1**	**99.9**	**99.9**	**98.1**	**98.1**	**97.3**	**99.9**	**98.1**	**99.7**	**98.8**	**97.8**	---	**1/1375**	**26/1397**	**24/1379**	**6/ 1398**
19. Isolate KNN50-13c	**99.9**	**98.9**	**98.8**	**98.6**	**100.0**	**100.0**	**98.6**	**100.0**	**99.9**	**98.6**	**98.6**	**98.6**	**100.0**	**98.6**	**99.8**	**98.9**	**97.9**	**99.9**	---	**19/1376**	**20/1376**	**5/1376**
20. Isolate KNN50-12c	**98.8**	**98.1**	**98.0**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	**100.0**	**100.0**	**98.6**	**98.9**	**99.9**	**98.7**	**99.9**	**99.0**	**98.1**	**98.6**	---	**1/1381**	**31/1398**
21. Isolate KNN50-9b	**98.6**	**98.2**	**98.1**	**99.9**	**98.3**	**98.3**	**99.7**	**98.1**	**98.1**	**99.7**	**99.9**	**99.6**	**98.7**	**99.9**	**98.6**	**99.9**	**98.8**	**98.3**	**98.6**	**99.9**	---	**30/1383**
22. Isolate KNN50-7d	**99.5**	**98.8**	**98.1**	**97.9**	**99.6**	**99.7**	**97.8**	**99.6**	**99.5**	**97.8**	**97.8**	**97.1**	**99.6**	**97.7**	**99.2**	**98.3**	**97.5**	**99.6**	**99.6**	**97.8**	**97.8**	---
23. Isolate KNN50-6e	**99.9**	**98.9**	**98.6**	**98.3**	**100.0**	**100.0**	**98.2**	**100.0**	**99.9**	**98.2**	**98.2**	**97.4**	**100.0**	**98.1**	**99.6**	**98.7**	**97.9**	**99.9**	**100.0**	**98.2**	**98.2**	**99.6**
24. Isolate KNN50-5c	**99.9**	**98.9**	**98.8**	**98.6**	**100.0**	**100.0**	**98.6**	**100.0**	**99.9**	**98.6**	**98.6**	**98.6**	**100.0**	**98.5**	**99.8**	**98.9**	**97.9**	**99.9**	**100.0**	**98.6**	**98.5**	**99.6**
25. Isolate KNN50-2e	**98.8**	**97.8**	**97.9**	**100.0**	**98.3**	**98.1**	**100.0**	**98.1**	**98.1**	**100.0**	**100.0**	**98.4**	**98.9**	**99.6**	**98.7**	**99.9**	**99.0**	**98.1**	**98.6**	**100.0**	**99.9**	**97.7**
26. Isolate KNN50-1a	**98.6**	**98.1**	**98.0**	**100.0**	**98.3**	**98.2**	**99.8**	**98.0**	**97.9**	**99.8**	**99.9**	**98.5**	**98.9**	**99.9**	**98.7**	**99.9**	**98.8**	**98.1**	**98.6**	**100.0**	**99.9**	**97.7**
27. Isolate KNN49-10b	**99.9**	**98.9**	**98.6**	**98.3**	**100.0**	**100.0**	**98.3**	**100.0**	**99.9**	**98.3**	**98.3**	**98.3**	**100.0**	**98.2**	**99.6**	**98.7**	**97.9**	**99.9**	**100.0**	**98.3**	**98.2**	**99.6**
28. Isolate KNN49-1h	**99.9**	**98.8**	**98.6**	**98.3**	**100.0**	**100.0**	**98.1**	**99.9**	**99.9**	**98.1**	**98.1**	**97.4**	**100.0**	**98.1**	**99.8**	**98.9**	**97.9**	**99.9**	**100.0**	**98.1**	**98.3**	**99.7**

Supplemented Table (cont.)

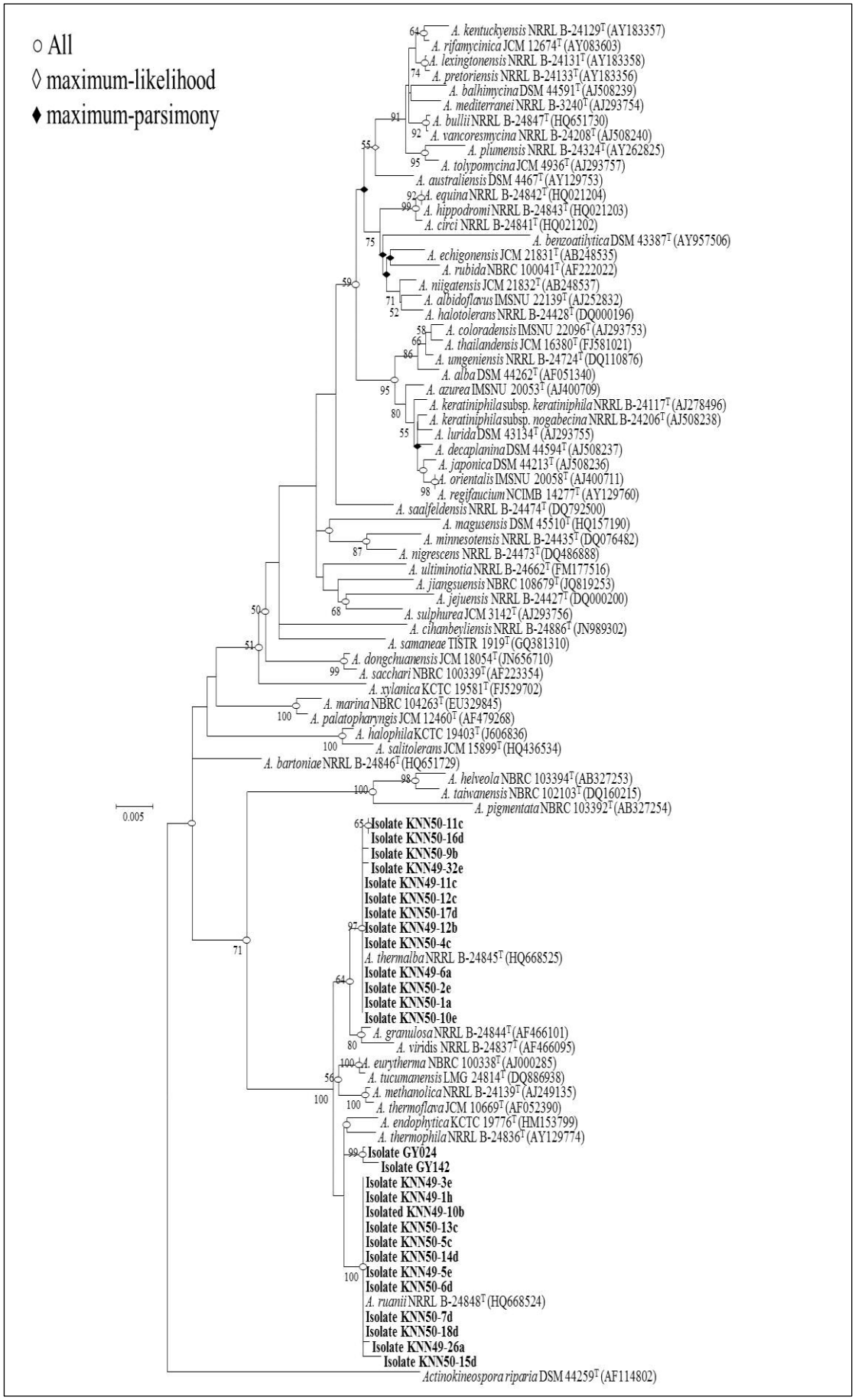
Isolate	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1.Isolate KNN50-8b	2/1317	2/1314	16/1314	18/1317	2/1316	2/1312	0/1317	18/1317	16/1316	18/1317	19/1317	19/1316	20/1317	23/1316	26/1316	24/1313	68/1315	67/1317	66/1315	84/1313	87/1314
2. Isolate GY024	15/1370	15/1348	31/1375	26/1370	15/1352	16/1369	15/1395	15/1328	26/1394	17/1380	19/1394	17/1394	18/1395	35/1351	39/1353	20/1390	72/1412	63/1411	62/1429	68/1395	85/1394
3. Isolate GY142	20/1397	17/1373	29/1394	28/1397	19/1380	19/1392	18/1410	17/1369	29/1409	20/1409	22/1409	21/1409	22/1410	40/1392	47/1381	29/1396	80/1408	70/1409	68/1407	74/1406	95/1407
4. Isolate KNN49-6a	24/1382	20/1377	0/1383	0/1383	24/1381	24/1380	24/1382	0/1357	28/1382	26/1382	18/1382	22/1382	23/1383	26/1381	25/1380	32/1378	83/1381	79/1382	76/1380	86/1378	98/1379
5. Isolate KNN49-5e	0/1382	0/1377	24/1382	24/1382	0/1381	0/1380	0/1382	14/1357	16/1381	18/1381	19/1381	19/1381	20/1382	31/1380	33/1379	24/1377	70/1380	65/1381	64/1379	83/1378	86/1379
6. Isolate KNN49-3e	0/1382	0/1375	26/1382	25/1381	0/1380	0/1383	0/1382	14/1355	16/1381	18/1381	19/1381	19/1381	20/1382	31/1378	35/1379	24/1377	70/1380	65/1381	64/1379	83/1378	86/1379
7. Isolate KNN50-10e	25/1399	20/1375	0/1398	3/1402	24/1382	26/1394	28/1401	3/1359	32/1401	30/1401	22/1401	26/1401	27/1402	29/1383	27/1383	36/1397	87/1400	83/1401	80/1399	90/1397	101/1398
8. Isolate KNN50-4c	0/1402	0/1377	26/1400	28/1403	0/1384	1/1397	3/1404	17/1361	19/1403	21/1403	22/1403	22/1403	23/1404	34/1384	37/1385	27/1399	73/1402	68/1403	67/1401	86/1400	89/1401
9. Isolate KNN49-26a	1/1401	1/1376	27/1398	29/1401	1/1383	2/1396	4/1402	18/1360	20/1401	22/1401	23/1401	23/1401	24/1402	35/1383	38/1384	28/1397	74/1400	69/1401	68/1399	87/1398	89/1399
10. Isolate KNN49-12b	25/1401	20/1377	0/1400	3/1404	24/1384	26/1396	28/1403	3/1361	32/1403	30/1403	22/1403	26/1403	27/1404	29/1385	27/1385	36/1399	87/1402	83/1403	80/1401	90/1399	102/1400
11.Isolate KNN49-11c	25/1401	20/1377	0/1400	2/1403	24/1384	26/1396	27/1402	2/1360	31/1402	29/1402	21/1402	25/1402	26/1403	28/1384	27/1385	35/1398	86/1401	82/1402	79/1400	89/1398	101/1399
12. Isolate KNN49-32e	37/1397	19/1375	22/1400	21/1398	23/1379	37/1396	39/1399	1/1355	43/1399	41/1399	33/1399	37/1399	38/1400	33/1381	34/1383	47/1395	99/1400	94/1401	92/1399	101/1395	113/1397
13. Isolate KNN50-18d	0/1313	0/1310	14/1310	16/1313	0/1312	0/1308	2/1313	16/1313	14/1312	16/1313	17/1313	17/1312	18/1313	21/1312	24/1312	22/1309	66/1311	65/1313	64/1311	82/1309	85/1310
14. Isolate KNN50-17d	26/1397	21/1376	5/1403	1/1399	25/1380	27/1395	27/1403	1/1356	31/1403	29/1403	21/1403	25/1403	26/1404	27/1380	26/1381	35/1399	85/1402	82/1403	79/1401	89/1399	101/1400
15. Isolate KNN50-15d	5/1314	3/1311	17/1311	17/1315	5/1313	3/1309	3/1316	1/1316	15/1315	17/1316	18/1316	18/1315	19/1316	22/1315	25/1313	23/1312	66/1314	66/1316	65/1314	83/1312	86/1313
16.Isolate KNN50-16d	17/1313	15/1311	1/1311	1/1314	17/1312	15/1309	15/1315	1/1315	18/1314	17/1315	9/1315	12/1314	13/1315	21/1314	22/1312	22/1311	69/1313	72/1315	69/1313	77/1311	88/1312
17. Isolate KNN50-11c	29/1369	29/1366	14/1367	16/1370	29/1368	29/1364	31/1369	1/1359	33/1369	32/1370	24/1370	27/1369	28/1370	38/1368	38/1368	37/1365	87/1368	88/1370	85/1368	92/1365	103/1366
18. Isolate KNN50-14d	1/1398	1/1376	27/1398	26/1397	1/1381	1/1398	1/1398	15/1356	17/1397	19/1397	20/1397	20/1397	21/1398	32/1379	36/1381	25/1393	71/1396	66/1397	65/1395	84/1394	87/1395
19. Isolate KNN50-13c	0/1376	0/1376	19/1376	19/1376	0/1375	0/1374	0/1376	14/1357	16/1375	18/1375	19/1375	19/1375	20/1376	26/1374	28/1373	24/1371	70/1374	65/1375	64/1373	83/1372	86/1373
20.Isolate KNN50-12c	25/1398	20/1377	0/1399	0/1399	24/1381	26/1396	25/1398	0/1357	29/1398	27/1398	19/1398	23/1398	24/1399	26/1381	25/1382	33/1394	84/1397	80/1398	77/1396	87/1394	99/1395
21. Isolate KNN50-9b	25/1383	21/1377	1/1381	1/1385	25/1382	23/1378	23/1384	1/1361	27/1384	25/1384	17/1384	21/1384	22/1385	27/1383	26/1381	31/1380	82/1383	78/1384	75/1382	85/1380	97/1381
22. Isolate KNN50-7d	6/1402	5/1377	32/1400	33/1402	5/1384	4/1397	8/1403	21/1360	24/1402	26/1402	27/1402	27/1402	28/1403	38/1383	42/1385	32/1398	78/1401	73/1402	72/1400	91/1399	94/1400
23.Isolate KNN50-6e	---	0/1377	26/1399	27/1401	0/1384	1/1397	2/1402	16/1360	18/1401	20/1401	21/1401	21/1401	22/1402	33/1383	37/1385	26/1397	72/1400	67/1401	66/1399	85/1398	88/1399
24. Isolate KNN50-5c	100.0	---	20/1377	20/1377	0/1376	0/1375	0/1377	14/1357	16/1376	18/1376	19/1376	19/1376	20/1377	27/1375	29/1374	24/1372	70/1375	65/1376	64/1374	83/1373	86/1374
25. Isolate KNN50-2e	98.1	98.6	---	0/1400	24/1381	27/1397	30/1404	0/1357	34/1404	32/1404	24/1404	28/1404	29/1405	26/1381	26/1383	38/1400	89/1403	85/1404	82/1402	92/1400	104/1401
26. Isolate KNN50-1a	98.1	98.6	100.0	---	26/1384	26/1396	25/1403	0/1361	29/1403	27/1403	19/1403	23/1403	24/1404	26/1385	25/1385	33/1399	84/1402	80/1403	77/1401	87/1399	99/1400
27. Isolate KNN49-10b	100.0	100.0	98.3	98.1	---	0/1380	2/1384	16/1359	18/1383	20/1383	21/1383	21/1383	22/1384	33/1382	35/1381	26/1379	71/1382	66/1383	65/1381	84/1380	88/1381
28. Isolate KNN49-1h	99.93	100.0	98.1	98.1	100.0	---	1/1397	14/1355	17/1396	19/1396	20/1396	20/1396	21/1397	31/1378	35/1380	25/1392	71/1395	66/1396	65/1394	84/1393	87/1394

Supplemented Table (cont.)

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
29. *A_ruanii_*	**100.0**	**98.9**	**98.7**	**98.3**	**100.0**	**100.0**	**98.0**	**99.9**	**99.7**	**98.0**	**98.1**	**97.2**	**99.9**	**98.1**	**99.8**	**98.9**	**97.7**	**99.9**	**100.0**	**98.2**	**98.3**	**99.4**	**99.9**
30. *A_thermalba*	**98.6**	**98.9**	**98.8**	**100.0**	**99.0**	**99.0**	**99.8**	**98.8**	**98.7**	**99.9**	**99.9**	**99.9**	**98.8**	**99.9**	**99.9**	**99.9**	**99.9**	**98.9**	**99.0**	**100.0**	**99.9**	**98.5**	**98.8**
31. *A_thermoflava*	**98.8**	**98.1**	**97.9**	**98.0**	**98.8**	**98.8**	**97.7**	**98.7**	**98.6**	**97.7**	**97.8**	**96.9**	**98.9**	**97.8**	**98.9**	**98.6**	**97.6**	**98.8**	**98.8**	**97.9**	**98.1**	**98.3**	**98.7**
32. *A_endophytica*	**98.6**	**98.8**	**98.6**	**98.1**	**98.7**	**98.7**	**97.9**	**98.5**	**98.4**	**97.9**	**97.9**	**97.1**	**98.8**	**97.9**	**98.7**	**98.7**	**97.7**	**98.6**	**98.7**	**98.1**	**98.2**	**98.2**	**98.6**
33. *A_methanolica*	**98.6**	**98.6**	**98.4**	**98.7**	**98.6**	**98.6**	**98.4**	**98.4**	**98.4**	**98.4**	**98.5**	**97.6**	**98.7**	**98.5**	**98.6**	**99.3**	**98.3**	**98.6**	**98.6**	**98.6**	**98.8**	**98.1**	**98.5**
34. *A_eurytherma*	**98.6**	**98.8**	**98.5**	**98.4**	**98.6**	**98.6**	**98.1**	**98.4**	**98.4**	**98.2**	**98.2**	**97.4**	**98.7**	**98.2**	**98.6**	**99.1**	**98.0**	**98.6**	**98.6**	**98.4**	**98.5**	**98.1**	**98.5**
35. *A_tucumanensis*	**98.5**	**98.7**	**98.4**	**98.3**	**98.6**	**98.6**	**98.1**	**98.4**	**98.3**	**98.1**	**98.2**	**97.3**	**98.6**	**98.2**	**98.6**	**99.0**	**98.0**	**98.5**	**98.6**	**98.3**	**98.4**	**98.0**	**98.4**
36. *A_granulosa*	**98.3**	**97.4**	**97.1**	**98.1**	**97.8**	**97.8**	**97.9**	**97.5**	**97.5**	**97.9**	**98.0**	**97.6**	**98.4**	**98.0**	**98.3**	**98.4**	**97.2**	**97.7**	**98.1**	**98.1**	**98.1**	**97.3**	**97.6**
37. *A_viridis*	**98.0**	**97.1**	**96.6**	**98.2**	**97.6**	**97.5**	**98.1**	**97.3**	**97.3**	**98.1**	**98.1**	**97.5**	**98.2**	**98.1**	**98.1**	**98.3**	**97.2**	**97.4**	**98.0**	**98.2**	**98.1**	**97.0**	**97.3**
38. *A_thermophila*	**98.2**	**98.6**	**97.9**	**97.7**	**98.3**	**98.3**	**97.4**	**98.1**	**98.0**	**97.4**	**97.5**	**96.6**	**98.3**	**97.5**	**98.3**	**98.3**	**97.3**	**98.2**	**98.3**	**97.6**	**97.6**	**97.7**	**98.1**
39. *A_pigmentata*	**94.8**	**94.9**	**94.3**	**94.0**	**94.9**	**94.9**	**93.8**	**94.8**	**94.7**	**93.8**	**93.9**	**92.9**	**95.0**	**93.9**	**95.0**	**94.7**	**93.6**	**94.9**	**94.9**	**94.0**	**94.1**	**94.4**	**94.9**
40. *A_helveola*	**94.9**	**95.5**	**95.0**	**94.3**	**95.3**	**95.3**	**94.1**	**95.2**	**95.1**	**94.1**	**94.2**	**93.3**	**95.1**	**94.2**	**95.0**	**94.5**	**93.6**	**95.3**	**95.3**	**94.3**	**94.4**	**94.8**	**95.2**
41. *A_taiwanensis*	**95.0**	**95.7**	**95.8**	**94.5**	**95.4**	**95.4**	**94.3**	**95.2**	**95.1**	**94.3**	**94.4**	**93.4**	**95.1**	**94.4**	**95.1**	**94.7**	**93.8**	**95.3**	**95.3**	**94.5**	**94.6**	**94.9**	**95.3**
42. *A_orientalis*	**93.6**	**95.1**	**94.7**	**93.8**	**94.0**	**94.0**	**93.6**	**93.9**	**93.8**	**93.6**	**93.6**	**92.8**	**93.7**	**93.6**	**93.8**	**94.1**	**93.3**	**94.0**	**94.0**	**93.8**	**93.8**	**93.5**	**93.9**
43. *Actinokineospora riparia*	**93.4**	**93.9**	**93.3**	**92.9**	**93.8**	**93.8**	**92.8**	**93.7**	**93.6**	**92.7**	**92.8**	**91.9**	**93.5**	**92.8**	**93.5**	**93.3**	**92.5**	**93.76**	**93.7**	**92.9**	**93.0**	**93.3**	**93.7**

Isolate	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
29. *A_ruanii_*	**100.0**	**97.9**	**98.2**	**99.9**	**99.9**	**---**	14/1392	16/1456	18/1441	19/1460	19/1456	20/1461	31/1414	35/1385	24/1426	70/1455	65/1456	64/1458	83/1453	87/1457
30. *A_thermalba*	**99.0**	**100.0**	**100.0**	**98.8**	**99.0**	**99.0**	---	17/1389	16/1389	8/1392	11/1389	12/1392	20/1390	21/1359	21/1360	70/1388	71/1390	68/1390	77/1386	88/1389
31. *A_thermoflava*	**98.8**	**97.6**	**97.9**	**98.7**	**98.8**	**98.9**	98.8	---	20/1442	13/1457	16/1459	17/1460	34/1416	41/1388	24/1429	79/1455	74/1456	71/1454	86/1452	94/1452
32. *A_endophytica*	**98.7**	**97.7**	**98.1**	**98.6**	**98.6**	**98.8**	98.9	98.6	---	12/1443	16/1442	17/1443	37/1413	44/1386	23/1413	76/1440	72/1441	70/1439	82/1437	97/1438
33. *A_methanolica*	**98.6**	**98.3**	**98.7**	**98.5**	**98.6**	**98.7**	99.4	99.1	99.2	---	10/1457	11/1478	31/1415	38/1386	22/1427	77/1455	78/1456	73/1468	80/1452	93/1471
34. *A_eurytherma*	**98.6**	**98.0**	**98.4**	**98.5**	**98.6**	**98.7**	99.2	98.9	98.9	99.3	---	1/1460	36/1416	43/1388	26/1429	75/1455	78/1456	75/1454	80/1452	96/1452
35. *A_tucumanensis*	**98.6**	**97.9**	**98.3**	**98.4**	**98.5**	**98.6**	99.1	98.8	98.8	99.3	99.9	---	37/1418	44/1389	27/1430	76/1456	79/1457	76/1469	81/1453	97/1472
36. *A_granulosa*	**98.0**	**98.1**	**98.1**	**97.6**	**97.8**	**97.8**	98.6	97.6	97.4	97.8	97.5	97.4	---	11/1387	39/1386	77/1414	69/1415	72/1414	92/1409	101/1412
37. *A_viridis*	**97.9**	**98.1**	**98.2**	**97.5**	**97.5**	**97.5**	98.5	97.1	96.8	97.8	96.9	96.8	99.2	---	44/1384	80/1386	74/1387	79/1385	100/1381	102/1383
38. *A_thermophila*	**98.3**	**97.3**	**97.6**	**98.1**	**98.2**	**98.3**	98.5	98.3	98.4	98.5	98.2	98.1	97.2	96.8	---	77/1425	75/1426	71/1424	86/1422	100/1422
39. *A_pigmentata*	**94.9**	**93.7**	**94.0**	**94.9**	**94.9**	**95.2**	95.0	94.6	94.7	94.7	94.9	94.8	94.6	94.2	94.6	---	46/1475	44/1475	110/1451	110/1453
40. *A_helveola*	**95.3**	**94.0**	**94.3**	**95.2**	**95.3**	**95.5**	94.9	94.9	95.0	94.6	94.6	94.6	95.1	94.7	94.7	96.9	---	17/1475	91/1452	111/1454
41. *A_taiwanensis*	**95.3**	**94.2**	**94.5**	**95.3**	**95.3**	**95.6**	95.1	95.1	95.1	95.0	94.8	94.8	94.9	94.3	95.0	97.0	98.	---	94/1450	113/1466
42. *A_orientalis*	**94.0**	**93.4**	**93.8**	**93.9**	**94.0**	**94.3**	94.4	94.1	94.3	94.5	94.5	94.4	93.5	92.8	94.0	92.4	93.73	93.5	---	97/1452
43. *Actinokineospora riparia*	**93.7**	**92.6**	**92.9**	**93.6**	**93.8**	**94.0**	93.66	93.53	93.25	93.68	93.39	93.41	92.85	92.62	92.97	92.43	92.37	92.29	93.32	---

Strain codes, as given in Figure 4.1.


○ All
◊ maximum-likelihood
♦ maximum-parsimony
0.005
A. kentuckyensis NRRL B-24129T (AY183357)
A. rifamycinica JCM 12674T (AY083603)
A. lexingtonensis NRRL B-24131T (AY183358)
A. pretoriensis NRRL B-24133T (AY183356)
A. balhimycina DSM 44591T (AJ508239)
A. mediterranei NRRL B-3240T (AJ293754)
A. bullii NRRL B-24847T (HQ651730)
A. vancoresmycina NRRL B-24208T (AJ508240)
A. plumensis NRRL B-24324T (AY262825)
A. tolypomycina JCM 4936T (AJ293757)
A. australiensis DSM 4467T (AY129753)
A. equina NRRL B-24842T (HQ021204)
A. hippodromi NRRL B-24843T (HQ021203)
A. circi NRRL B-24841T (HQ021202)
A. benzoatilytica DSM 43387T (AY957506)
A. echigonensis JCM 21831T (AB248535)
A. rubida NBRC 100041T (AF222022)
A. niigatensis JCM 21832T (AB248537)
A. albidoflavus IMSNU 22139T (AJ252832)
A. halotolerans NRRL B-24428T (DQ000196)
A. coloradensis IMSNU 22096T (AJ293753)
A. thailandensis JCM 16380T (FJ581021)
A. umgeniensis NRRL B-24724T (DQ110876)
A. alba DSM 44262T (AF051340)
A. azurea IMSNU 20053T (AJ400709)
A. keratiniphila subsp. keratiniphila NRRL B-24117T (AJ278496)
A. keratiniphila subsp. nogabecina NRRL B-24206T (AJ508238)
A. lurida DSM 43134T (AJ293755)
A. decaplanina DSM 44594T (AJ508237)
A. japonica DSM 44213T (AJ508236)
A. orientalis IMSNU 20058T (AJ400711)
A. regifaucium NCIMB 14277T (AY129760)
A. saalfeldensis NRRL B-24474T (DQ792500)
A. magusensis DSM 45510T (HQ157190)
A. minnesotensis NRRL B-24435T (DQ076482)
A. nigrescens NRRL B-24473T (DQ486888)
A. ultiminotia NRRL B-24662T (FM177516)
A. jiangsuensis NBRC 108679T (JQ819253)
A. jejuensis NRRL B-24427T (DQ000200)
A. sulphurea JCM 3142T (AJ293756)
A. cihanbeyliensis NRRL B-24886T (JN989302)
A. samaneae TISTR 1919T (GQ381310)
A. dongchuanensis JCM 18054T (JN656710)
A. sacchari NBRC 100339T (AF223354)
A. xylanica KCTC 19581T (FJ529702)
A. marina NBRC 104263T (EU329845)
A. palatopharyngis JCM 12460T (AF479268)
A. halophila KCTC 19403T (J606836)
A. salitolerans JCM 15899T (HQ436534)
A. bartoniae NRRL B-24846T (HQ651729)
A. helveola NBRC 103394T (AB327253)
A. taiwanensis NBRC 102103T (DQ160215)
A. pigmentata NBRC 103392T (AB327254)
Isolate KNN50-11c
Isolate KNN50-16d
Isolate KNN50-9b
Isolate KNN49-32e
Isolate KNN49-11c
Isolate KNN50-12c
Isolate KNN50-17d
Isolate KNN49-12b
Isolate KNN50-4c
A. thermalba NRRL B-24845T (HQ668525)
Isolate KNN49-6a
Isolate KNN50-2e
Isolate KNN50-1a
Isolate KNN50-10e
A. granulosa NRRL B-24844T (AF466101)
A. viridis NRRL B-24837T (AF466095)
A. eurytherma NBRC 100338T (AJ000285)
A. tucumanensis LMG 24814T (DQ886938)
A. methanolica NRRL B-24139T (AJ249135)
A. thermoflava JCM 10669T (AF052390)
A. endophytica KCTC 19776T (HM153799)
A. thermophila NRRL B-24836T (AY129774)
Isolate GY024
Isolate GY142
Isolate KNN49-3e
Isolate KNN49-1h
Isolated KNN49-10b
Isolate KNN50-13c
Isolate KNN50-5c
Isolate KNN50-14d
Isolate KNN49-5e
Isolate KNN50-6d
A. ruanii NRRL B-24848T (HQ668524)
Isolate KNN50-7d
Isolate KNN50-18d
Isolate KNN49-26a
Isolate KNN50-15d
Actinokineospora riparia DSM 44259T (AF114802)

Supplemented figure. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (~1350 bp) showing relationships between the *Amycolatopsis* isolates and and between them and the all type strains of *Amycolatopsis* species. The symbol "o" indicates branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods, the symbols "◊" and the symbol "♦" branches that were recovered with maximum-likelihood and maximum-parsimony tree-making algorithms, repectively. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets;only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Chapter 5. Biosystematic studies on *Modestobacter* strains isolated from extreme hyper-arid desert soil and from historic buildings

5.1. Abstract

A polyphasic study was undertaken to establish the taxonomic status of 17 *Modestobacter* strains isolated either from an extreme hyper-arid Atacama Desert soil or from deteriorated sandstone from historic buildings in Salamanaca, Spain. The isolates were shown to have chemotaxonomic and morphological properties consistent with their classification in the genus *Modestobacter*. Three of the isolates from the Atacama Desert soil and four from deteriorated sandstone formed a distinct 16S rRNA gene subclade. These isolates had chemotaxonomic and many common phenotypic properties, were most closely related to the type strain of *Modestobacter marinus*, but were readily distinguished from the latter using genotypic and phenotypic data and hence may represent one or more novel *Modestobacter* species. The majority of the isolates from the extreme hyper-arid soil formed a well delineated subclade in the *Modestobacter* 16S rRNA gene tree, shared many phenotypic features and may form one or more new centres of taxonomic variation in the genus *Modestobacter*. The two remaining strains, isolates MDVD 1 and MON 3.1, from deteriorated sandstone, constituted well defined lineages in the *Modestobacter* 16S rRNA gene tree and were readily distinguished from one another and from all of the type strains using a broad range of phenotypic properties. On the basis of these data, it is proposed that these isolates be classified in the genus *Modestobacter* as *Modestobacter lapidis* sp. nov. and *Modestobacter muralis* sp. nov. with isolates MDVD1 (=DSM……= NCIMB…..= NRRL…..) and MON 3.1 (=DSM…..=NCIMB……..=NRRL……) as the respective type strains.

5.2. Introduction

The genus *Modestobacter* (Mevs *et al*., 2000) and the genera *Blastococcus* (Ahrens & Moll, 1970) and *Geodermatophilus* (Luedemann, 1968) comprise the family

Geodermatophilaceae (Normand, 2006; Normand & Benson, 2012a) which belongs to the order *Frankiales* (Normand & Benson, 2012b). *Geodermatophilaceae* strains have a characteristic 16S rRNA gene signature pattern, may form rudimentary hyphae, have modest growth requirements and have been isolated frequently from extreme habitats characterised by dry conditions, such as those associated with desert and high altitude soils and with the surfaces of rocks and ancient monuments (Eppard *et al*., 1996; Urzì *et al*., 2001; Nie *et al*., 2012). *Modestobacter* strains form a distinct clade in the *Geodermatophilus* 16S rRNA gene tree (Qin *et al*., 2013) and can also be distinguished from the genera *Blastococcus* and *Geodermatophilus* using 16S rRNA targeted oligonucleotide primers (Urzì *et al*., 2004), by phenotypic and physiological properties (Normand & Benson, 2012b), and notably by how they have adapted to resisting stress in extreme habitats (Essoussi *et al*., 2010; Gtari *et al*., 2012). The presence of *Geodermatophilaceae* strains on rock and building surfaces is often associated with black, grey and orange patinas and spots and with additional phenomena, such as biopitting, crumbling and powdering (Urzì & Realini, 1998; Urzì *et al*., 2001, 2004).

The genus currently encompasses four species, *Modestobacter multiseptatus* (Mevs *et al*., 2000), the type species, *Modestobacter marinus* (Xiao *et al*., 2011), *Modestobacter roseus* (Qin *et al*., 2013) and *Modestobacter versicolor* (Reddy *et al*., 2007), which were isolated from regolithic soil from the Linnaeus Terrace of the Asgard Range in the Transantarctic Mountains, a deep-sea sediment sample from the Atlantic Ocean, from surface-sterilised roots of the coastal halophyte *Salicornia europaea* and from a biological soil crust sample from the Colorado Plateau of the USA, respectively.

Modestobacter strains are Gram-positive, non-spore-forming actinobacteria which form rod and coccoid-shaped elements, and have a tendency to form short multiseptate filaments and to grow on oligotrophic media; the wall peptidoglycan contains *meso*-diaminopimelic acid (*meso*-A_2pm), major fatty acids include $C_{18:1}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$, the predominant respiratory quinone is tetrahydrogenated with nine isoprene uints (MK_9[H4]) and the major polar lipids include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, uncharactrised aminophospholipids and an unknown uncharacterised phospholipid (Xiao *et al.*, 2011; Qin *et al*., 2013).

The primary aim of the present study was to establish the taxonomic status of *Modestobacter* strains isolated from an extreme hyper-arid Atacama Desert soil and from deteriorated sandstone scrapped from the surfaces of historic buildings. The isolates were compared with one another and with the type strains of *M. multiseptatus*,

M. marinus, M. roseus and *M. versicolor* using a polyphasic taxonomic approach. Most of the isolates were assigned to two distinct subclades in the *Modestobacter* 16S rRNA but two strains from the deteriorated sandstone, namely isolates MON 3.1 and MDVD 1, formed the nuclei of two new *Modestobacter* species for which the names *Modestobacter lapidis* sp. nov. and *Modestobacter muralis* sp. nov. are proposed.

5.3. Materials and Methods

5. 3. 1. Isolation of *Modestobacter* strains

Modestobacter strains were sought from an extreme hyper-arid soil sample collected from the Yungay region of the Atacama Desert (24^{o} 06' 18.6" S / 70^{o} 01' 55.6" W) using a range of selective media (Table 5.1). The media were incubated at 28^{o}C for 21 days following inoculation with a 10^{-1} suspension of the soil. The number of *Modestobacter* strains growing on the isolation plates were counted as colony forming units (cfu) per gram dry weight soil. In contrast, isolates CAT N4, MON1.1, MON3.1, MDVD1 and MDVD4 were isolated on Luedemann's agar (Luedemann, 1971) and isolate CMB2 from *Microlunatus* agar (Nakamura *et al*., 1995), these media were inoculated with 10^{-3} and 10^{-4} suspensions of deteriorated sandstone scrapped from the surfaces of historic buildings in Salamanca, Spain (40° 57' 45" N) and incubated at 28°C for 5 weeks.

5. 3. 2. Test strains, maintenance and cultural conditions

Eleven representative isolates from the extreme hyper-arid Yungay soil with the typical appearance of *Modestobacter* colonies were taken from the isolation plates, subcultured onto modified Bennett's agar (Jones, 1949), incubated at 28^{o}C for 3 weeks and checked for purity by examination of Gram-stained smears. These strains, the six isolates from the biodeteriorated sandstone and the type strains of *M. multiseptatus, M. marinus, M. roseus* and *M. versicolor* were maintained on modified Bennett's agar slopes at room temperature and in 20% glycerol (v/v) at -20^{o}C and -80^{o}C. Biomass for most of the chemotaxonomic analyses was prepared in shake flasks (200 resolutions per minute) of yeast extract-malt extract broth (International *Streptomyces* Project [ISP] medium 2; Shirling & Gottlieb, 1966) after incubation for 14 days at 28^{o}C, whereas those for the fatty acid analyses were harvested after 3 to 5 days or until good growth was obtained

from the third quadrant (Sasser *et al.*, 1990). Biomass for the molecular systematic studies was prepared in the same way, but was stored at -20^{o}C as washed preparations.

5. 3. 3. Phylogeny

Genomic DNA was extracted from eleven representative strains isolated from the Yungay soil and from the six isolates recovered from the deteriorated sandstone. PCR amplification and 16S rRNA gene sequencing was carried out following the procedure of Kim & Goodfellow (2002). 16S rRNA gene sequences of the isolates (1386-1394 nucleotides [nt]) were aligned using MEGA version 5 software (Tamura *et al*., 2011) against corresponding sequences of the *Modestobacter* type strains taken from the GenBank database using the EzTaxon-e server (Kim *et al*., 2012). Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighnour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the MEGA 5 software; an evolutionary distance matrix for the neighbour-joining analysis was constructed after Jukes and Cantor (1969). The topologies of the inferred evolutionary trees were evaluated in bootstrap analyses (Felsenstein, 1985) based on a 1000 resamplings of the neighbour-joining dataset using MEGA 5 software. The root positions of the unrooted trees were estimated using the sequence of *Geodermatophilus obscurus* DSM 43160^{T} (GenBank accession number CP 001807) as the outgroup. The 16S rRNA gene sequences of all of the isolates were examined for the present of 16S rRNA nucleotide signatures found to be characteristic for *Geodermatophilaceae* strains (Normand & Benson, 2012a).

5. 3. 4. Chemotaxonomy

All of the isolates were examined for the presence of isomers of diaminopimelic acid (A_2pm) using the procedure described by Hasegawa *et al.* (1983). In addition, two representatives from the Yungay soil, isolates KNN45-2b, KNN46-4b, and isolates MDVD1 and MON3.1 from deteriorated sandstone were examined for other chemotaxonomic properties considered to be characteristic for *Modestobacter* strains (Normand & Benson, 2012b). Standard procedures were used to determine the menaquinone (Minnikin *et al*., 1984a), muramic acid type (Uchida *et al*., 1999), diagnostic whole-cell sugars (Schaal, 1985) and polar lipids (Minnikin *et al*., 1984). Mycolic acids were sought from the isolates using the acid methanolysis procedure

described by Minnikin *et al*. (1980). Fatty acids were extracted, methylated and analysed according to the standard protocol of the Sherlock Microbial Identification System (MIDI) and peaks were named using the database SACTIN6. The G+C mol% of the DNA of isolate KNN45-2b was determined following the procedure described by Gonzalez and Saiz-Jimenez (2002).

5. 3. 5. DNA:DNA relatedness

DNA:DNA relatedness values between isolate KNN45-2b and *M. marinus* DSM 45201^T were determined, using triplicate samples, by applying the fluorimetric method of Gonzalez & Saiz-Jimenez (2005); the optimal temperature for reassociation (Tm) was calculated by using the equation T_{or} - 0.51 (GC%) + 41. The melting temperature (Tm) at which 50% of the initial double-stranded DNA denatured into single stranded DNA for isolate KNN45-2b and for the isolate KNN45-2b : *M. marinus* DSM 45201^T hybrid DNA were compared and the differences (ΔTm) determined.

5. 3. 6. Cultural and micromorphological properties

The micromorphological properties of all of the isolates were observed from methylene blue stained smears prepared from growth taken from ISP 2 agar plates (Shirling & Gottlieb, 1966), that had been incubated at 28^{o}C for 7-10 days, and examined under the light microscope. The isolates were examined for cultural properties following growth on glucose-yeast extract malt-extract, glycerol-asparagine , inorganic salts-starch, oatmeal, peptone-yeast extract-iron, tryptone-yeast extract and tyrosine agars (ISP media 2, 5, 4, 3, 6, 1 and 7; Shirling & Gottlieb, 1966) after incubation at 28^{o}C for 14 days. A modified hanging drop method was used to determine whether the isolates were motile (Rohde, 2011).

5. 3. 7. Phenotypic tests

The seventeen isolates and the type strains of the four *Modestobacter* species were examined for a broad range of phenotypic properties. The enzyme profiles of the strains were determined using API ZYM strips (BioMerieux) and biochemical, carbon utilisation and inhibitory properties using Biolog GENIII microplates, in each case following the manufacturer's instructions; a standard inoculum equivalent to 5.0 on the McFarland scale (Murray *et al*., 1999) was used to inoculate the BIOLOG microplates

and the API-ZYM kits. The strains were examined for their ability to grow over a range of pH and temperatures following growth on ISP2 for 21 days and for their ability to grow in the presence of 5% carbon dioxide as a sole carbon source using a Thermo Forma Series II Water Jacket CO_2 incubator and carbon utilisation agar plates (ISP medium 9; Shirling & Gottlieb, 1966) that were incubated at 28^{o}C for 14 days.

5. 3. 8. Generation of whole-genome sequence of isolate KNN45-2b and genomic analysis

Isolate KNN45-2b (neé ASC16) was grown on tryptone soya broth supplemented with 10% sucrose-yeast extract-malt extract medium (1:1, v/v) with 5 mM $MgCl_2$ and 0.5 % glycine at 30^{o}C for 48 hours. Cells were resuspended in 10 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA and incubated with lysozyme at 37^{o}C for 1 to 30 minutes until they were lysed. Sodium dodecyl sulphate (0.5 %, w/v final concentration) and proteinase K (40 µg) were added and the cell extract incubated at 50^{o}C for 6 hours when a standard phenol/chloroform extraction was performed on the lysate. The extract was adjusted to 0.3 M sodium acetate (pH 5.5) and the DNA was spooled with a glass rod upon addition of 2 volumes of 96 % ethanol. After washing and drying, the DNA was dissolved in TE buffer. DNA quality was verified by *Sal*I digestion and agarose gel electrophoresis. Illumina/Solexa sequencing on Genome Analyzer IIx was outsourced (ServiceSX, Leiden, The Netherlands) and 100-nt paired-end-reads were obtained. The quality of the short reads was verified using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and, depending on the quality, reads were trimmed at both ends. Processed raw reads were used as input for the Velvet assembly algorithm version 1.206 (Zerbino & Birney 2008). The genome was annotated using the RAST server (Aziz *et al*., 2008) with default options. Predictions of gene clusters for natural products were performed using antiSMASH (Medema *et al*., 2011). The genome sequence has been deposited at DDBJ/EMBL/GenBank under the accession number JPMX00000000.

5.4. Results

Selective isolation, enumeration and initial classification. Small numbers of strains growing on the isolation plates were assigned to the genus *Modestobacter* as they formed characteristic round, slightly mucoid colonies that were initially orange to beige

in colour but later turned black (Table 5.1). The highest count, 6.0 x 10^1 cfu/g dry weight soil, was recorded from the humic acid-vitamin agar plates.

Table 5.1. *Modestobacter* strains taken to represent those growing on the selective isolation media after incubation for 21 days at 28^{o}C.

Media	**Target organism(s)**	**Number of *Modestobacter* isolates (cfu/g dry weight soil)**	**Selected isolates**
Gause's No.1 agar supplemented with nalidixic acid (10 µg/ml) (Zakharova *et al.*,2003)	Rare or uncommon actinobacteria	$4x10^1$	KNN45-1a*, KNN46-6a,* KNN46-7a*, KNN46-8a*
Geodermatophilus obscurus agar supplemented with nystatin (25 µg/ml) (Uchida &Seino, 1997)	*Geodermatophilus* spp.	$1.5x10^1$	KNN46-11f, KNN46-12f
Humic acid-vitamin agar (Hayakawa & Nonomura, 1987)	*Streptosporangiaceae* spp.	$6 x10^1$	KNN45-2b*, KNN45-3b*, KNN45-4b, KNN46-1b, KNN46-2b*, KNN46-5b
Luedemann's agar supplemented with nystatin (25 µg/ml) (Luedemann, 1971)	*Modestobacter* spp.	$1x10^1$	KNN46-10g*
Microlunatus agar supplemented with nystatin (25 µg/ml) (Nakamura *et al*., 1995)	*Modestobacter* spp.	$1x10^1$	KNN46-4b*
Minimal medium agar (Johnson *et al.*, 1981)	Rare and uncommon actinobacteria	$1x10^1$	KNN46-9c*
R2A agar supplemented with nystatin (25 µg/ml) (Reasoner & Geldreich, 1985)	*Modestobacter* spp.	$1x10^1$	KNN46-3b*

All of the media were supplemented with cycloheximide (25 µg ml^{-1}).

*Strains included in 16S rRNA gene sequence study.

Phylogeny. The assignment of the seventeen isolates to the *Modestobacter* 16S rRNA gene tree was supported by all of the tree-making algorithms and by a 100% bootstrap value (Figure 5.1). Fifteen of the isolates were recovered in two 16S rRNA phyletic lines, subclades 1 and 2, the taxonomic integrity of which was underpinned by all of the tree-making algorithms and by high bootstrap values. Strains MDVD 1 and MON 3.1, organisms isolated from deteriorated sandstone, formed distinct lineages in the *Modestobacter* 16S rRNA gene tree. All of the isolates were found to have the 16S rRNA gene signatures characteristic of members of the family *Geodermatophilaceae* (Normand & Benson, 2012a).

Eight out of the eleven strains isolated from the extreme hyper-arid Yungay soil were assigned to 16S rRNA subclade 1(Figure 5.1). These strains shared 16S rRNA gene similarities within the range 99.1 to 99.5 %, values that corresponded to 7 to 12 nt differences at 1385 to 1392 locations (Table 5.2); three of the strains, isolates KNN46-

4b, KNN46-7a and KNN46-8a, had identical 16S rRNA gene sequences. The isolates in this subclade were most closely related to the type strain of *M. versicolor* sharing 16S rRNA gene similarities with the latter within the range 99.1 to 99.5%, values that corresponded to between 7 and 9 nt differences at between 1386 and 1392 locations.

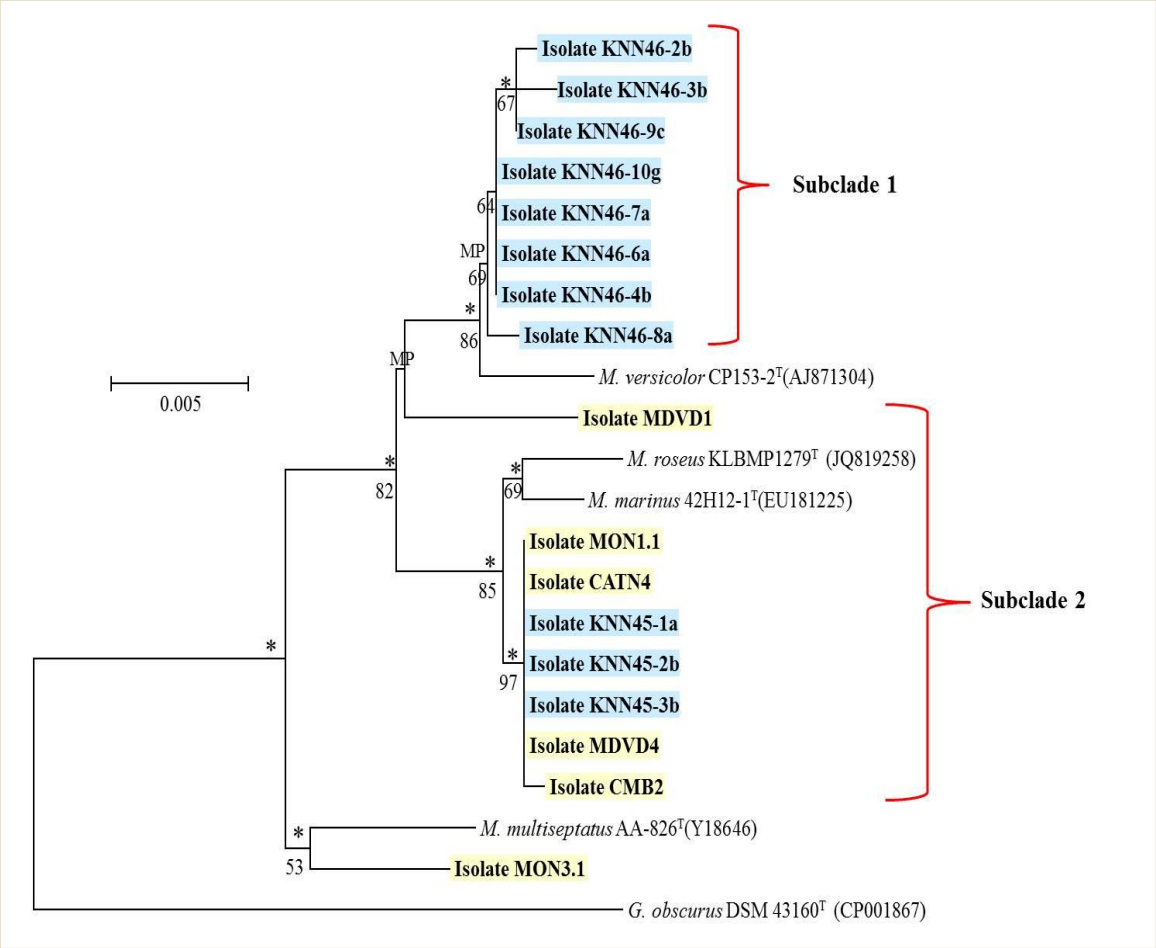


Figure 5.1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (~1390 bp) showing relationships between strains isolated from the extreme-hyper-arid Yungay soil and deteriorated sandstone and between them and the type strains of *Modestobacter* species. Asterisks indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. MP indicates branches of the tree that were supported by the maximum-parsimony method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position. Blue and yellow coloured indicated strains isolated from the Yungay soil and from deteriorated sandstone, respectively.

16S rRNA subclade 2 encompassed the three remaining isolates from the extreme hyper-arid Yungay soil and isolates CAT N4, CMB 2, MDVD 4 and MON 1.1 from deteriorated sandstone. All of these isolates, apart from strain CMB 2, had identical 16S rRNA gene sequences and were most closely related to the type strain of *M. marinus* sharing similarities with the latter of 99.6%, values equivalent to 5-6 nt differences at between 1390 and 1393 sites. The isolates were also closely related to *M. roseus* KCBMP 1279^T sharing 16S rRNA gene similarities with the latter of 99.4-99.5% values equivalent to 7-8 nt differences at between 1385-1391 locations. All of these strains formed a distinct 16S rRNA gene subclade, the taxonomic integrity of which was supported the three tree-making algorithms and by a 85% bootstrap value (Table 5.1).

The two remaining strains, isolates MDVD 1 and MON 3.1, were sharply separated from one another and from most of the other strains in the *Modestobacter* 16S rRNA gene tree (Figure 5.1). Isolate MON 3.1 and the type strain of *M. multiseptatus* formed a 16S rRNA gene subclade that was underpinned by all of the tree-making algorithms though the bootstrap value was only 53%. The two strains shared a 16S rRNA gene similarity of 98.9%, which corresponds to 16 nt differences at 1392 locations. The final isolate, strain MDVD 1, was most closely related to isolates KNN46-4b, KNN46-7a and KNN46-10g, members of subclade 1, sharing 16S rRNA gene similarities with the latter of 99.0%, a value equivalent to 14 nt differences at 1394 sites.

Chemotaxonomy. All of the isolates contained *meso*-A_2pm as the major diamino acid. Isolates KNN45-2b and KNN46-4b were found to contain galactose, glucose and xylose in whole-cell hydrolysates, N-acetyl muramic acid, tetrahydrogenated menaquinones as the sole isoprenologue, but lacked mycolic acids. They also contained major amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol, as exemplified in Figure 5.2. Fatty acid profiles of most of the isolates and the marker strains are shown tin Tables 5.3 and 5.4. The fatty acid profiles of the strains showed considerable qualitative and quantitative differences but with only two exceptions, *M. marinus* DSM 45201^T and *M. multiseptatus* DSM 44406^T, the predominant component was *iso*-$C_{16:0}$. The isolates related to the type strain of *M. versicolor*, unlike the latter, contained major amounts of $C_{17:1}$ ω9c.

Table 5.2. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from the hyper-arid Yungay soil and from deteriorated sandstone and between them and the type-strains of *Modestobacter* species.

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1.Isolate KNN46-2b	---	**4/1390**	**14/ 1388**	**14/ 1383**	**2/ 1390**	**14/ 1389**	**3/ 1390**	**2/ 1390**	**4/ 1390**	**1/ 1390**	**2/ 1390**	**9/ 1386**	**19/ 1387**	**20/ 1386**	**21/ 1385**	**14/ 1387**	**16/ 1388**	**15/ 1386**	**14/ 1387**	**14/ 1385**	**20/ 1390**	**56/ 1386**
2. Isolate KNN46-3b	**99.7**	---	**17/ 1391**	**17/ 1386**	**5/ 1393**	**17/ 1392**	**4/ 1393**	**5/ 1393**	**7/ 1393**	**3/ 1393**	**5/ 1393**	**12/ 1389**	**22/ 1390**	**23/ 1389**	**24/ 1388**	**17/ 1390**	**19/ 1391**	**17/ 1389**	**17/ 1390**	**16/ 1388**	**23 /1393**	**60/ 1389**
3.Isolate KNN45-1a	**99.0**	**98.8**	---	**0/ 1388**	**12/ 1394**	**0/ 1394**	**13/ 1394**	**12/ 1394**	**13/ 1394**	**14/ 1394**	**12 /1394**	**16/ 1390**	**5/ 1392**	**25/ 1390**	**7/ 1390**	**0/ 1392**	**16/ 1392**	**1/ 1391**	**0/ 1392**	**1/ 1390**	**21/ 1394**	**56/ 1391**
4. Isolate KNN45-2b	**99.0**	**98.8**	**100.0**	---	**12/ 1389**	**0/ 1389**	**13/ 1389**	**12/ 1389**	**13/ 1389**	**14/ 1389**	**12/ 1389**	**16/ 1385**	**5/ 1387**	**25/ 1385**	**7/ 1385**	**0/ 1387**	**16/ 1387**	**1/ 1386**	**0/ 1387**	**1/ 1385**	**21/ 1389**	**56/ 1386**
5. Isolate KNN46-4b	**99.9**	**99.6**	**99.1**	**99.1**	---	**12/ 1395**	**1/ 1396**	**0/ 1396**	**2/ 1396**	**2/ 1396**	**0/ 1396**	**7/ 1392**	**17/ 1393**	**19/ 1392**	**19/ 1391**	**12/ 1393**	**14/ 1394**	**13/ 1392**	**12 /1393**	**12/ 1391**	**18/ 1396**	**55/ 1392**
6. isolate KNN45-3b	**99.0**	**98.8**	**100.0**	**100.0**	**99.1**	---	**13 /1395**	**12/ 1395**	**13/ 1395**	**14/ 1395**	**12/ 1395**	**16/ 1391**	**5/ 1393**	**25/ 1391**	**7/ 1391**	**0/ 1393**	**16/ 1393**	**1/ 1392**	**0/ 1393**	**1/ 1391**	**21/ 1395**	**56/ 1392**
7. Isolate KNN46-6a	**99.8**	**99.7**	**99.1**	**99.1**	**99.9**	**99.1**	---	**1/ 1396**	**3/ 1396**	**3/ 1396**	**1/ 1396**	**8/ 1392**	**18/ 1393**	**20/ 1392**	**20/ 1391**	**13/ 1393**	**15/ 1394**	**13/ 1392**	**13/ 1393**	**12/ 1391**	**19/ 1396**	**56/ 1392**
8.Isolate KNN46-7a	**99.9**	**99.6**	**99.1**	**99.1**	**100.0**	**99.1**	**99.9**	---	**2/ 1396**	**2/ 1396**	**0/ 1396**	**7/ 1392**	**17/ 1393**	**19/ 1392**	**19/ 1391**	**12/ 1393**	**14/ 1394**	**13/ 1392**	**12/ 1393**	**12/ 1391**	**18/ 1396**	**55/ 1392**
9. Isolate KNN46-8a	**99.7**	**99.5**	**99.1**	**99.1**	**99.9**	**99.1**	**99.8**	**99.9**	---	**4/ 1396**	**2/ 1396**	**9/ 1392**	**18/ 1393**	**20/ 1392**	**20/ 1391**	**13/ 1393**	**15/ 1394**	**14/ 1392**	**13/ 1393**	**13/ 1391**	**19/ 1396**	**56/ 1392**
10. Isolate KNN46-9c	**99.9**	**99.8**	**99.0**	**99.0**	**99.9**	**99.0**	**99.8**	**99.9**	**99.7**	---	**2/ 1396**	**9/1392**	**19/ 1393**	**21/ 1392**	**21/ 1391**	**14/ 1393**	**16/ 1394**	**15/ 1392**	**14/ 1393**	**14/ 1391**	**20/ 1396**	**57/ 1392**
11. Isolate KNN46-10g	**99.9**	**99.6**	**99.1**	**99.1**	**100.0**	**99.1**	**99.9**	**100.0**	**99.9**	**99.9**	---	**7/ 1392**	**17/ 1393**	**19/ 1392**	**19/ 1391**	**12/ 1393**	**14/ 1394**	**13/ 1392**	**12/ 1393**	**12/ 1391**	**18/ 1396**	**55/ 1392**
12. *M. versicolor*	**99.4**	**99.1**	**98.9**	**98.8**	**99.5**	**98.9**	**99.4**	**99.5**	**99.4**	**99.4**	**99.5**	---	21/ 1391	20/ 1390	23/ 1391	**16/ 1391**	**18/ 1392**	**17/ 1389**	**16/ 1391**	**17/ 1389**	**25/ 1392**	**60/ 1388**
13. *M. marinus*	**98.6**	**98.4**	**99.6**	**99.6**	**98.8**	**99.6**	**98.7**	**98.8**	**98.7**	**98.6**	**98.8**	98.5	---	28/ 1391	8/ 1391	**5/ 1393**	**21/ 1391**	**6/ 1391**	**5/ 1393**	**6/ 1391**	**22/ 1393**	**57/ 1390**
14. *M. multiseptatus*	**98.6**	**98.3**	**98.2**	**98.2**	**98.6**	**98.2**	**98.6**	**98.6**	**98.6**	**98.5**	**98.6**	98.6	98.0	---	32/ 1389	**25/ 1391**	**23/ 1390**	**26/ 1389**	**25/ 1391**	**25/ 1389**	**16/ 1392**	**59/ 1392**
15. *M. roseus*	**98.5**	**98.3**	**99.5**	**99.5**	**98.6**	**99.5**	**98.6**	**98.6**	**98.6**	**98.5**	**98.6**	98.4	99.4	97.7	---	**7/ 1391**	**23/ 1391**	**8/ 1389**	**7/ 1391**	**8/ 1389**	**28/ 1391**	**51/ 1388**
16. Isolate MDVD4	**99.0**	**98.8**	**100.0**	**100.0**	**99.1**	**100.0**	**99.1**	**99.1**	**99.1**	**99.0**	**99.1**	**98.9**	**99.6**	**98.2**	**99.5**	---	**16/ 1391**	**1/ 1391**	**0/ 1393**	**1/ 1391**	**21/ 1393**	**56/ 1390**
17.Isolate MDVD1	**98.9**	**98.6**	**98.9**	**98.9**	**99.0**	**98.9**	**98.9**	**99.0**	**98.9**	**98.9**	**99.0**	**98.7**	**98.5**	**98.4**	**98.4**	**98.9**	---	**17/ 1390**	**16/ 1391**	**16/ 1389**	**23/ 1394**	**57/ 1390**
18. Isolate CMB2	**98.9**	**98.8**	**99.9**	**99.9**	**99.1**	**99.9**	**99.1**	**99.1**	**99.0**	**98.9**	**99.1**	**98.8**	**99.6**	**98.1**	**99.4**	**99.9**	**98.8**	---	**1/ 1391**	**1/ 1389**	**22/ 1392**	**57/ 1389**
19. Isolate CATN4	**99.0**	**98.8**	**100.0**	**100.0**	**99.1**	**100.0**	**99.1**	**99.1**	**99.1**	**99.0**	**99.1**	**98.9**	**99.6**	**98.2**	**99.5**	**100.0**	**98.9**	**99.9**	---	**1/ 1391**	**21/ 1393**	**56/ 1390**
20. Isolate MON1.1	**99.0**	**98.9**	**99.9**	**99.9**	**99.1**	**99.9**	**99.1**	**99.1**	**99.1**	**99.0**	**99.1**	**98.8**	**99.6**	**98.2**	**99.4**	**99.9**	**98.9**	**99.9**	**99.9**	---	**21/ 1391**	**56/ 1388**
21. Isolate MON3.1	**98.6**	**98.4**	**98.5**	**98.5**	**98.7**	**98.5**	**98.6**	**98.7**	**98.6**	**98.6**	**98.7**	**98.2**	**98.4**	**98.9**	**98.0**	**98.5**	**98.4**	**98.4**	**98.5**	**98.5**	---	**51/ 1392**
22. *G. obscurus*	**96.0**	**95.7**	**96.0**	**96.0**	**96.1**	**96.0**	**96.0**	**96.1**	**96.0**	**95.9**	**96.1**	95.7	95.9	95.8	96.3	**96.0**	**95.9**	**95.9**	**96.0**	**96.0**	**96.3**	---

Table 5.3. Fatty aicds (%) of *Modestobacter* subclade 1 isolates and their nearest phylogenetic neighbour, the type strain of *Modestobacter versicolor*.

Fatty acid	KNN46-2b	KNN46-3b	KNN46-4b	KNN46-6a	KNN46-7	KNN46-8a	KNN46-9c	KNN46-10g	*M. versicolor* DSM 16678^T
Iso-$C_{12:0}$	-	-	-	-	0.3	0.2	-	-	-
$C_{12:0}$	-	0.5	0.3	0.3	-	-	-	-	-
Iso-$C_{14:0}$	0.6	0.4	2.0	0.8	2.3	0.9	1.1	0.6	1.0
$C_{14:0}$	0.2	0.7	0.5	0.6	0.5	0.4	0.8	0.4	0.3
iso-$C_{15:1}$ G	1.9	1.3	1.5	0.7	1.7	1.4	0.4	0.7	2.8
iso-$C_{15:0}$	6.2	3.6	3.4	2.3	3.6	3.4	4.3	4.2	19.9
anteiso-$C_{15:0}$	0.7	0.9	1.2	1.5	1.3	0.7	1.1	0.5	2.8
$C_{15:1}$ B	0.2	-	0.4	-	0.4	0.3	0.3	0.2	-
$C_{15:0}$	0.6	1.2	0.5	0.7	0.8	0.7	1.9	1.1	0.7
iso-$C_{16:1}$ H	4.0	1.8	8.5	3.1	9.9	5.1	2.0	1.7	-
iso-$C_{16:0}$	22.9	21.1	33.1	23.8	32.8	27.8	23.0	26.2	22.2
$C_{16:1}$ ω9c	9.8	8.4	13.0	10.0	13.1	10.9	6.4	5.0	1.3
$C_{16:0}$	4.3	9.4	7.1	9.8	5.8	4.6	9.3	4.6	3.2
9-Methyl $C_{16:0}$	3.4	0.7	1.1	1.3	1.3	1.4	0.5	1.2	2.0
anteiso-$C_{17:1}$ C	0.8	0.3	0.8	1.3	0.8	0.4	0.3	0.2	0.4
iso-$C_{17:0}$	4.5	2.5	1.2	2.6	1.2	2.0	2.3	3.5	8.7
anteiso-$C_{17:0}$	2.3	2.0	1.8	5.8	1.8	1.5	2.5	1.2	4.9
$C_{17:1}$ ω9c	16.8	14.1	9.5	10.1	11.1	14.8	16.0	16.0	5.5
$C_{17:0}$ cyclo	1.7	0.9	2.2	-	1.0	1.2	0.9	0.8	-

$C_{17:0}$	2.2	3.9	1.2	2.9	1.4	2.8	7.4	4.6	4.8
10-Methyl $C_{17:0}$	1.7	1.0	1.3	1.8	1.3	2.2	0.8	3.6	0.9
$C_{18:3}$ ω6,12,14c	-	3.1	1.1	1.1	1.4	1.1	-	0.9	1.0
iso-$C_{18:0}$	0.3	0.4	0.2	0.6	-	0.5	0.4	0.7	0.4
$C_{18:1}$ ω9c	9.7	14.6	4.6	15.9	4.1	12.6	10.0	15.5	9.8
$C_{18:0}$	0.9	2.5	0.4	2.3	0.4	1.1	3.5	1.8	3.9
$C_{17:0}$ *iso*-2OH	1.4	2.2	1.7	-	1.1	1.5	2.5	2.3	0.9
Summed feature 3	-	-	-	-	-	-	-	-	-
Summed feature 7	1.4	0.9	0.3	1.0	0.3	0.8	0.6	0.6	-
Summed feature 9	1.0	-	0.9	-	0.4	-	1.4	1.9	1.3

Summed feature 3: C16:1 ω7c , summed feature 7: $C_{18:1}$ ω7c/ $C_{18:1}$ ω9t/ $C_{18:1}$ ω12t. and summed feature 9: *iso*-C_{17} ω9c and/or 10-methyl $C_{16:0}$.

Table 5.4. Fatty acids (%) of *Modestobacter* subclade 2 isolates and strains MDVD 1 and MON 3.1 and their nearest phylogenetic neighbours, the type strains of *M. marinus*, *M. multiseptatus* and *M. roseus.*

Fatty acid	*Modestobacter* subclade 2							*M. marinus* DSM 45201^T	*M. roseus* DSM 45764^T	*M. multiseptatus* DSM 44406^T	MON3.1	MDVD1
	MDVD4	CATN4	MON1.1	CMB2	KNN45-1a	KNN45-2b	KNN45-3b					
iso-$C_{12:0}$	0.1	-	-	-	-	-	-	-	-	-	-	-
$C_{12:0}$	0.2	-	-	0.1	0.7	0.3	0.2	-	-	-	-	-
iso-$C_{13:0}$	-	-	-	0.0	-	-	-	0.2	-	-	0.10	-
$C_{13:0}$	0.2	0.3	-	0.1	-	-	-	0.4	-	-	-	-
iso-$C_{14:0}$	2.5	1.3	0.7	1.4	1.6	1.5	1.9	1.3	2.4	1.1	2.1	1.4
$C_{14:0}$	0.5	1.0	0.4	0.6	0.9	0.8	0.3	1.0	0.8	0.4	2.1	0.9
iso-$C_{15:1}$ G	0.6	0.4	1.3	1.2	0.7	0.8	1.5	0.7	-	1.7	5.3	-
iso-$C_{15:0}$	3.6	3.6	5.1	4.4	7.4	7.1	2.9	9.7	11.0	21.5	17.4	8.2
anteiso-$C_{15:0}$	0.9	1.3	2.6	0.9	1.0	0.9	0.6	4.2	4.5	3.7	2.5	1.5
$C_{15:1}$ B	0.5	0.5	0.2	0.5	0.3	0.4	0.4	0.7	-	-	1.2	-
$C_{15:0}$	4.3	4.9	0.8	2.0	1.7	1.5	0.6	5.6	1.3	1.3	2.3	3.9
iso-$C_{16:1}$ H	1.7	1.2	2.1	4.3	1.6	1.3	9.4	0.3	5.3	0.5	3.6	-
iso-$C_{16:0}$	34.6	16.4	24.6	28.1	22.6	23.4	39.1	10.3	21.9	19.7	21.8	16.1
$C_{16:1}$ ω9c	4.1	5.6	8.1	9.6	8.0	7.3	11.1	3.2	-	1.0	10.0	3.6
$C_{16:0}$	6.9	11.5	8.1	6.9	8.8	8.9	5.7	11.2	7.2	3.8	7.6	8.3
9-Methyl-$C_{16:0}$	0.2	-	0.9	0.7	0.8	0.7	0.9	0.3	-	1.2	0.5	-
anteiso-$C_{17:1}$ C	0.1	-	1.0	0.3	-	0.2	0.4	0.2	-	0.2	0.5	-

iso-$C_{17:0}$	0.8	1.3	2.8	1.3	2.8	3.1	0.9	2.8	3.1	8.8	1.0	3.2
anteiso-$C_{17:0}$	0.8	1.5	6.5	0.9	1.4	1.4	0.7	3.7	2.1	5.99	1.6	2.3
$C_{17:1}$ ω8c	-	-	-	-	-	-	-	-	15.0		-	-
$C_{17:1}$ ω9c	17.3	14.3	11.6	15.5	13.8	13.5	8.5	13.7	-	4.81	6.9	19.4
$C_{17:0}$ cyclo	0.7	1.5	1.6	2.8	0.7	1.0	2.5	0.2	1.0	-	2.3	-
$C_{17:0}$	10.3	16.4	4.1	4.7	5.3	5.2	1.2	20.0	9.6	8.6	1.5	17.2
10-Methyl-$C_{17:0}$	0.7	0.4	1.1	1.3	0.8	0.8	2.0	-	1.0	0.4	0.2	0.5
$C_{18:3}$ ω 6,12,14c	0.9	2.1	0.4	0.5	4.1	2.0	1.2	1.3	-	0.9	-	1.0
iso-$C_{18:0}$	1.3	0.5	0.7	0.3	0.5	0.5	0.3	-	-	0.5	-	-
$C_{18:1}$ ω9c	2.4	5.1	9.3	4.9	9.8	10.2	4.4	2.1	2.1	5.5	3.9	5.4
$C_{18:0}$	2.3	3.8	2.2	0.8	3.0	3.2	0.6	3.7	0.6	6.6	0.8	2.8
$C_{17:0}$ *iso*-2OH	1.5	3.7	1.9	3.7	1.8	2.1	1.6	2.6	-	0.7	2.7	3.2
Summed feature 3	-	-	-	-	-	-	-	-	8.4	-	-	-
Summed feature 7	-	0.4	0.4	0.7	-	0.5	0.2	-	0.4	-	0.1	-
Summed feature 9	0.3	1.3	1.5	1.4	-	1.5	0.9	0.4	1.5	0.8	1.6	1.0

Summed feature 3: $C_{16:1}$ ω7c and/or $C_{17:1}$ ω6c; summed feature 7: $C_{18:1}$ ω7c/ $C_{18:1}$ ω9t/ $C_{18:1}$ ω12c and summed feature 9: *iso*-C_{17} ω9c and/or 10-methyl-$C_{16:0}$.

and with two exceptions, isolates KNN46-4b and KNN46-7a, had similar proportions of $C_{18:1}\omega9c$ (Table 5.3). In general, the isolates belonging to 16S rRNA gene subclade 1 showed similar fatty acid profiles to one another and to a lesser extent with *M. marinus* DSM 45201^T. In contrast, isolate MON 3.1 and *M. multiseptatus* DSM 44406^T exhibited markedly different qualitative and quantitative fatty acid profiles. The type strain of *M. roseus* was readily distinguished from those of the other strains as it lacked $C_{17:1}\omega8c$ but contained a major amount of *iso*-$C_{16:0}$. The G+C content of the DNA of isolate KNN45-2b was 72.5±1%.

DNA:DNA relatedness. The Δ*T*m between isolate KNN45-2b g DNA and isolate KNN45-2b : *M. marinus* DSM 45201^T hybrid DNA was 4.9±0.3%, a result which can be equated with a DNA:DNA similarity of 70.2±0.1% (Gonzalez & Saiz-Jimenez, 2005), a value very close to the cut-off point for assigning strains to the same genomic species (Figure 5.3).

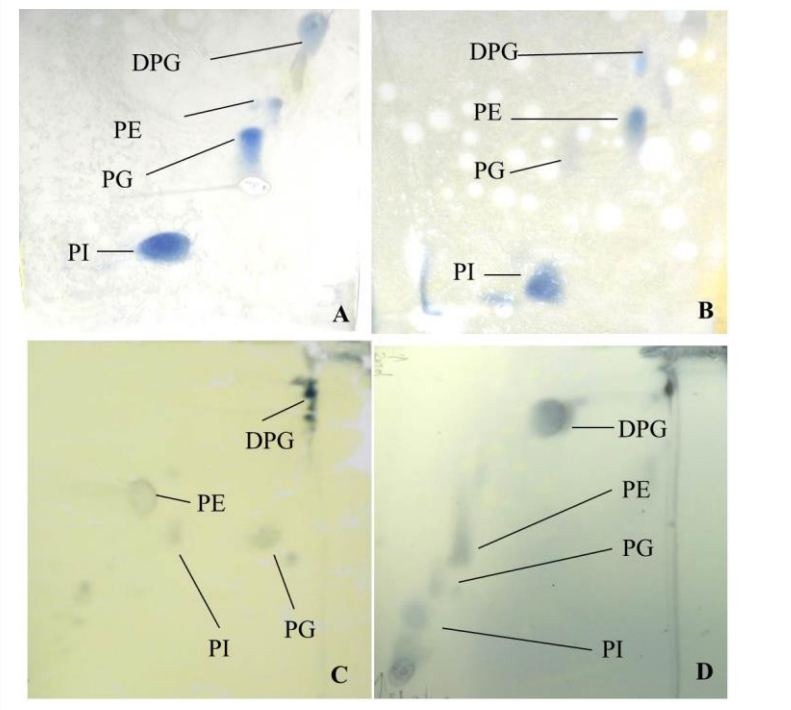


Figure 5.2. Two-dimensional thin-layer chromatography of polar lipids of isolates (A), KNN45-2b; (B), KNN46-4b; (C) MDVD 1 and (D) MON 3.1 stained with molybdenum blue spray (Sigma). Chloroform:methanol:water (32.5:12.5:2.0, v/v) was used in the first direction, followed by chloroform : acetic acid : methanol : water (40.0 : 7.5 : 6.0 : 2.0,v/v) in the second direction. Abbrevations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol and PI, phosphatidylinositol.

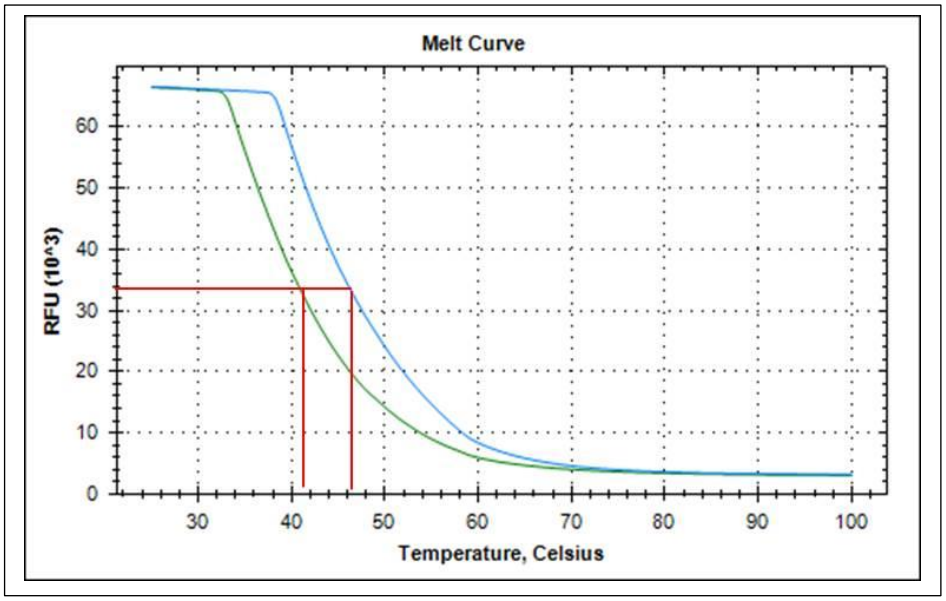


Figure 5.3. Thermal denaturation of genomic DNA from isolate KNN45-2b and isolate KNN45-2b : *M. marinus* DSM 45201^T hybrid DNA. The calculated Δ*T*m is 4.9^{o}C±0.3.

Cultural and morphological properties. All of the strains grew well on ISP media 1, 2, 3, 6 and 7, moderately on ISP medium 5 and poorly on ISP medium 4 (Table 5.5). Colony colours ranged from yellowish white to black; strains assigned to the *M. versicolor* 16S rRNA subclade produced diffusible pigments on ISP media 2 and 7. All of the isolates formed entire, flat, round, mucoid colonies with entire margins.

Table 5.5. Growth and cultural characteristics of isolates and *Modestobacter* type strains on ISP media after incubation for 14 days at 28^{o}C.

Media	Growth	Substrate mycelium colour	Diffusible pigment
Glycerol-asparagine agar (ISP 5)	++	Olivaceous black	None
Inorganic salts-starch agar (ISP 4)	+	Yellowish white	None
Oatmeal agar (ISP 3)	+++	Olivaceous black	None
Peptone-yeast extract-iron agar (ISP 6)	+++	Black / orange*	None/ Light yellow*
Tryptone-yeast extract agar (ISP 1)	+++	Yellowish white	None
Tyrosine agar (ISP 7)	+++	Yellowish white	None / Light yellow*
Yeast extract-malt extract agar (ISP 2)	+++++	Black	None / Light yellow*

++++ abundant growth; +++ very good growth; ++ good growth; + poor growth.
* Results for members of the *M. versicolor* DSM 16678^T and associated isolates from the Yungay soil(see Figure 5.1).

Phenotypic properties. Identical results were obtained for the duplicated strains in all of the phenotypic tests. The seventeen isolates and the *Modestobacter* type strains grew at 20 and 28^{o}C, at pH 7 and 8, produced acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine aryamidase (API ZYM tests), reduced nitrate to nitrite, hydrolysed urea, and used acetoacetic acid and dextrin as sole carbon sources, grew in the presence of minocycline, potassium tellurite and 1%, w/v sodium chloride (Biolog Gen III microplates) and showed scant growth in the presence of 5% carbon dioxide as a sole carbon source. In contrast, none of strains grew at 10 or 40^{o}C, at pH 4, 5, 9, 10 or 11, produced α-fucosidase, α- or β-galactosidases, N-acetyl-β-glucosamidase, or grew on α-amino-butyric acid, formic acid, α-methyl-D-glucoside, glycyl-proline or methyl pyruvate as sole carbon sources (Biolog GEN III microplates).

It can be seen from Table 5.6 that the *Modestobacter* type strains can be distinguished from one another using a broad range of phenotypic properties. With few exceptions the subclade 1 isolates from the extreme hyper-arid Yungay soil shared the same phenotypic profile, ones that readily distinguished them from all of other isolates and from the *Modestobacter* type strain (Table 5.6). Unlike *M. versicolor* DSM 16678^{T}, their nearest phylogenetic neighbour, these isolates produced alkaline phosphatase, cysteine arylamidase and trypsin, conversely only the *M. versicolor* type strain used D-aspartic acid, D-cellobiose, D-fructose-6-PO_4, D-galacturonic acid, gelatin, gentobiose, D-gluconic acid, 3-methyl glucose, α-D-glucose, L-glutaric acid, D- & L-lactic acid, D-malic acid and D-pectin as sole carbon sources.

The subclade 2 isolates had many phenotypic properties in common some of which distinguish them from the type strain of *M. marinus*, their closet phylogenetic neighbour (Table 5.6). The isolates, unlike *M. marinus* DSM 45201^{T}, grew at pH 5 and 6, in the presence of guadinine hydrochloride, conversely only the *M. marinus* strains used inosine and D-serine as sole carbon sources. In contrast, only the type strain of *M. marinus* used D-galactose, D-glycerol, D-mannose, stachyose and D-turanose, as sole carbon sources. These isolates can also be distinguished from the type strain of *M. roseus* by their ability to grow in the presence of azetreonam, gusanidine hydrochloride, lithium chloride, niaproof 4, at pH5 and used L-alanine and sodium butyrate as sole carbon compounds though the *M. roseus* strain showed a much greater ability to assimilate carbon compounds (Table 5.6).

Isolates MON3.1 and MDVD1, which formed distinct branches in the *Modestobacter* 16S rRNA gene tree, had phenotypic properties that readily distinguished them from one another and from all of the remaining strains (Table 5.6), including their nearest neighbours. Thus, isolate MON 3.1 produced cysteine arylamidase, and lipase (C14) and used acetic acid, D-cellobiose, α-ketoglutaric acid, D-trehalose and Tween 40 as sole carbon sources through, in general, the *M. multiseptatus* strain showed a much greater capacity to grow and to metabolise carbon compounds and grow in the presence of inhibitory agents. Similarly, isolate MDVD1 and the type strain of *M. multiseptatus* were readily distinguished, only the former produced alkaline phosphatase and cysteine arylamidase and only the latter α-chymotrypsin and α- and β-glucosidases. In addition, *M. multiseptatus* DSM 44406^T assimilated a much broader range of carbon compounds, as exemplified by its ability to use D-cellobiose, 3-methyl-glucose, D- and L-lactic acid, methyl ether, L-malic acid and D-salicin as sole carbon sources.

Table 5.6. Phenotypic properties that distinguish the *Modestobacter* isolates from one another and from the type strains of *Modestobacter*.

Characteristics	*M. multiseptatus* DSM 44406^T	*M. marinus* DSM 45201^T	*M. roseus* DSM 45764^T	*M. versicolor* DSM 16678^T	*Modestobacter* subclade 1 isolates	*Modestobacter* subclade 2 isolates	Isolate MDVD1	Isolate MON 3.1
API ZYM tests:								
Alkaline phosphatase	+	+	+	-	+	+	+	-
α-Chymotrypsin	+	+	+	+	+	+	-	+
Cysteine arylamidase	-	+	+	-	+	+	+	+
Esterase lipase (C8)	+	+	+	-	-	+	-	-
α-glucosidase	-	-	+	+	+	-	-	+
β-glucosidase	-	-	+	+	+	-	-	-
Trypsin	-	-	+	-	+	-	-	-
Micromorphology:								
Motility	Motile	Non-motile	Motile	Motile	Motile	Non-motile	Motile	Motile
Colony properties:	Mucoid	Round , mucoid	Flat, mucoid	Flat , mucoid	Mucoid	Round , mucoid	Mucoid	Flat, mucoid
Diffusible pigments:								
Glucose-yeast extract-malt extract agar	-	+/-	-	+	+	+/-	-	-
Peptone-yeast extract-iron agar	-	-	-	+	-	-	-	-
Tyrosine agar	-	-	-	+	-	-	-	-

Resistance to inhibitory compounds:								
Azetreonam	+	+	-	+	+	+	+	+
Fusidic acid	+	+	+	+	+	+	+	-
Guanidine HCl	+	-	-	+	-	+	-	-
Lincomycin	+	-	+	+	+	+	+	-
Lithium chloride	+	+	-	+	+	+	+	+
Nalidixic acid	+	+	+	+	+	+	+	-
Niaproof4	+	+	-	+	+	+	+	-
Rifamycim SV	+	-	+	+	+	-	+	+
Tetrazoliun blue	+	-	+	+	+	-	+	-
Tetrazoliun violet	+	+	+	+	+	+	+	-
Troleandomycin	+	-	+	+	+	-	+	-
Vancomycin	+	-	+	+	+	+	+	-
Growth at:								
pH5	+	-	-	+	+	+	+	-
pH6	+	-	+	+	+	+	+	+
pH8	+	+	+	+	+	+	+	+
pH9	+	+	+	+	+	+	+	+
Growth in the present of:								
4%, (w/v) NaCl	+	-	+	+	+	+	+	+
8%, (w/v) NaCl	+	-	-	+	+	-	+	+
Sole carbon sources:								
Acetic acid[1]	-	+	+	-	-	+	-	+
N-Acetyl-D-glucosamine	-	+	+	-	-	+	-	-
N-Acetyl-β-D-mannose	-	+	+	-	-	+	-	-
N-Acetyl-neuramic acid	+	+	+	-	-	+	-	-
L-Alanine	+	+	-	-	-	+	-	-
D-Arabitol	+	-	+	-	-	-	-	-
L-Arginine	+	-	-	-	-	-	-	-
D-Aspartic acid	+	+	+	+	-	+	-	+
L-Aspartic acid	+	-	+	-	-	-	-	+
α-keto-butyric acid	-	-	+	-	-	-	-	-
α-hydroxy butyric acid	+	-	-	+	+	-	-	-
β-hydroxy-D,L-butyric acid	+	-	-	-	-	-	-	+
D-Cellobiose	-	+	+	+	-	+[1]	-	+
Citric acid	-	-	+	-	-	-	-	-
D-Fructose	-	+	+	-	-	+	-	-
D-Fructose-6PO_4	+	+	+	+	-	+	-	+
D-Fucose	-	-	+	-		-	-	-
L-Fucose	+	-	+	-	-	-	-	-
D-Galactose	+	+	-	-	-	-	-	-
D-Galacturonic acid	+	+	+	+	-	+	-	-
L-Galacturonic acid lactone	+	-	+	-	-	-	-	-
Gelatin	-	-	+	+	-	-[2]	-	-
Gentiobiose	-	+	+	+	-	+	-	-
D-Gluconic acid	+	-	-	+	-	-[3]	+	+
D-Glucuronic acid	+	-	-	-	-	-[4]	+	-
Glucuronamide	+	-	+	-	-	-[5]	-	-
3-Methyl glucose	-	-	-	+	-	-[6]	-	-
α-D-Glucose	+	+	+	+	-	+[7]	-	-
D-Glucose-6-PO_4	+	+	+	-	-	+[8]	-	+
α-*keto*-Glutaric acid	-	-	+	-	-	-[9]	+	+
L-Glutamic acid	+	-	-	+	-	-	-	+
Glycerol	+	+	-	-	-	-	-	-
L-Histidiine	+	-	-	-	-	-	-	-
Inosine	+	-	+	+	+	+	+	+
Myo-Inositol	+	-	-	-	-	-	-	-
D-Lactose	-	+	+	-	-	+	-	-

L-Lactic acid	+	+	+	-	-	+	-	-
D- & L- Lactic acid methyl ether	+	+	-	+	-	+[10]	-	-
D-Malic acid	+	-	+	+	-	-[11]	+	-
L-Malic acid	+	-	+	+	+	-[12]	-	+
D-Maltose	+	-	+	-	-	-	-	+
D-Mannose	+	+	+	-	-	-	-	-
L-Mannose	-	+	+	-	-	+	-	-
D-Melibiose	+	+	+	-	-	+	-	-
Mucic acid	+	-	+	-	-	-	-	+
D-pectin	+	+	+	+	-	+[13]	-	-
ρ-Hydroxy-phenylacetic acid	-	-	+	-	-	-	-	-
Propionic acid	+	-	+	+	-	-[14]	-	-
L-pyroglutamic acid	+	-	+	+	-	-	-	-
Quinic acid	+	-	+	+	-	-	-	+
D-Raffinose	-	+	+	+	-	+	-	+
L-Rhamnose	+	-	+	-	-	-	-	-
D-Saccharic acid	+	-	+	-	-	-	-	-
D-Salicin	+	-	+	+	-	-	-	-
D-Serine	+	-	+	-	-	+	-	-
L-Serine	+	+	+	-	-	+	-	+
Stachyose	-	+	-	-	-	-	-	-
Sodium butyrate	+	-	-	+	+	+	+	-
Sodium bromide	+	-	-	+	+	-	+	+
1% sodium lactate	+	-	+	+	+	-	+	+
D. Sorbitol	-	-	+	-	-	-	-	-
Bromo-succinic acid	-	-	+	-	-	-	-	-
D-Sucrose	+	+	+	-	-	+	-	+
D-Trehalose	-	+	-	-	-	+[15]	-	+
D-Turanose	-	+	+	-	-	-	-	+
Tween 40	+	+	+	+	+	+	+	+

+ : positive; -: negative; +/- variable. *Codes for these isolates are shown in Figure 5.1.
Positive results were recorded for :[1]isolates KNN46-7a, KNN46-8a and KNN46-10g; [2]isolates KNN46-3b and KNN46-6a; [3]isolates KNN46-6a and KNN46-10g; [4]isolates KNN46-6a; KNN46-8a, KNN46-9c; [5]isolates KNN46-3b, KNN46-4b, KNN46-6a and KNN46-8a; [6]isolates KNN46-6a and KNN46-10g; [7]isolates KNN46-8a, KNN46-9c and KNN46-10g; [8]isolates KNN46-4b; KNN46-6a and KNN46-8a; [9]isolates KNN46-4b, KNN46-6a and KNN46-8a; [10]isolates KNN46-3b, KNN46-4b, KNN46-7a and KNN46-8a; [11]isolates KNN46-4b, KNN46-6a and KNN46-8a; [12]isolates KNN46-2b, KNN46-3b, KNN46-9c; [13]isolates KNN46-3b, KNN46-6a, KNN46-8a and KNN46-10g ; [14]isolates KNN46-3b, KNN46-4b, KNN46-6a and KNN46-7a and [15]isolates KNN46-9c and KNN46-10g .

Analysis of whole genome sequence of isolate KNN45-2b. Full genome sequencing of strain KNN45-2b (GenBank accession number JPMX00000000) using Illumina methods led to an assembly of 140 contigs for a total genome size of ~4.96 Mb, predicted to encode 4,683 proteins (Figure 5.4). The functions of the genes were catalogued into different functional classes. The relative distribution of the different classes is similar to that of the model strains "*Streptomyces coelicolor"* A3(2) (Bentley *et al*., 2002) and "*Streptomyces lividans"* (Cruz-Morales *et al.*, 2013). RAST is a widely used annotation tool that allows good initial prediction of gene functions. However, for a more detailed prediction regarding the specific functions of secondary metabolism-

related genes, a dedicated algorithm was required such as antiSMASH (Medema *et al*., 2011). Only a handful of natural product clusters were predicted (two terpene-, one siderophore-, two polyketide synthase-type clusters and one of unknown type). This can be explained by the relatively small size of this genome when compared to those other actinomycetes (~ 5 Mb *vs.* 8-10 Mb).

5.5. Discussion

All of the isolates were Gram-positive, formed rod- and coccoid-shaped cells that tended to be aggregated together, produced beige to orange colonies which on prolonged incubation turned black, and had whole cell hydrolysates rich in *meso*-A_2pm, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1974). The representative isolates were found to contain tetrahydrogenated menaquinones with nine isoprene units as sole isoprenologues, and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine (diagnostic phospholipid) and phosphatidylinositol as major polar lipids (phospholipid type 2; Lechevalier *et al*., 1977, 1981), but lacked mycolic acids, and with two exceptions contained *iso*-$C_{16:0}$ as the predominant fatty acid. The G+C content of the DNA of isolate KNN45-2b was 72.5$\pm$1% All of these properties are consistent with the classification of the isolates in the genus *Modestobacter* (Mevs *et al*., 2000; Normand & Benson, 2012b; Qin *et al*., 2013).

Three out of the eleven representatives of the *Modestobacter* strains isolated from the extreme hyper-arid Yungay soil and four out of the six strains from deteriorated sandstone shared high 16S rRNA gene similarities, had similar fatty acid profiles and many phenotypic properties in common. They were most closely related to *M. marinus* DSM 45201^T but can be distinguished from the latter on the basis of their fatty acid profile and by a broad range of phenotypic properties. Further comparative taxonomic studies are needed to clarify relationships between the subclade 2 isolates to determine whether they represent one or more *Modestobacter* species.

The remaining isolates from the Yungay soil showed high 16S rRNA gene similarities (99.1 to 99.5%) and were found to have many phenotypic properties in common. These isolates were readily distinguished from all of the remaining *Modestobacter* strains, including *M. versicolor*, their nearest phylogenetic neighbour, using a combination of phenotypic properties. Further studies need to be carried out on

these strains to establish whether they belong to one or more novel *Modestobacter* species.

The two remaining isolates, strains MON 3.1^T and MDVD 1^T, merit recognition as novel *Modestobacter* species as they formed well delineated branches in the *Modestobacter* 16S rRNA gene tree and can be distinguished from all of the remaining strains, including their nearest phylogenetic neighbours, using a battery of phenotypic properties. It is, therefore, proposed that isolates MON 3.1 and MDVD 1 be recognised as new *Modestobacter* species, namely as *Modestobacter lapidis* sp. nov. and *Modestobacter muralis* sp. nov.

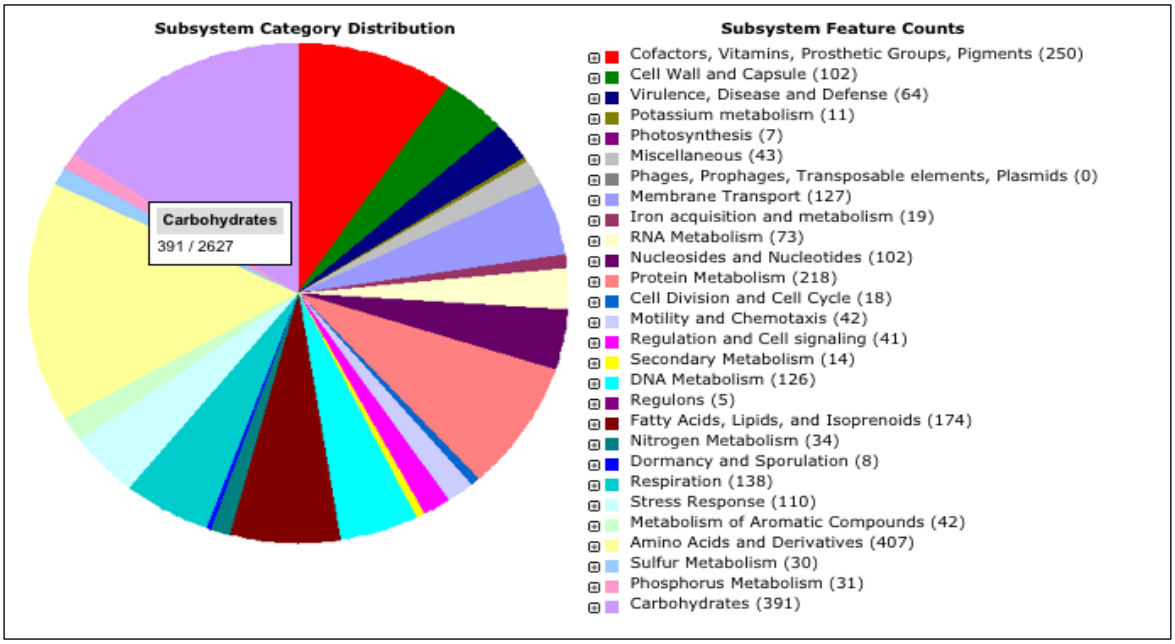


Figure 5.4. Overview of *Modestobacter* strain KNN45-2b subsystem gene functions as generated by analysis on the RAST server at (http://rast.nmpdr.org).

Relatively little is known about the distribution, numbers, kinds and activities of *Modestobacter* strains in natural habitats. However, members of this taxon, like *Blastococcus* and *Geodermatophilus* strains, tend to be associated with extreme habitats characterised by dryness, low nutrient availability and high UV radiation (Eppard *et al*., 1996; Urzì & Realini, 1998; Urzì, *et al*., 2001; Salazar *et al*., 2006). In the present study the isolation of small numbers of *Modestobacter* strains from the extreme hyper-arid Yungay soil is further evidence that these organisms are present in dry, nutrient poor habitats subject to high UV radiation. To the best of our knowledge this is the first report on the isolation of *Modestobacter* strains from arid desert soils. Furthermore, the circumscription of two novel *Modestobacter* strains from deteriorated sandstone is in

line with the view that isolates associated with the family *Geodermatophilacea*e show a relatively marked genotypic and phenotypic diversity on exposed and arid rock-surfaces (Eppard *et al.*, 1996; Urzì, *et al*., 2001;Essoussi *et al*., 2010).

The ability of *Modestobacter* strains to survive under harsh conditions such as those outlined above, may be associated with their pleomorphic morphology, thick walls and ability to synthesise melanin-like pigments (Essoussi *et al*., 2010). The observation that *Geodermatophilus* strains can be transported over long distances, and resist radiation and desiccation while in the high atmosphere (Chuvochina *et al*., 2011), similarly may account for the isolation of the subclade 2 isolates from environmental samples collected as far apart as Chile and Spain. However, additional research is needed to build upon these pioneering studies, notably by establishing the ecological roles that *Modestobacter* strains play in harsh environments. Detailed annotation of the KNN45-2b genome, particularly of the stress response genes, followed by appropriate physiological studies should enable the chemical ecology of these and other members of the family *Geodermatophilaceae* recovered from the Atacama Desert to be defined.

Description of *Modestobacter lapidis* sp. nov.

Modestobacter lapidis sp. nov. (la.pi'dis). L. gen. n. *lapidis* of a stone.

Aerobic, Gram-positive, motile, non-spore forming actinobacterium which forms short rods and cocci (1.5-2.8 x 1.7-3.0 µm). Grows well on ISP medium 2 producing black mucoid colonies. Grows from 20 to 37^{o}C, optimum temperature is 28^{o}C, from pH 6-9 and in the presence of 8% NaCl (w/v). Additional phenotypic test results are cited in the text and in Tables 5.3 and 5.4. Chemotaxonomic properties are typical of the genus.

The type strain MON 3.1^{T} (= DSM……., = NCIMB……, = NRRL…….) was isolated from a deteriorated sandstone historic building in Salamanaca, Spain. The description is based on a single strain and hence serves as the description of the type strain. The GenBank accession number for the 16S rRNA gene sequence of strain MON 3.1^{T} is …….

Description of *Modestobacter muralis* sp. nov.

Modestobacter muralis sp. nov. (mu.ra'lis. L. adj. *muralis* pertaining or belonging to walls.

Aerobic, Gram-positive, non-spore forming actinobacterium that forms slightly curved, short rods (0.5-1.2 x 1.0-3.0 µm). Grows well on ISP medium 2 producing flat, black mucoid, colonies. Grows from 20 to 37^{o}C, optimal temperature is 28^{o}C, from pH 6-9

and in the presence of 8%, w/v NaCl. Additional phenotypic test results are cited in the text and in Tables 5.3 and 5.4. Chemotaxonomic properties are typical of the genus.

The type strain MDVD 1^T (= DSM……., = NCIMB……, = NRRL…….) was isolated from deteriorated sandstone from a historic building in Salamanaca, Spain. The description is based on a single strain and hence serves as a description of the type strain. The GenBank accession number for the 16S rRNA gene sequence of strain MDVD 1^T is …….

Acknowledgements

The fatty acid data and the 16S rRNA gene sequence data on isolates MDVD1, MDVD4, CAT N4, CMB2, MON1.1 and MON3.1 were provided by Professor Martha Trujillo (University of Salamanca, Spain). The full genome sequence data on isolate KNN45-2b was generated by Dr. Geneviève Girard and Professor Gilles van Wezel (Institute of Biology, University of Leiden, Leiden, The Netherlands).

Chapter 6. Polyphasic Studies on Presumptive *Streptomyces* Strains isolated from Hyper-arid and Extreme Hyper-arid Atacama Desert Soils

6.1. Abstract

Twenty-five presumptive novel streptomycetes isolated from hyper-arid and extreme hyper-arid Atacama Desert soils were the subject of polyphasic studies designed to establish their taxonomic status. All of the isolates were found to exhibit chemotaxonomic, cultural and morphological properties consistent with their classification in the genus *Streptomyces*. Five of the isolates formed a well supported subclade in the *Streptomyces* 16S rRNA gene tree together with the type strain of *Streptomyces fimbriatus*. Three of these isolates were considered to be membere of this species based on genotypic and phenotypic criteria; an emended description of *S. fimbriatus* is given. Seven of the isolates formed a well delineated subclade in the *Streptomyces pseudogriseolus* 16S rRNA gene subclade, but further studies are needed to establish whether they merit species status. The remaining strains, including isolate C34, formed a distinct subclade in the *Streptomyces* 16S rRNA gene tree and shared a wealth of phenotypic features. Multilocus sequence analyses (MLSA) based on five house-keeping gene alleles underpinned the separation of isolate C34^T and related strains from all of their nearest neighbours, apart from the type strains of *Streptomyces chiangmaiensis* and *Streptomyces hyderabadensis* which are not currently in the MLSA database. Strain C34^T and the other members of the subclade were distinguished readily from the *S. chiangmaiensis* and *S. hyderabadensis* strains using a combination of cultural and morphological data. Consequently, strain C34^T and the other fourteen members of the well-delineated 16S rRNA subclade are considered to form a new species of the genus *Streptomyces* for which the name *Streptomyces leeuwenhoekii* sp. nov. is proposed. The type strain is C34^T (=DSM 42122^T = NRRL B-24963^T). Analysis of the whole-genome sequence of *S. leeuwenhoekii* C34^T, with 6,780 predicted open reading frames and total genome size of around 7.86 Mb, revealed a high potential for natural product biosynthesis.

6.2. Introduction

A major attraction of strains assigned to the genus *Streptomyces* is their unique capacity to synthesise new specialised metabolites, notably antibiotics, that can be developed as resources for healthcare (Hopwood, 2007; Bérdy, 2012). Streptomycetes account for about 40% of all known natural products and have genomes that typically contain over twenty biosynthetic gene clusters that encode for known or predicted specialised metabolites (Goodfellow & Fiedler, 2010; Becerril-Espinosa *et al*., 2013). It is, however, difficult to find new chemical entities from know *Streptomyces* species as screening them tends to lead to the costly rediscovery of known bioactive compounds (Busti *et al*., 2006; Williams, 2008) Consequently, innovative strategies are needed to selectively isolate, dereplicate and identify new *Streptomyces* species for pharmaceutical screening programmes, as illustrated by the taxonomic approach to drug discovery recommended by Goodfellow and Fiedler (2010).

The taxonomic approach to drug discovery has been shown to be particularly effective in the isolation of novel streptomycetes from extreme habitats, as exemplified by the discovery of caboxamycin, a new bensoxazole antibiotic from extracts of a *Streptomyces* strain isolated from an Atlantic Ocean deep sea sediment (Hohmann *et al*., 2009) and warkmycin, a novel angucycline antibiotic, produced by a *Streptomyces* strain isolated from an embryonic sand dune (Helaly *et al*., 2013). This strategy has recently been applied in studies of actinobacterial communities in Atacama Desert soils in Northern Chile (Bull & Asenjo, 2013). This, the oldest and driest desert on the planet has evolved over several million years of aridity and hyper-aridity (Gomez-Silva *et al*., 2008).

Despite, the extreme conditions of the Atacama Desert, phylogenetically novel actinobacteria, notably streptomycetes, have been isolated from soils taken from hyper-arid extreme hyper-arid regions of the desert (Okoro *et al*., 2009; Bull & Asenjo, 2013). Three of the putatively novel streptomycetes have been validly named as *Streptomyces atacamensis*, *Streptomyces bullii* and *Streptomyces deserti* (Santhanam *et al*., 2012a, b, 2013). In addition, *Streptomyces* isolates C34, C58 and C79, representatives of a large, well delineated 16S rRNA gene subclade (Okoro *et al*., 2009) are the source of novel antibiotics, namely the chaxalactins and chaxamycins from the former (Rateb *et al*., 2011a, b), four new specialised metabolites from the latter (Fiedler *et al*., unpublished) and a novel lasso peptide from isolate C58 (Jaspars *et al*., unpublished). Another putatively novel *Streptomyces* strain from high altitude Atacama Desert soil produces

novel aminobenzoquinones, the abenquines, which show inhibitory activity against bacteria and dermatophilic fungi (Schulz *et al*., 2011).

The primary aim of the present study was to establish the taxonomic status of isolates C34, C58 and C79 and additional putatively novel *Streptomyces*, strains isolated from extreme hyper-arid and hyper-arid Atacama Desert soils using a polyphasic approach. Most of the isolates, including strains C34, C58 and C79, were shown to form a new centre of taxonomic variation in the genus *Streptomyces*, this taxon was designated *Streptomyces leeuwenhoekii* with isolate C34 as the type strain. The remaining isolates formed a putatively novel *Streptomyces* species or belonged to, or were closely related, to *Streptomyces fimbriatus*.

6.3. Materials and Methods

6. 3. 1. Selective isolation, maintenance and cultural conditions

All but two of the 28 putatively novel *Streptomyces* strains, including isolates C34, C38, C58 and C79, were recovered from a hyper-arid soil collected from the Chaxa de Laguna, Salar de Atacama of the Atacama Desert (23^{o} 170/S, 68^{o} 100N), near Tacanao. The fresh strains were taken from a range of selective isolation media (Table 6.1) following inoculation with a 10^{-1} soil suspension that had been held at 55^{o}C for 6 minutes, as described by Okoro *et al*. (2009). Similarly, the two remaining isolates, strains KNN13a and KNN42f, were isolated from an extreme hyper-arid soil sample taken from Yungay region of the Atacama Desert (24^{o} 06’ 18.6”S, 70^{o}01’55.6”W) on Gause’s No.1 agar (Gause *et al*., 1957) and *Microlunatus* agar (Nakamura *et al*., 1995), respectively. All of the strains were maintained on modified Bennett’s agar slopes (Jones, 1949) and as suspensions of hyphal fragments and spores in 20% glycerol (v/v) at -80^{o}C. Biomass for the molecular systematic and most of the chemotaxonomic studies was scapped from 14 day-old modified Bennett’s agar plates incubated at 28^{o}C and washed twice in distilled water; biomass for most of the chemotaxonomic analyses was freeze-dried and that for the molecular systematic studies stored at -20^{o}C. Cells for the fatty acid analyses were harvested from yeat extract-malt extract broth (International *Streptomyces* Project [ISP] medium 2; Shirling & Gottlieb (1966) after 3 days at 25^{o}C.

Table 6.1. Media used for the selective isolation of actinobacteria from Atacama Desert environmental samples.

Media	Selective agents (µg ml^{-1})	Target organism(s)	Selected isolates
Gause's No.1 agar (Gause *et al*. 1957; Zakharova *et al.*, 2003)	Nalidixic acid (10)	Rare or uncommon actinobacteria	KNN2-6a, KNN6-9a, KNN6-11a, KNN10-5a, KNN11-1a, KNN13a,
Geodermatophilus obscurus agar (Uchida &Seino, 1997)	Nystatin (25)	*Geodermatophilus* spp.	KNN42f
Glucose-yeast extract agar (Athalye *et al.*, 1981)	Rifampicin (20)	*Actinomadura* spp.	
HV agar (Hayakawa & Nonomura , 1987)	Humic acid (1g L^{-1})	*Streptosporangiaceae* spp.	KNN6-6b, KNN24-1b, KNN26b, KNN35-1b, KNN35-2b, KNN38-1b, KNN64-5b
Luedemann's agar (Luedemann, 1971)	Nystatin (25)	*Modestobacter* spp.	
Microlunatus agar (Nakamura *et al*., 1995)	Nystatin (25)	*Modestobacter* spp.	
Minimal medium agar (Johnson *et al.*, 1981)	Nystatin (25)	Rare or uncommon actinobacteria	KNN48-6d
Oligotrophic agar (Senechkin *et al.*, 2010)	Low carbon and nitrogen content	Rare and uncommon actinobacteria	
R2A (Reasoner & Geldreich, 1985)	Nystatin (25)	*Modestobacter* spp.	
Starch-casein agar (Küster &Williams, 1964)	Nystatin (25)	*Streptomyces* spp.	
SM1 (Tan *et al.*, 2006)	Neomycin (1) and nystatin (25)	*Amycolatopsis* spp.	KNN48-3e, KNN83e

All of the media were supplemented with cycloheximide (25 µg ml^{-1}).

6. 3. 2. Chemotaxonomy and morphology

All of the isolates were examined for the presence of isomers of diaminopimelic acid (A_2pm) following the procedure described by Hasegawa *et al.* (1983). Strains KNN24-1b, KNN26b, KNN35-2b and KNN48-1c were also examined for diagnostic

menaquinones and whole organism sugars using standard procedures (Hasegawas *et al*., 1983; Collins *et al*., 1985). Cellular fatty acids extracted from isolates C34^T, C38, C58, C59 and C79 were methylated and analysed by gas chromatography (Hewlett Packard model 6890) following the recommended procedure of the Sherlock Microbial Identification System (MIDI, Sasser 1990). The resultant fatty acid methyl esters were identified and quantified using the MIDI ACTINO1 database (version 6.10).

The micromorphology of all of the isolates were observed on oatmeal agar plates (ISP medium 3; Shirling & Gottlieb, 1966) after 14 days at 28^{o}C, using the coverslip technique described by Kawato and Shinobu (1959). Spore chain morphology and spore surface ornamentation of isolates C34, C38, C59 and KNN35-2b were detected by examining gold coated, dehydrated specimens taken from the oatmeal agar plates , using an electron microscope (Cambridge Stereoscan 240 instrument) and the procedure described by O'Donnell *et al*. (1993). Cultural characteristics of all of the isolates were determined using ISP media (Shirling & Gottlieb, 1966) after incubation at 28^{o}C for 14 days.

6. 3. 3. Phylogenetic analyses

Genomic DNA was extracted from biomass of all the isolates and PCR-mediated amplification of 16S rRNA purified gene products realised, as described by Kim and Goodfellow (2002). The resultant almost complete 16S rRNA gene sequences were submitted to the EzTAXON server (http://eztaxon-e.ezbiocloud.net/; Kim *et al*. (2012) and aligned with corresponding 16S rRNA gene sequences of the type strains of the most closely related *Streptomyces* species using CLUSTAL W version 1.8 software (Thompson *et al*., 1994). Phylogenetic trees were generated from each set of aligned sequences using the maximum-likelihood (Felsenstein 1981), maximum-parsimony (Fitch 1971) and neighbour-joining algorithms (Saitou & Nei, 1987) drawn from the MEGA 5 and PHYML software packages (Guindon & Gascuel, 2003; Tamura *et al*., 2011); evolutionary distance matrices for the neighbour-joining analyses were prepared using the Jukes and Cantor (1969) model. The topology of the inferred evolutionary trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset using MEGA 5 software. The root positions of the unrooted trees were estaminted using the sequence of *Streptomyces albus* subspecies *albus* DSM 40313^T (GenBank accession number AJ 621602) as the out group.

6. 3. 4. Phenotypic tests

All of isolates assigned to the *S. fimbriatus* and *S. leeuwenhoekii* 16S rRNA gene trees (Figures 6.1 and 6.2) were examined for an extensive range of biochemical, degradative and physiological properties of value in streptomycete systematics (Kämpfer, 2012). The enzyme profiles of the strains were determined using API ZYM strips (BioMerieux) and their ability to use a broad range of carbon sources determined using Biolog GEN III Microplates, in each case following the manufacturer's instructions; a standard inoculum equivalent to 5.0 on the McFarland scale (Murray *et al*., 1999) was used to inoculate both the microplates and the API ZYM strips. All of the tests were carried out in duplicate.

6. 3. 5. Generation of whole-genome sequence of isolate C34^T and genome analysis

Isolate C34^T was grown on TSBS-YEME (1:1, v/v) with 5 mM $MgCl_2$ and 0.5% glycine at 30^{o}C for 48 hours. Cells were resuspended in 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and incubated with lyzozyme at 37^{o}C for 1 to 30 minutes until they were lysed. Sodium dodecyl sulphate (0.5% final concentration) and proteinase K (40 µg) were added and the cell extract incubated at 50^{o}C for 6 hours when a standard phenol/chloroform extraction was performed on the lysate. The extract was adjusted to 0.3 M NaOAC (pH 5.5) and DNA was spooled with a glass rod upon addition of 2 volumes of 96% ethanol. After washing and drying, the DNA was dissolved in TE buffer. DNA quality was verified by SalI digestion and agarose gel electrophoresis.

Illumina/Solexa sequencing on Genome Analyzer IIx was outsourced (ServiceSX, Leiden, The Netherlands) and 100-nt paired-end-reads were obtained. The quality of the short reads was verified using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), and depending on the quality, reads were trimmed at both ends. Processed raw reads were then used as input for the Velvet assembly algorithm (Zerbino & Birney, 2008). The genome was annotated using the RAST server (Aziz *et al.*, 2008) with default options. Predictions of gene clusters for natural products were performed using antiSMASH (Medema *et al*., 2011). The genome sequence has been deposited at DDBJ/EMBL/GenBank under the accession number AZSD0000000.

6. 3. 6. Multilocus Sequence Analysis

Multilocus sequence analysis was based on previously described methods (Guo *et al*., 2008; Rong *et al.*, 2009; Labeda, 201; Rong & Huang, 2010, 2012). Genomic DNA was extracted isolates C34^T, C38, C58 and C79 and from the strains listed in Table 6.2 using UltraClean® microbial DNA isolation kits (MoBio Labs, Carlsbad, CA) following the instructions of the manufacturer. Partial sequences of the house-keeping genes *atpD* (ATP synthase F1, β subunit), *gyrB* (DNA gyrase β subunit) and *rpoB* (RNA polymerase β subunit) were amplified and sequenced using the primers and protocols described previously (Guo *et al*., 2008; Rong et *al*., 2009), as well as modified primers designed to optimise amplification and sequencing of the house-keeping genes *recA* (recombinase A) and *trpB* (tryptophan synthetase, β subunit) for a broader range of *Streptomyces* species (Labeda *et al*., 2014). Amplified products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced using BigDye 3.1 on an ABI model 3730 sequencer in the NCAUR core sequencing facility. Sequence data for the five house-keeping loci for each strain were deposited in Genbank with the accession numbers shown in Table 6.2. House-keeping gene sequences for species of the genus *Streptomyces* were organized using Bacterial Isolate Genomic Sequence Database (BIGSdb) version 1.6.3 (Jolley & Maiden, 2010) on the ARS Microbial Genomic Sequence Database server at http://199.133.98.43. The alleles of the house-keeping loci for strain C34^T, C38, C58 and C79 were found within the draft genome using the genome sequence scan function in BIGSdb. The alleles were tagged within the genome sequence and added to the sequence database. The sequences of the loci for each strain were concatenated head to tail and exported in FASTA format, providing a dataset of 152 strains and 2575 positions. Sequences were aligned using MUSCLE (Edgar, 2004) and phylogenetic relationships constructed in MEGA 5.2 (Tamura *et al*., 2011) using maximum-likelihood based on the General Time Reversible model (Nei & Kumar, 2000), determined to be the optimal model for these data using jmodeltest2 (Darriba *et al*., 2012; Guindon & Gascuel, 2003). The phylogenetic relationships of the strains were also determined using maximum-parsimony and neighbour-joining analyses. MLSA evolutionary distances were determined using MEGA 5.2 to calculate the Kimura 2-parameter distance (Kimura, 1980).

Table 6.2. *Streptomyces* strains house-keeping gene sequences deposited for the present study.

Species	Strain	*atpD*	*gyrB*	*recA*	*rpoB*	*trpB*
S. leeuwenhoekii	NRRL B-24963^T (= DSM 42122^T)	KJ137029	KJ137046	KJ137063	KJ137080	KJ137097
S. chryseus	NRRL B-12347^T	KJ137020	KJ137037	KJ137054	KJ137071	KJ137088
S. daghestanicus	NRRL B-5418^T	KJ137021	KJ137038	KJ137055	KJ137072	KJ137089
S. fimbriatus	NRRL B-3175^T	KJ137022	KJ137039	KJ137056	KJ137073	KJ137090
S. fumanus	NRRL B-3898^T	KJ137023	KJ137040	KJ137057	KJ137074	KJ137091
S. fumigatiscleroticus	NRRL B-3856^T	KJ137024	KJ137041	KJ137058	KJ137075	KJ137092
S. ghanaensis	NRRL B-12104^T	KJ137025	KJ137042	KJ137060	KJ137076	KJ137093
S. glaucus	NRRL B-16368^T	KJ137026	KJ137043	KJ137059	KJ137077	KJ137094
S. griseomycini	NRRL B-5421^T	KJ137027	KJ137044	KJ137061	KJ137078	KJ137095
S. hirsutus	NRRL B-2713^T	KJ137028	KJ137045	KJ137062	KJ137079	KJ137096
S. lusitanus	NRRL B-5637^T	KJ196366	KJ196368	KJ196370	KJ196372	KJ196374
S. mexicanus	NRRL B-24196^T	KJ137030	KJ137047	KJ137064	KJ137081	KJ137098
S. parvulus	NRRL B-1628^T	KJ196367	KJ196369	KJ196371	KJ196373	KJ196374
S. recifensis	NRRL B-3811^T	KJ137031	KJ137048	KJ137065	KJ137082	KJ137099
S. seoulensis	NRRL B-24310^T	KJ137032	KJ137049	KJ137066	KJ137083	KJ137100
S. thermocoprophilus	NRRL B-24314^T	KJ137033	KJ137050	KJ137067	KJ137084	KJ137101
S. thermodiastaticus	NRRL B-5316^T	KJ137034	KJ137051	KJ137068	KJ137085	KJ137102
S. thermovulgaris	NRRL B-12375^T	KJ137035	KJ137052	KJ137069	KJ137086	KJ137103
S. thermovulgaris	NRRL B-12375^T	KJ137036	KJ137053	KJ137070	KJ137087	KJ137104

6.4. Results

All of the strains isolated from the hyper-arid and extreme hyper-arid Atacama Desert soils produced an extensively branched mycelium which carried aerial hyphae that differentiated into chains of spores, and produced whole-organism hydrolysates rich in *LL*-A_2pm, glucose and xylose and contained octahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue. Representative strains, isolates C34^T, C38, C58, C59 and C79 contained major amounts of saturated, *iso*- and *anteiso*-fatty acids (Table 6.3). In addition, all of the isolates assigned to *S. leeuwenhoekii* 16S rRNA subclade grew well on the ISP media producing a variety of pigments responsible for the colour of the substrate and aerial mycelial; none of the isolates formed melanin pigments on peptone-yeast extract-iron agar (Tables 6.7, 6.10 and 6.13). All of these properties are consistent with the classification of the isolates in the genus *Streptomyces* (Kämpfer, 2012).

Table 6.3. Fatty acid profile (%) of representatives of the *Streptomyces leeuwenhoekii* and *Streptomyces fimbriatus* 16S rRNA gene subclade.

Fatty acid	*S. leeuwenhoekii*				*S. fimbriatus*
	C34^T	C38	C58	C79	C59
iso- $C_{11:0}$	-	0.1	-	-	-
$C_{12:0}$	0.2	-	-	-	0.5
iso- $C_{12:0}$	-	0.2	0.1	0.2	-
iso- $C_{13:0}$	0.4	0.4	0.4	0.5	0.29
anteiso- $C_{13:0}$	0.2	0.4	0.2	0.2	0.23
$C_{13:0}$	-	-	-	0.1	-
$C_{13:0}$ 2OH	-	-	-	-	0.26
iso- $C_{14:0}$	4.3	5.6	4.1	4.0	2.09
$C_{14:0}$	1.1	0.3	0.4	0.8	1.45
iso- $C_{15:0}$	5.5	7.4	8.0	7.5	6.83
anteiso- $C_{15:0}$	29.2	30.0	25.9	30.4	10.07
$C_{15:0}$ 20H	0.2	-	-	0.2	-
iso-$C_{15:1}$ H/13:0 3OH	-	-	-	-	0.31
iso- $C_{16:1}$ H	0.4	2.0	2.4	0.6	6.32
iso- $C_{16:0}$	12.5	24.9	21.0	15.4	18.33
$C_{16:0}$	19.1	4.1	5.4	10.4	5.47
anteiso- $C_{17:1}$ ω9c	1.1	1.3	3.7	1.4	5.4
iso- $C_{17:0}$	4.4	5.3	5.0	6.0	7.41
anteiso- $C_{17:0}$	13.8	15.1	14.5	14.5	12.05
$C_{17:1}$ ω8c	0.2	-	0.5	0.3	1.62
cyclo- $C_{17:0}$	-	0.5	0.6	0.3	1.11
$C_{17:0}$	3.5	0.6	0.9	2.2	1.55
$C_{17:0}$ OH	-	-	0.1	-	-
$C_{17:0}$10-methyl	-	-	0.4	-	0.91
iso- $C_{18:0}$	0.5	0.6	-	0.5	0.64
$C_{18:0}$	0.9	0.2	-	0.8	2.39
$C_{18:1}$ ω7c	-	-	-	-	0.5
$C_{18:1}$ ω9c	-	-	0.9	0.5	0.92
iso- $C_{18:1}$ H	-	-	0.9	0.5	1.24
Summed features:					
$C_{16:1}$ ω7c / $C_{16:1}$ ω6c	1.5	0.6	1.5	1.1	4.19
iso-$C_{17:1}$ ω9c/10-methyl $C_{16:0}$	1.0	0.7	4.0	1.6	0.5
$C_{16:0}$ / *iso*- $C_{17:1}$ ω9c	-	-	0.1	0.4	7.92

$C_{18:1}$ ω7c / $C_{18:1}$ ω6c	-	-	0.1	1.6	-

***Streptomyces leeuwenhoekii* 16S rRNA gene subclade.** Fifteen of the isolates from the hyper-arid Salar de Atacama soil, including isolate C34^T, formed a well delineated subclade in the *Streptomyces* 16S rRNA gene tree, a taxon that was supported by all of the tree-making algorithms and by an 82% bootstrap value. The isolates shared 16S rRNA gene sequence similarities within the range 99.5-99.9%, values corresponding to between 2 to 6 nt differences at between 1313-1372 sites (Table 6.4). These isolates were most closely related to *Streptomyces mexicanus* CH-M-1035^T sharing 16S rRNA gene similarities with the latter within the range 98.7-99.0% , values equivalent to 14-17 nt differences at between 1308-1367 sites. The isolates were also closely related to the type strains of *Streptomyces althioticus* (98.7-98.9%, 15-20 nt differences), *Streptomyces chiangmaiensis* (98.5-98.9%, 15-20 nt differences), *Strerptomyces lusitanus* (98.7-99.0%, 13-18 nt differences), *Streptomyces parvulus* (98.6-99.0%, 14-19 nt differences) and *Streptomyces speibonae* (98.6-99.0%, 14-18 nt differences).

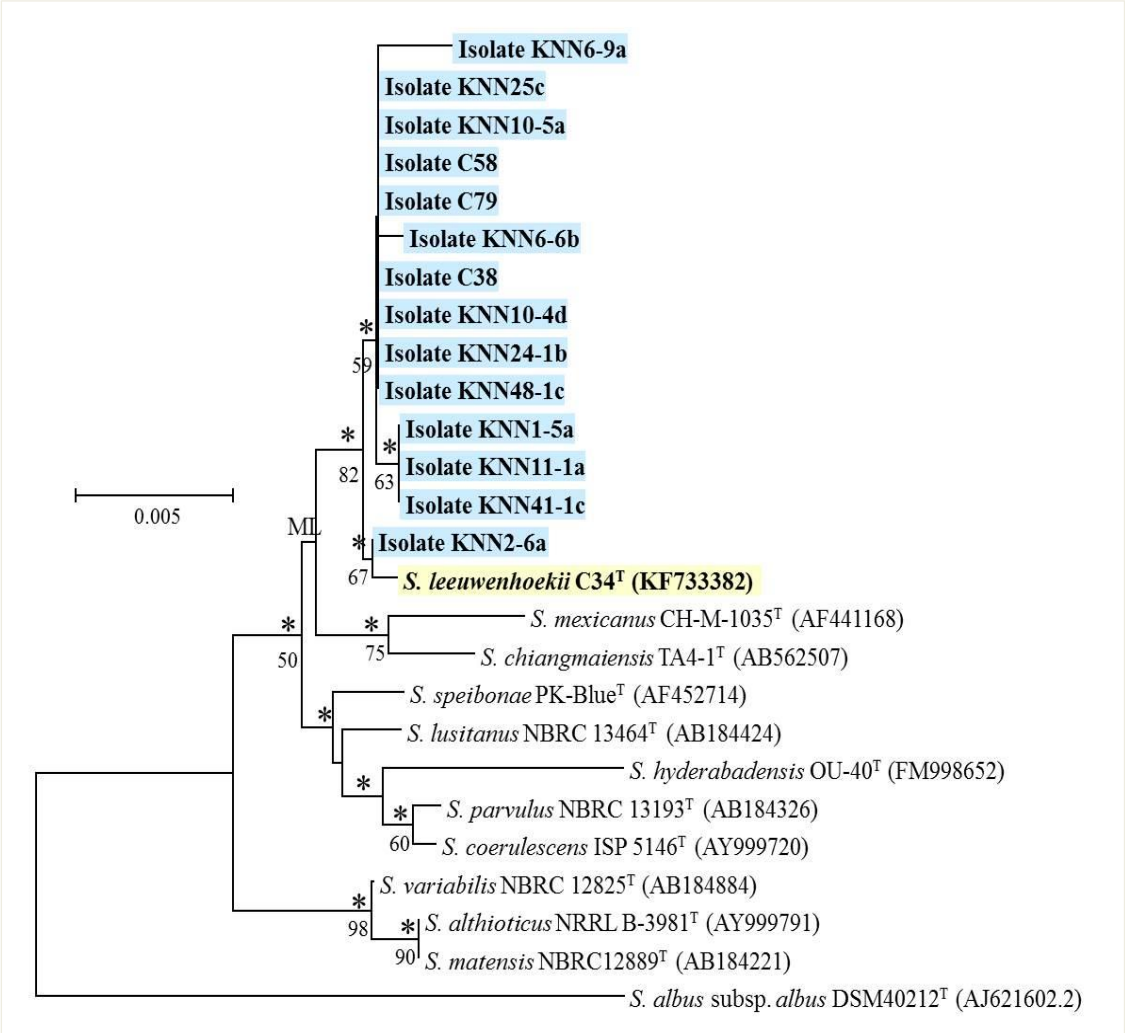


Figure 6.1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences (1313-1372) showing relationships between *Streptomyces leeuwenhoekii* C34^T, and related strains isolated from hyper-arid Salar de Atacama soil and between them and the type strains of closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 sampled datasets, only values above 50% are given. The root position of the tree was determined using *Streptomyces albus* subsp. *albus* DSM 40313^T. Bar, 0.002 substitutions per nucleotide position.

The phylogenetic relationships of isolates C34^T, C38, C58 and C79 to one another and to other *Streptomyces* species based on an analysis of the sequence alignments resulting from concatenation of the house-keeping genes head to tail is shown in Figure 6.2 and in the expanded version of this tree (Figure S1a-1c). It is apparent that isolates C34^T, C38, C58 and C79 gave identical results but were not phylogeneticaly closely rrelated to any *Streptomyces* type strains for which sequences of the house-keeping loci are available, but appear to be most closely related to *Streptomyces thermocoprophilus* NRRL B-24314^T based on the present analysis. This relationship is not supported by significant bootstrap values nor by the stability of the relationship when different phylogenetic algorithms are used, as in the maximum-parsimony and neighbour-joining analyses. MLSA evolutionary distances were determined using MEGA 5.2 to calculate Kimura 2-parameter distances (Kimura, 1980), as shown in Table 6.5. Isolates C34^T, C38, C58 and C79 were found to have an MLSA distance greater than 0.007 with all of the phylogenetically near species therby supporting the proposal that these strains represent a new species since the cut-off point empirically determined by Rong and Huang (2012) stated that this distance corresponds to 70% DNA:DNA relatedness.

Table 6.4. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between *Streptomyces leeuwenhoekii* C34^T and related strain isolated from the hyper-arid Salr de Atacama soil and between them and the type strains of closely related *Streptomyces* species.

Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. Isolate C38	---	**1/1362**	**1/1370**	**1/1362**	**4/1365**	**1/1331**	**3/1334**	**0/1324**	**0/1353**	**3/1330**	**0/1313**	**2/1372**	**1/1362**
2. Isolate C58	**99.9**	---	**0/1362**	**1/1352**	**4/1355**	**1/1321**	**4/1325**	**0/1314**	**0/1343**	**3/1320**	**0/1303**	**3/1362**	**1/1352**
3. Isolate C79	**99.9**	**100.0**	---	**1/1360**	**4/1363**	**1/1329**	**3/1332**	**0/1322**	**0/1351**	**3/1328**	**0/1311**	**3/1370**	**1/1360**
4. Isolate KNN1-5a	**99.9**	**99.9**	**99.9**	---	**2/1362**	**2/1331**	**4/1334**	**1/1324**	**1/1353**	**2/1330**	**1/1313**	**1/1362**	**0/1362**
5. Isolate KNN2-6a	**99.7**	**99.7**	**99.7**	**99.9**	---	**2/1331**	**4/1334**	**1/1324**	**1/1353**	**4/1330**	**1/1313**	**3/1365**	**2/1362**
6. Isolate KNN6-6b	**99.9**	**99.9**	**99.9**	**99.9**	**99.9**	---	**4/1331**	**1/1324**	**1/1331**	**4/1330**	**1/1313**	**1/1331**	**2/1331**
7. Isolate KNN6-9a	**99.8**	**99.7**	**99.8**	**99.7**	**99.7**	**99.7**	---	**3/1324**	**3/1334**	**6/1330**	**3/1313**	**3/1334**	**4/1334**
8. Isolate KNN10-4d	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	---	**0/1324**	**1/1324**	**0/1312**	**0/1324**	**1/1324**
9. Isolate KNN10-5a	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	---	**3/1330**	**0/1313**	**0/1353**	**1/1353**
10. Isolate KNN11-1a	**99.8**	**99.8**	**99.8**	**99.9**	**99.7**	**99.7**	**99.6**	**99.9**	**99.8**	---	**1/1313**	**3/1330**	**2/1330**
11. Isolate KNN24-1b	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	**100.0**	**99.9**	---	**0/1313**	**1/1313**
12. Isolate KNN25c	**99.9**	**99.8**	**99.8**	**99.9**	**99.8**	**99.9**	**99.8**	**100.0**	**100.0**	**99.8**	**100.0**	---	**1/1362**
13. Isolate KNN41-1c	**99.9**	**99.9**	**99.9**	**100.0**	**99.9**	**99.9**	**99.7**	**99.9**	**99.9**	**99.9**	**99.9**	**99.9**	---
14. Isolate KNN48-1c	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	**100.0**	**99.8**	**100.0**	**100.0**	**99.9**
15. *S. leeuwenhoekii*	**99.7**	**99.9**	**99.8**	**99.7**	**99.6**	**99.7**	**99.5**	**99.8**	**99.8**	**99.6**	**99.8**	**99.6**	**99.7**
16. *S. mexicanus*	98.9	99.0	99.0	98.9	98.8	98.9	98.7	98.9	99.0	98.7	98.9	98.8	98.9
17. *S. hyderabadensis*	98.4	98.5	98.5	98.4	98.2	98.3	98.2	98.4	98.5	98.2	98.4	98.3	98.4
18. *S. parvulus*	98.8	99.0	98.9	99.0	98.6	98.8	98.7	98.9	98.9	98.8	98.9	98.7	99.0
19. *S. lusitanus*	98.9	99.0	99.0	98.9	98.7	98.9	98.7	98.9	99.0	98.7	98.9	98.8	98.9
20. *S. speibonae*	98.8	99.0	98.9	98.8	98.6	98.8	98.7	98.9	98.9	98.7	98.9	98.7	98.8
21. *S. chiangmaiensis*	98.7	98.9	98.8	98.7	98.6	98.7	98.5	98.7	98.7	98.5	98.7	98.5	98.7
22. *S. coerulescens*	98.7	98.8	98.8	98.8	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.5	98.8
23. *S. althioticus*	98.8	98.9	98.8	98.9	98.5	98.7	98.6	98.8	98.8	98.7	98.8	98.6	98.9
24. *S. matensis*	98.7	98.8	98.8	98.8	98.5	98.6	98.5	98.7	98.7	98.6	98.7	98.5	98.8
25. *S. variabilis*	98.7	98.8	98.8	98.8	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.5	98.8
26. *S. albus* subsp. *albus*	96.9	96.8	96.8	96.8	96.8	96.8	96.6	96.8	96.9	96.6	96.8	96.7	96.8

Type strain codes, as given in Figure 6.1.

Table 6.4. (2)

Isolates	14	15	16	17	18	19	20	21	22	23	24	25
1. Isolate C38	**0/1335**	**4/1372**	**15/1367**	**22/1370**	**16/1371**	**15/1371**	**16/1371**	**18/1369**	**18/1371**	**17/1364**	**18/1364**	**18/1371**
2. Isolate C58	**0/1325**	**2/1363**	**14/1357**	**20/1360**	**14/1361**	**13/1361**	**14/1361**	**15/1359**	**16/1361**	**15/1354**	**16/1354**	**17/1361**
3. Isolate C79	**0/1333**	**3/1370**	**14/1365**	**21/1368**	**15/1369**	**14/1369**	**15/1369**	**17/1367**	**17/1369**	**16/1362**	**17/1362**	**17/1369**
4. Isolate KNN1-5a	**1/1335**	**4/1362**	**15/1357**	**22/1360**	**14/1361**	**15/1361**	**16/1361**	**18/1359**	**16/1361**	**15/1354**	**16/1354**	**16/1361**
5. Isolate KNN2-6a	**1/1335**	**5/1365**	**16/1360**	**25/1363**	**19/1364**	**18/1364**	**19/1364**	**19/1362**	**21/1364**	**20/1357**	**21/1357**	**21/1364**
6. Isolate KNN6-6b	**1/1331**	**4/1331**	**15/1326**	**22/1329**	**16/1330**	**15/1330**	**16/1330**	**18/1330**	**18/1330**	**17/1323**	**18/1323**	**18/1330**
7. Isolate KNN6-9a	**3/1334**	**7/1335**	**17/1329**	**24/1332**	**18/1333**	**17/1333**	**18/1333**	**20/1333**	**20/1333**	**19/1326**	**20/1326**	**20/1333**
8. Isolate KNN10-4d	**0/1324**	**3/1324**	**14/1319**	**21/1322**	**15/1323**	**14/1323**	**15/1323**	**17/1323**	**17/1323**	**16/1316**	**17/1316**	**17/1323**
9. Isolate KNN10-5a	**0/1335**	**3/1353**	**14/1348**	**21/1351**	**15/1352**	**14/1352**	**15/1352**	**17/1351**	**17/1352**	**16/1345**	**17/1345**	**17/1352**
10. Isolate KNN11-1a	**3/1330**	**6/1330**	**17/1325**	**24/1328**	**16/1329**	**17/1329**	**18/1329**	**20/1329**	**18/1329**	**17/1322**	**18/1322**	**18/1329**
11. Isolate KNN24-1b	**0/1313**	**3/1313**	**14/1308**	**21/1311**	**15/1312**	**14/1312**	**15/1312**	**17/1312**	**17/1312**	**16/1305**	**17/1305**	**17/1312**
12. Isolate KNN25c	**0/1335**	**6/1372**	**17/1367**	**24/1370**	**18/1371**	**17/1371**	**18/1371**	**20/1369**	**20/1371**	**19/1364**	**20/1364**	**20/1371**
13. Isolate KNN41-1c	**1/1335**	**4/1362**	**15/1357**	**22/1360**	**14/1361**	**15/1361**	**16/1361**	**18/1359**	**16/1361**	**15/1354**	**16/1354**	**16/1361**
14. Isolate KNN48-1c	**---**	**3/1335**	**14/1330**	**21/1333**	**15/1334**	**14/1334**	**15/1334**	**17/1334**	**17/1334**	**16/1327**	**17/1327**	**17/1334**
15. *S. leeuwenhoekii*	**99.8**	**---**	15/1367	23/1370	17/1371	16/1371	17/1371	16/1369	19/1371	18/1364	19/1364	20/1371
16. *S. mexicanus*	99.0	98.9	---	23/1365	19/1366	21/1366	18/1366	15/1364	19/1366	23/1359	24/1360	24/1366
17. *S. hyderabadensis*	98.4	98.3	98.3	---	15/1371	24/1371	25/1371	26/1368	24/1371	27/1364	27/1364	32/1371
18. *S. parvulus*	98.9	98.8	98.6	98.9	---	14/1372	19/1372	18/1369	12/1372	15/1365	16/1365	22/1372
19. *S. lusitanus*	99.0	98.8	98.5	98.3	99.0	---	14/1372	21/1369	8/1372	9/1365	9/1365	17/1372
20. *S. speibonae*	98.9	98.8	98.7	98.2	98.6	99.0	---	19/1369	15/1372	15/1365	16/1365	15/1372
21. *S. chiangmaiensis*	98.7	98.8	98.9	98.1	98.7	98.5	98.6	---	20/1369	22/1362	23/1362	26/1369
22. *S. coerulescens*	98.7	98.6	98.6	98.3	99.1	99.4	98.9	98.5	---	12/1365	12/1365	14/1372
23. *S. althioticus*	98.8	98.7	98.3	98.0	98.9	99.3	98.9	98.4	99.1	---	0/1364	4/1365
24. *S. matensis*	98.7	98.6	98.2	98.0	98.8	99.3	98.8	98.3	99.1	100.0	---	5/1365
25. *S. variabilis*	98.7	98.5	98.2	97.7	98.4	98.8	98.9	98.1	99.0	99.7	99.6	---
26. *S. albus* subsp. *albus*	96.8	96.8	96.7	96.3	96.2	96.5	96.4	96.3	96.3	96.9	96.8	96.7

Type strain codes, as given in Figure 6.1.

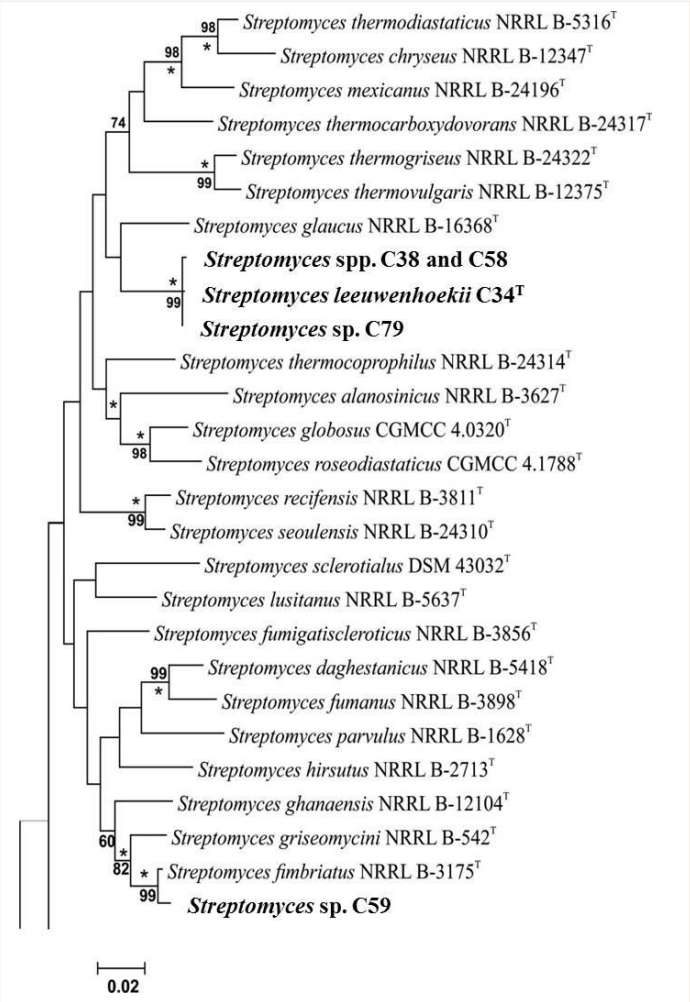


Figure 6.2. Subtree from the phylogenetic tree inferred from concatenated partial sequences of the house-keeping genes *atpD, gyrB, recA, rpoB* and *trpB* in MEGA 5.2 (Tamura *et al.*, 2011) using the maximum-likelihood method based on the General Time Reversible model (Nei & Kumar, 2000). There were 2575 positions and 152 strains in the final dataset. Trees were also inferred using the Tamura-Nei evolutionary distance method (Tamura & Nei, 1993) with the neighbour-joining algorithm of Saitou & Nei (1987), neighbour-joining and maximum parsimony models in MEGA 5.2, correspond with branches in all methods are marked with an asterisk. Percentages at the nodes represent levels of bootstrap support from 1000 re-sampled datasets (Felsenstein, 1985) with values less than 60% not shown. The proposed new species, *Streptomyces leeuwenhoekii* DSM 42122^T is indicated with a bold node label. Bar marker equals number of substitutions per site.

Table 6.5. MLSA distances for strains phylogenetically near to *S. leeuwenhoekii* C34^T and related isolates (values in bold text). The distances between the isolates and the type strains are greater than 0.007 which was defined by Rong and Huang (2012) as equivalent to 70% genomic DNA similarity.

Strain	MLSA (Kimura 2-parameter) Distance											
S. albus NRRL B-1811^T	-											
S. thermocarboxydovorans NRRL B-24317^T	0.141											
S. thermocoprophilus NRRL B-24314^T	0.136	0.073										
S. thermodiastaticus NRRL B-5316^T	0.148	0.063	0.074									
S. thermogriseus NRRL B-24322^T	0.137	0.071	0.073	0.073								
S. thermovulgaris NRRL B-12375^T	0.138	0.075	0.075	0.069	0.020							
S. sclerotialus DSM 43032^T	0.145	0.086	0.084	0.089	0.096	0.098						
S. globosus CGMCC 4.0320^T	0.145	0.070	0.053	0.066	0.071	0.075	0.078					
S. roseodiastaticus CGMCC 4.1788^T	0.140	0.069	0.063	0.065	0.076	0.069	0.088	0.037				
S. alanosinicus NRRL B-3627^T	0.139	0.088	0.073	0.095	0.089	0.092	0.090	0.065	0.068			
***S. leeuwenhoekii* C34^T, C38, C58 and C79**	**0.146**	**0.062**	**0.054**	**0.073**	**0.076**	**0.082**	**0.071**	**0.061**	**0.069**	**0.070**		
S. chryseus NRRL B-12347^T	0.141	0.066	0.078	0.032	0.077	0.072	0.086	0.076	0.070	**0.095**	0.076	
S. daghestanicus NRRL B-5418^T	0.129	0.063	0.060	0.068	0.078	0.080	0.081	0.063	0.065	**0.074**	0.053	0.071
S.. fimbriatus NRRL B-3175^T	0.141	0.060	0.047	0.067	0.075	0.075	0.076	0.054	0.065	**0.076**	0.042	0.069
S. fumanus NRRL B-3898^T	0.134	0.072	0.069\8	0.070	0.087	0.088	0.080	0.065	0.072	**0.080**	0.061	0.078
S. fumigatiscleroticus NRRL B-3856^T	0.134	0.069	0.062	0.073	0.076	0.077	0.070	0.055	0.065	**0.070**	0.056	0.075
S. ghanaensis NRRL B-12104^T	0.143	0.063	0.065	0.073	0.080	0.080	0.077	0.059	0.070	**0.075**	0.060	0.078
S. glaucus NRRL B-16368^T	0.138	0.062	0.060	0.066	0.076	0.070	0.082	0.064	0.054	**0.083**	0.055	0.067
S. griseomycini NRRL B-5421^T	0.144	0.056	0.055	0.064	0.075	0.077	0.069	0.058	0.063	**0.074**	0.041	0.071
S. hirsutus NRRL B-2713^T	0.152	0.072	0.069	0.083	0.084	0.085	0.076	0.072	0.072	**0.085**	0.064	0.082
S. lusitanus NRRL B-5637^T	0.135	0.070	0.069	0.081	0.083	0.087	0.069	0.068	0.077	**0.147**	0.065	0.084
S. mexicanus NRRL B-24196^T	0.147	0.065	0.068	0.042	0.077	0.074	0.083	0.059	0.055	**0.086**	0.066	0.060
S. parvulus NRRL B-1628^T	0.134	0.079	0.075	0.083	0.085	0.088	0.077	0.083	0.085	**0.083**	0.072	0.085
S. recifensis NRRL B-3811^T	0.146	0.078	0.066	0.073	0.083	0.087	0.085	0.058	0.066	**0.087**	0.073	0.080

S. seoulensis NRRL B-24310^T	0.146	0.078	0.064	0.073	0.083	0.086	0.082	0.061	0.068	**0.087**	0.069	0.079
S. daghestanicus NRRL B-5418^T	-											
S.. fimbriatus NRRL B-3175^T	0.058											
S. fumanus NRRL B-3898^T	0.034	0.058										
S. fumigatiscleroticus NRRL B-3856^T	0.058	0.044	0.055									
S. ghanaensis NRRL B-12104^T	0.052	0.040	0.052	0.053								
S. glaucus NRRL B-16368^T	0.060	0.047	0.064	0.061	0.060							
S. griseomycini NRRL B-5421^T	0.056	0.027	0.059	0.050	0.042	0.057						
S. hirsutus NRRL B-2713^T	0.060	0.051	0.062	0.063	0.052	0.068	0.053					
S. lusitanus NRRL B-5637^T	0.058	0.060	0.060	0.057	0.054	0.072	0.059	0.065				
S. mexicanus NRRL B-24196^T	0.067	0.058	0.070	0.064	0.066	0.064	0.061	0.076	0.071			
S. parvulus NRRL B-1628^T	0.054	0.066	0.061	0.070	0.064	0.066	0.066	0.063	0.060	0.079		
S. recifensis NRRL B-3811^T	0.069	0.067	0.070	0.066	0.075	0.073	0.069	0.070	0.066	0.071	0.070	
S. seoulensis NRRL B-24310^T	0.069	0.063	0.069	0.062	0.072	0.068	0.069	0.071	0.064	0.069	0.066	0.019

The distance between the isolates and all of the *Streptomyces* type strains is greater than 0.007 which was defined by Rong & Huang (2012) as equivalent to 70 % genomic DNA similarity.

Identical results were obtained between the duplicated cultures of each of the fifteen isolates for all of the phenotypic tests. The isolates were found to have many phenotypic properties in common, showing pronounced enzymatic activity and an ability to assimilate a broad range of carbon sources (Table 6.6). All of the isolates grew well on ISP media, notably on oatmeal and yeast extract-malt extract agar (Table 6.7). In general, they formed yellowish white substrate mycelia, an olivaceous gray green aerial spore mass and, when formed, yellowish diffusible pigments. The three representative strains, isolates C34^T, C58 and C79, produced spiral chains of smooth surfaced spores borne on aerial mycelia (Figure 6.3).

Table 6.6. Phenotypic properties of isolate C34^T and other isolates assigned to *Streptomyces leeuwenhoekii* 16S rRNA gene cluster.

Test type	Results
All strains giving positive results	
API ZYM tests:	Acid phosphatase, N-acetyl-β-glucosamidase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), α-fucosidase, α-

	galactosidase, β-glucosidase, lipase, leucine amylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine amylamidase
Bio-chemical tests:	NO_3 reductase and urease
Growth on carbon sources:	Acetoacetic acid, α-keto-butyric acid, Dextrin, D-fructose , D-fructose-6-PO_4, D-galacturonic acid , D-gluconic acid, α-D-glucose , α-keto-glutaric acid , D-mannose , pectine, quicnic acid, L-rhamnose and Tween 40
Growth at or in the presence of:	pH5, pH6, pH10, 1% NaCl and 7% NaCl
All strains giving negative results	
API ZYM tests:	β-galactosidase, β-glucuronidase, α-glucosidase and α-mannosidase
Bio-chemical tests:	NO_2 reductase and oxidase
Growth on carbon sources:	N-acetyl-β-D-mannosamine, N-acetyl neuraminic acid, D-arabitol, L-arginine, γ-aminobutyric acid, β-hydroxy butyric acid, citric acid, D-fucose , L-fucose formic acid, D-galacturonic acid lactone , gelatin , glycyl-L-proline, glucoronamide, β-methyl-D-glucoside , D-glucose-6-PO_4, glycerol, L-pyroglutamic acid, L-glutamic acid, inosine , *myo*-inositol , L-lactic acid, α-D-lactose , D-maltose , D-malic acid , L-malic acid , D-mannitol , D-melibiose, mucic acid, ρ-hydroxyphenyl acetic acid , methyl pyruvate, D-raffinose , D-sorbitol , D-saccharic acid , D-salicin, D-serine, L-serine, D-stachyose, bromo-succinic acid, D-sucrose, D-trehalose and D-turanose
*Strains growing variable results (see details in supplemented Table S1)	
Growth on sole carbon sources:	Acetic acid, N-acetyl-β-D-mannosamine, N-acetyl-D-glucosamine, L-alanine, L-aspartic acid, α-hydroxy butyric acid, α-hydroxy butyric acid, D-cellobiose, D-galactose, formic acid, gentiobose, gelatin, D-gluconic acid, Glucoronamide, 3-methyl-glucose, L-histidine, D-lactic acid methyl ether, D-maltose and propionic acid

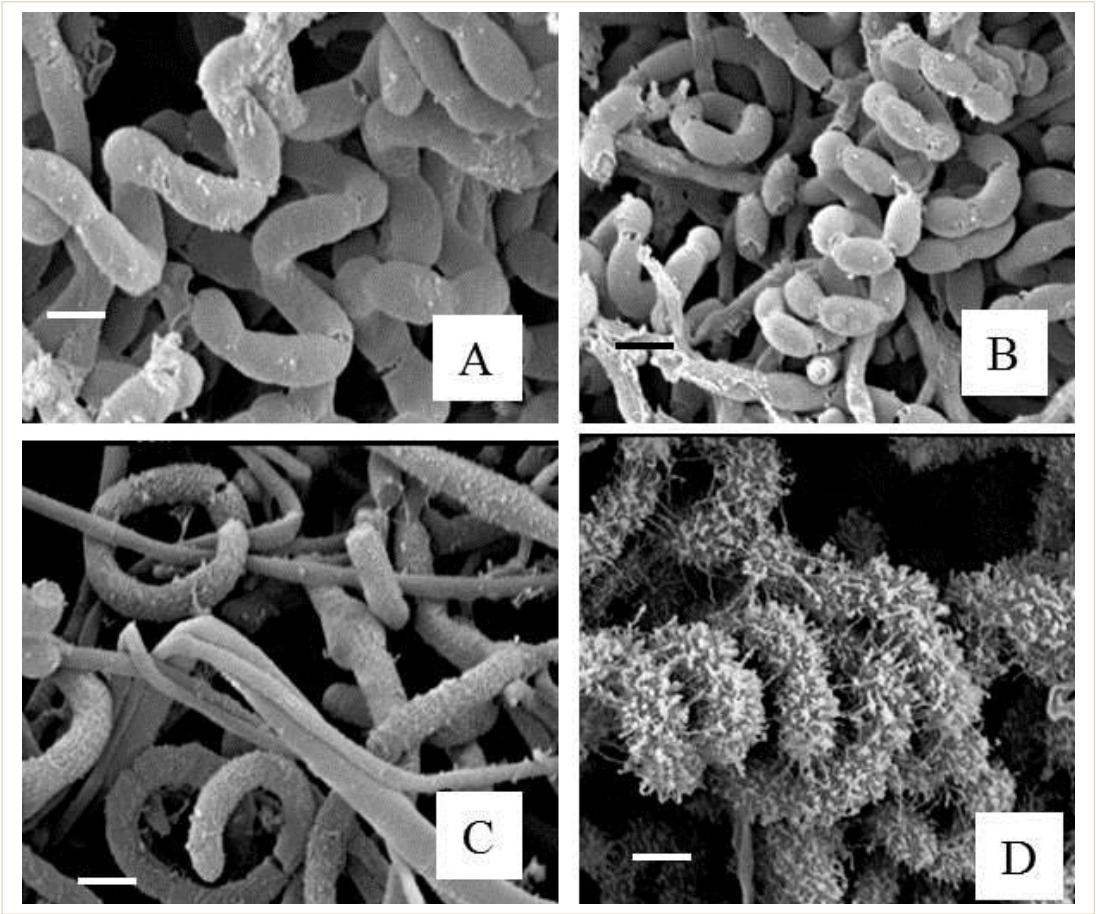
*The detailed results are given in Table S1.

Table 6.7. Growth and cultural characteristics of strains assigned to the *Streptomyces leeuwenhoekii* 16S rRNA gene subclade on ISP media after incubation for 14 days at 28^{o}C.

Media	Growth	Substrate mycelial colour	Aerial spore mass colour	Diffusible pigment
Glycerol-asparagine agar (ISP 5)	+++	Yellowish white	Olivaceous gray green	None
Inorganic salts-starch agar (ISP 4)	+++	Yellowish white	Olivaceous gray green	Yellowish
Oatmeal agar (ISP 3)	++++	Yellowish white	Olivaceous gray green	Yellowish
Peptone-yeast extract-iron agar (ISP 6)	+++	Gray yellow	Olivaceous gray green	Gray yellow
Tryptone-yeast extract agar (ISP 1)	+++	Yellowish white	Olivaceous gray green	None
Tyrosine agar (ISP 7)	+++	Yellowish white	Olivaceous gray green	Yellowish
Yeast extract-malt extract agar (ISP 2)	++++	Gray yellow	Gray yellowish green	Pale yellow

++++ abundant growth; +++ very good growth.

Figure 6.3. Scanning electron micrographs of representative streptomycetes grown on oatmeal agar after 14 days at 28°C. (A, B, C), *Streptomyces* isolates C34^T, C58 and KNN35-2b showing smooth ornamented spores in spiral spore chains, (D) isolate C59 showing spiral chains of hairy ornamented spores. Scale bars : 1µm.



Full genome sequencing of strain C34^T (GenBank accession number AZSD0000000) using Illumina led to an assembly of 658 contigs for a total genome size of 7.86 Mb, predicted to encode 77 RNAs and 6,780 proteins. The functions of the genes were catalogued into different functional classes (Figure 6.4). The relative distribution of the different classes is similar to that of the model strains *S. coelicolor* A3(2) (Bentley *et al*., 2002) and "*S. lividans* 66" (Cruz-Morales *et al*., 2013). RAST is a widely used annotation tool that allows good initial prediction of gene functions. However, for a more detailed prediction regarding the specific functions of secondary metabolism-related genes, a dedicated algorithm is required such as antiSMASH (Medema *et al*., 2011). In particular, the AHBA gene linked to chaxamycin synthesis in strain C34^T (Rateb *et al*., 2011a) was found by BLAST search among several polyketide synthase (PKS) type 1 genes in a cluster of genes with similarity to genes of the

rifamycin biosynthetic pathway (sequence 0641). However, many more PKS gene clusters were identified by antiSMASH (Table 6.8), including the genes that seem likely to be responsible for the biosynthesis of chaxamycin and chaxalactin.

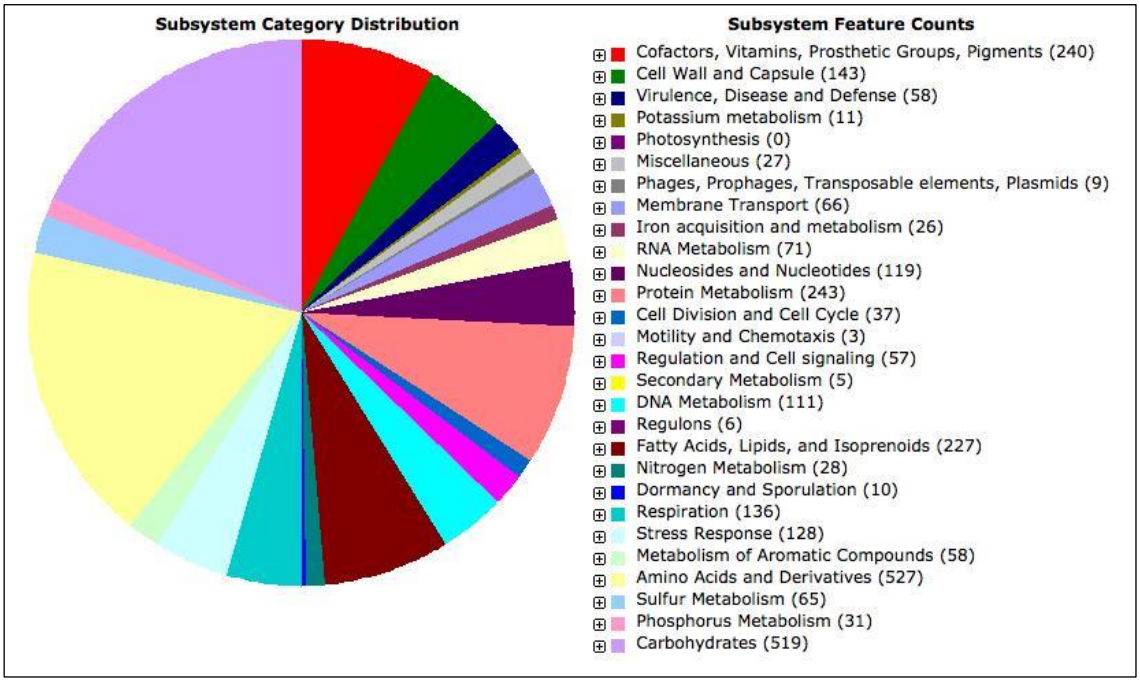


Figure 6.4. Overview of *Streptomyces* isolate C34^T subsystem gene functions as generated by analysis on the RAST server at (http://rast.nmpdr.org).

Table 6.8. Predicted natural products gene clusters in strain C34^T.

Contig	gene cluster type	compound with gene cluster of highest homology
c34_sequence_116	"t1pks-nrps"	
c34_sequence_124	"transatpks-nrps"	"leinamycin"
c34_sequence_141	"terpene"	"albaflavenone"
c34_sequence_142	"t1pks"	"oxazolomycin"
c34_sequence_143	"butyrolactone"	"lactonamycin"
c34_sequence_155	"t1pks"	
c34_sequence_17	"nrps"	"daptomycin"
c34_sequence_201	"amglyccycl"	"cetoniacytone A"
c34_sequence_23	"t1pks"	
c34_sequence_241	"t1pks"	
c34_sequence_246	"terpene"	
c34_sequence_326	"terpene"	
c34_sequence_347	"siderophore"	"geosmin"
c34_sequence_364	"other"	
c34_sequence_37	"t3pks"	
c34_sequence_368	"melanin"	"melanin"
c34_sequence_396	"other"	"melanin"
c34_sequence_418	"t1pks"	"macbecin"
c34_sequence_457	"t3pks"	
c34_sequence_541	"nrps"	
c34_sequence_542	"other"	"kirromycin"

c34_sequence_552	"terpene"	
c34_sequence_60	"transatpks-t2pks"	"leinamycin"
c34_sequence_6	"t1pks-siderophore"	"geosmin"
c34_sequence_70	"ectoine"	
c34_sequence_640	"terpene"	
c34_sequence_642	"t1pks"	"rubradirin"
c34_sequence_78	"other"	
c34_sequence_7	"siderophore"	
c34_sequence_84	"siderophore"	"desferrioxamine"

***Streptomyces fimbriatus* 16S rRNA gene subclade.** Five strains isolated from the hyper-arid Salar de Atacama soil, a single isolate from the extreme hyper-arid Yungay soil and *S. fimbriatus* NBRC 15411^T formed a subclade in the *Streptomyces* 16S rRNA gene tree, the taxonomic status of which was underpinned by all of the tree-making algorithms and by a 99% bootstrap value (Figure 6.5). Similarly, isolates C59, KNN13a, KNN26b and the *S. fimbriatus* strain formed a well supported branch in the *S. fimbriatus* 16S rRNA gene subclade; these strains shared identical or almost identical 16S rRNA gene similarities (Table 6.9). Isolate C59 was also found to be closely related to the type strain of *S. fimbriatus* based upon the analyses of the sequence alignments resulting from the concatenation of house-keeping genes head to tail is shown in Figure 6.2. The two remaining isolates, strains KNN38-1b and KNN64-5b, formed a second branch in the *S. fimbriatus* 16S rRNA gene clade that was supported by all of the tree-making algorithms and by a 99% bootstrap value. However, these isolates shared a relatively low 16S rRNA gene similarity, namely 98.2%, a value corresponding to 25 nt differences at 1352 locations. Indeed, isolate KNN64-5b was most closely related to isolate KNN26b; these strains shared a 16S rRNA gene similarity of 98.9%, a value equivalent to 15 nt differences at 1352 locations.

The strains assigned to the *S. fimbriatus* 16S rRNA gene subclade were most closely, albeit quite distinctly related to the type strain of *Streptomyces werraensis*, sharing 16S rRNA gene similarities with the latter within the range 97.0 to 98.9%, values that corresponded to between 15 and 40 nt differences at between 1352-1353 locations. It is also interesting that the type strains of *Streptomyce griseomycini* and *Streptomyces griseostramineus* were found to have identical 16S rRNA gene sequences; these strains formed a well delineated branch in the *Streptomyces* 16S rRNA gene tree (Figure 6.5).

All of the strains assigned to the *S. fimbriatus* 16S rRNA gene subclade grew well on the ISP media producing a range of aerial and substrate mycelial pigments and, when formed, yellowish diffusible pigments (Table 6.10). These organisms also had a

broad range of phenotypic properties in common with identical results recorded for each set of duplicated strains. All of the strains produced acid and alkaline phosphatases, N-acetyl-glucosamidase, esterase (C4), esterase lipase (C8), β-glucosidase and leucine arylamidase (API-ZYM tests), nitrate reductase and urease, and assimilated acetoacetic acid, citric acid, D-galacturonic acid and α-D-glucose, grew from pH 5.0-11.0 and in the presence of 1% NaCl (w/v) (Biolog GEN III Microplates). In contrast, none of strains produced α-chymotrypsin, α-fucosidase, β-glucoronidase, α-mannosidase or trypsin (API-ZYM tests), nitrate reductase or oxidase, nor did they assimilate dextrin, L-histidine, L-lactic acid, D-lactic acid methyl ether, N-acetyl-galactosamine, L-pyrogalacturonic acid, quinic acid, raffinose, D-salicin, D-serine, sodium bromide, sodium butyrate, sodium lactate or D-stachyose or grew in the presence of azetreonan, fusidic acid, guanidine hydrochloride, lincomycin, minocycline, niaproof 4, rifampicin SV, 4% NaCl (w/v), troleandomycin or vancomycin (Biolog GEN III Microplates).

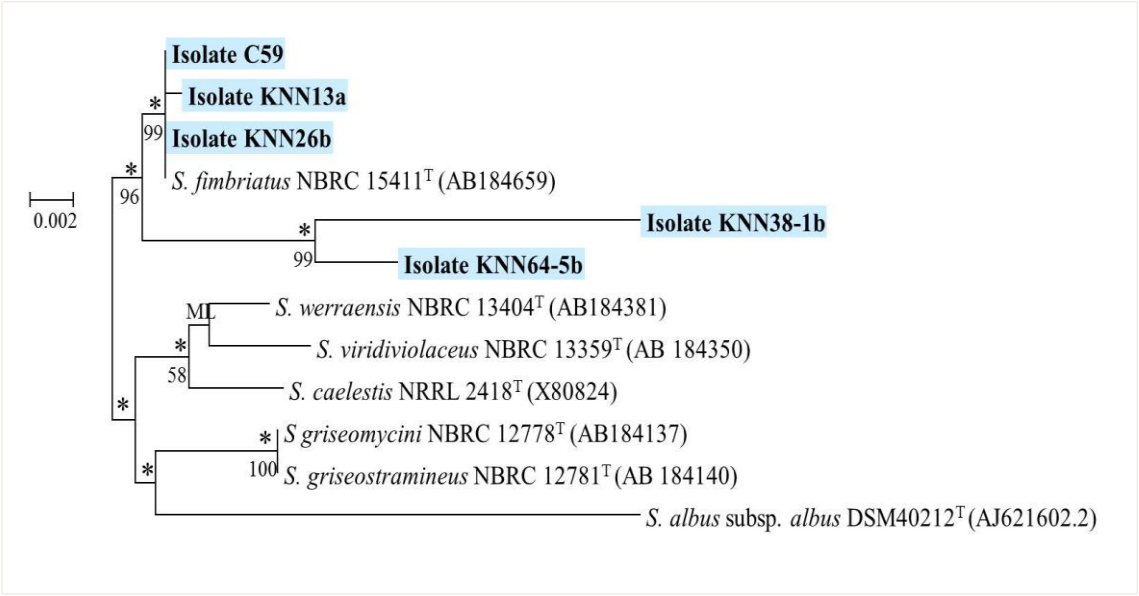


Figure 6.5. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from +hyper-arid and *extreme hyper-arid Atacama Desert soils and between them and the type strains of closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. ML indicates a branch of the tree that was supported by the maximum-likelihood tree-making method. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 sampled datasets, only values above 50% are given. The root position of the tree was determined using *Streptomyces albus* subsp. *albus* DSM 40313^T. Bar, 0.002 substitutions per nucleotide position.

Table 6.9. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from hyper-arid and extreme hyper-arid Atacama Desert soils and between them and the type strains of closely related *Streptomyces* species.

Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. Isolate C59	---	**1/ 1356**	**0/ 1356**	**34/ 1353**	**15/ 1355**	**0/ 1356**	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
2. Isolate KNN13a	**99.9**	---	**1/ 1356**	**35/ 1353**	**16/ 1355**	**1/ 1356**	**16/ 1352**	**17/ 1353**	**17/ 1353**	**19/ 1352**	**16/ 1352**	**36/ 1350**
3. Isolate KNN26b	**100.0**	**99.9**	---	**34/ 1353**	**15/ 1355**	**0/ 1356**	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
4. Isolate KNN38-1b	**97.5**	**97.4**	**97.5**	---	**25/ 1352**	**34/ 1353**	**40/ 1352**	**41/ 1353**	**41/ 1353**	**43/ 1352**	**46/ 1352**	**64/ 1347**
5. Isolate KNN64-5b	**98.9**	**98.8**	**98.9**	**98.2**	---	**15/ 1355**	**30/ 1351**	**31/ 1352**	**31/ 1352**	**33/ 1351**	**30/ 1351**	**50/ 1349**
6. *S. fimbriatus*	**100.0**	**99.9**	**100.0**	**97.5**	**98.9**	---	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
7. *S. werraensis*	98.9	98.8	98.9	97.0	97.8	98.9	---	19/ 1352	19/ 1352	11/ 1352	10/ 1352	41/ 1346
8. *S. griseostramineus*	98.8	98.7	98.8	97.0	97.7	98.8	98.6	---	0/ 1353	14/ 1352	21/ 1352	37/ 1347
9. *S. griseomycini*	98.8	98.7	98.8	97.0	97.7	98.8	98.6	100.0	---	14/ 1352	21/ 1352	37/ 1347
10. *S. viridiviolaceus*	98.7	98.6	98.7	96.8	97.6	98.7	99.2	99.0	99.0	---	15/ 1351	41/ 1346
11. *S. caelestis*	98.9	98.8	98.9	96.6	97.8	98.9	99.3	98.5	98.5	98.9	---	41/ 1346

Type strain codes, as given in Figure 6.5.

Identical results were also obtained between the duplicated set of strains in the balance of the phenotypic tests. *Streptomyces fimbriatus* NRRL B-3175^T and isolates C59, KNN13a and KNN26b have a phenotypic profile that readily distinguishes them from isolates KNN38-1b and KNN64-5b (Table 6.11). It is al so clear that the *S. fimbriatus* type strain and its closest phylogenetic neighbours together with isolate KNN38-1b metabolise a much broader rangeof sole carbon compounds than isolate KNN38-1b. Strain C59, a representative of the isolates found to be most closely related to the type strain of *S. fimbriatus*, produced hairy ornamented spores in tight spiral chains (Figure 6.3) and contained major amounts (>10%) of *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and *antieso*-$C_{17:0}$ (Table 6.3).

Table 6.10. Growth and cultural characteristics of strains assigned to the *Streptomyces fimbriatus* 16S rRNA gene tree on ISP media after incubation for 14 days at 28^{o}C.

Media	Growth	Substrate mycelium colour	Aerial spore mass colour	Diffusible pigment
Glycerol-asparagine agar (ISP 5)	+++	Dark gray	Dark gray	None
Inorganic salts-starch agar (ISP 4)	+++	Dark gray	Dark gray	Light yellowish brown
Oatmeal agar (ISP 3)	++++	Dark gray	Dark gray	Light yellowish

				brown
Peptone-yeast extract-iron agar (ISP 6)	+++	Gray yellow	Olivaceous gray green	Gray yellow
Tryptone-yeast extract agar (ISP 1)	+++	Yellowish white	Olivaceous gray green	None
Tyrosine agar (ISP 7)	+++	Yellowish white	Olivaceous gray green	Yellowish
Yeast extract-malt extract agar (ISP 2)	++++	Dark gray	Dark gray	Light yellowish brown

++++ abundant growth; +++ very good growth.

Table 6.11. Phenotypic tests that distinguish between members of the *Streptomyces fimbriatus* 16S rRNA gene subclade.

Tests	*S. fimbriatus* NRRL B-3175^T, C59, KNN13a and KNN26b	Isolate KNN38-1b	Isolate KNN64-5b
API-ZYM tests:			
α-galactosidase	-	+	-
α-glucosidase	2+	+	+
Lypase (C14)	1+ (KNN26b)	+	-
Naphthol-AS-BI-phosphohydrolase	-	+	+
Valine arylamidase	-	+	+
Biolog GEN III microplate test			
Growth on sole carbon sources:			
Acetic acid	+	+	-
ρ-hydroxyphenyl acetic acid	+	+	-
L-alanine	+	+	-
D-arabitol	+	+	-
L-arginine	+	+	-
γ-aminobutyric acid	+	+	-
L-aspartic acid	+	+	-
α-hydroxy butyric acid	+	+	-
β-hydroxy butyric acid	+	+	-
α-keto-butyric acid	+	+	-
D-cellobiose	+	+	-
Formic acid	-	+	-
D-fructose-6-PO_4	+	+	-
D-fucose	-	+	+
L-fucose	+	+	-
D-fructose	+	+	-
D-galactose	-	+	+
D-galacturonic acid	+	+	-
D-galacturonic acid lactone	-	+	-
Gentiobose	+	+	-
Gelatin	+	+	-
Glycyl-L-proline	+	+	-
Glucoronamide	+	+	-
β-methyl-D-glucoside	+	+	-
D-glucose-6-PO_4	2+	+	-
3-methyl-glucose	+	+	-
Glycerol	+	+	-

N-acetyl-D-glucosamine	+	+	-
α-*keto*-glutaric acid	-	+	-
L-glutamic acid	+	+	-
Inosine	+	+	-
Myo-inositol	+	+	-
α-D-lactose	+	+	-
D-maltose	+	+	-
D-malic acid	+	+	-
L-malic acid	1+ (KNN26b)	+	-
D-mannitol	+	+	-
D-mannose	+	+	-
N-acetyl-β-D-mannosamine	-	+	-
D-melibiose	-	+	-
Mucic acid	+	+	-
N-acetyl neuraminic acid	-	+	-
Pectine	+	+	-
Propionic acid	+	+	-
Methyl pyruvate	-	+	-
L-rhamnose	+	+	-
D-sorbitol	-	+	-
D-turanose	-	+	-
D-saccharic acid	2+	+	-
L-serine	2+	+	-
D-sucrose	-	+	-
Bromo-succinic acid	2+	+	-
D-trehalose	+	+	-
Tween 40	+	+	-

+, positive; - negative;; $^{2+}$, *S. fimbriatus* NRRL B-3175^{T} and isolate C59 positive

***Streptomyces pseudogriseolus* 16S rRNA gene subclade.** Seven strains isolated from either hyper-arid or extreme hyper-arid Atacama Desert soils formed a distinct branch in the *Streptomyces pseudogriseolus* 16S rRNA gene subclade, an association that was supported by all of the tree-making algorithms and by a 90% bootstrap value (Figure 6.6). The isolates exhibited identical or almost identical 16S rRNA gene similarities (Table 6.12) and were most closely related to a second branch in the *S. pseudogriseolus* 16S rRNA gene tree composed of the type strains of *Streptomyces capillispiralis*, *Streptomyces gancidicus* and *S. pseudogriselous*, sharing 16S rRNA gene similarities with the latter with in the range 99.4-99.6%, values that corresponded to between 5 and 7 nt differences at between 1369 and 1381 locations. In turn, the *S. capillispiralis*, *S. gancidicus* and *S. pseudogriseolus* strains were found to have identical or almost identical 16S rRNA gene similarities (Table 6.12).

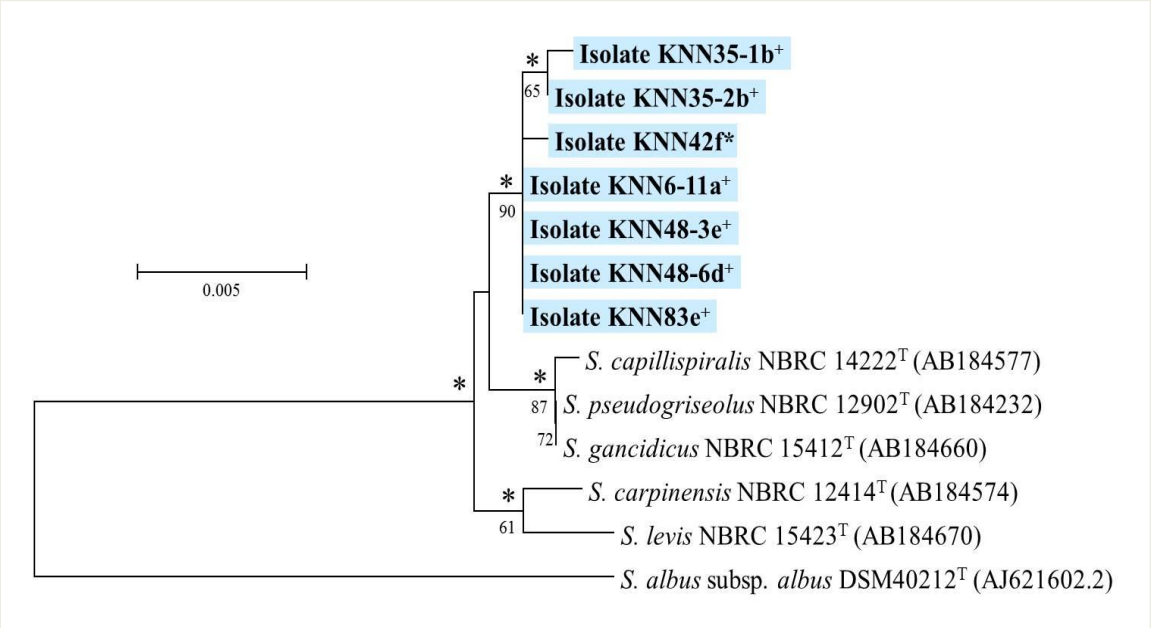


Figure 6.6. Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between strains isolated from +hyper-arid and *extreme hyper-arid and Atacama Desert soils and between them and the type strains of closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 sampled datasets, only values above 50% are given. The root position of the tree was determined using *Streptomyces albus* subsp. *albus* DSM 40313^T. Bar, 0.005 substitutions per nucleotide position.

Table 6.12. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains isolated from hyper-arid and extreme hyper-arid Atacama Desert soils and between them and the type strains of closely related *Streptomyces* species.

Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. Isolate KNN6-1a	**---**	**2/ 1381**	**1/ 1381**	**1/ 1373**	**0/ 1370**	**0/ 1381**	**0/ 1381**	**5/ 1381**	**5/ 1380**	**6/ 1381**	**7/ 1381**	**8/ 1381**
2. Isolate KNN35-1b	**99.9**	**---**	**1/ 1381**	**3/ 1373**	**2/ 1370**	**2/ 1381**	**2/ 1381**	**7/ 1381**	**7/ 1380**	**8/ 1381**	**9/ 1381**	**10/ 1381**
3. Isolate KNN35-2b	**99.9**	**99.9**	**---**	**2/ 1373**	**1/ 1370**	**1/ 1381**	**1/ 1381**	**6/ 1381**	**6/ 1380**	**7/ 1381**	**8/ 1381**	**9/ 1381**
4. Isolate KNN42f	**99.9**	**99.8**	**99.9**	**---**	**1/ 1363**	**1/ 1373**	**1/ 1373**	**6/ 1373**	**6/ 1372**	**7/ 1373**	**8/ 1373**	**9/ 1373**
5. Isolate KNN48-3e	**100.0**	**99.9**	**99.9**	**99.9**	**---**	**0/ 1370**	**0/ 1370**	**5/ 1370**	**5/ 1369**	**6/ 1370**	**7/ 1370**	**8/ 1370**
6. Isolate KNN48-6d	**100.0**	**99.9**	**99.9**	**99.9**	**100.0**	**---**	**0/ 1381**	**5/ 1381**	**5/ 1380**	**6/ 1381**	**7/ 1381**	**8/ 1381**
7. Isolate KNN83e	**100.0**	**99.9**	**99.9**	**99.9**	**100.0**	**100.0**	**---**	**5/ 1381**	**5/ 1380**	**6/ 1381**	**7/ 1381**	**8/ 1381**
8. *S.*	99.6	99.5	99.6	99.6	99.6	99.6	99.6	---	0	1/	10/	9/

pseudogriseolus									/1381	1382	1381	1381
9. *S. gancidicus*	99.6	99.5	99.6	99.6	99.6	99.6	99.6	100.0	---	1/ 1381	10/ 1380	9/ 1380
10. *S. capillispiralis*	99.6	99.4	99.5	99.5	99.6	99.6	99.6	99.9	99.9	---	11/ 1381	8/ 1381
11. *S. carpinensis*	99.5	99.4	99.4	99.4	99.5	99.5	99.5	99.3	99.3	99.2	---	7/ 1381
12. *S. levis*	99.4	99.3	99.4	99.3	99.4	99.4	99.4	99.4	99.4	99.4	99.5	---

Type strain codes, as given in Figure 6.6.

All of the isolates grew well on the ISP media, especially on oatmeal and yeast-extract malt-extract agar (Table 6.13). In general, the substrate mycelia were gray to yellowish white and the aerial spore mass grayish yellow or light orange yellow, as were the diffusible pigments when produced.

Table 6.13. Growth and cultural characteristics isolates forming a branch in the *Streptomyces pseudogriseolus* 16S rRNA gene tree on ISP media after incubation for 14 days at 28^{o}C.

Media	Growth	Substrate mycelium colour	Aerial spore mass colour	Diffusible pigment
Glycerol-asparagine agar (ISP 5)	+++	Dark gray	Dark gray	None
Inorganic salts-starch agar (ISP 4)	+++	Yellowish white	Light orange yellow	Llight orange yellow
Oatmeal agar (ISP 3)	++++	Yellowish white	Light orange yellow	Llight orange yellow
Peptone-yeast extract-iron agar (ISP 6)	+++	Gray yellow	Olivaceous gray green	Gray yellow
Tryptone-yeast extract agar (ISP 1)	+++	Yellowish white	Light orange yellow	Llight orange yellow
Tyrosine agar (ISP 7)	+++	Yellowish white	Light orange yellow	Llight orange yellow
Yeast extract-malt extract agar (ISP 2)	++++	White	Dark orange yellow	Yellowish gray

++++ abundant growth; +++ very good growth.

6.5. Discussion

Although the natural product chemistry of Atacama Desert streptomycetes is still at a pioneering stage, it is encouraging that strains isolated from hyper-arid Salar de Atacama soil and recovered in a taxonomically distinct subclade in the *Streptomyces* 16S rRNA gene tree by Okoro *et al*., (2009) have been found to be a source of novel antibiotics (Bull & Asenjo, 2013). To date, it has been shown that four representatives

of this taxon produce novel specialised metabolites; *Streptomyces* isolate C34^T synthesise new bioactive ansamycin type polyketides, the chaxamycins, and rare 22-membered macrolactone polyketides, the chaxalactins (Rateb *et al*., 2011a, 2011b), *Streptomyces* isolate C38, antitumour macrolactones (Nachtigall *et al*., 2011), *Streptomyces* isolate C58, a novel lasso peptide (Jaspars *et al*., unpublished) and *Streptomyces* isolate C79, four new but uncharacterised, bioactive metabolites (Fiedler *et al*., unpublished). It is important to establish the taxonomic provenance of such potentially useful streptomycetes as part of an integrated approach to exploitable microbiology and with this in mind to isolate additional representatives of such creative taxa.

In the present study additional strains isolated from the hyper-arid Salar de Atacama soil were recovered in the well delineated 16S rRNA gene subclade recognised by Okoro *et al.* (2009), a result that suggests that members of this taxon are common in hyper-arid soils from this region of the Atacama Desert. Indeed, the distinctness of this taxon was underpinned by the results of the MLSA analysis based on concatenated sequences of five house-keeping genes as the MLST distances between C34^T, C38, C58 and C79 and their nearest phylogenetic neighbours was greater that the 0.007 cut-off point that is considered to be equivalent 70% DNA:DNA homology (Rong & Huang, 2012). In addition, all of the representatives of the 16S rRNA gene subclade shared many phenotypic features in common while representative strains were found to produce smooth surfaced spores in spiral chains. Some of these properties can be weighted to distinguish between members of the subclade and the type strains of closely related *Streptomyces* species, including *Streptomyces chiangmaiensis* JCM 16578^T and *Streptomyces hyderabadensis* CCTCC-A 209024^T that are not represented in the MLSA database. Members of the *S. leeuwenhoekii* 16S rRNA gene subclade, unlike the latter, produce diffusible pigments on inorganic salts-starch, oatmeal and tyrosine agars (Reddy *et al*., 2011; Promnuan *et al*., 2013). They can also be separated from the *S. hyderabadensis* strain by their ability to form spiral, as opposed to, straight chains of spores. Properties such as these have been considered to be of particular value in distinguishing between *Streptomyces* species (Labeda *et al*., 2012).

It can be concluded from this combination of genotypic and phenotypic data that the representatives of the *S. leeuwenhoekii* 16S rRNA gene subclade can be distinguished readily from phylogenetically related species that are validly named. It is, therefore proposed that members of the subclade be recognised as a new *Streptomyces* species, *Streptomyces leeuwenhoekii* sp. nov. It is clear from the whole genome

sequence that type strain, isolate C34^T, contains a large number of gene clusters involved in natural product synthesis.

Streptomyces fimbriatus (Millard & Burr, 1926) Waksman and Lechevalier 1953 is an excellent example of a rare actinomycete as the species description is based on a single strain (Kämpfer, 2012). Millard and Burr's original single isolate, which is no longer extant, was obtained from a case of common potato scrab. The neotype strain, which forms a distinct branch in the *Streptomyces* 16S rRNA gene tree, produces septacidin, an antitumour and antifungal antibiotic (Dutcher *et al*., 1964; van Saltza & Pansy, 1964). Given this pedigree, it is especially interesting that five strains isolated from the Atacama Desert soils formed well-delineated subclade in the *Streptomyces* 16S rRNA gene tree.

Three out of the five Atacama Desert strains assigned to the *S. fimbriatus* 16S rRNA gene clade not only had identical or almost identical 16S rRNA gene sequences to *S. fimbriatus* NBRC 15411^T but also shared many phenotypic properties with the latter and hence can be considered to be *bona fide* members of this species. One of these strains, isolate C59, clustered with the type strain of *S. fimbriatus* in the phylogenetic tree based on the concatenated gene sequences of the five house-keeping geness. In light, of these results, an emended description of *S. fimbriatus* is given here. The two remaining strains, isolates KNN38-1b and KNN64-5b may represent new *Streptomyces* species but further comparative studies are need to confirm their taxonomic status.

Further comparative studies are also needed to resolve the taxonomic status of the seven Atacama Desert strains that formed a well supported branch in the *Streptomyces pseudogriseolus* 16S rRNA gene subclade. Unscrambling the relationships between members of this taxon and the type strain of *Streptomyces capillispiralis* (Mertz & Higgens, 1982), *Streptomyces ganidicus* (Suzuki, 1957) and *Streptomyces pseudogriseolus* is compounded by the fact that these strains also need to be studied further as they have identical 16S rRNA gene sequences a point highlighted in this study but made earlier by Kämpfer (2012).

Description of *Streptomyces leeuwenhoekii* sp. nov.

Streptomyces leeuwenhoekii (le.e.u.wen'ho.e.ki.i. of Leeuwenhoek, named after Antonie van Leeuwenhoek (1632-1723), the father of microbiology).

Aerobic, Gram-positive, catalase-positive actinomycete which forms an extensively branched substrate mycelium that carries aerial hyphae which differentiate

into spiral chains of smooth surfaced spores (0.7-0.9 x 0.8-1.0 µm) on oatmeal agar. Light yellowish brown diffusible pigments are formed on inorganic salts-starch, oatmeal, tyrosine and yeast extract-malt extract agars. Grows from 4 to 50^{o}C, optimally ~ 30^{o}C , from pH 6.0 to 11, optimally ~7.0, and in the presence of 10%, w/v sodium chloride. Additional phenotypic properties are cited in the text and in Tables 6.7 and 6.9. Chemotaxonomic properties are typical of the genus. Produces novel bioactive secondary metabolites, the chaxalactins and chaxamycins. The DNA G+C composition is 72.6 mol%.

The type strain C34^{T} (=DSM 42122^{T}; NRRL B-24963^{T}) together with additional strains was isolated from a hyper-arid soil collected from the Chaxa de Laguna, of the Salar de Atacama of the Atacama Desert, near Tocanao, Chile. The species description is based on all of these strains. The GenBank accession number for the 16S rRNA gene sequence of the type strain is KF 733382.

Emended description of *Streptomyces fimbriatus* (Millard & Burr, 1936) Waksman 1953.

The species description is based on the present study and on the earlier work of Kämpfer (2012).

Aerobic, Gram-positive, catalase-positive actinomycetes which form an extremely branched substrate mycelium that bear aerial hyphae that differentiate into spiral chains of hairly ornamented spores (0.8-1.2 x 1.5-1.8 µm) on oatmeal agar. Dark gray substrate and aerial mycelia are formed on oatmeal agar, as is a light yellowish brown diffusible pigment. Grow from 20-40^{o}C, optimum temperature ~ 30^{o}C, from pH 5-11 and in presences of 4% , w/v of NaCl. Additional phenotypic properties are cited in the text and in Tables 6.10 and 6.11. Chemotaxonomic properties are typical of the genus. Produces septacidin, an antitumour and antifungal purine antibiotic

The source of the type strains DSM 40942^{T} (=NBRC 15411^{T} = NRRL B-3175^{T}) is not known. The additional of the species isolated from hyper-arid and extreme hyper-arid Atacama Desert soils.

Acknowledgements

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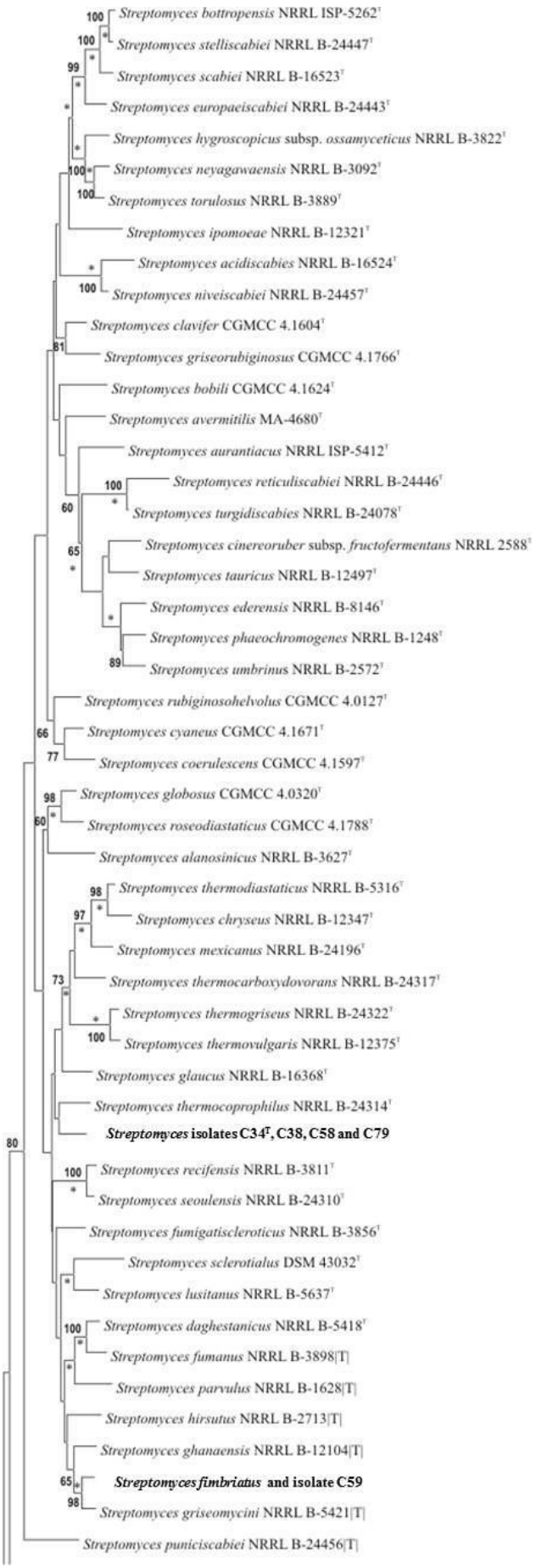
D.P. Labeda (National Center for Agriculture Utilization Research, Peoria, USA) for undertaking the MLSA analyses.

Supplemental Table. Phenotypic properties of *Streptomyces leeuenhoekii* C34^T and related strains (isolates C38, C58, C79, KNN1-5a, KNN2-6a, KNN6-6b, KNN6-9a, KNN10-4d, KNN10-5a, KNN11-1a, KNN24-1b, KNN25c, KNN33a, KNN41-1c and KNN48-1c).

Tests	Strains															
	KNN1-5a	KNN2-6a	KNN6-6b	KNN6-9a	KNN10-4d	KNN10-5a	KNN11-1a	KNN24-1b	KNN25c	KNN33a	KNN41-1c	KNN48-1c	C34^T	C38	C58	C79
Sole Carbon																
Acetic acid	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+
N-acetyl-β-D-mannosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
N-acetyl-D-glucosamine	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
L-alanine	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
L-aspartic acid	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
α-hydroxy butyric acid	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-
D-cellobiose	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
D-galactose	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Formic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobose	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
D-gluconic acid	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Glucoronamide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-methyl-glucose	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
L-histidine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
D-lactic acid methyl ether	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
D-maltose	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Propionic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-

Supplemental Figure1a-c Phylogenetic tree inferred from concatenated partial sequences of the house-keeping genes *atpD, gyrB, recA,* r*poB* and *trpB* in MEGA 5.2 (Tamura *et al.,* 2011) using the maximum-likelihood method based on the General Time Reversible model (Nei & Kumar 2000). There were 2575 positions and 152 strains in the final dataset. Trees were also inferred using the Tamura-Nei evolutionary distance method (1993) with the neighbour-joining algorithm of Saitou & Nei (1987) neighbour-joining and maximum-parsimony models in MEGA 5.2 and conserved branches in all methods are marked with an asterisk. Percentages at the nodes represent levels of bootstrap support from 1000 re-sampled datasets (Felsenstein 1985) with values less than 60% not shown. The proposed new species *Streptomyces leeuwenhoekii* DSM 42122^{T} is indicated with a bold node label. Bar marker equals number of substitutions per site.

Suppl. Fig 1a.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Streptomyces reticuliscabiei NRRL B-24446T
Streptomyces turgidiscabies NRRL B-24078T
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Streptomyces ederensis NRRL B-8146T
Streptomyces phaeochromogenes NRRL B-1248T
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Streptomyces globosus CGMCC 4.0320T
Streptomyces roseodiastaticus CGMCC 4.1788T
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Streptomyces thermodiastaticus NRRL B-5316T
Streptomyces chryseus NRRL B-12347T
Streptomyces mexicanus NRRL B-24196T
Streptomyces thermocarboxydovorans NRRL B-24317T
Streptomyces thermogriseus NRRL B-24322T
Streptomyces thermovulgaris NRRL B-12375T
Streptomyces glaucus NRRL B-16368T
Streptomyces thermocoprophilus NRRL B-24314T
Streptomyces isolates C34T, C38, C58 and C79
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Streptomyces fumigatiscleroticus NRRL B-3856T
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Streptomyces daghestanicus NRRL B-5418T
Streptomyces fumanus NRRL B-3898[T]
Streptomyces parvulus NRRL B-1628[T]
Streptomyces hirsutus NRRL B-2713[T]
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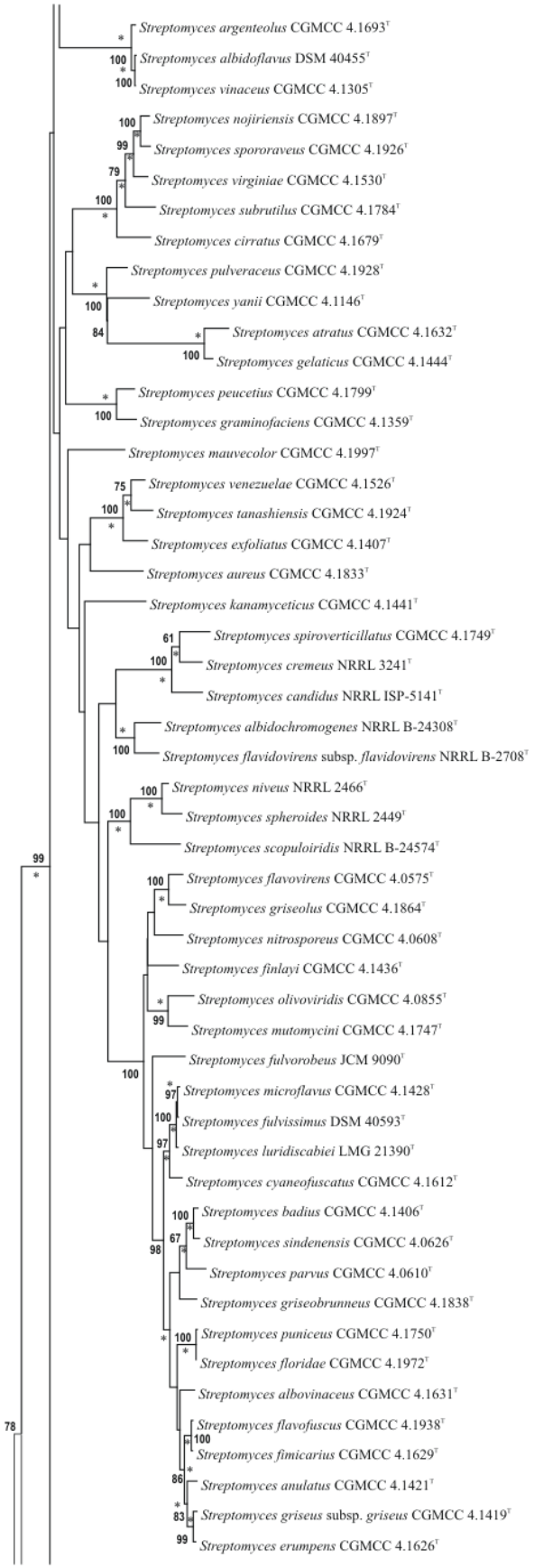
Streptomyces griseomycini NRRL B-5421[T]
Streptomyces puniciscabiei NRRL B-24456[T]

Suppl. Fig 1b.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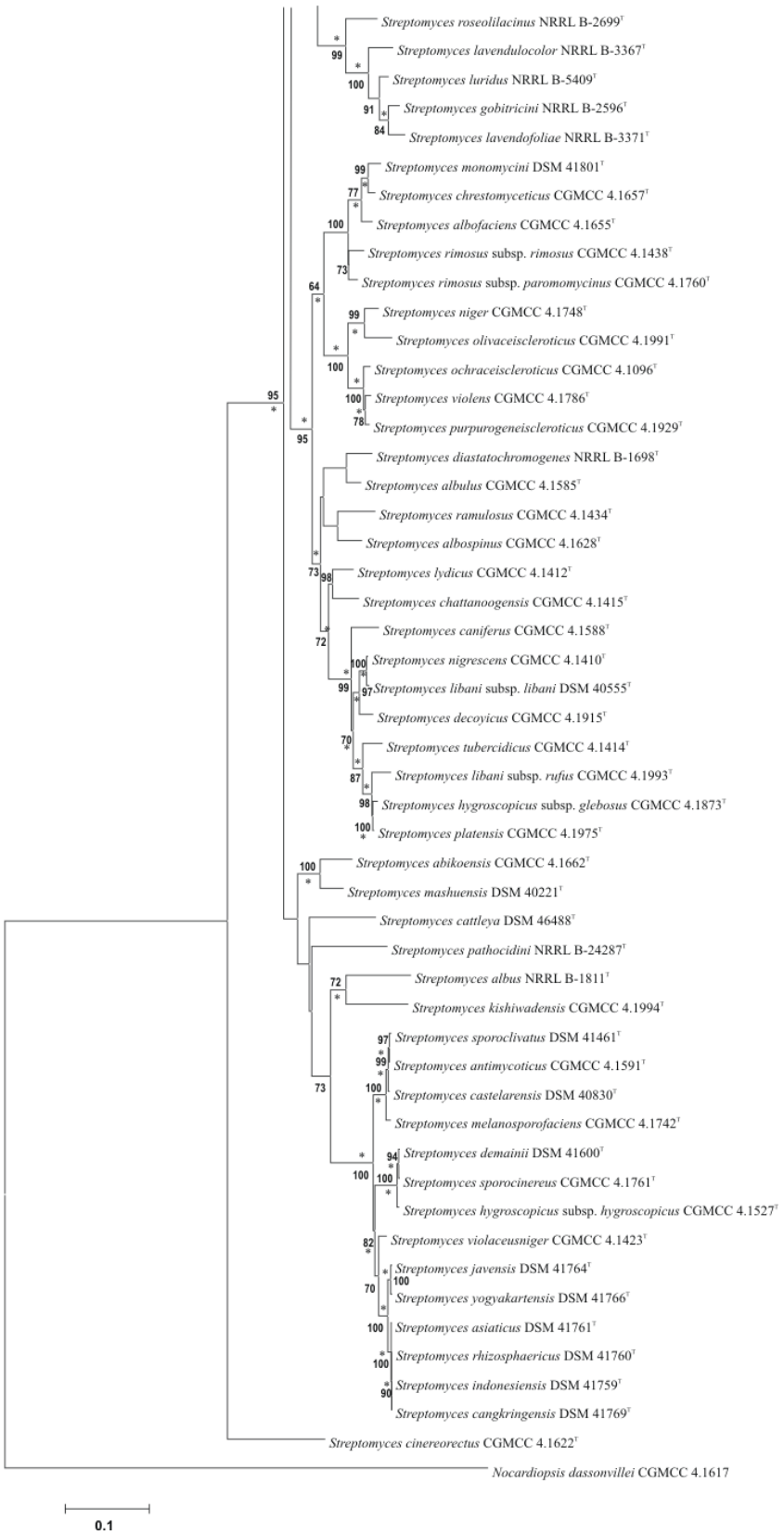
Suppl. Fig 1c.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Streptomyces yogyakartensis DSM 41766T
Streptomyces asiaticus DSM 41761T
Streptomyces rhizosphaericus DSM 41760T
Streptomyces indonesiensis DSM 41759T
Streptomyces cangkringensis DSM 41769T
Streptomyces cinereorectus CGMCC 4.1622T
Nocardiopsis dassonvillei CGMCC 4.1617
0.1

Chapter 7. General Discussion and Prospectives for Future Works

7.1. General discussion

The primary aims of the project were realiased as (a), a high quality library of taxonomically diverse filamentous actinobacteria with the capacity to synthesise a broad range of bioactive compounds isolated from two contrasting locations in the Atacama Desert, namely hyper-arid and extreme hyper-arid soils collected from the Salar de Atacama and Yungay regions, respectively and (b), selected isolates of interest from bioprospecting and ecological perspectives were shown to belong to novel or presumptively novel actinobacterial species using polyphasic taxonomic precedures and 16S rRNA gene sequence data. These results provide further evidence that novel and rare actinobacteria can be isolated from arid Atacama Desert soils using a combination of selective isolation, dereplication and characterisation procedures.

The results of the present study confirm and extend those reported by Okoro and her colleagues in their pioneering studies on cultivable actinobacteria isolated hyper-arid extreme hyper-arid Atacama Desert soils (Okoro *et al*., 2009, 2010). Once again, most isolates were assigned to new centres of taxonomic variation in the *Streptomyces* 16S rRNA gene tree. It was particularly interesting that additional isolates from the hyper-arid Salar de Atacama soil were recovered in the deep-rooted *Streptomyces* 16S rRNA gene subclade discovered by Okoro *et al.* (2009), especially since some members of this taxon now know to synthesise novel antibiotics, notably the atacamycins (Nachtigall *et al*., 2011) and chaxalactins and chaxamycins (Rateb *et al*., 2011a, 2011b; Bull & Asenjo, 2013). Indeed, it was conclusively shown from the results of a wide-ranging polyphasic study that members of this taxon merited recognition as a new species, *Streptomyces leeuwenhoekii* sp. nov. The whole-genome sequence of the type strain of this species was found to contain many polyketide synthase genes, including those that seem likely to be responsible for the biosynthesis of the chaxalactins and chaxamycins.

Other polyphasic studies on selected isolates yielded interesting results. Representative *Amycolatopsis* strains isolated from the hyper-arid Salar de Atacama soil were found to belong to two known, albeit rare species, namely *A. ruanii* and *A. thermalba*, taxa based on single strains isolated from Australian desert soils (Zucchi *et al*., 2012b). In contrast, representative *Modestobacter* strains isolated from the extreme

hyper-arid Yungay soil were shown to belong to at least two novel species though additional comparative taxonomic studies are needed to confirm this. It was also interesting that the full genome sequence of a representative of one of these taxa, unlike the *S. leeuwenhoekii* type strain, contained few natural product gene clusters. These contrasting results suggest that Atacama Desert actinobacteria may have developed, through evolutionary processes, different strategies for surviving the harsh environmental conditions that prevail in the desert.

The comparative 16S rRNA gene sequence analyses showed that the hyper-arid Salar de Atacama soil contained representatives of taxa other than the genera *Amycolatopsis*, *Lechavalieria* and *Streptomyces* that were detected by Okoro *et al*. (2009). In the present study the selective isolation strategies led to the recovery from Salar de Atacama soil of representatives of the genera *Actinomadura*, *Kribbella*, *Nonomuraea* and *Saccharothrix*, rare taxa known to be the source of novel specialised (secondary) metabolites (Tiwari & Gupta, 2012a, b). Similarly, members of the genera *Blastococcus*, *Couchioplanes*, *Geodermatophilus*, *Modestobacter* and *Pseudonocardia*, as well as *Streptomyces* strains, were recovered from the extreme hyper-arid Yungay soil. It was interesting that members of all but one of these genera contain as putatively new species as they shared relatively low 16S rRNA gene similarities with the type strains of their nearest neighbours. The exception, the genus *Amycolatopsis*, was of interest as the strains assigned to this taxon belonged to the thermophilic and poorly studied *A. methanolica* 16S rRNA gene subclade.

It is evident from the phylogenetic, and from the more comprehensive polyphasic studies, that hyper- and extreme hyper-arid Atacama Desert soils contain many novel actinobacteria, notably streptomycetes, that can be isolated using appropriate selective isolation, dereplication strategies and recognised using a combination of genotypic and phenotypic procedures. It is apparent from the plug assays, especially the one based on *B. subtilis* reporter genes, that representative novel and putatively novel actinobacteria are the source of a broad range of interesting bioactive compounds with the potential to be developed into drug leads. So, once again, the premise that extreme habitats are a rich source of novel actinobacterial taxa with the capacity to produce novel specilaised metabolites has been realised.

However, it has to be conceded that both polyphasic taxonomic studies and comparative 16S rRNA gene analyses have serious limitations. The former by their very nature cannot be applied to large number of strains, as exemplified by the fact that only four novel *Streptomyces* species and three novel *Lechevalieria* species have been

proposed for strains isolated from hyper- and extreme hyper-arid Atacama Desert soils (Okoro *et al*., 2009; Santhanam *et al*., 2012a, b, 2013; Busarakam *et al*., 2014). At the present rate of progress it would take several decades to formally name all of putatively novel taxa highlighted in the present study.

16S rRNA gene sequencing studies have greatly enhanced our understanding of actinobacterial diversity (Goodfellow *et al*., 2012) and can be expected to do so in the near future (Kim & Chun, 2014; Yarza & Munoz, 2014). Nevertheless, this approach to unravelling prokaryotic diversity does have drawbacks, notably by providing insufficient resolution for distinguishing between closely related species and for detecting the closest neighbours of deep seated lineages in taxonomically complex genera, both phenomena were appaarent in the present study with particular respect to relationships within the genus *Streptomyces*. Thus, in the present investigation, the analysis of sequence alignments derived from concatenation of five conserved house-keeping genes showed that isolates assigned to the *S. leeuwenhoekii* 16S rRNA gene subclade formed a distinct branch in *Streptomyces* gene trees (Busarakam *et al*., 2014). Indeed, multi-locus sequence analyses based on such conserved genes are clarifying relationships between closely related *Streptomyces* species, including ones with identical 16S rRNA gene sequences (Rong & Huang, 2008, 2010; Rong *et al*., 2009, 2010, 2014).

It seems likely that colossal taxonomic bottlenecks such as these touched upon above will increasingly be resolved now that whole-genome sequences are increasingly being used in prokaryotic systematics (Zhi *et al*., 2012), as exemplified in the present study with respect to the type strain of *S. leeuwenhoekii*. This logical extension of the polyphasic taxonomic concept will not only provide a wealth of high quality taxonomic data (Klenk & Göker, 2010; Sutcliffe *et al*., 2012; Sentausa & Fournier, 2013) but will provide an insight into the ecology, evolution, physiology and biotechnological potential of prokaryotic species (Gao & Gupta, 2012; Chandra & Chater, 2014; Girard *et al*., 2014). However, while systematists should "embrace the genome" it is important that future developments should build upon sound taxonomic practice, notably the nomenclatural type concept (Goodfellow & Fiedler, 2010; Jensen, 2010, Whitman, 2014).

At present, the widespread use of whole-genome sequence is restricted as such data are only available for 1,725 out of nearly 11,000 archaeal and bacterial type strains (Chun & Rainey, 2014) whereas; in contrast nearly a full complement of 16s rRNA gene sequences are available (Kim & Chun, 2014; Yarza & Munoz, 2014). However,

the relative lack of whole-genome sequence data is being addressed by the one thousand microbial genomes (KMG-1) project (Genomic Encycopedia of Type Strains, Phase 1, Kyrpides *et al*., 2014). Once whole genome sequences are available for all type strains, there will be an opportunity for them to serve as a companion to all further isolates thereby dispensing with the need to maintain costly service collections, a vision promulgated by Whitman (2014), who also pointed out that this approach was in line with the principles of the Bacteriological Code (Lapage *et al*., 1990), which only requires that species be unique and completely identified prior to naming.

7.2. Pospectives for future work

Ecology:

- Culture-independent studies will be carried out to establish the key genera present in the hyper-arid and extreme hyper-arid soils used in the present study.
- Inovative and effective taxonomic approaches will be used to isolate members of dominant members of actinobacterial communities detected in the culture-independent studies.
- Emphasis will also be placed on the selective isolation of rare genera, such as *Modestobacter* which predominate in extreme hyper-arid surface soils and on rock surfaces given their ability to produce melanin-like pigments.
- Biogeographical studies will be undertaken on selected genera found to be discontinuously distributed in the Salar de Atacama and Yungay soils.
- Whole genome sequencing studies of representative novel isolates will be generated to gain an insight into how such taxa cope with the harsh environmental conditions that prevail in arid Atacama Desert soils.

Systematics:

- Formal naming of putatively novel *Modestobacter* strains isolated from the extreme hyper-arid Yungay soil following the completion of a polyphasic taxonomic study.
- Completion of the taxonomic studies on representatives of the *S. fimbriatus, S. leeuwenhoekii* and *S. radiopugnans* 16S rRNA gene subclades with the aim of formally naming new species.

- Comprehensive genotypic and phenotypic studies on representatives of rare genera recovered from the hyper-arid Salar de Atacama soil in order to formally name them as new species.

Bioprospecting:

- The *Streptomyces leeuwenhoekii* C34 gene clusters responsible for the biosynthesis of the chaxamycins and chaxalactins will be identified, and expressed heterologously in "*Streptomyces coelicolor*" A(3)2. Similarly approaches will be taken to express and characterise other novel specilaised metabolite gene clusters identified in the *S. leeuwenhoekii* C34 genome sequence.
- Screening of representatives of rare genera isolated from the hyper-arid Salar de Atacama soil for putatively novel bioactive compounds using the *Bacillus subtilis* reporter genes with interesting leads pursued using chemical screens.
- Genome scanning of selected representatives of rare novel genera isolated from the hyper-arid Atacama Desert soil to identify novel pathways.
- Selective isolation, dereplication and screening of additional rare actinobacterial genera highlighted in the culture-independent studies on the Salar de Atacama and Yungay soils.

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Appendix 1. Assignment of isolates to colour-groups

Assignment of strains isolated from the Salar de Atacama and Yungay environmental samples to multi- and single-membered colour-groups based on their ability to produce aerial spore mass, substrate mycelial and diffusible pigments on oatmeal agar and melanin pigments on peptone-yeast extract agar after incubation at 28^{o}C for 4 weeks and 3 days, respectively.

Growth on oatmeal agar				Number of isolates	Strain numbers and source of isolates			
					Salar de Atacama		Yungay	
Colour group code	Aerial spore mass colour	Substrate mycelial colour	Colour of diffusible pigments		Codes	*Melanin production	Codes	*Melanin production
KNN1	white (263)	yellowish white (92)	None	5	KNN1-1a, KNN1-2c, KNN1-3b, KNN1-4c and KNN1-5f	-	None	-
KNN2	medium gray (265)	olivaceous black (114)	light olivaceous gray (112)	23	KNN2-1b, KNN2-2c, KNN2-3c, KNN2-4c, KNN2-5c, KNN2-6c, KNN2-7d, KNN2-8b, KNN2-9d, KNN2-10d, KNN2-11d, KNN2-12d, KNN2-13a, KNN2-14c, KNN2-15a, KNN2-16c, KNN2-17d, KNN2-18b C34, C38, C58, C59, C79,	-		-
KNN3	white (263)	yellow white (92)	none	19	KNN3-1b, KNN3-2b, KNN3-3c, KNN3-4c, KNN3-5c, KNN3-6c, KNN3-7c, KNN3-8c, KNN3-9c, KNN3-10e, KNN3-11d, KNN3-12d, KNN3-13b, KNN3-14d, KNN3-15d, KNN3-16d, KNN3-17d, KNN3-18b and KNN3-19b	-	None	
KNN4	white (263)	yellow white (92)	yellow white (92)	4	KNN4-1b, KNN4-2e, KNN4-3b and KNN4-4a	-	None	
KNN5	medium gray (265)	gray greenish yellow (105)	pale greenish yellow (104)	25	KNN5-1a, KNN5-2a, KNN5-3c, KNN5-4c, KNN5-5a,KNN5-6a, KNN5-7a, KNN5-8b, KNN5-9b, KNN5-10b, KNN5-11b, KNN5-12b,KNN5-13b, KNN5-14b, KNN5-15b, KNN5-16d, KNN5-17d, KNN5-18d, KNN5-19d, KNN5-20b, KNN5-21b, KNN5-22d, KNN5-23c, KNN5-24b, and KNN5-25d	+	None	-
KNN6	medium gray (265)	gray olive 9110)	light orange yellow (70)	11	KNN6-1a, KNN6-2a, KNN6-3a, KNN6-4a, KNN6-5a, KNN6-6b, KNN6-7d, KNN6-8d, KNN6-9a, KNN6-10b and KNN6-11a	-		-
KNN7	very orange (48)	slightly orange (50)	none	4	None		KNN7-1b, KNN7-2b, KNN7-3b, KNN7-4b	-
KNN8	medium gray (265)	gray greenish yellow (105)	light gray olive (109)	10	KNN8-1b, KNN8-2d, KNN8-3a, KNN8-4b, KNN8-5b, KNN8-6a, KNN8-7a, KNN8-8a, KNN8-9c and KNN8-10e	-	None	
KNN9	light gray (264)	gray greenish yellow (105)	light oliveceous gray (112)	3	KNN9-1a, KNN9-2c and KNN9-3b	-	None	
KNN10	light gray (264)	gray greenish yellow (105)	light oliveceous gray (112)	5	KNN10-1a, KNN10-2b, KNN10-3b, KNN10-4d and KNN10-5a	-	None	
KNN11	yellowish white (92)	yellowish white (92)	none	6	KNN11-1a, KNN11-2a, KNN11-3c, KNN11-4b, KNN11-5a and KNN11-6a	-	None	-

KNN12	gray yellow (90)	light brown (57)	medium yellow (87)	2	-		KNN12-1a and KNN12-2a	
KNN13	medium gray (265)	gray olivaceous green (127)	none	1	None		KNN13a	-
KNN14	light gray (264)	pale yellow (89)	light brown (57)	3	KNN14-1f, KNN14-2e and KNN14-3e	-	None	
KNN15	brilliant yellow (83)	light yellow (86)	none	2	None		KNN15-1a and KNN15-2a	-
KNN16	pale yellow (61)	yellowish white (92)	none	2	KNN16-1c	+	KNN16-2c	+
KNN17	very light purple (222)	blackish purple (230)	very deep purplish Red (257)	2	KNN17-1c and KNN17-2b	+	None	
KNN18	light gray (264)	pale yellow (89)	none	3	KNN18-1b, KNN18-2c and KNN18-3d	-	None	
KNN19	white (263)	light yellow (86)	light yellow (86)	2	None		KNN19-1b and KNN19-2c	-
KNN20	slightly orange (50)	slightly orange (50)	none	1	None		KNN20c	-
KNN21	light gray (264)	yellowish white (92)	light gray yellowish brown (79)	1	None		KNN21a	-
KNN22	brown pink (33)	dark yellowish brown (75)	light orange (52)	1	None	-	KNN22a	
KNN23	yellowish white (92)	deep orange yellow (72)	gray yellow (90)	2	None	-	KNN23-1b, KNN23-2b	
KNN24	yellowish white (92)	yellowish white (92)	none	8	KNN24-1b, KNN24-2c, KNN24-3c, KNN24-4c, KNN24-5a, KNN24-6c, KNN24-7e and KNN24-8e	-	None	
KNN25	dark yellowish brown (78)	middle orange yellow (71)	pale orange yellow (70)	1	KNN25c	-	None	
KNN26	dark gray (75)	dark gray (75)	light yellowish brown (76)	1	KNN26b	-	None	
KNN27	white (263)	yellowish white (92)	none	1	KNN27a	-	None	

KNN28	yellowish white (92)	light orange yellow (70)	dark yellow (88)	1	KNN28a	-	None	
KNN29	yellowish white (92)	yellowish white (92)	yellowish white (92)	2	KNN29a	+	KB36a and KB44a	+
KNN30	Medium gray (265)	dark yellowish brown (78)	light brown (57)	1	KNN30a	-	None	
KNN31	light gray (264)	brown gray (61)	gray yellowish brown	1	KNN31d	-	None	
KNN32	gray (265)	dark red gray (23)	pale yellowish green (121)	2	KNN32-1a, KNN32-2b	-	None	
KNN33	pale yellow (89)	pale yellow (89)	pale yellow (89)	1	KNN33a	-	None	
KNN34	white (263)	light orange yellow (70)	light yellow (86)	1	KNN34c	-	None	
KNN35	white (263)	dark orange yellow (72)	yellowish gray (90)	2	KNN35-1b, KNN35-2b	+	None	
KNN36	light orange yellow (70)	light orange yellow (70)	light yellowish brown (76)	3	KNN36-1c, KNN36-2c and KNN36-3c	+	None	
KNN37	medium gray (265)	middle yellowish brown (77)	medium gray (265)	5	KNN37-1e, KNN37-2a, KNN37-3a, KNN37-4a and KNN37-5a	-	None	
KNN38	dark gray (75)	dark gray (75)	light yellowish brown (76)	5	KNN38-1b, KNN38-2d, KNN38-3a, KNN38-4b and KNN38-5b	-	None	
KNN39	yellowish white (92)	light orange yellow (70)	light orange yellow (70)	1	KNN39c	-	None	
KNN40	yellowish white (92)	light yellow (86)	none	1	None		KNN40b	-
KNN41	medium	olivaceous black	light olivaceous	2	KNN41-1b, KNN41-2a	-	None	

	gray (265)	(114)	gray (112)					
KNN42	dark gray (75)	dark gray (75)	light yellowish brown (76)	1	None		KNN42f	-
KNN43	gray yellowish pink (32)	very dark red (17)	bright pink	1	KNN43b	-	None	
KNN44	black (267)	black (267)	light yellowish brown (76)	4	None		KNN44-1b, KNN44-2a, KNN44-3b, KNN44-4b	+
KNN45	black (267)	black (267)	none	4	None		KNN45-1a, KNN45-2b, KNN45-3b and KNN45-4b	-
KNN46	black (267)	black (267)	none	12	None		KNN46-1b, KNN46-2b, KNN46-3b, KNN46-4b, KNN46-5b, KNN46-6a, KNN46-7a, KNN46-8a, KNN46-9c, KNN46-10g, KNN46-11f, KNN46-12f	-
KNN47	black (267)	black (267)	none	1	None	-	KNN47b	-
KNN48	yellowish white (92)	light orange yellow (70)	light orange yellow (70)	20	KNN48-1c, KNN48-2a, KNN48-3e, KNN48-4e, KNN48-5e	-	KNN48-6d, KNN48-7d, KNN48-8d, KNN48-9d, KNN48-10b, KNN48-11b, KNN48-12b, KNN48-13a, KNN48-14a,KNN48-15c, KNN48-16c, KNN48-17c, KNN48-18c, KNN48-19c, KNN48-20c	-
KNN49	yellowish white (92)	yellowish white (92)	none	32	KNN49-1h, KNN49-2e, KNN49-3e, KNN49-4e, KNN49-5e, KNN49-6a, KNN49-7c, KNN49-8c, KNN49-5c, KNN49-6b, KNN49-7c, KNN49-8b, KNN49-9c, KNN49-10b, KNN49-11c, KNN49-12b, KNN49-13b, KNN49-14b, KNN49-15b, KNN49-16c, KNN49-17b, KNN49-18b, KNN49-19a, KNN49-20b, KNN49-21c, KNN49-22d, KNN49-23d, KNN49-24d, KNN49-25d, KNN49-26a, KNN49-27a, KNN49-28a, KNN49-29a, KNN49-30b, KNN49-31b, KNN49-32e	-	None	
KNN50	yellowish white (92)	yellowish white (92)	none	18	KNN50-1a, KNN50-2e, KNN50-3c, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-12c, KNN50-13c, KNN50-14d, KNN50-15d, KNN550-16d, KNN50-17d and KNN50-18d	-	None	
KNN51	yellowish	yellowish white	none	1	None	-	KNN51b	-

	white (92)	(92)						
KNN52	light olive brown (94)	dark gray yellow (91)	dark gray yellow (91)	3	KNN52-1c, KNN52-2b and KNN52-3b	-	-	-
KNN53	White (263)	yellowish white (92)	yellowish white (92)	3	KNN53-1a, KNN53-2a and KNN53-3a	-	-	-
KNN54	gray yellowish brown (32)	dark yellowish brown (78)	dark yellowish brown (78)	2	KNN54-1a and KNN54-2b	-	None	-
KNN55	pink white (9)	very dark red (7)	middle brown (58)	2	-	-	KNN55-1b and KNN55-2b	
KNN56	none	light yellowish brown (76)	none	1	KNN56a	-	None	-
KNN57	brown pink (33)	dark yellowish brown (75)	light orange (52)	2	KNN57-1b and KNN57-2b	-	-	-
KNN58	yellowish white (92)	dark orange yellow (72)	light orange yellow (70)	2	KNN58-1b and KNN58-2c	-		-
KNN59	yellowish white (92)	gray yellow (90)	gray yellow (90)	1	KNN59e	-	-	-
KNN60	pale yellow (89)	middle yellowish brown (77)	none	2	KNN60-1c and KNN60-2d	-	-	-
KNN61	light gray (264)	light gray yellowish brown (79)	light gray yellowish brown (79)	4	KNN61-1a, KNN61-2e, KNN61-3e, KNN61-4b and KNN61-2c	-	-	-
KNN62	dark gray purple (229)	blackish purple (230)	pale gray (233)	1	KNN62b	-	-	-
KNN63	yellowish white (92)	yellowish white (92)	none	18	KNN63-1a, KNN63-2b, KNN63-3b, KNN63-4b, KNN63-5a, KNN63-6b, KNN63-7b, KNN63-8d, KNN63-9d, KNN63-10d, KNN63-11b, KNN63-12b, KNN63-13b, KNN63-14b, KNN63-15b, KNN63-16b, KNN63-17b	-	-	-

					and KNN63-18b			
KNN64	dark gray (75)	dark gray (75)	light yellowish brown (76)	6	KNN64-1a, KNN64-2a, KNN64-3a, KNN64-4b, KNN64-5b and KNN64-6b	-	-	-
KNN65	white (263)	middle orange yellow (71)	Gray yellow (90)	6	KNN65-1f, KNN65-2a, KNN65-3c, KNN65-4c, KNN65-5d and KNN65-6b	-	-	-
KNN66	yellowish white (92)	light gray yellowish brown (79)	light gray yellowish brown (79)	1	KNN66b	-	-	-
KNN67	none	light orange yellow (70)	none	4	KNN67-1b, KNN67-2b, KNN67-3b and KNN67-4b	-	-	-
KNN68	yellowish white (92)	middle yellowish brown (77)	dark orange yellow (72)	4	KNN68-1b, KNN68-2b, KNN68-3b and KNN68-4b	-	-	-
KNN69	slightly yellowish pink (26)	light brown (57)	light brown (57)	3	KNN69-1e, KNN69-2a and KNN69-3a	-	-	-
KNN70	very yellowish pink (25)	light gray yellowish brown (79)	light gray yellowish brown (79)	1	KNN80b	-	-	-
KNN71	yellowish white (92)	light orange yellow (70)	light orange yellow (70)	2	KNN71-1a and KNN71-2a	-	-	-
KNN72	yellowish white (92)	light gray yellowish brown (79)	light gray yellowish brown (79)	1	KNN72a	-	-	-
KNN73	yellowish white (92)	light orange yellow (70)	gray yellow (90)	4	KNN73-1a, KNN73-2a , KNN73-3d and KNN73-4d	-	-	-
KNN74	yellowish white (92)	dark yellowish brown (78)	light gray yellowish brown (79)	2	KNN74-1c and KNN74-2c	-	-	-

KNN75	white (263)	yellowish white (92)	none	4	KNN75-1c, KNN75-2b, KNN75-3b and KNN75-4b	-	-	-
KNN76	yellowish white (92)	light orange yellow (70)	light orange yellow (70)	1	KNN76-1b	-	-	-
KNN77	yellowish white (92)	middle yellowish brown (77)	dark orange yellow (72)	6	-	-	KNN77-1a, KNN77-2a, KNN77-3a, KNN77-1a, KNN77-5a and KNN77-6a	-
KNN78	yellowish White (92)	yellowish White (92)	gray yellow (90)	2	KNN78-1e and KNN78-2e	-	-	-
KNN79	yellowish white (92)	deep yellowish brown (75)	middle yellowish brown (77)	3	KNN79-1b, KNN79-2b and KNN79-3d	-	-	-
KNN80	none	White yellow(92)	pale yellow (89)	2	KNN80-1c and KNN80-2d	-	-	-
KNN81	pale yellowish pink (31)	Light orange (52)	middle yellowish brown (77)	4	KNN81-1c, KNN81-2b, KNN81-3d and KNN81-4d	-	-	-
KNN82	yellowish white (92)	blackish purple (230)	gray reddish brown (46)	2	KNN82-1a and KNN82-2c	-	-	-
KNN83	white (263)	yellowish White (92)	none	1	KNN83e	-	-	-
KNN84	dark yellowish brown (78)	middle orange yellow (71)	pale orange yellow (70)	1	KNN84c	-	-	-
KNN85	yellowish white (92)	middle yellow (87)	dark gray yellow (91)	8	KNN85-1f, KNN85-2b, KNN85-3c, KNN85-4c, KNN85-5c, KNN85-6c, KNN85-7b, KNN85-8a	-	-	-
KNN86	black (267)	black (267)	none	5	-	-	KNN86-1b, KNN86-2b, KNN86-3b, KNN86-4a and KNN86-5a	-
KNN87	dark pale gray (234)	blackish purple (230)	light reddish brown (45)	1	KNN87b	+	-	-

KNN88	yellowish white (92)	light gray yellowish brown (79)	light gray yellowish brown (79)	4	-	-	KNN88-1a, KNN88-2a, KNN88-3a and KNN88-4a	-
KNN89	medium gray (265)	olivaceous black (114)	light olivaceous gray (112)	1	-	-	KNN89a	-
KNN90	yellowish white (92)	light gray yellowish brown (79)	light gray yellowish brown (79)	1	-	-	KNN90a	-
KNN91	black (267)	black (267)	light yellowish brown (76)	6	-	-	KNN91-1a, KNN91-2a, KNN91-3a, KNN91-4a, KNN91-5a and KNN91-6a	-
KNN92	dark pale gray (234)	blackish purple (230)	light reddish brown (45)	7	-	-	KNN92-1a, KNN92-2a, KNN92-3a, KNN92-4a, KNN92-5a, KNN92-6a and KNN92-7a	-
KNN93	white (263)	yellowish White (92)	none	4	-	-	KNN93-1a, KNN93-2a, KNN93-3a and KNN93-4a	-
KNN94	white (263)	yellowish white (92)	None	1	KNN94e	-	-	-
KNN95	white (263)	middle orange yellow (71)	Gray yellow (90)	7	-	-	KNN95-1b, KNN95-2b, KNN95-3b, KNN95-4b, KNN95-5b, KNN995-6b and KNN95-7b	-
KNN96	white (263)	yellowish White (92)	none	1	-	-	KNN96a	-
KNN97	yellowish White (92)	yellowish White (92)	gray yellow (90)	1	-	-	KNN97a	-
KNN98	yellowish white (92)	dark yellowish brown (78)	light gray yellowish brown (79)	1	-	-	KNN98a	-

Codes exemplified by strain KNN2-1b, this organism was isolated by Kanungnid Busarakam (KNN), assigned to colour-group 2 as the first member of group and was isolated on a humic acid-vitamin agar isolation plate. The C-coded isolates represent a colour-group delineated by Okoro *et al*. (2009).

Selective isolation media : (a), Gause No.1 agar; (b), HVA, humic acid-vitamin agar; (c), Oligotrophic agar; (d), minimal medium agar; (e), SM1 agar; (f) *Geodermatophilus obscurus* agar; (g) Luedemann's agar, (h), SCAV agar.

Appendix 2. Reagents and buffers

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 added to make a final volume of 100ml. The working solution was prepared by diluting the stock solution to give 0.1X SSC buffer.

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 give a final volume of 100 ml.

Lugol's iodine for the detection of starch degradation
Iodine……. 5 g
Potassium iodide…… 10 g
Distilled water…… 100 ml
Iodine and potassium iodide were 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

Potassium phosphate buffer
Solution A: 0.1 M potassium dihydrogen phosphate (KH_2PO_4)
Solution B: 0.1 M dipotassium hydrogen phosphate (K_2HPO_4)

Desired pH	Solution A (ml)	Solution B (ml)
5.7	98.5	6.5
5.8	92.0	8.0
5.9	90.0	10.0
6.0	87.7	12.3
6.1	85.0	15.0
6.2	81.5	18.5
6.3	77.5	22.5
6.4	73.5	26.5
6.5	68.5	31.5
6.6	62.5	37.5
6.7	56.5	43.5
6.8	51.0	49.0

Desired pH	Solution A (ml)	Solution B (ml)
6.9	45.0	55.0
7.0	39.0	61.0
7.1	33.0	67.0
7.2	28.0	72.0
7.3	23.0	77.0
7.4	19.0	81.0
7.5	16.0	84.0
7.6	13.0	87.0
7.7	10.5	90.5
7.8	8.5	91.5
7.9	7.0	93.0
8.0	5.3	94.7

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.

Appendix 3. 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). Media were autioclaved at 121°C for 20 minutes unless otherwise stated.

1) Culture media

Carbon utilisation medium (ISP medium 9, Pridham & Gottlieb, 1948**)**

$(NH_4)_2SO_4$……2.64 g
KH_2PO_4 ……...2.38 g
$K_2HPO_4.3H_2O$ ……5.65 g
$MgSO_4.7H_2O$ …….. 1.0 g
Pridham & Gottlieb trace salts* ……1.0 ml
Agar ……. 15.0 g
dH_2O……...1 L
pH 6.8-7.0

Pridham & Gottlieb trace salts*
$CuSO_4.5H_2O$ ……0.64 g
$FeSO_4.7H_2O$ ……... 0.11 g
$MnCl_2.4H_20$ ……. 0.79 g
$ZnSO_4.7H_2O$ ……...0.15 g
dH_2O ……...100.0 ml

Carbohydrate solutions were prepared, separately, Tyndallised (steam at 100^oC between 8 to 24 hours) individual carbon sources were mixed thoroughly with the molten basal medium to give the appropriate final concentration.

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

Glucose……..…… 4.0 g
Yeast extract……..... 4.0 g
Malt extract…….…… 10.0 g
$CaCO_3$…….……..... ….2.0 g
Agar……. 15.0 g
dH_2O……...1.0 L
pH……. 7.2 ± 0.2

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

L-asparagine ……..... 1.0 g
Glycerol…….. 10.0 g
KH_2PO_4..... 1.0 g
Trace salt solution* …….……..1.0 ml
Agar……... 15.0 g
dH_2O……...1.0 L
pH……..…… 6.8 ± 0.2

Trace salt solution*
$FeSO_4.7H_2O$…… ……...0.001 g
$MnCl_2.4H_2O$…….. …….0.001 g
$ZnSO_4.7H_2O$…… …….……..0.001 g
pH 7.4±0.2

Inorganic salts 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 was added to 500 ml of distilled water and the preparation mixed well to prevent clumping then sterilsed.

Luria-Bertani medium (Miller, 1972)
Tryptone…….…. 10.0 g
Yeast extract…… 5.0 g
NaCl……..…... 10.0 g
dH_2O……...1.0 L

Bennett's agar (Jones, 1949)
Yeast extract …….. 1.0 g
Lab-LEMCO……... 0.8 g
Bacto-Casitone……..... 2.0 g
Glycerol……. 10.0 g
Agar……... 15.0 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 to 1000 ml of distilled water and boil for 20 minutes. The mixture is 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
dH_2O……...1.0 L
pH……. 7.0 ±0.2

Tryptone-yeast extract agar (ISP medium 1; Shirling & Gottlieb, 1996)
Bacto-Tryptone ……..…….. 5.0 g
Bacto-Yeast extract……..... 3.0 ml
Agar……..…… 15.0 g
dH_2O……...1.0 L

pH…….. 7.0 -7.2

Tyrosine agar (ISP medium 7; Shirling & Gottlieb, 1966)
Glycerol……... 15.0 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
dH_2O……...1.0 L
Trace salt solution (see ISP3) …..…….... 1.0 ml
Agar……..... 20.0 g
pH……..…… 7.2 - 7.4

Modified Bennett's agar (Jones, 1949)
This composition of Bennett's Agar followed the original formulation of Jones (1949) but 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).
Glucose ……...…… 10.0 g
Bacto-Casitone (Difco) …… 2.0 g
Yeast-extract …… 1.0 g
Lab-Lemco (Oxoid) ……..…... 0.8 g
Agar …… 15.0 g
dH_2O……...1.0 L
pH……..…… 7.2 - 7.4

2) Phenotypic test media
Aesculin/ Arbutin degradation (Kutzner, 1976)
Basal medium
Yeast extract……..…... 3.0 g
Ferric ammonium citrate …….. 0.5 g
Agar…….. 7.5 g
pH……. 7.2
Aesculin and arbutin were tyndallised in distilled water then added to the melted basal medium to give a final concentration of 0.1% (w/v).

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
dH_2O……...…..1.0 L
pH…….…… 6.8
Autoclaved at 121°C for 20 minutes. The allantoin was tyndallised for 24 hours 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 tests

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. The pH of the media were tested using pH paper and adjusted with sterile 1 N NaOH or 1 N HCl to the desired pH.

Chitin degradation (Hsu & Lockwood, 1975)

Colloidal chitin……..... 4.0 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……..… 20.0 g
dH_2O……...1 L
pH……. 8.0

DNase test agar for DNA degradation (Difco)

Tryptose…… 20.0 g
DNA…….**.....** 2.0 g
NaCl……...… 5.0 g
Agar……..... 12.0 g
dH_2O……...1.0 L
pH…… 7.2

Nitrate reduction medium (Gordon & Mihm, 1962)

KNO_3…….. 1.0 g
Lab LEMCO…….…….. 2.4 g
dH_2O…….……...1.0 L
pH…….…….. 7.0
Three ml amounts of the media were dispensed into bijoux bottles.

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

Tryptone ……. 20.0 g
NaCl……..……....…. 5.0 g
dH_2O…….1.0 L
Agar……..... 12.0 g
pH…… 7.2
RNA (3 g) was tyndallised and added to the molten 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 ……..…….. 10.0 g
NaCl…… .5.0 g
$CaCl_2.H_2O$……..... …..0.1 g
Agar…… 15.0 g
dH_2O……...1.0 L

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).

Tributyrin agar
Tributyrin agar base (Sigma T3688)……..... 23.0 g
Tributyrin (Sigma T8626)……..... 10.0 ml
Distilled water……..…. 990 ml
Tributyrin agar base was dissolved in 990 ml distilled water by heating and stirring on a heated magnetic stirrer. Tributyrin was added to the medium while it was being stirred. After autoclaving the molten medium was cooled in a 50°C water bath and mixed by gentle swirling while pouring in order to maintain uniform turbidity in the agar.

Urea degradation (Krieg & Padgett, 2011)
Basal medium
KH_2PO_4……..…… 9.1 g
Na_2HPO_4……..... .9.5 g
Yeast extract……..… 0.1 g
Phenol red…… 0.01 g
dH_2O……...1 L
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 of the medium were aseptically dispensed into bijoux bottles.

3) Isolation media
Gause's agar No.1 (Gause *et al*., 1957)
Starch……20.0 g
$FeSO_4.7H_2O$…….……0.01 g
KNO_3 …….……..……..1.0 g
K_2HPO_4……...……... 0.5 g
$MgSO_4.7H_2O$ ……..…0.5 g
NaCl……...…..... 0.5 g
Agar……..……..15.0 g
dH_2O……...1.0 L
pH ……..17.2+0.2

***Geodermatophilus obscurus* agar (Uchida & Seino, 1997)**
Soluble starch ……..10.0 g
Yeast extract……..……4.0 g
Tryptone……..…….4.0 g
Sucrose……..……2.0 g
Agar……..……...15.0 g
dH_2O……1.0 L
pH……7.0

Humic acid vitamin agar (Hayakawa & Nonomura, 1987)
Humic acid*……1.0 g
Vitamin solution**……..……1.0 ml
$CaCO_3$ ……0.02 g
$FeSO_4.7H_2O$……....…0.01 g

KCl……....1.7 g
$MgSO_4.7H_2O$ ……0.05 g
Na_2HPO_4……... 0.5 g
Agar……...…15.0 g
dH_2O……1.0 L
pH……7.2±0.2
*Humic acid was dissolved in 10 ml of 0.2 N NaOH

****Vitamin 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 (B5) ……...… 10 mg
Pyridoxine (B6) …… 10 mg
Riboflavin Na choline (B2) ……..……...……10 mg
Thiamin (B1) …… 10 mg
Cyanocobalamin (B12) ……..... 0.5 mg
Folic acid ……...……... 0.5 mg

***Microlunatus* agar (Nakamura *et al*., 1995)**

Glucose……..……0.5 g
Peptone……..…...……0.5 g
Yeast extract……..……...……0.5 g
Na-glutamate……...……0.5 g
KH_2PO_4……..…..……0.5 g
$MgSO_4.7H_2O$……..……..…0.1 g
dH_2O……..……...1.0 L
pH……..……..7.0

Minimal medium (Johnson *et al.*, 1981)

KH_2PO_4……..……1.5 g
K_2HPO_4……..……2.9 g
Urea……..……..2.1 g
$MgCl_2.6H_2O$……...1.0 g
$CaCl_2$……150 mg
$FeSO_4.6H_2O$……1.25 mg
Cysteine hydrochloride……..……...1.0 g
Resazurin……..…..2.0 mg
Cellobiose……..5.0 g
Morpholinopropane sulfonic acid…….……10.0 g
Pyridoxamine hydrochloride…….2.0 mg
Biotin…….0.2 mg
ρ-Aminobenzoic acid…….……0.4 mg
Vitamin B_{12}…….……...0.2 mg
Sodium citrate.H_2O……..……..3.0 g
dH_2O……..……...1.0 L

Oligotrophic medium (Senechkin *et al*., 2010)

$MgSO_4.7H_2O$……...0.5 g
KNO_3……..….0.5 g

$KH_2PO_4.3H_2O$…….1.3 g
$Ca(NO_3)_2.4H_2O$…….0.06 g
Glucose……..…….2.5 g
Enzymetic casein hydrolysate……...……..0.2 g
Agar……..……..15.0 g
dH_2O……..……1.0 L
pH……..……..……..7.2

R2A agar (Reasoner & Geldriech, 1985)
Proteose peptone……..……..…….0.5 g
Casamino acid…….…….……..0.5 g
Yeast extract……..……..…0.5 g
Dextrose……..…….…..0.5 g
Soluble starch ……..…..…0.5 g
K_2HPO_4……0.3 g
$MgSO_4.7H_2O$……..…....0.005 g
Sodium pyruvate……..……...……0.3 g
Agar……...…..…..15.0 g
dH_2O……..……..1.0 L
pH…….. 7.2±0.2

SM1 (Tan *et al*., 2006)
Basal medium*……..…..100.0 ml
Sterile molten agar (15%w/v) ……..……..…900.0 ml
*Basal medium: yeast nitrogen base (Difco) ……..…… 67.0 g
Casamino acid (Difco)…… 100.0 mg
dH_2O…….....1.0 L
Basal medium were steriled by cellulose filter (0.20 µm). Then add to sterilised K_2HPO_4 (10%w/v) 200 ml. Followed by filter sterilized of
D(-)-sorbitol (final concentration)…….1.0 %w/v
Cycloheximide……50.0 µg/ml
Neomycin sulphate……4.0 µg/ml
Nystatin……50.0 µg/ml

Starch casein-vitamin agar (Küster & Williams, 1964)
Difco-vitamin-free casein……..…0.3 g
KNO_3 ……..……..2.0 g
NaCl…….….…….…2.0 g
$MgSO_4.7H_2O$……...……0.05 g
$CaCO_3$ …….……....0.02 g
$FeSO_4.7H_2O$……..…....0.01 g
Soluble starch ……..……..10.0 g
Agar……..…..15.0 g
dH_2O……....1.0 L
pH 7.0±0.2

Appendix 4. Nucleotide similarity and differences tables

Table 1 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains KNN34c, KNN53-1a and KNN53-3d isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Actinomadura* species.

Isolate	1	2	3	**4**	5	6	7	8	9	10	11	12	13	14	**15**	16	17	18	19	20
1. Isolate KNN34	---	**42/1363**	**39/1400**	**15/1393**	**22/1399**	**20/1400**	**25/1343**	**25/1401**	**23/1396**	**26/1401**	**28/1397**	**25/1400**	**27/1391**	**39/1401**	**52/1394**	**36/1398**	**30/1401**	**32/1400**	**28/1401**	**25/1400**
2.Isolate KNN53-3	**96.9**	---	**3/1365**	**54/1360**	**51/1363**	**39/1364**	**37/1336**	**47/1364**	**38/1361**	**37/1365**	**50/1359**	**40/1364**	**43/1355**	**8/1366**	**18/1363**	**19/1363**	**31/1366**	**32/1364**	**38/1365**	**40/1364**
3.Isolate KNN53-1	**97.2**	**99.8**	---	**52/1401**	**52/1407**	**36/1413**	**41/1344**	**47/1402**	**36/1409**	**37/1414**	**52/1409**	**36/1413**	**41/1404**	**9/1415**	**17/1404**	**19/1407**	**28/1415**	**29/1413**	**34/1414**	**36/1413**
4. *A. rugatobispora*	**98.9**	**96.0**	**96.3**	---	**24/1405**	**26/1405**	**34/1340**	**37/1397**	**30/1402**	**37/1405**	**41/1399**	**34/1405**	**32/1396**	**52/1405**	**56/1404**	**47/1402**	**42/1405**	**42/1405**	**40/1405**	**34/1405**
5. *A. vinacea*	**98.4**	**96.3**	**96.3**	**98.3**	---	**25/1441**	**34/1343**	**31/1429**	**32/1423**	**34/1438**	**50/1421**	**33/1432**	**32/1432**	**51/1438**	**61/1405**	**47/1428**	**44/1441**	**39/1427**	**35/1418**	**33/1432**
6. *A. bangladensis*	**98.6**	**97.1**	97.5	98.2	98.3	---	20/1343	25/1431	15/1437	18/1455	58/1438	19/1444	7/1449	40/1454	50/1406	34/1429	25/1458	27/1442	26/1434	19/1444
7. *A. napierensis*	**98.1**	**97.2**	97.0	97.5	97.5	98.5	---	25/1343	25/1340	27/1344	37/1339	29/1343	24/1334	36/1344	52/1342	34/1342	36/1344	30/1343	30/1344	29/1343
8. *A. xylanlytica*	**98.2**	**96.6**	96.7	97.4	97.8	98.3	98.1	---	25/1412	26/1441	41/1416	31/1424	31/1440	47/1432	57/1398	39/1418	42/1452	27/1420	27/1411	31/1424
9. *A. meyeae*	**98.4**	**97.2**	97.4	97.9	97.8	99.0	98.1	98.2	---	12/1435	44/1425	22/1433	22/1428	40/1435	50/1403	38/1421	33/1438	31/1433	27/1429	22/1433
10. *A. geliboluensis*	**98.1**	**97.3**	97.4	97.4	97.6	98.8	98.0	98.2	99.2	---	54/1440	27/1445	28/1455	42/1457	51/1407	36/1427	39/1466	32/1444	28/1436	27/1445
11. *A. flavaba*	**98.0**	**96.3**	96.3	97.1	96.5	96.0	97.2	97.1	96.9	96.3	---	53/1434	64/1429	79/1450	63/1400	56/1417	66/1440	56/1435	54/1426	53/1434
12. *A. madurae*	**98.2**	**97.1**	97.5	97.6	97.7	98.7	97.8	97.8	98.5	98.1	96.3	---	26/1435	40/1445	51/1406	35/1426	34/1445	31/1439	32/1431	0/1445
13. *A. chokoriensis*	**98.1**	**96.8**	97.1	97.7	97.8	99.5	98.2	97.9	98.5	98.1	95.5	98.2	---	45/1446	56/1397	37/1420	30/1467	33/1433	32/1425	26/1435
14. *A. phis*	**97.2**	**99.4**	99.4	96.3	96.5	97.3	97.3	96.7	97.2	97.1	94.6	97.2	96.9	---	23/1408	19/1428	35/1458	32/1445	39/1436	40/1445
15. *A. rifamicini*	**96.3**	**98.7**	98.8	96.0	95.7	96.4	96.1	95.9	96.4	96.4	95.5	96.4	96.0	98.4	---	26/1407	40/1408	39/1406	47/1407	51/1406
16. *A. cremea*	**97.4**	**98.6**	98.7	96.7	96.7	97.6	97.5	97.3	97.3	97.5	96.1	97.6	97.4	98.7	98.2	---	26/1431	35/1426	43/1417	35/1426
17. *A. sediminis*	**97.9**	**97.7**	98.0	97.0	97.0	98.3	97.3	97.1	97.7	97.3	95.4	97.7	98.0	97.6	97.2	98.2	---	37/1445	44/1436	34/1445
18. *A. flabrosa*	**97.7**	**97.7**	98.0	97.0	97.3	98.1	97.8	98.1	97.8	97.8	96.1	97.9	97.7	97.8	97.2	97.6	97.4	---	19/1434	31/1439
19. *A. nitritigenes*	**98.0**	**97.2**	97.6	97.2	97.5	98.2	97.8	98.1	98.1	98.1	96.2	97.8	97.8	97.3	96.7	97.0	96.9	98.7	---	32/1431
20. A. madurae	**98.2**	**97.1**	97.5	97.6	97.7	98.7	97.8	97.8	98.5	98.1	96.3	100	98.2	97.2	96.4	97.6	97.7	97.0	97.8	---

Strain codes, as given in Figure 3.1.

Table 2 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains KNN49-1h, KNN49-3e, KNN49-5e, KNN49-6a, KNN49-10b, KNN49-11c, KNN49-12b, KNN49-26a, KNN49-32e, KNN50-1a, KNN50-2e, KNN50-4c, KNN50-5c, KNN50-6e, KNN50-7d, KNN50-8b, KNN50-9b, KNN50-10e, KNN50-11c, KNN50-13c, KNN50-14d, KNN50-15d, KNN50-12c, KNN50-16d, KNN50-17d and KNN 50-18d isolated from the hyper-arid Salar de Atacama environmental sample and the type strains of closely related *Amycolatopsis* species.

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Isoalte KNN50-8b	---	**17/ 1285**	**21/ 1315**	**16/ 1314**	**2/ 1314**	**2/ 1312**	**16/ 1315**	**2/ 1317**	**3/ 1316**	**16/ 1317**	**16/ 1317**	**17/ 1312**	**2/ 1313**	**16/ 1314**	**7/ 1314**	**19/ 1313**	**17/ 1316**	**3/ 1313**	**2/ 1314**	**16/ 1314**	**19/ 1317**	**7/ 1317**
2. Isolate GY024	**98.7**	---	**4/ 1366**	**25/ 1353**	**15 /1353**	**15/ 1354**	**26/ 1369**	**15/ 1371**	**16/ 1370**	**26/ 1370**	**26/ 1370**	**40/ 1372**	**15/ 1281**	**28/ 1374**	**18/ 1282**	**16/ 1282**	**30/ 1338**	**16/ 1370**	**15/ 1347**	**26/ 1369**	**24/ 1351**	**16/ 1371**
3. Isolate GY142	**98.4**	**99.7**	---	**27/ 1378**	**17/ 1378**	**18/ 1378**	**31/ 1395**	**21/ 1398**	**22/ 1397**	**31/ 1397**	**30/ 1396**	**39/ 1392**	**19/ 1311**	**29/ 1392**	**20/ 1314**	**18/ 1313**	**34/ 1365**	**19/ 1393**	**17/ 1372**	**28/ 1393**	**26/ 1380**	**26/ 1397**
4. Isolate KNN49-6a	**98.8**	**98.2**	**98.0**	---	**24 /1382**	**24/ 1380**	**0/ 1381**	**24/ 1382**	**25/ 1381**	**0/ 1383**	**0/ 1383**	**7/ 1381**	**14/ 1310**	**1/ 1382**	**17/ 1311**	**1/ 1311**	**14/ 1367**	**25/ 1381**	**19/ 1376**	**0/ 1383**	**1/ 1381**	**29/ 1382**
5. Isolate KNN49-5e	**99.9**	**98.9**	**98.8**	**98.3**	---	**0/ 1380**	**24/ 1380**	**0/ 1382**	**1/ 1381**	**24/ 1382**	**24/ 1382**	**23/ 1380**	**0/ 1310**	**25/ 1381**	**3/ 1311**	**15/ 1311**	**29/ 1366**	**1/ 1381**	**0/ 1376**	**24/ 1382**	**23/ 1380**	**5/ 1382**
6. Isolate KNN49-3e	**99.9**	**98.9**	**98.7**	**98.3**	**100.0**	---	**25/ 1379**	**0/ 1382**	**1/ 1381**	**25/ 1381**	**25/ 1381**	**25/ 1381**	**0/ 1308**	**26/ 1380**	**3/ 1309**	**15/ 1309**	**29/ 1364**	**0/ 1383**	**0/ 1374**	**25/ 1381**	**23/ 1378**	**4/ 1382**
7. Isolate KNN50-10e	**98.8**	**98.1**	**97.8**	**100.0**	**98.3**	**98.2**	---	**25/ 1401**	**26/ 1399**	**0/ 1402**	**0/ 1401**	**21/ 1396**	**14/ 1311**	**1/ 1397**	**20/ 1313**	**4/ 1312**	**14/ 1368**	**26/ 1395**	**19/ 1374**	**0/ 1397**	**4/ 1383**	**31/ 1400**
8. Isolate KNN50-4c	**99.9**	**98.9**	**98.5**	**98.3**	**100.0**	**100.0**	**98.2**	---	**1/ 1402**	**25/ 1403**	**25/ 1402**	**38/ 1398**	**0/ 1313**	**26/ 1398**	**6/ 1315**	**18/ 1314**	**29/ 1369**	**1/ 1398**	**0/ 1376**	**25/ 1398**	**26/ 1384**	**6/ 1403**
9. Isolate KNN49-26a	**99.8**	**98.8**	**98.4**	**98.2**	**99.9**	**99.9**	**98.1**	**99.9**	---	**26/ 1401**	**26/ 1400**	**38/ 1396**	**1/ 1312**	**27/ 1396**	**7/ 1314**	**19/ 1313**	**30/ 1369**	**2/ 1397**	**1/ 1375**	**26/ 1397**	**27/ 1383**	**7/ 1401**
10. Isolate KNN49-12b	**98.8**	**98.1**	**97.8**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	---	**0/ 1403**	**21/ 1398**	**14/ 1313**	**1/ 1399**	**20/ 1315**	**4/ 1314**	**14/ 1370**	**26/ 1397**	**19/ 1376**	**0/ 1399**	**4/1385**	**31/ 1402**
11. Isolate KNN49-11c	**98.8**	**98.1**	**97.9**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	**100.0**	---	**21/ 1398**	**14/ 1313**	**1/ 1399**	**19/ 1314**	**3/ 1313**	**14/ 1370**	**26/ 1397**	**19/ 1376**	**0/ 1399**	**3/ 1384**	**31/ 1402**
12. Isolate KNN49-32e	**98.7**	**97.1**	**97.2**	**99.5**	**98.3**	**98.2**	**98.5**	**97.3**	**97.3**	**98.5**	**98.5**	---	**15/ 1308**	**23/ 1398**	**18/ 1309**	**2/ 1309**	**15/ 1365**	**38/ 1397**	**19/ 1374**	**20/ 1397**	**6/ 1379**	**41/ 1398**
13. Isolate KNN50-18d	**99.9**	**98.8**	**98.6**	**98.9**	**100.0**	**100.0**	**98.9**	**100.0**	**99.92**	**98.9**	**98.9**	**98.9**	---	**14/ 1310**	**5/ 1312**	**17/ 1312**	**15/ 1312**	**1/ 1309**	**0/ 1310**	**14/ 1310**	**17/ 1313**	**5/ 1313**
14. Isolate KNN50-17d	**98.8**	**98.0**	**97.9**	**99.9**	**98.2**	**98.1**	**99.9**	**98.1**	**98.1**	**99.9**	**99.9**	**98.4**	**98.9**	---	**17/ 1311**	**1/ 1311**	**15/ 1366**	**27/ 1396**	**20/ 1375**	**1/ 1398**	**2/ 1380**	**32/ 1398**
15. Isolate KNN50-15d	**99.5**	**98.6**	**98.5**	**98.7**	**99.8**	**99.8**	**98.5**	**99.5**	**99.5**	**98.5**	**98.6**	**98.6**	**99.6**	**98.7**	---	**18/1314**	**20/1313**	**4/1310**	**3/1311**	**17/1311**	**18/1315**	**10/1314**
16. Isolate KNN50-16d	**98.6**	**98.8**	**98.6**	**99.9**	**98.9**	**98.9**	**99.7**	**98.6**	**98.6**	**99.7**	**99.8**	**99.9**	**98.7**	**99.9**	**98.6**	---	**2/1312**	**16/1310**	**15/1311**	**1/1311**	**2/1314**	**22/1313**
17. Isolate KNN50-11c	**98.7**	**97.8**	**97.5**	**99.0**	**97.88**	**97.9**	**99.0**	**97.9**	**97.8**	**99.0**	**99.0**	**98.9**	**98.9**	**98.9**	**98.5**	**99.9**	---	**30/1365**	**29/1366**	**14/1367**	**17/1370**	**34/1369**
18. Isolate KNN50-14d	**99.8**	**98.8**	**98.6**	**98.2**	**99.9**	**100.0**	**98.1**	**99.9**	**99.9**	**98.1**	**98.1**	**97.3**	**99.9**	**98.1**	**99.7**	**98.8**	**97.8**	---	**1/1375**	**26/1397**	**24/1379**	**6/ 1398**
19. Isolate KNN50-13c	**99.9**	**98.9**	**98.8**	**98.6**	**100.0**	**100.0**	**98.6**	**100.0**	**99.9**	**98.6**	**98.6**	**98.6**	**100.0**	**98.6**	**99.8**	**98.9**	**97.9**	**99.9**	---	**19/1376**	**20/1376**	**5/1376**
20. Isolate KNN50-12c	**98.8**	**98.1**	**98.0**	**100.0**	**98.3**	**98.2**	**100.0**	**98.2**	**98.1**	**100.0**	**100.0**	**98.6**	**98.9**	**99.9**	**98.7**	**99.9**	**99.0**	**98.1**	**98.6**	---	**1/1381**	**31/1398**
21. Isolate KNN50-9b	**98.6**	**98.2**	**98.1**	**99.9**	**98.3**	**98.3**	**99.7**	**98.1**	**98.1**	**99.7**	**99.9**	**99.6**	**98.7**	**99.9**	**98.6**	**99.9**	**98.8**	**98.3**	**98.6**	**99.9**	---	**30/1383**
22. Isolate KNN50-7d	**99.5**	**98.8**	**98.1**	**97.9**	**99.6**	**99.7**	**97.8**	**99.6**	**99.5**	**97.8**	**97.8**	**97.1**	**99.6**	**97.7**	**99.2**	**98.3**	**97.5**	**99.6**	**99.6**	**97.8**	**97.8**	---
23. Isolate KNN50-6e	**99.9**	**98.9**	**98.6**	**98.3**	**100.0**	**100.0**	**98.2**	**100.0**	**99.9**	**98.2**	**98.2**	**97.4**	**100.0**	**98.1**	**99.6**	**98.7**	**97.9**	**99.9**	**100.0**	**98.2**	**98.2**	**99.6**
24. Isolate KNN50-5c	**99.9**	**98.9**	**98.8**	**98.6**	**100.0**	**100.0**	**98.6**	**100.0**	**99.9**	**98.6**	**98.6**	**98.6**	**100.0**	**98.5**	**99.8**	**98.9**	**97.9**	**99.9**	**100.0**	**98.6**	**98.5**	**99.6**
25. Isolate KNN50-2e	**98.8**	**97.8**	**97.9**	**100.0**	**98.3**	**98.1**	**100.0**	**98.1**	**98.1**	**100.0**	**100.0**	**98.4**	**98.9**	**99.6**	**98.7**	**99.9**	**99.0**	**98.1**	**98.6**	**100.0**	**99.9**	**97.7**
26. Isolate KNN50-1a	**98.6**	**98.1**	**98.0**	**100.0**	**98.3**	**98.2**	**99.8**	**98.0**	**97.9**	**99.8**	**99.9**	**98.5**	**98.9**	**99.9**	**98.7**	**99.9**	**98.8**	**98.1**	**98.6**	**100.0**	**99.9**	**97.7**
27. Isolate KNN49-10b	**99.9**	**98.9**	**98.6**	**98.3**	**100.0**	**100.0**	**98.3**	**100.0**	**99.9**	**98.3**	**98.3**	**98.3**	**100.0**	**98.2**	**99.6**	**98.7**	**97.9**	**99.9**	**100.0**	**98.3**	**98.2**	**99.6**
28. Isolate KNN49-1h	**99.9**	**98.8**	**98.6**	**98.3**	**100.0**	**100.0**	**98.1**	**99.9**	**99.9**	**98.1**	**98.1**	**97.4**	**100.0**	**98.1**	**99.8**	**98.9**	**97.9**	**99.9**	**100.0**	**98.1**	**98.3**	**99.7**

Strain codes, as given in Figure 3.2.

Table2 (cont.)

Isolate	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1.Isolate KNN50-8b	2/1317	2/1314	16/1314	18/1317	2/1316	2/1312	0/1317	18/1317	16/1316	18/1317	19/1317	19/1316	20/1317	23/1316	26/1316	24/1313	68/1315	67/1317	66/1315	84/1313	87/1314
2. Isolate GY024	15/1370	15/1348	31/1375	26/1370	15/1352	16/1369	15/1395	15/1328	26/1394	17/1380	19/1394	17/1394	18/1395	35/1351	39/1353	20/1390	72/1412	63/1411	62/1429	68/1395	85/1394
3. Isolate GY142	20/1397	17/1373	29/1394	28/1397	19/1380	19/1392	18/1410	17/1369	29/1409	20/1409	22/1409	21/1409	22/1410	40/1392	47/1381	29/1396	80/1408	70/1409	68/1407	74/1406	95/1407
4. Isolate KNN49-6a	24/1382	20/1377	0/1383	0/1383	24/1381	24/1380	24/1382	0/1357	28/1382	26/1382	18/1382	22/1382	23/1383	26/1381	25/1380	32/1378	83/1381	79/1382	76/1380	86/1378	98/1379
5. Isolate KNN49-5e	0/1382	0/1377	24/1382	24/1382	0/1381	0/1380	0/1382	14/1357	16/1381	18/1381	19/1381	19/1381	20/1382	31/1380	33/1379	24/1377	70/1380	65/1381	64/1379	83/1378	86/1379
6. Isolate KNN49-3e	0/1382	0/1375	26/1382	25/1381	0/1380	0/1383	0/1382	14/1355	16/1381	18/1381	19/1381	19/1381	20/1382	31/1378	35/1379	24/1377	70/1380	65/1381	64/1379	83/1378	86/1379
7. Isolate KNN50-10e	25/1399	20/1375	0/1398	3/1402	24/1382	26/1394	28/1401	3/1359	32/1401	30/1401	22/1401	26/1401	27/1402	29/1383	27/1383	36/1397	87/1400	83/1401	80/1399	90/1397	101/1398
8. Isolate KNN50-4c	0/1402	0/1377	26/1400	28/1403	0/1384	1/1397	3/1404	17/1361	19/1403	21/1403	22/1403	22/1403	23/1404	34/1384	37/1385	27/1399	73/1402	68/1403	67/1401	86/1400	89/1401
9. Isolate KNN49-26a	1/1401	1/1376	27/1398	29/1401	1/1383	2/1396	4/1402	18/1360	20/1401	22/1401	23/1401	23/1401	24/1402	35/1383	38/1384	28/1397	74/1400	69/1401	68/1399	87/1398	89/1399
10. Isolate KNN49-12b	25/1401	20/1377	0/1400	3/1404	24/1384	26/1396	28/1403	3/1361	32/1403	30/1403	22/1403	26/1403	27/1404	29/1385	27/1385	36/1399	87/1402	83/1403	80/1401	90/1399	102/1400
11.Isolate KNN49-11c	25/1401	20/1377	0/1400	2/1403	24/1384	26/1396	27/1402	2/1360	31/1402	29/1402	21/1402	25/1402	26/1403	28/1384	27/1385	35/1398	86/1401	82/1402	79/1400	89/1398	101/1399
12. Isolate KNN49-32e	37/1397	19/1375	22/1400	21/1398	23/1379	37/1396	39/1399	1/1355	43/1399	41/1399	33/1399	37/1399	38/1400	33/1381	34/1383	47/1395	99/1400	94/1401	92/1399	101/1395	113/1397
13. Isolate KNN50-18d	0/1313	0/1310	14/1310	16/1313	0/1312	0/1308	2/1313	16/1313	14/1312	16/1313	17/1313	17/1312	18/1313	21/1312	24/1312	22/1309	66/1311	65/1313	64/1311	82/1309	85/1310
14. Isolate KNN50-17d	26/1397	21/1376	5/1403	1/1399	25/1380	27/1395	27/1403	1/1356	31/1403	29/1403	21/1403	25/1403	26/1404	27/1380	26/1381	35/1399	85/1402	82/1403	79/1401	89/1399	101/1400
15. Isolate KNN50-15d	5/1314	3/1311	17/1311	17/1315	5/1313	3/1309	3/1316	1/1316	15/1315	17/1316	18/1316	18/1315	19/1316	22/1315	25/1313	23/1312	66/1314	66/1316	65/1314	83/1312	86/1313
16.Isolate KNN50-16d	17/1313	15/1311	1/1311	1/1314	17/1312	15/1309	15/1315	1/1315	18/1314	17/1315	9/1315	12/1314	13/1315	21/1314	22/1312	22/1311	69/1313	72/1315	69/1313	77/1311	88/1312
17. Isolate KNN50-11c	29/1369	29/1366	14/1367	16/1370	29/1368	29/1364	31/1369	1/1359	33/1369	32/1370	24/1370	27/1369	28/1370	38/1368	38/1368	37/1365	87/1368	88/1370	85/1368	92/1365	103/1366
18. Isolate KNN50-14d	1/1398	1/1376	27/1398	26/1397	1/1381	1/1398	1/1398	15/1356	17/1397	19/1397	20/1397	20/1397	21/1398	32/1379	36/1381	25/1393	71/1396	66/1397	65/1395	84/1394	87/1395
19. Isolate KNN50-13c	0/1376	0/1376	19/1376	19/1376	0/1375	0/1374	0/1376	14/1357	16/1375	18/1375	19/1375	19/1375	20/1376	26/1374	28/1373	24/1371	70/1374	65/1375	64/1373	83/1372	86/1373
20.Isolate KNN50-12c	25/1398	20/1377	0/1399	0/1399	24/1381	26/1396	25/1398	0/1357	29/1398	27/1398	19/1398	23/1398	24/1399	26/1381	25/1382	33/1394	84/1397	80/1398	77/1396	87/1394	99/1395
21. Isolate KNN50-9b	25/1383	21/1377	1/1381	1/1385	25/1382	23/1378	23/1384	1/1361	27/1384	25/1384	17/1384	21/1384	22/1385	27/1383	26/1381	31/1380	82/1383	78/1384	75/1382	85/1380	97/1381
22. Isolate KNN50-7d	6/1402	5/1377	32/1400	33/1402	5/1384	4/1397	8/1403	21/1360	24/1402	26/1402	27/1402	27/1402	28/1403	38/1383	42/1385	32/1398	78/1401	73/1402	72/1400	91/1399	94/1400
23.Isolate KNN50-6e	---	0/1377	26/1399	27/1401	0/1384	1/1397	2/1402	16/1360	18/1401	20/1401	21/1401	21/1401	22/1402	33/1383	37/1385	26/1397	72/1400	67/1401	66/1399	85/1398	88/1399
24. Isolate KNN50-5c	100.0	---	20/1377	20/1377	0/1376	0/1375	0/1377	14/1357	16/1376	18/1376	19/1376	19/1376	20/1377	27/1375	29/1374	24/1372	70/1375	65/1376	64/1374	83/1373	86/1374
25. Isolate KNN50-2e	98.1	98.6	---	0/1400	24/1381	27/1397	30/1404	0/1357	34/1404	32/1404	24/1404	28/1404	29/1405	26/1381	26/1383	38/1400	89/1403	85/1404	82/1402	92/1400	104/1401
26. Isolate KNN50-1a	98.1	98.6	100.0	---	26/1384	26/1396	25/1403	0/1361	29/1403	27/1403	19/1403	23/1403	24/1404	26/1385	25/1385	33/1399	84/1402	80/1403	77/1401	87/1399	99/1400
27. Isolate KNN49-10b	100.0	100.0	98.3	98.1	---	0/1380	2/1384	16/1359	18/1383	20/1383	21/1383	21/1383	22/1384	33/1382	35/1381	26/1379	71/1382	66/1383	65/1381	84/1380	88/1381
28. Isolate KNN49-1h	99.93	100.0	98.1	98.1	100.0	---	1/1397	14/1355	17/1396	19/1396	20/1396	20/1396	21/1397	31/1378	35/1380	25/1392	71/1395	66/1396	65/1394	84/1393	87/1394

Strain codes, as given in Figure 3.2.

Table2 (cont.)

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
29. *A_ruanii_*	**100.0**	**98.9**	**98.7**	**98.3**	**100.0**	**100.0**	**98.0**	**99.9**	**99.7**	**98.0**	**98.1**	**97.2**	**99.9**	**98.1**	**99.8**	**98.9**	**97.7**	**99.9**	**100.0**	**98.2**	**98.3**	**99.4**	**99.9**
30. *A_thermalba*	**98.6**	**98.9**	**98.8**	**100.0**	**99.0**	**99.0**	**99.8**	**98.8**	**98.7**	**99.9**	**99.9**	**99.9**	**98.8**	**99.9**	**99.9**	**99.9**	**99.9**	**98.9**	**99.0**	**100.0**	**99.9**	**98.5**	**98.8**
31. *A_thermoflava*	**98.8**	**98.1**	**97.9**	**98.0**	**98.8**	**98.8**	**97.7**	**98.7**	**98.6**	**97.7**	**97.8**	**96.9**	**98.9**	**97.8**	**98.9**	**98.6**	**97.6**	**98.8**	**98.8**	**97.9**	**98.1**	**98.3**	**98.7**
32. *A_endophytica*	**98.6**	**98.8**	**98.6**	**98.1**	**98.7**	**98.7**	**97.9**	**98.5**	**98.4**	**97.9**	**97.9**	**97.1**	**98.8**	**97.9**	**98.7**	**98.7**	**97.7**	**98.6**	**98.7**	**98.1**	**98.2**	**98.2**	**98.6**
33. *A_methanolica*	**98.6**	**98.6**	**98.4**	**98.7**	**98.6**	**98.6**	**98.4**	**98.4**	**98.4**	**98.4**	**98.5**	**97.6**	**98.7**	**98.5**	**98.6**	**99.3**	**98.3**	**98.6**	**98.6**	**98.6**	**98.8**	**98.1**	**98.5**
34. *A_eurytherma*	**98.6**	**98.8**	**98.5**	**98.4**	**98.6**	**98.6**	**98.1**	**98.4**	**98.4**	**98.2**	**98.2**	**97.4**	**98.7**	**98.2**	**98.6**	**99.1**	**98.0**	**98.6**	**98.6**	**98.4**	**98.5**	**98.1**	**98.5**
35. *A_tucumanensis*	**98.5**	**98.7**	**98.4**	**98.3**	**98.6**	**98.6**	**98.1**	**98.4**	**98.3**	**98.1**	**98.2**	**97.3**	**98.6**	**98.2**	**98.6**	**99.0**	**98.0**	**98.5**	**98.6**	**98.3**	**98.4**	**98.0**	**98.4**
36. *A_granulosa*	**98.3**	**97.4**	**97.1**	**98.1**	**97.8**	**97.8**	**97.9**	**97.5**	**97.5**	**97.9**	**98.0**	**97.6**	**98.4**	**98.0**	**98.3**	**98.4**	**97.2**	**97.7**	**98.1**	**98.1**	**98.1**	**97.3**	**97.6**
37. *A_viridis*	**98.0**	**97.1**	**96.6**	**98.2**	**97.6**	**97.5**	**98.1**	**97.3**	**97.3**	**98.1**	**98.1**	**97.5**	**98.2**	**98.1**	**98.1**	**98.3**	**97.2**	**97.4**	**98.0**	**98.2**	**98.1**	**97.0**	**97.3**
38. *A_thermophila*	**98.2**	**98.6**	**97.9**	**97.7**	**98.3**	**98.3**	**97.4**	**98.1**	**98.0**	**97.4**	**97.5**	**96.6**	**98.3**	**97.5**	**98.3**	**98.3**	**97.3**	**98.2**	**98.3**	**97.6**	**97.6**	**97.7**	**98.1**
39. *A_pigmentata*	**94.8**	**94.9**	**94.3**	**94.0**	**94.9**	**94.9**	**93.8**	**94.8**	**94.7**	**93.8**	**93.9**	**92.9**	**95.0**	**93.9**	**95.0**	**94.7**	**93.6**	**94.9**	**94.9**	**94.0**	**94.1**	**94.4**	**94.9**
40. *A_helveola*	**94.9**	**95.5**	**95.0**	**94.3**	**95.3**	**95.3**	**94.1**	**95.2**	**95.1**	**94.1**	**94.2**	**93.3**	**95.1**	**94.2**	**95.0**	**94.5**	**93.6**	**95.3**	**95.3**	**94.3**	**94.4**	**94.8**	**95.2**
41. *A_taiwanensis*	**95.0**	**95.7**	**95.8**	**94.5**	**95.4**	**95.4**	**94.3**	**95.2**	**95.1**	**94.3**	**94.4**	**93.4**	**95.1**	**94.4**	**95.1**	**94.7**	**93.8**	**95.3**	**95.3**	**94.5**	**94.6**	**94.9**	**95.3**
42. *A_orientalis*	**93.6**	**95.1**	**94.7**	**93.8**	**94.0**	**94.0**	**93.6**	**93.9**	**93.8**	**93.6**	**93.6**	**92.8**	**93.7**	**93.6**	**93.8**	**94.1**	**93.3**	**94.0**	**94.0**	**93.8**	**93.8**	**93.5**	**93.9**
43. *Actinokineospora riparia*	**93.4**	**93.9**	**93.3**	**92.9**	**93.8**	**93.8**	**92.8**	**93.7**	**93.6**	**92.7**	**92.8**	**91.9**	**93.5**	**92.8**	**93.5**	**93.3**	**92.5**	**93.76**	**93.7**	**92.9**	**93.0**	**93.3**	**93.7**

Strain codes, as given in Figure 3.2.

Isolate	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
29. *A_ruanii_*	**100.0**	**97.9**	**98.2**	**99.9**	**99.9**	**---**	14/1392	16/1456	18/1441	19/1460	19/1456	20/1461	31/1414	35/1385	24/1426	70/1455	65/1456	64/1458	83/1453	87/1457
30. *A_thermalba*	**99.0**	**100.0**	**100.0**	**98.8**	**99.0**	**99.0**	---	17/1389	16/1389	8/1392	11/1389	12/1392	20/1390	21/1359	21/1360	70/1388	71/1390	68/1390	77/1386	88/1389
31. *A_thermoflava*	**98.8**	**97.6**	**97.9**	**98.7**	**98.8**	**98.9**	98.8	---	20/1442	13/1457	16/1459	17/1460	34/1416	41/1388	24/1429	79/1455	74/1456	71/1454	86/1452	94/1452
32. *A_endophytica*	**98.7**	**97.7**	**98.1**	**98.6**	**98.6**	**98.8**	98.9	98.6	---	12/1443	16/1442	17/1443	37/1413	44/1386	23/1413	76/1440	72/1441	70/1439	82/1437	97/1438
33. *A_methanolica*	**98.6**	**98.3**	**98.7**	**98.5**	**98.6**	**98.7**	99.4	99.1	99.2	---	10/1457	11/1478	31/1415	38/1386	22/1427	77/1455	78/1456	73/1468	80/1452	93/1471
34. *A_eurytherma*	**98.6**	**98.0**	**98.4**	**98.5**	**98.6**	**98.7**	99.2	98.9	98.9	99.3	---	1/1460	36/1416	43/1388	26/1429	75/1455	78/1456	75/1454	80/1452	96/1452
35. *A_tucumanensis*	**98.6**	**97.9**	**98.3**	**98.4**	**98.5**	**98.6**	99.1	98.8	98.8	99.3	99.9	---	37/1418	44/1389	27/1430	76/1456	79/1457	76/1469	81/1453	97/1472
36. *A_granulosa*	**98.0**	**98.1**	**98.1**	**97.6**	**97.8**	**97.8**	98.6	97.6	97.4	97.8	97.5	97.4	---	11/1387	39/1386	77/1414	69/1415	72/1414	92/1409	101/1412
37. *A_viridis*	**97.9**	**98.1**	**98.2**	**97.5**	**97.5**	**97.5**	98.5	97.1	96.8	97.8	96.9	96.8	99.2	---	44/1384	80/1386	74/1387	79/1385	100/1381	102/1383
38. *A_thermophila*	**98.3**	**97.3**	**97.6**	**98.1**	**98.2**	**98.3**	98.5	98.3	98.4	98.5	98.2	98.1	97.2	96.8	---	77/1425	75/1426	71/1424	86/1422	100/1422
39. *A_pigmentata*	**94.9**	**93.7**	**94.0**	**94.9**	**94.9**	**95.2**	95.0	94.6	94.7	94.7	94.9	94.8	94.6	94.2	94.6	---	46/1475	44/1475	110/1451	110/1453
40. *A_helveola*	**95.3**	**94.0**	**94.3**	**95.2**	**95.3**	**95.5**	94.9	94.9	95.0	94.6	94.6	94.6	95.1	94.7	94.7	96.9	---	17/1475	91/1452	111/1454
41. *A_taiwanensis*	**95.3**	**94.2**	**94.5**	**95.3**	**95.3**	**95.6**	95.1	95.1	95.1	95.0	94.8	94.8	94.9	94.3	95.0	97.0	98.	---	94/1450	113/1466
42. *A_orientalis*	**94.0**	**93.4**	**93.8**	**93.9**	**94.0**	**94.3**	94.4	94.1	94.3	94.5	94.5	94.4	93.5	92.8	94.0	92.4	93.73	93.5	---	97/1452
43. *Actinokineospora riparia*	**93.7**	**92.6**	**92.9**	**93.6**	**93.8**	**94.0**	93.66	93.53	93.25	93.68	93.39	93.41	92.85	92.62	92.97	92.43	92.37	92.29	93.32	---

Strain codes, as given in Figure 3.2.

Table 3 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between 16 strains isolated from the extreme hyper-arid Yungay environmental sample and the type strains of closely related *Blastococcus, Geodermatophilus* and *Modestobacter* species.

Isolate	1	2	3	4	5	6	7	8	9	10	11	12
1. Isolate KNN45-3	---	**12/1389**	**0/1405**	**0/1405**	**3/1405**	**3/1405**	**1/1405**	**14/1394**	**3/1398**	**12/1396**	**12/1396**	**7/1401**
2. Isolate KNN46-3	**99.1**	---	**12/1389**	**12/1389**	**14/1389**	**15/1389**	**13/1389**	**1/1381**	**13/1383**	**0/1388**	**0/1388**	**17/1386**
3. Isolate KNN46-6	**100**	**99.1**	---	**6/1419**	**3/1410**	**3/1411**	**1/1421**	**14/1403**	**3/1398**	**12/1401**	**12/1401**	**8/1404**
4. Isolate KNN46-10	**100**	**99.1**	**99.6**	---	**3/1411**	**3/1412**	**7/1420**	**14/1401**	**3/1398**	**12/1402**	**12/1402**	**8/1402**
5. Isolate KNN46-9	**99.8**	**99**	**99.8**	**99.8**	---	**4/1411**	**4/1411**	**17/1399**	**3/1398**	**14/1402**	**14/1402**	**4/1401**
6. Isolate KNN46-8	**99.8**	**98.9**	**99.8**	**99.8**	**99.7**	---	**2/1412**	**15/1400**	**4/1398**	**15/1402**	**15/1402**	**7/1402**
7. Isolate KNN46-5	**99.9**	**99.1**	**99.9**	**99.5**	**99.7**	**99.9**	---	**13/1403**	**2/1398**	**13/1402**	**13/1402**	**7/1404**
8. Isolate KNN45-4	**99**	**99.9**	**99**	**99**	**98.8**	**98.9**	**99.1**	---	**13/1387**	**3/1393**	**3/1393**	**18/1394**
9. Isolate KNN46-4	**99.8**	**99.1**	**99.8**	**99.8**	**99.8**	**99.7**	**99.9**	**99.1**	---	**14/1390**	**14/1390**	**3/1397**
10. Isolate KNN45-2	**99**	**100**	**99.1**	**99.1**	**99.0**	**98.9**	**99.1**	**99.8**	**99**	---	**0/1403**	**18/1393**
11. Isolate KNN45-1	**99**	**100**	**99.1**	**99.1**	**99.0**	**98.9**	**99.1**	**99.8**	**99**	**100**	---	**18/1393**
12. Isolate KNN46-2	**99.5**	**98.8**	**99.4**	**99.4**	**99.7**	**99.5**	**99.5**	**98.7**	**99.8**	**98.7**	**98.7**	---
13. *M. versicolor*	99	98.5	99.1	99.1	99.0	98.9	99.1	98.3	99.0	98.5	98.5	98.7
14. *M. marinus*	98.8	99.6	98.4	98.8	98.6	98.6	98.3	99.4	98.6	99.6	99.6	98.2
15. *M. roseus*	98.3	99.1	97.9	98.3	98.1	98.1	97.8	98.9	98.1	99.1	99.1	97.7
16. *M. multiseptatus*	98.6	98.1	98.2	98.6	98.4	98.4	98.1	97.9	98.4	98.1	98.1	98.0
17. Isolate KNN44-1	**96.5**	**96.4**	**96.5**	**96.5**	**96.6**	**96.6**	**96.5**	**96.4**	**96.5**	**96.4**	**96.4**	**96.7**
18. Isolate KNN44-3	**96**	**95.8**	**95.6**	**96**	**95.8**	**95.8**	**95.5**	**95.7**	**95.8**	**95.9**	**95.9**	**95.5**
19. Isolate KNN44-4	**96.3**	**96.1**	**96.3**	**96.3**	**96.3**	**96.3**	**96.3**	**96.1**	**96.3**	**96.1**	**96.1**	**96.3**
20. *G. normandii*	95.6	96.1	95.2	95.6	95.4	95.4	95.1	96.0	95.4	96.1	96.1	95.0
21. *G. solani*	94.9	94.6	94.9	94.9	94.8	94.7	94.9	94.5	94.9	94.6	94.6	94.7
22. *G. ruber*	95.2	95.2	94.9	95.3	95.0	95.0	94.8	95.1	95.1	95.3	95.3	94.6
23. *G. telluris*	94.2	94.7	93.9	94.3	94.0	94.0	93.8	94.7	94.1	94.8	94.8	93.6
24. *G. obscurus*	96	96	95.6	96.1	95.8	95.8	95.7	95.9	95.8	96.1	96.1	95.4
25. *G. terrae*	95.6	95.3	95.5	95.5	95.4	95.4	95.5	95.2	95.8	95.2	95.2	95.6
26. *G. saharensis*	95.9	96.5	95.6	96	95.7	95.7	95.5	96.4	95.8	96.5	96.5	95.4
27. *G. siccatus*	95.8	95.7	95.4	95.9	95.6	95.6	95.4	957	95.6	95.8	95.8	95.2
28. *G. arenarius*	95.6	95.2	95.2	95.6	95.4	95.4	95.1	95.0	95.4	95.2	95.2	95.0
29. *G. nigrescens*	95.7	95.4	95.3	95.8	95.5	95.5	95.3	95.3	95.5	95.4	95.4	95.1
30. *G. taihuensis*	93.9	93.9	93.5	93.4	93.6	93.5	93.3	93.1	93.9	93.6	93.6	93.3
31. Isolate KNN47	**96.6**	**96.3**	**96.3**	**96.7**	**96.4**	**96.4**	**96.2**	**96.1**	**96.4**	**96.4**	**96.4**	**96.1**
32. *B. jejuensis*	96.7	96.5	96.7	96.7	96.6	96.5	96.6	96.3	96.6	96.6	96.6	96.3
33. *B. saxobsidens*	96.6	96.3	96.3	96.7	96.4	96.4	96.2	96.1	96.5	96.4	96.4	96.1
34. *B. endophyticus*	96.7	96.3	96.3	96.6	96.4	96.5	96.2	96.2	96.5	96.4	96.4	96.1
35. *B. aggregatus*	96	95.9	96	96	95.9	95.8	95.9	95.5	96	95.8	95.8	95.6

Strain codes, as given in Figure 3.3.

Table 3 (continue)

Isolate	13	14	15	16	17	18	19	20	21	22	23	24
1. Isolate KNN45-3	**12/1393**	**17/1402**	**24/1400**	**20/1401**	**45/1298**	**56/1398**	**49/1322**	**62/1399**	**70/1358**	**67/1401**	**81/1401**	**56/1401**
2. Isolate KNN46-3	**21/1385**	**5/1387**	**12/1385**	**26/1385**	**47/1298**	**58/1383**	**51/1322**	**54/1384**	**74/1358**	**66/1386**	**73/1386**	**55/1386**
3. Isolate KNN46-6	**12/1398**	**23/1418**	**30/1416**	**26/1417**	**45/1298**	**62/1413**	**49/1322**	**68/1415**	**70/1358**	**73/1417**	**87/1417**	**62/1417**
4. Isolate KNN46-10	**12/1400**	**17/1423**	**24/1416**	**20/1422**	**45/1298**	**57/1418**	**49/1322**	**62/1420**	**70/1358**	**67/1422**	**81/1422**	**56/1422**
5. Isolate KNN46-9	**14/1399**	**20/1408**	**27/1406**	**23/1407**	**44/1298**	**59/1404**	**49/1322**	**65/1405**	**71/1358**	**70/1407**	**84/1407**	**59/1407**
6. Isolate KNN46-8	**15/1399**	**20/1409**	**27/1407**	**23/1408**	**44/1298**	**59/1404**	**49/1322**	**65/1406**	**72/1358**	**70/1408**	**84/1408**	**59/1408**
7. Isolate KNN46-5	**13/1399**	**24/1419**	**31/1417**	**27/1418**	**45/1298**	**63/1414**	**49/1322**	**69/1416**	**70/1358**	**74/1418**	**88/1418**	**63/1418**
8. Isolate KNN45-4	**24/1389**	**8/1402**	**15/1400**	**29/1400**	**47/1291**	**60/1397**	**51/1315**	**56/1399**	**74/1350**	**69/1401**	**75/1401**	**58/1401**
9. Isolate KNN46-4	**14/1387**	**20/1395**	**27/1393**	**22/1394**	**45/1298**	**59/1392**	**49/1322**	**64/1392**	**70/1358**	**69/1394**	**83/1394**	**58/1394**
10. Isolate KNN45-2	**21/1398**	**5/1401**	**12/1399**	**26/1399**	**47/1297**	**58/1397**	**51/1321**	**54/1398**	**74/1357**	**66/1400**	**73/1400**	**55/1400**
11. Isolate KNN45-1	**21/1398**	**5/1401**	**12/1399**	**26/1399**	**47/1297**	**58/1397**	**51/1321**	**54/1398**	**74/1357**	**66/1400**	**73/1400**	**55/1400**
12. Isolate KNN46-2	**18/1390**	**25/1401**	**32/1399**	**28/1400**	**43/1298**	**63/1396**	**49/1322**	**70/1398**	**72/1358**	**75/1400**	**89/1400**	**64/1400**
13. *M. versicolor*	---	25/1446	23/1432	26/1408	51/1298	66/1397	55/1320	64/1400	72/1356	76/1410	84/1442	66/1443
14. *M. marinus*	98.3	---	11/1451	29/1439	47/1298	62/1424	50/1322	55/1428	75/1358	72/1444	73/1476	56/1477
15. *M. roseus*	98.4	99.2	---	38/1426	44/1298	59/1415	48/1320	46/1419	71/1356	66/1429	66/1448	54/1448
16. *M. multiseptatus*	98.2	98	97.3	---	45/1298	61/1422	48/1320	68/1428	70/1356	70/1435	86/1436	55/1436
17. Isolate KNN44-1	**96.1**	**96.4**	**96.6**	**96.5**	**---**	**4/1300**	**1/1288**	**31/1297**	**39/1296**	**25/1300**	**43/1299**	**13/1300**
18. Isolate KNN44-3	**95.3**	**95.7**	**95.8**	**95.7**	**99.7**	**---**	**1/1324**	**39/1423**	**49/1356**	**31/1428**	**55/1427**	**20/1428**
19. Isolate KNN44-4	**95.8**	**96.2**	**96.4**	**96.4**	**99.9**	**99.9**	**---**	**31/1319**	**44/1320**	**24/1324**	**46/1323**	**13/1324**
20. *G. normandii*	95.4	96.2	96.8	95.2	97.6	97.3	97.7	---	60/1357	46/1429	30/1431	30/1429
21. *G. solani*	94.7	94.5	94.8	94.8	97	96.4	96.8	95.6	---	59/1358	69/1359	53/1358
22. *G. ruber*	94.6	95	95.4	95.1	98.1	97.8	98.2	96.8	95.7	---	66/1447	35/1448
23. *G. telluris*	94.2	95	95.4	94	97	96.2	96.5	97.9	94.9	95.4	---	46/1479
24. *G. obscurus*	95.4	96.2	96.3	96.2	99.0	98.6	99.0	97.9	96.1	97.6	96.9	---
25. *G. terrae*	94.9	95	95	95.4	96.8	96.2	96.5	95.3	97.7	95.3	94.1	95.9
26. *G. saharensis*	95.7	96.9	97	95.7	97.6	97	97.7	98.5	95.3	96.3	97.4	97.7
27. *G. siccatus*	95.2	96	96	96.1	98.8	98.5	98.9	97.8	96.2	97.9	96.7	99.1
28. *G. arenarius*	95.5	95.5	95.9	95.3	97.5	96.8	97.3	97.7	95.4	96.3	97.6	97.6
29. *G. nigrescens*	95.6	95.6	96	95.2	97.2	96.9	97.3	98.6	95.2	96.3	97.1	97.6
30. *G. taihuensis*	92.6	91.8	92.1	93.1	96.9	95	96.5	94.3	99.1	93.9	92.6	93.8
31. Isolate KNN47	**96.3**	**96.2**	**96.6**	**96.2**	**96.7**	**96.3**	**96.6**	**96.6**	**96**	**95.9**	**95.7**	**96.5**
32. *B. jejuensis*	96.5	96.6	96.6	96.7	97.8	97.5	97.8	96.1	95.5	96.9	94.8	97.4
33. *B. saxobsidens*	96.1	96.4	96.5	96.3	96.8	96.6	96.8	96.9	95.7	96	95.5	97.3
34. *B. endophyticus*	96.4	96.2	96.9	95.7	97.3	96.9	97.2	96.6	96.6	96.5	95.6	96.7
35. *B. aggregatus*	96	95.8	96.1	95.9	96.7	95.9	96.4	95.1	95.7	95.8	94.4	96

Strain codes, as given in Figure 3.3.

Table 3 (continue)

Isolate	25	26	27	28	29	30	31	32	33	34	35
1. Isolate KNN45-3	**59/1353**	**57/1402**	**59/1401**	**62/1402**	**60/1398**	**85/1397**	**47/1398**	**46/1392**	**47/1399**	**47/1401**	**56/1392**
2. Isolate KNN46-3	**63/1353**	**49/1387**	**59/1386**	**67/1387**	**64/1383**	**84/1382**	**51/1383**	**48/1384**	**51/1384**	**51/1386**	**57/1383**
3. Isolate KNN46-6	**61/1355**	**63/1418**	**65/1417**	**68/1418**	**66/1414**	**92/1408**	**53/1413**	**46/1397**	**53/1415**	**53/1417**	**56/1397**
4. Isolate KNN46-10	**61/1355**	**57/1423**	**59/1422**	**62/1423**	**60/1419**	**93/1408**	**47/1416**	**46/1399**	**47/1420**	**49/1420**	**56/1399**
5. Isolate KNN46-9	**63/1355**	**60/1408**	**62/1407**	**65/1408**	**63/1404**	**90/1403**	**50/1403**	**48/1398**	**50/1405**	**50/1406**	**58/1398**
6. Isolate KNN46-8	**63/1355**	**60/1409**	**62/1408**	**65/1409**	**63/1405**	**92/1404**	**50/1403**	**49/1398**	**50/1406**	**50/1407**	**59/1398**
7. Isolate KNN46-5	**61/1355**	**64/1419**	**66/1418**	**69/1419**	**67/1415**	**94/1409**	**54/1413**	**47/1398**	**54/1416**	**54/1417**	**57/1398**
8. Isolate KNN45-4	**65/1348**	**51/1402**	**61/1401**	**70/1402**	**66/1398**	**97/1397**	**54/1397**	**51/1389**	**54/1399**	**54/1401**	**62/1389**
9. Isolate KNN46-4	**57/1347**	**59/1395**	**61/1394**	**64/1395**	**62/1391**	**85/1390**	**50/1392**	**47/1386**	**49/1392**	**49/1394**	**56/1386**
10. Isolate KNN45-2	**65/1354**	**49/1401**	**59/1400**	**67/1401**	**64/1397**	**90/1396**	**51/1396**	**48/1398**	**51/1398**	**51/1399**	**59/1397**
11. Isolate KNN45-1	**65/1354**	**49/1401**	**59/1400**	**67/1401**	**64/1397**	**90/1396**	**51/1396**	**48/1398**	**51/1398**	**51/1399**	**59/1397**
12. Isolate KNN46-2	**60/1350**	**65/1401**	**67/1400**	**70/1401**	**68/1397**	**93/1396**	**54/1396**	**52/1389**	**55/1398**	**55/1400**	**61/1389**
13. *M. versicolor*	69/1354	62/1440	67/1400	65/1440	64/1442	105/1424	51/1394	49/1401	55/1426	50/1395	58/1437
14. *M. marinus*	68/1354	46/1470	58/1434	67/1474	65/1470	118/1440	54/1422	48/1404	53/1460	55/1429	61/1441
15. *M. roseus*	68/1354	42/1449	57/1419	59/1449	58/1447	114/1438	48/1412	48/1402	51/1445	44/1414	56/1430
16. *M. multiseptatus*	62/1354	61/1433	56/1432	68/1437	69/1431	98/1416	54/1420	46/1402	53/1436	62/1427	58/1405
17. Isolate KNN44-1	**42/1295**	**31/1297**	**16/1300**	**33/1300**	**36/1296**	**40/1296**	**43/1298**	**29/1298**	**42/1298**	**35/1298**	**43/1298**
18. Isolate KNN44-3	**52/1350**	**43/1424**	**22/1428**	**46/1428**	**44/1422**	**70/1410**	**52/1419**	**35/1400**	**49/1424**	**44/1421**	**58/1400**
19. Isolate KNN44-4	**46/1316**	**31/1320**	**15/1324**	**36/1324**	**36/1318**	**46/1320**	**45/1322**	**29/1322**	**42/1322**	**37/1322**	**48/1321**
20. *G. normandii*	64/1353	21/1434	31/1429	33/1430	20/1434	81/1413	49/1419	55/1400	45/1427	49/1428	69/1400
21. *G. solani*	31/1343	64/1358	52/1358	63/1358	65/1356	12/1359	55/1358	61/1358	59/1358	46/1358	59/1357
22. *G. ruber*	64/1353	54/1440	30/1438	53/1448	53/1438	87/1424	59/1422	44/1403	58/1444	50/1431	60/1413
23. *G. telluris*	80/1353	38/1471	48/1437	36/1477	43/1472	106/1441	61/1421	73/1403	65/1459	63/1430	80/1440
24. *G. obscurus*	56/1353	34/1469	13/1438	35/1477	35/1471	90/1440	50/1422	37/1404	40/1460	47/1431	58/1441
25. *G. terrae*	---	60/1354	55/1353	71/1353	63/1352	41/1352	62/1351	65/1352	60/1352	59/1353	64/1351
26. *G. saharensis*	95.6	---	35/1430	36/1470	30/1472	102/1440	55/1420	57/1402	48/1454	57/1429	72/1439
27. *G. siccatus*	95.9	97.6	---	37/1438	33/1428	75/1414	50/1422	40/1403	43/1434	47/1431	58/1403
28. *G. arenarius*	94.8	97.6	97.4	---	24/1468	100/1440	45/1422	60/1404	51/1460	48/1431	63/1441
29. *G. nigrescens*	95.3	98.0	97.7	98.4	---	103/1438	50/1418	60/1400	46/1452	52/1427	68/1437
30. *G. taihuensis*	97.0	92.9	94.7	93.1	92.8	---	76/1408	71/1402	101/1440	68/1409	95/1427
31. Isolate KNN47	**95.4**	**96.1**	**96.5**	**96.8**	**96.5**	**94.6**	---	**32/1397**	**14/1422**	**25/1422**	**30/1397**
32. *B. jejuensis*	95.2	95.9	97.2	95.7	95.7	94.9	97.7	---	28/1404	39/1398	35/1403
33. *B. saxobsidens*	95.6	96.7	97.0	96.5	96.8	93.0	99.0	98.0	---	33/1429	37/1429
34. *B. endophyticus*	95.6	96.0	96.7	96.7	96.4	95.2	98.2	97.2	97.7	---	33/1398
35. *B. aggregatus*	95.3	95.0	95.9	95.6	95.3	93.3	97.9	97.5	97.4	97.6	---

Strain codes, as given in Figure 3.3.

Table 4. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains C59, KNN26b, KNN38-1b, KNN42f and KNN64-5b isolated from the Atacama Desert environmental samples and between them and the type strains of closely related *Streptomyces* species.

Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. Isolate C59	---	**1/ 1356**	**0/ 1356**	**34/ 1353**	**15/ 1355**	**0/ 1356**	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
2. Isolate KNN13a	**99.9**	---	**1/ 1356**	**35/ 1353**	**16/ 1355**	**1/ 1356**	**16/ 1352**	**17/ 1353**	**17/ 1353**	**19/ 1352**	**16/ 1352**	**36/ 1350**
3. Isolate KNN26b	**100.0**	**99.9**	---	**34/ 1353**	**15/ 1355**	**0/ 1356**	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
4. Isolate KNN38-1b	**97.5**	**97.4**	**97.5**	---	**25/ 1352**	**34/ 1353**	**40/ 1352**	**41/ 1353**	**41/ 1353**	**43/ 1352**	**46/ 1352**	**64/ 1347**
5. Isolate KNN64-5b	**98.9**	**98.8**	**98.9**	**98.2**	---	**15/ 1355**	**30/ 1351**	**31/ 1352**	**31/ 1352**	**33/ 1351**	**30/ 1351**	**50/ 1349**
6. *S. fimbriatus*	**100.0**	**99.9**	**100.0**	**97.5**	**98.9**	---	**15/ 1352**	**16/ 1353**	**16/ 1353**	**18/ 1352**	**15/ 1352**	**35/ 1350**
7. *S. werraensis*	98.9	98.8	98.9	97.0	97.8	98.9	---	19/ 1352	19/ 1352	11/ 1352	10/ 1352	41/ 1346
8. *S. griseostramineus*	98.8	98.7	98.8	97.0	97.7	98.8	98.6	---	0/ 1353	14/ 1352	21/ 1352	37/ 1347
9. *S. griseomycini*	98.8	98.7	98.8	97.0	97.7	98.8	98.6	100.0	---	14/ 1352	21/ 1352	37/ 1347
10. *S. viridiviolaceus*	98.7	98.6	98.7	96.8	97.6	98.7	99.2	99.0	99.0	---	15/ 1351	41/ 1346
11. *S. caelestis*	98.9	98.8	98.9	96.6	97.8	98.9	99.3	98.5	98.5	98.9	---	41/ 1346

Strain codes, as given in Figure 6.5

Table 5. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains C34, C38, C58, KNN2-6c, KNN6-6b, KNN6-9a, KNN9-3b, KNN10-4d, KNN10-5a, KNN11-1a, KNN11-5a, KNN24-1b, KNN25c, KNN41-1b and KNN48-1c isolated from the Atacama Desert environmental sample and between them and the type strains of closely related *Streptomyces* species.

Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. Isolate C38	---	**1/1362**	**1/1370**	**1/1362**	**4/1365**	**1/1331**	**3/1334**	**0/1324**	**0/1353**	**3/1330**	**0/1313**	**2/1372**	**1/1362**
2. Isolate C58	**99.9**	---	**0/1362**	**1/1352**	**4/1355**	**1/1321**	**4/1325**	**0/1314**	**0/1343**	**3/1320**	**0/1303**	**3/1362**	**1/1352**
3. Isolate C79	**99.9**	**100.0**	---	**1/1360**	**4/1363**	**1/1329**	**3/1332**	**0/1322**	**0/1351**	**3/1328**	**0/1311**	**3/1370**	**1/1360**
4. Isolate KNN1-5a	**99.9**	**99.9**	**99.9**	---	**2/1362**	**2/1331**	**4/1334**	**1/1324**	**1/1353**	**2/1330**	**1/1313**	**1/1362**	**0/1362**
5. Isolate KNN2-6a	**99.7**	**99.7**	**99.7**	**99.9**	---	**2/1331**	**4/1334**	**1/1324**	**1/1353**	**4/1330**	**1/1313**	**3/1365**	**2/1362**
6. Isolate KNN6-6b	**99.9**	**99.9**	**99.9**	**99.9**	**99.9**	---	**4/1331**	**1/1324**	**1/1331**	**4/1330**	**1/1313**	**1/1331**	**2/1331**
7. Isolate KNN6-9a	**99.8**	**99.7**	**99.8**	**99.7**	**99.7**	**99.7**	---	**3/1324**	**3/1334**	**6/1330**	**3/1313**	**3/1334**	**4/1334**
8. Isolate KNN10-4d	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	---	**0/1324**	**1/1324**	**0/1312**	**0/1324**	**1/1324**
9. Isolate KNN10-5a	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	---	**3/1330**	**0/1313**	**0/1353**	**1/1353**
10. Isolate KNN11-1a	**99.8**	**99.8**	**99.8**	**99.9**	**99.7**	**99.7**	**99.6**	**99.9**	**99.8**	---	**1/1313**	**3/1330**	**2/1330**
11. Isolate KNN24-1b	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	**100.0**	**99.9**	---	**0/1313**	**1/1313**
12. Isolate KNN25c	**99.9**	**99.8**	**99.8**	**99.9**	**99.8**	**99.9**	**99.8**	**100.0**	**100.0**	**99.8**	**100.0**	---	**1/1362**
13. Isolate KNN41-1c	**99.9**	**99.9**	**99.9**	**100.0**	**99.9**	**99.9**	**99.7**	**99.9**	**99.9**	**99.9**	**99.9**	**99.9**	---
14. Isolate KNN48-1c	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**99.9**	**99.8**	**100.0**	**100.0**	**99.8**	**100.0**	**100.0**	**99.9**
15. C34	**99.7**	**99.9**	**99.8**	**99.7**	**99.6**	**99.7**	**99.5**	**99.8**	**99.8**	**99.6**	**99.8**	**99.6**	**99.7**
16. *S. mexicanus*	98.9	99.0	99.0	98.9	98.8	98.9	98.7	98.9	99.0	98.7	98.9	98.8	98.9
17. *S. hyderabadensis*	98.4	98.5	98.5	98.4	98.2	98.3	98.2	98.4	98.5	98.2	98.4	98.3	98.4
18. *S. parvulus*	98.8	99.0	98.9	99.0	98.6	98.8	98.7	98.9	98.9	98.8	98.9	98.7	99.0
19. *S. lusitanus*	98.9	99.0	99.0	98.9	98.7	98.9	98.7	98.9	99.0	98.7	98.9	98.8	98.9
20. *S. speibonae*	98.8	99.0	98.9	98.8	98.6	98.8	98.7	98.9	98.9	98.7	98.9	98.7	98.8
21. *S. chiangmaiensis*	98.7	98.9	98.8	98.7	98.6	98.7	98.5	98.7	98.7	98.5	98.7	98.5	98.7
22. *S. coerulescens*	98.7	98.8	98.8	98.8	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.5	98.8
23. *S. althioticus*	98.8	98.9	98.8	98.9	98.5	98.7	98.6	98.8	98.8	98.7	98.8	98.6	98.9
24. *S. matensis*	98.7	98.8	98.8	98.8	98.5	98.6	98.5	98.7	98.7	98.6	98.7	98.5	98.8
25. *S. variabilis*	98.7	98.8	98.8	98.8	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.5	98.8
26. *S. albus* subsp. *albus*	96.9	96.8	96.8	96.8	96.8	96.8	96.6	96.8	96.9	96.6	96.8	96.7	96.8

Strain codes, as given in Figure 6.1

Table 5. (2)

Isolates	14	15	16	17	18	19	20	21	22	23	24	25
1. Isolate C38	**0/1335**	**4/1372**	**15/1367**	**22/1370**	**16/1371**	**15/1371**	**16/1371**	**18/1369**	**18/1371**	**17/1364**	**18/1364**	**18/1371**
2. Isolate C58	**0/1325**	**2/1363**	**14/1357**	**20/1360**	**14/1361**	**13/1361**	**14/1361**	**15/1359**	**16/1361**	**15/1354**	**16/1354**	**17/1361**
3. Isolate C79	**0/1333**	**3/1370**	**14/1365**	**21/1368**	**15/1369**	**14/1369**	**15/1369**	**17/1367**	**17/1369**	**16/1362**	**17/1362**	**17/1369**
4. Isolate KNN1-5a	**1/1335**	**4/1362**	**15/1357**	**22/1360**	**14/1361**	**15/1361**	**16/1361**	**18/1359**	**16/1361**	**15/1354**	**16/1354**	**16/1361**
5. Isolate KNN2-6a	**1/1335**	**5/1365**	**16/1360**	**25/1363**	**19/1364**	**18/1364**	**19/1364**	**19/1362**	**21/1364**	**20/1357**	**21/1357**	**21/1364**
6. Isolate KNN6-6b	**1/1331**	**4/1331**	**15/1326**	**22/1329**	**16/1330**	**15/1330**	**16/1330**	**18/1330**	**18/1330**	**17/1323**	**18/1323**	**18/1330**
7. Isolate KNN6-9a	**3/1334**	**7/1335**	**17/1329**	**24/1332**	**18/1333**	**17/1333**	**18/1333**	**20/1333**	**20/1333**	**19/1326**	**20/1326**	**20/1333**
8. Isolate KNN10-4d	**0/1324**	**3/1324**	**14/1319**	**21/1322**	**15/1323**	**14/1323**	**15/1323**	**17/1323**	**17/1323**	**16/1316**	**17/1316**	**17/1323**
9. Isolate KNN10-5a	**0/1335**	**3/1353**	**14/1348**	**21/1351**	**15/1352**	**14/1352**	**15/1352**	**17/1351**	**17/1352**	**16/1345**	**17/1345**	**17/1352**
10. Isolate KNN11-1a	**3/1330**	**6/1330**	**17/1325**	**24/1328**	**16/1329**	**17/1329**	**18/1329**	**20/1329**	**18/1329**	**17/1322**	**18/1322**	**18/1329**
11. Isolate KNN24-1b	**0/1313**	**3/1313**	**14/1308**	**21/1311**	**15/1312**	**14/1312**	**15/1312**	**17/1312**	**17/1312**	**16/1305**	**17/1305**	**17/1312**
12. Isolate KNN25c	**0/1335**	**6/1372**	**17/1367**	**24/1370**	**18/1371**	**17/1371**	**18/1371**	**20/1369**	**20/1371**	**19/1364**	**20/1364**	**20/1371**
13. Isolate KNN41-1c	**1/1335**	**4/1362**	**15/1357**	**22/1360**	**14/1361**	**15/1361**	**16/1361**	**18/1359**	**16/1361**	**15/1354**	**16/1354**	**16/1361**
14. Isolate KNN48-1c	**---**	**3/1335**	**14/1330**	**21/1333**	**15/1334**	**14/1334**	**15/1334**	**17/1334**	**17/1334**	**16/1327**	**17/1327**	**17/1334**
15. *S. leeuwenhoekii*	**99.8**	**---**	15/1367	23/1370	17/1371	16/1371	17/1371	16/1369	19/1371	18/1364	19/1364	20/1371
16. *S. mexicanus*	99.0	98.9	---	23/1365	19/1366	21/1366	18/1366	15/1364	19/1366	23/1359	24/1360	24/1366
17. *S. hyderabadensis*	98.4	98.3	98.3	---	15/1371	24/1371	25/1371	26/1368	24/1371	27/1364	27/1364	32/1371
18. *S. parvulus*	98.9	98.8	98.6	98.9	---	14/1372	19/1372	18/1369	12/1372	15/1365	16/1365	22/1372
19. *S. lusitanus*	99.0	98.8	98.5	98.3	99.0	---	14/1372	21/1369	8/1372	9/1365	9/1365	17/1372
20. *S. speibonae*	98.9	98.8	98.7	98.2	98.6	99.0	---	19/1369	15/1372	15/1365	16/1365	15/1372
21. *S. chiangmaiensis*	98.7	98.8	98.9	98.1	98.7	98.5	98.6	---	20/1369	22/1362	23/1362	26/1369
22. *S. coerulescens*	98.7	98.6	98.6	98.3	99.1	99.4	98.9	98.5	---	12/1365	12/1365	14/1372
23. *S. althioticus*	98.8	98.7	98.3	98.0	98.9	99.3	98.9	98.4	99.1	---	0/1364	4/1365
24. *S. matensis*	98.7	98.6	98.2	98.0	98.8	99.3	98.8	98.3	99.1	100.0	---	5/1365
25. *S. variabilis*	98.7	98.5	98.2	97.7	98.4	98.8	98.9	98.1	99.0	99.7	99.6	---
26. *S. albus* subsp. *albus*	96.8	96.8	96.7	96.3	96.2	96.5	96.4	96.3	96.3	96.9	96.8	96.7

Strain codes, as given in Figure 6.1.

Table 6. Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between strains KNN6-11a, KNN35-1b, KNN35-2b, KNN42f, KNN48-3b, KNN48-6d and KNN83e isolated from the Atacama Desert environmental sample and between them and the type strains of closely related *Streptomyces* species.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Isolate KNN35-1b	---	**1/1382**	**2/1373**	**2/1370**	**2/1382**	**2/1381**	**2/1382**	**7/1382**	**7/1381**	**8/1382**	**9/1382**	**10/1382**
2. Isolate KNN35-2b	**99.9**	---	**2/1373**	**1/1371**	**1/1385**	**1/1382**	**1/1383**	**6/1391**	**6/1390**	**7/1391**	**8/1391**	**9/1391**
3. Isolate KNN42f	**99.9**	**99.9**	---	**1/1363**	**1/1373**	**1/1373**	**1/1373**	**6/1373**	**6/1372**	**7/1373**	**8/1373**	**9/1373**
4. Isolate KNN48-3b	**99.9**	**99.9**	**99.9**	---	**0/1388**	**0/1407**	**0/1407**	**9/1403**	**5/1392**	**6/1398**	**7/1403**	**8/1389**
5. Isolate KNN48-6d	**99.9**	**99.9**	**99.9**	**100.0**	---	**0/1399**	**0/1400**	**6/1402**	**5/1401**	**6/1402**	**7/1402**	**8/1402**
6. Isolate KNN6-11a	**99.9**	**99.9**	**99.9**	**100.0**	**100.0**	---	**0/1418**	**9/1414**	**5/1403**	**6/1409**	**7/1414**	**8/1400**
7. Isolate KNN83e	**99.9**	**99.9**	**99.9**	**100.0**	**100.0**	**100.0**	---	**9/1415**	**5/1404**	**6/1410**	**7/1415**	**8/1401**
8. *S. pseudogriseolus*	99.5	99.6	99.6	99.4	99.6	99.4	99.4	---	1/1452	2/1458	14/1462	10/1448
9. *S. gancidicus*	99.5	99.6	99.6	99.6	99.6	99.6	99.6	99.9	---	1/1452	10/1451	9/1447
10. *S. capillispiralis*	99.4	99.5	99.5	99.6	99.6	99.6	99.6	99.9	99.9	---	11/1457	8/1448
11. *S. carpinensis*	99.4	99.4	99.4	99.5	99.5	99.5	99.5	99.0	99.3	99.3	---	7/1448
12. *S. levis*	99.3	99.4	99.3	99.4	99.4	99.4	99.4	99.3	99.4	99.5	99.5	---

Strain codes, as given in Figure 6.6.

Appendix 5. Figures

Figure 1. Sampling site at the Chaxa de Laguna,Salar de Atacama, Atacama Desert, Chile.

Figure 2. Sampling site in the region of the Yungay, Atacama Desert Chile

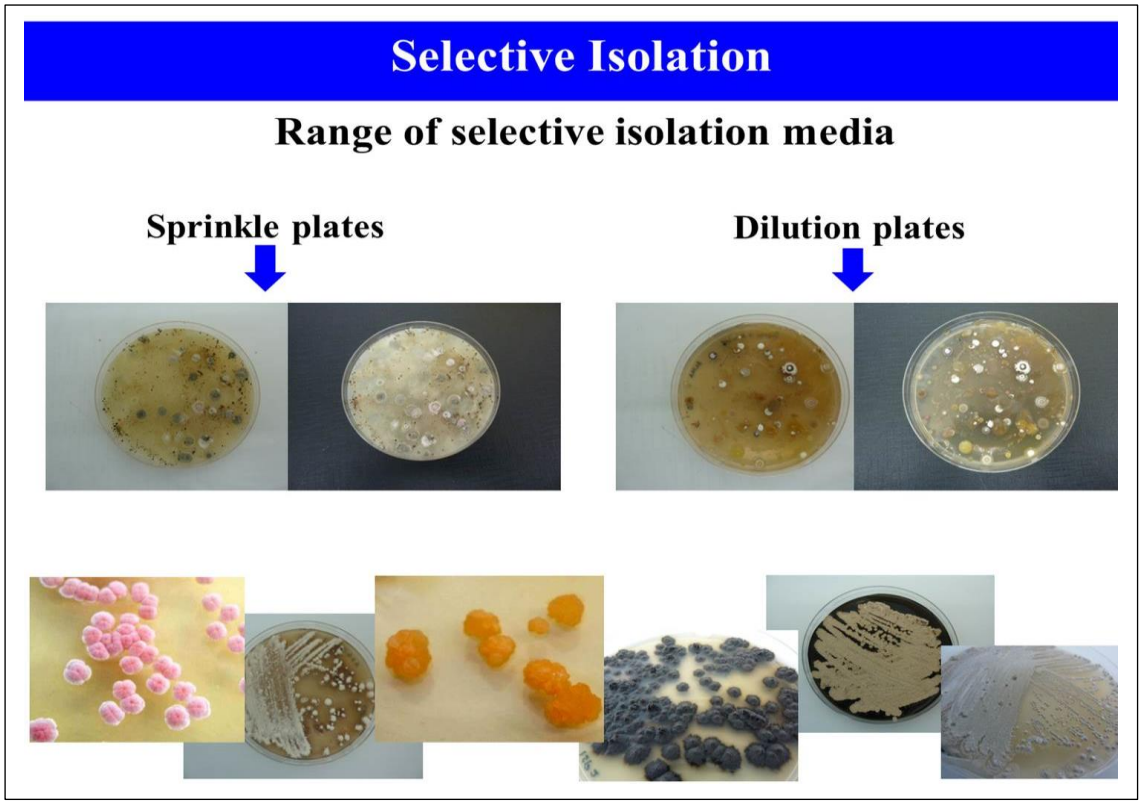

Figure 3. Selective isolation of filamentous actinobacteria from soil samples.

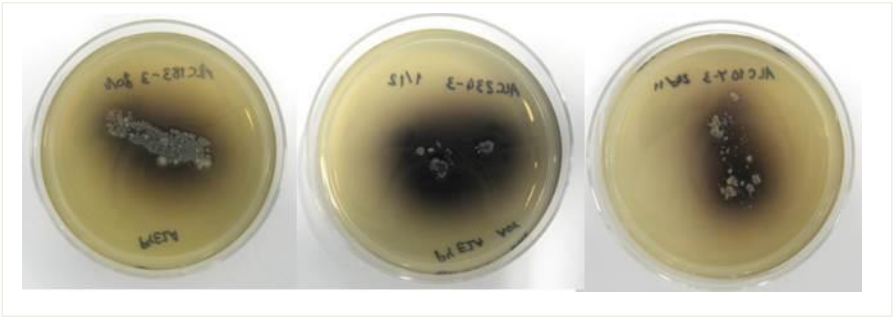

Figure 4. Production of melanin pigment on peptone yeast-extract-iron agar (ISP6 medium)

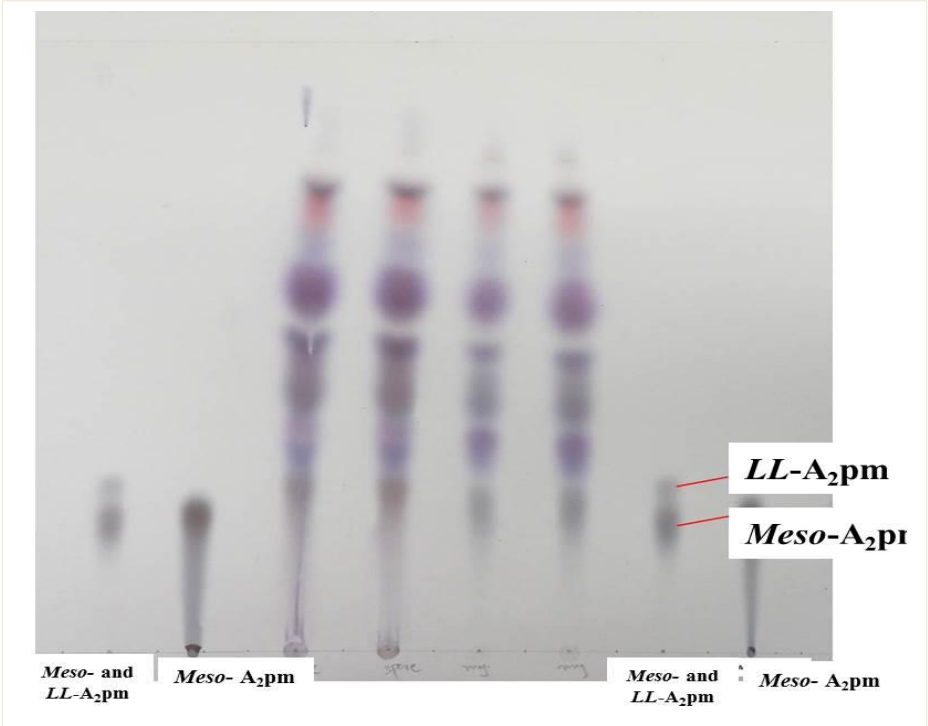

Figure 5. One dimensional thin layer chromatography to detect isomer of diaminopimelic analysis.

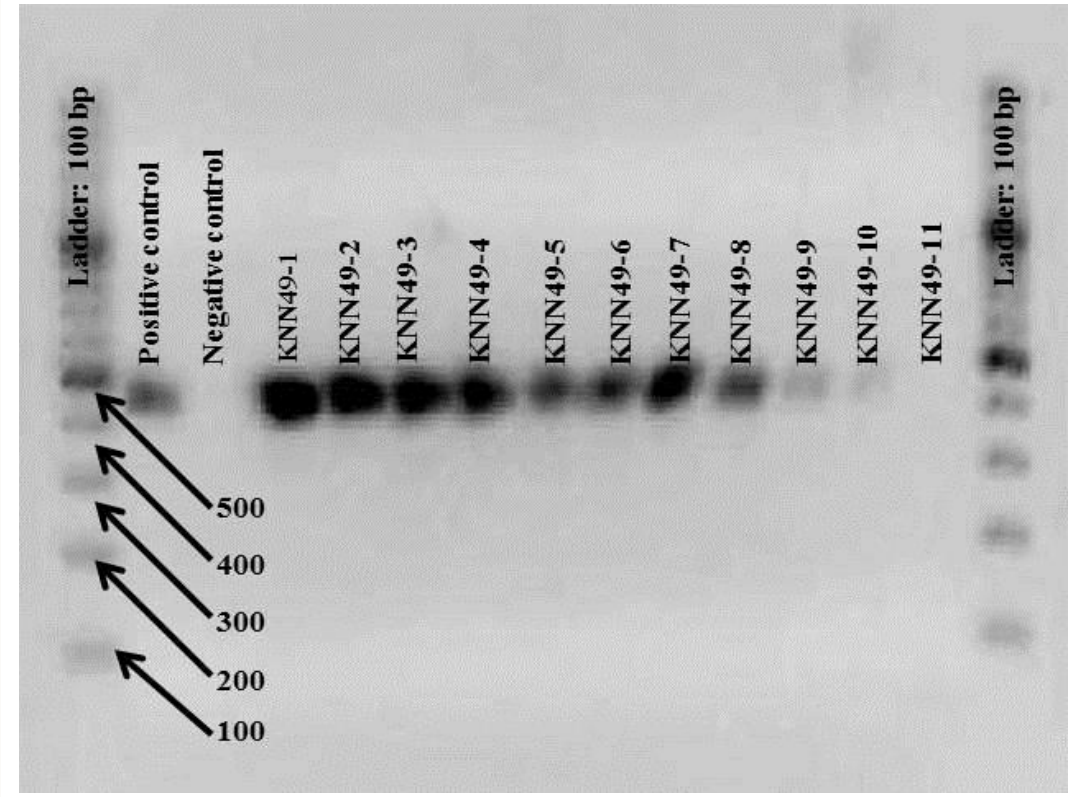

Figure 6. The primer pair AMY2 and ATOP were used to amplify a 435 bp product that is characteristic of members of the genus *Amycolatopsis*.

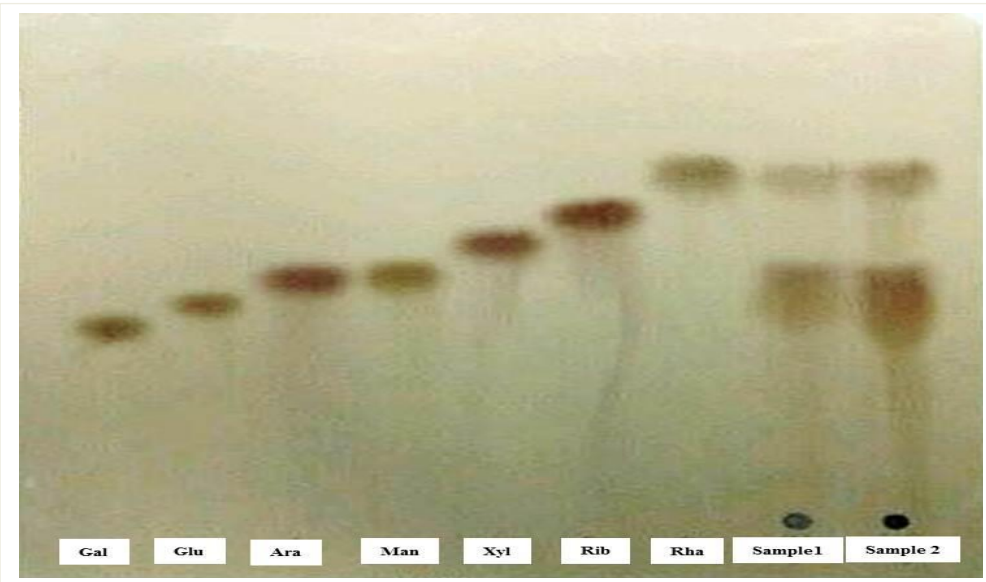

Figure 7. One dimensional thin layer chromatography to detect diagraphic whole-cell sugar analysis.

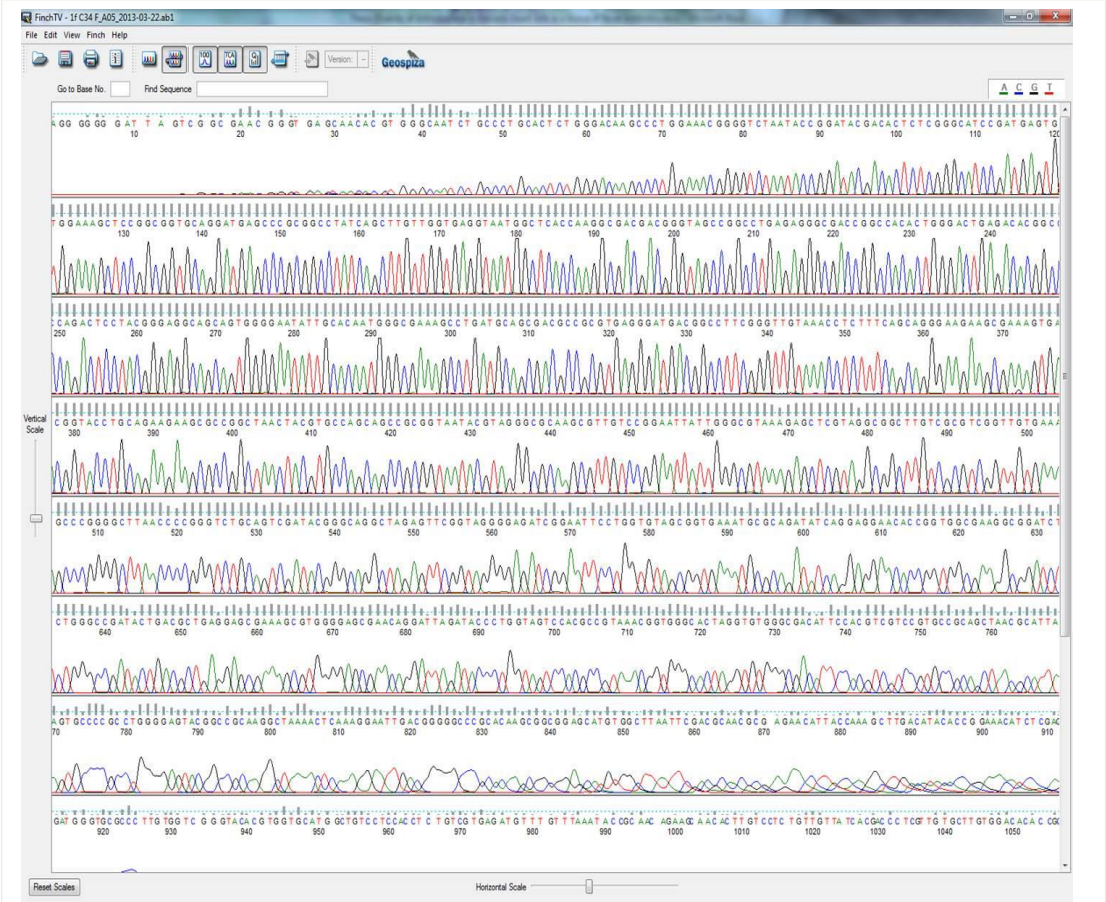

Figure 8. ABI format-chromatogram of *Streptomyces leeuwenhoekii* C34^{T}.

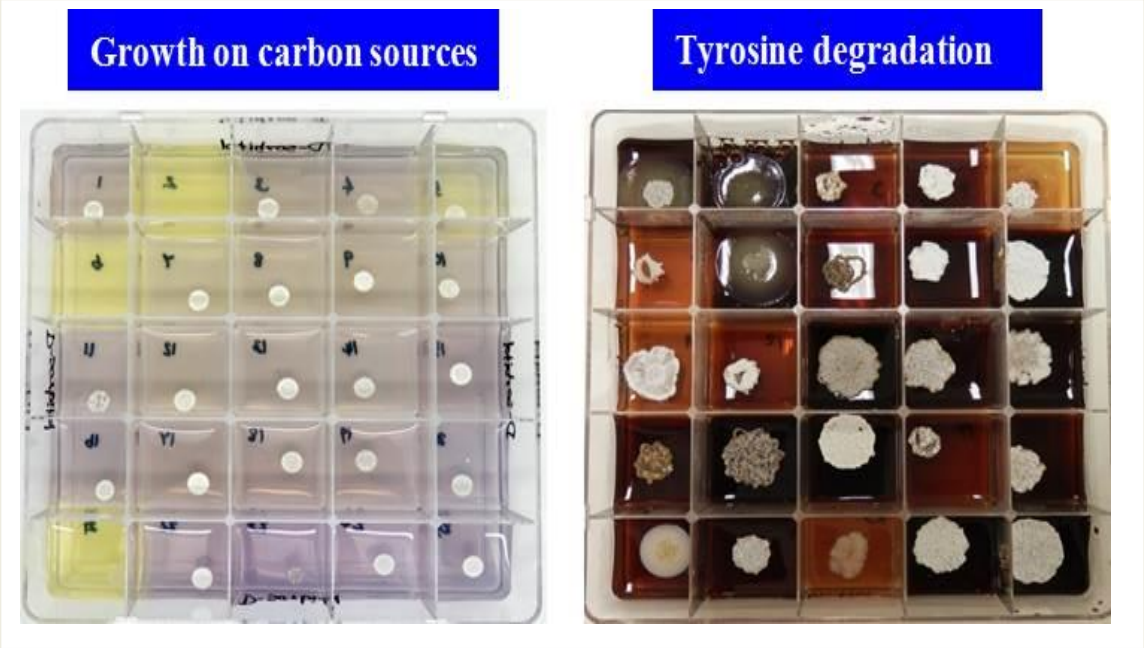

Figure 9. growth of isolates on sole carbon sources and degradation of tyrosine after incubated at 28^{o}C for 7 days

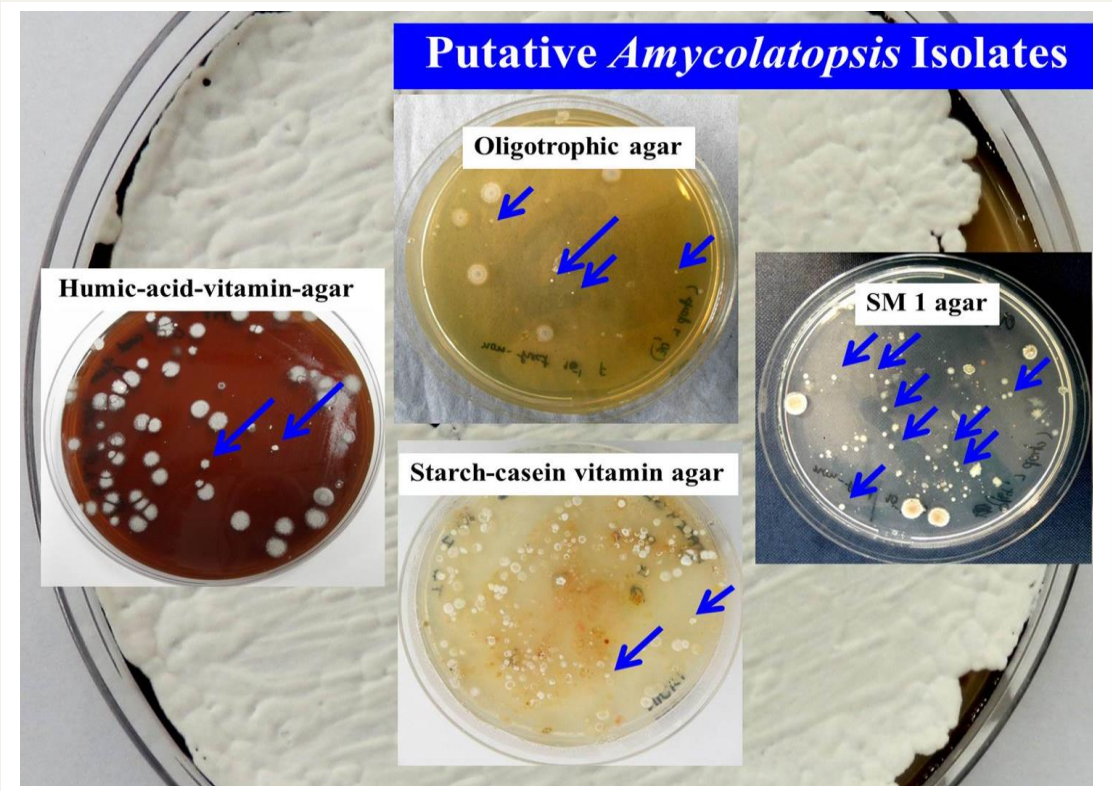

Figure 10. *Amycolatopsis* colonies growing on selective characters on isolation media after incubation at 28^{o}C for 14 days.

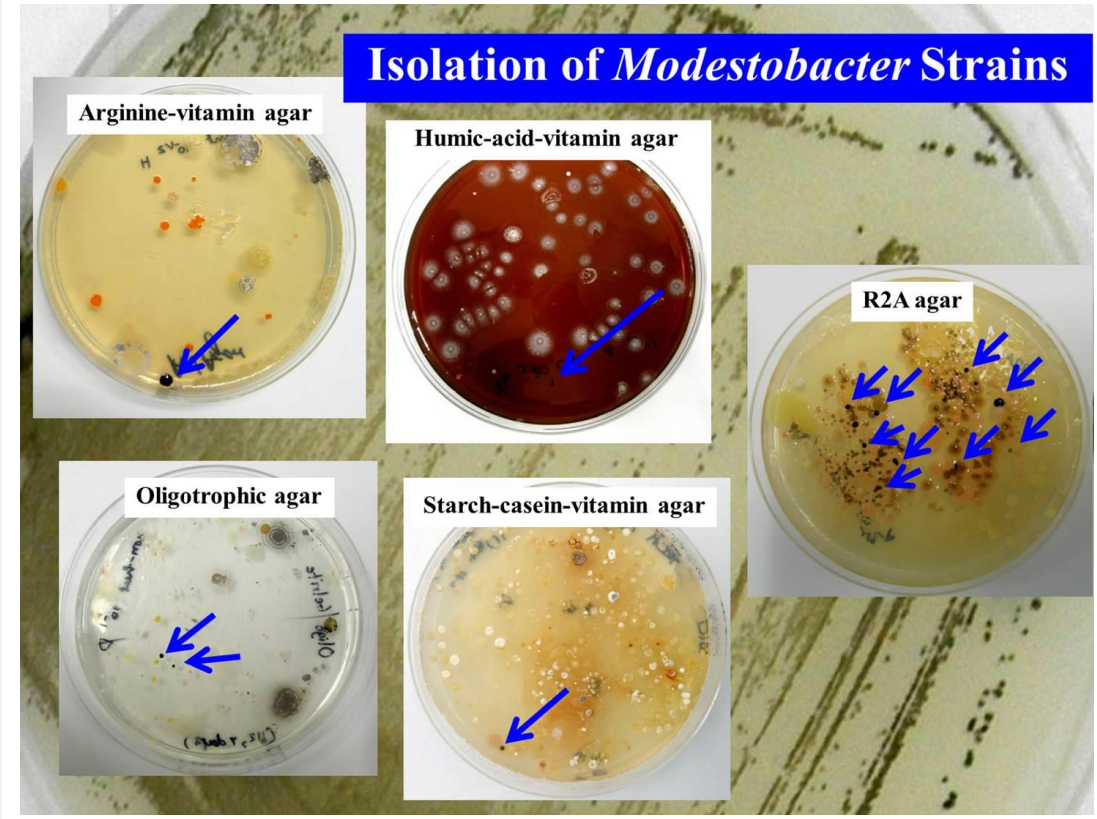

Figure 11. *Modestobacer* colonies growing on selective isolation media after incubation at 28°C for 14 days.

Figure 12. *Streptomyces* colonies growing on selective isolation media after incubated at 28°C for 14 days.