Actinobacterial Diversity in Atacama Desert Habitats as a Road Map to Biodiscovery

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Abstract

The Atacama Desert of Northern Chile, the oldest and driest nonpolar desert on the planet, is known to harbour previously undiscovered actinobacterial taxa with the capacity to synthesize novel natural products. In the present study, culture-dependent and culture-independent methods were used to further our understanding of the extent of actinobacterial diversity in Atacama Desert habitats. The culture-dependent studies focused on the selective isolation, screening and dereplication of actinobacteria from high altitude soils from Cerro Chajnantor. Several strains, notably isolates designated H9 and H45, were found to produce new specialized metabolites. Isolate H45 synthesized six novel metabolites, lentzeosides A-F, some of which inhibited HIV-1 integrase activity. Polyphasic taxonomic studies on isolates H45 and H9 showed that they represented new species of the genera *Lentzea* and *Streptomyces,* respectively; it is proposed that these strains be designated as *Lentzea chajnantorensis* sp. nov. and *Streptomyces aridus* sp. nov.. Additional isolates from sampling sites on Cerro Chajnantor were considered to be nuclei of novel species of *Actinomadura, Amycolatopsis, Cryptosporangium* and *Pseudonocardia.*

A majority of the isolates produced bioactive compounds that inhibited the growth of one or more strains from a panel of six wild type microorganisms while those screened against *Bacillus subtilis* reporter strains inhibited sporulation and cell envelope, cell wall, DNA and fatty acid synthesis.

Initial culture-independent studies were carried out to establish the extent of actinobacterial diversity in a range of hyper- and extreme hyper-arid Atacama Desert soils. Community DNA extracted from soil collected from the sampling sites was surveyed for actinobacteria by 454 pyrosequencing; rarefaction analyses indicated good coverage at most of the sites. The results revealed an amazing and unexpected taxonomic diversity at the ranks of order, family and genus, much of it novel. The total number of genera, for instance, is 328, of which around 40% could not be assigned to validly published genera. Rank abundancy profiles indicated that much of this diversity can be attributed to low abundancy taxa. Similar results were obtained from community DNA extracted from surface and subsurface soil samples collected at three different altitudes on Cerro Chajnantor. Actinobacterial community structure at these sampling sites was influenced by altitude and sampling depth, as well as several environmental variables that included conductivity, pH, redox potential and organic matter content.

It is evident from these studies that the Atacama Desert landscape abounds in novel actinobacterial taxa that synthesize a broad range of specialized metabolites that can be developed as drug leads.

Author's Declaration

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

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Publications Related to the Thesis

Publication:

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R. Brown, **H. Idris**, I. Singleton, A.T. Bull and M. Goodfellow (2014). Microbial life at high altitude: Actinobacterial diversity of surface and subsurface environmental samples from the Atacama Desert. *XVII International Symposium on the Biology of Actinomycetes*. Kusadasi, Turkey.

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Chapter 1. General Introduction

1.1 Background

Seventy years ago Sir Alexander Fleming, who discovered penicillin, warned in his Nobel Prize acceptance speech that the misuse of antibiotics would lead to the emergence of drug resistant microbial pathogens (Fleming 1945). Fleming's prediction has come to pass as antibiotic-resistant microbial pathogens now threaten the foundations of modern medicine. Routine medical procedures such as heart bypass surgery, cancer therapy, trauma surgery and intensive care treatments depend on the effective use of antibiotics. Indeed, some patients, such as those with cystic fibrosis, require regular drug therapy for this and many other infections by antibiotic-resistant pathogens that can be fatal.

The selective pressures on microorganisms led to the emergence and spread of multiple drug resistant (MDR) pathogens, as exemplified by the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species) all of which are now resistant to most antibiotics (Payne et al. 2007; Boucher et al. 2009; Genilloud 2014). MDR Gram-negative bacteria, in particular, are frequently associated with high mortality rates and a lack of treatment options for hospital acquired infections (Silver 2011).This problem extends to foodborne diseases caused by *Clostridium difficile, Escherichia coli* and *Salmonella* species and to pathogens like *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* (Wright 2012). It seems somewhat counterintuitive that the number of therapeutic drug leads is in sharp decline given the prospect of a return to the pre-antibiotic days of medicine (Talbot et al. 2006; Spellberg et al. 2008; Donadio et al. 2010; Butler and Cooper 2011; Genilloud 2014).

Microbial natural products remain the most promising source of new therapeutic drugs (Baltz 2007, 2008; Cragg and Newman 2013). This is partly because alternative approaches, such as target-based screening of chemical libraries have been found wanting, but also because most antibiotics are produced by microbes that have been evolving for around a billion years hence their fitness has been tested by an ability to pass through outer membranes and inhibit target enzymes, micromolecules and macromolecular structures (Baltz 2008). It is now evident that natural product libraries encompass much greater structural diversity than chemical libraries derived from combinatorial chemistry (Dobson 2004; Drees et al. 2004). More recently vast amounts of genomic data generated from whole-genome sequencing projects can be exploited in

the search for novel drug leads (Corre and Challis 2009; Walsh and Fischbach 2010; Winter et al. 2011).

The ability of microorganisms to synthesize natural products is unevenly distributed across the microbial world. Amongst prokaryotes, organisms classified in the phylum *Actinobacteria* (Goodfellow 2012b) are a rich source of bioactive compounds (Hopwood 2007; Newman and Cragg 2012). Indeed, about 75% of antibiotics are produced by filamentous bacteria belonging to the class *Actinobacteria* (Goodfellow 2012a), though most are made by members of a single taxon, the genus *Streptomyces* (Bérdy 2012; Demain 2014). The genomes of filamentous actinobacteria, unlike those of most prokaryotes, are rich in biosynthetic gene clusters that code for known or predicted specialized metabolites (Goodfellow and Fiedler 2010; Tang et al. 2015), a development that renewed interest in these organisms as a source of new antibiotics (Baltz 2007, 2008; Donadio et al. 2010; Gomez-Escribano and Bibb 2014). Culture-independent surveys make it clear that a vast array of taxonomically novel actinobacteria are present in natural ecosystems (Das et al. 2007; Yang et al. 2012; Serkebaeva et al. 2013; Prieto-Davó et al. 2013), an unseen majority potentially available for bioprospecting campaigns (Whitman et al. 1998; Bull 2004; Genilloud 2014).

Despite the developments outlined above it has become increasingly difficult to discover novel drug leads from filamentous actinobacteria isolated from well trawled terrestrial habitats as screening such organisms leads to the costly rediscovery of known compounds (Baltz 2007; Busti et al. 2006; Genilloud 2014). This problem can be addressed by the application of new search and discovery strategies, as exemplified by the taxonomic approach to bioprospecting recommended by Goodfellow and Fiedler (2010). The key steps involved in this culture-dependent strategy are shown in Figure 1.1. The first step in the antibiotic pipeline involves the choice of environmental samples followed by the selective isolation and recognition of putatively novel filamentous actinobacteria. Subsequent steps include the detection of interesting bioactive compounds from dereplicated strain libraries, primary screening of fermentation broths and mycelial extracts using chemical procedures such as HPLC diode array screening. The final steps are focused on the detection of putatively novel specialized metabolites and their structural elucidation. The premise underlying this taxonomic approach to bioprospecting is that extreme environmental conditions give rise to a unique actinobacterial diversity which is the basis of novel chemistry (Bull and Stach 2007; Bull 2011; Goodfellow et al. 2013).

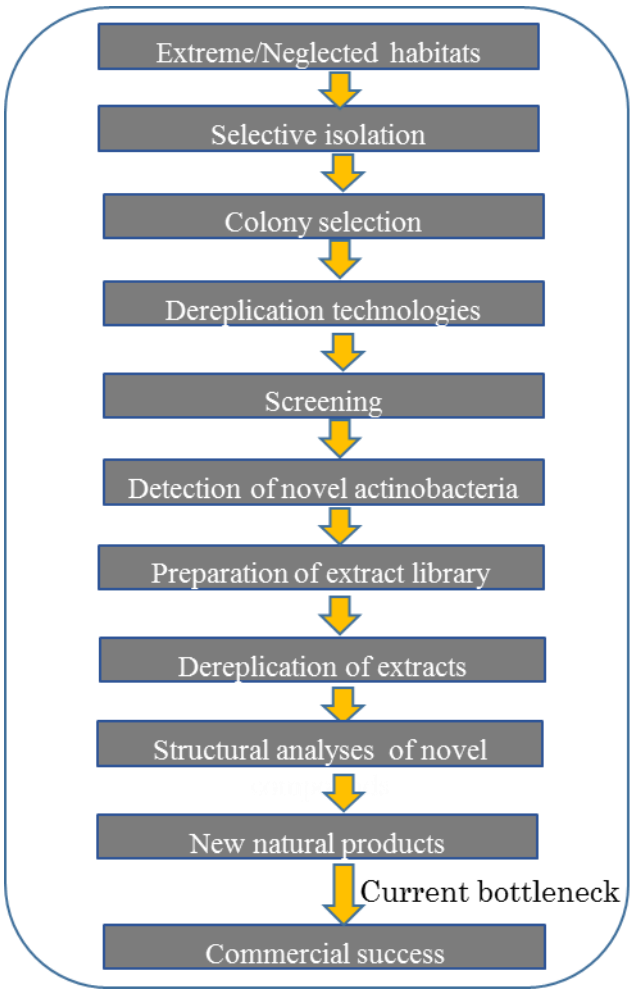

Figure 1.1 Culture-dependent bioprospecting strategy (modified from Goodfellow & Fiedler, 2010).

In practice, novel actinobacteria, especially streptomycetes, have been isolated from marine habitats using the strategy outlined in Figure 1.1 and representatives of dereplicated taxa shown to produce novel antibiotics with unique modes of action (Bull et al. 2005; Fiedler et al. 2005; Goodfellow and Fiedler 2010). Novel actinobacteria from deep sea sediments are a particularly good source of new specialized metabolites, as illustrated by the discovery of a new family of polyketides, the abyssomycins, from *Verrucosispora maris* AB-18-032^T (Riedlinger et al. 2004; Goodfellow et al. 2012b), the anticancer drug, salinosporamide from *Salinispora tropica* CNB-440^T (Jensen et al. 2007; Fenical et al. 2009) and the dermacozines, from *Dermacoccus abyssi* MT1.1^T and MT 1.2, non-filamentous, piezzotolerant strains isolated from the Challenger Deep of the Mariana Trench (Pathom-Aree et al. 2006a; Abdel-Mageed et al. 2010; Wagner et al. 2014). Strong support for culture-dependent approaches to bioprospecting come from extensive surveys of the obligate marine genus *Salinispora* (Jensen et al. 2005; Freel et al. 2012; Ahmed et al. 2013), notably from representative strains which show clear

evidence of coupling between taxonomic and chemical diversity (Jensen 2010; Ziemert et al. 2014).

More recently, the taxonomic approach to the discovery of new specialized metabolites from novel actinobacteria was extended to another extreme biome, the temperate Atacama Desert, the oldest and driest desert on Earth which is located in Northern Chile (Bull and Asenjo 2013; Bull et al. 2016). In initial studies, small but taxonomically diverse populations of presumptively novel filamentous actinobacteria were isolated from hyper-arid and extreme hyper-arid soils collected from the Salar de Atacama and Yungay Core regions of the desert, respectively (Okoro et al. 2009; Busarakam 2014). Subsequently, several new species of *Lechevalieria* and *Streptomyces* were validly published (Okoro et al. 2010; Santhanam et al. 2012b; 2012a; 2013), notably *Streptomyces leeuwenhoekii* (Busarakam et al. 2014), the type strain of this species produces new polyketide antibiotics, the chaxalactins (Rateb et al. 2011b) and chaxamycins (Rateb et al. 2011a) while additional members synthesize 22-membered macrolactone antibiotics, the atacamycins (Nachtigall et al. 2011; Elsayed et al. 2015) and chaxapeptin, a new lasso peptide (Elsayed et al. 2015). Rateb et al. (2013) reported the induction of biosynthetic pathways in an *Aspergillus fumigatus* strain when grown in the presence of *Streptomyces bullii* $C2^T$ leading to the isolation of ergosterol, seven metabolites belonging to the diketopiperazine class of alkaloids and two metabolites from the rare class of pseurotins.

1.2 Objectives and content of thesis

This project was designed to build upon and extend pioneering work on Atacama Desert actinobacteria carried out by Okoro et al. (2009) and Busarakam (2014), the results of which have been considered within the broader context of Atacama Desert microbiology (Bull and Asenjo 2013; Bull et al. 2016). A biphasic approach was adopted in this investigation as environmental samples collected from a range of Atacama Desert habitats were examined using culture-dependent and culture-independent procedures, as outlined in Figure 1.2. The culture-independent studies were intended to establish the extent of actinobacteria diversity in Atacama Desert habitats with an emphasis on determining and explaining actinobacterial community structure in soil samples collected at altitudes between *ca*. 3000 and 5000 m. up to the Chajnantor Plateau at the Atacama Large Millimeter Array (ALMA) Observation site. The culture-dependent studies were focused on the isolation, dereplication and screening of actinobacteria isolated from the ALMA

samples and from extreme hyper-arid soil collected from Lomas Bayas, possibly the driest area in the Atacama Desert. Selected isolates from these and earlier studies were the subject of polyphasic taxonomic analyses while extracts of judiciously chosen isolates showing bioactivity in plug assays were screened for novel chemistry by Professor Marcel Jaspars.

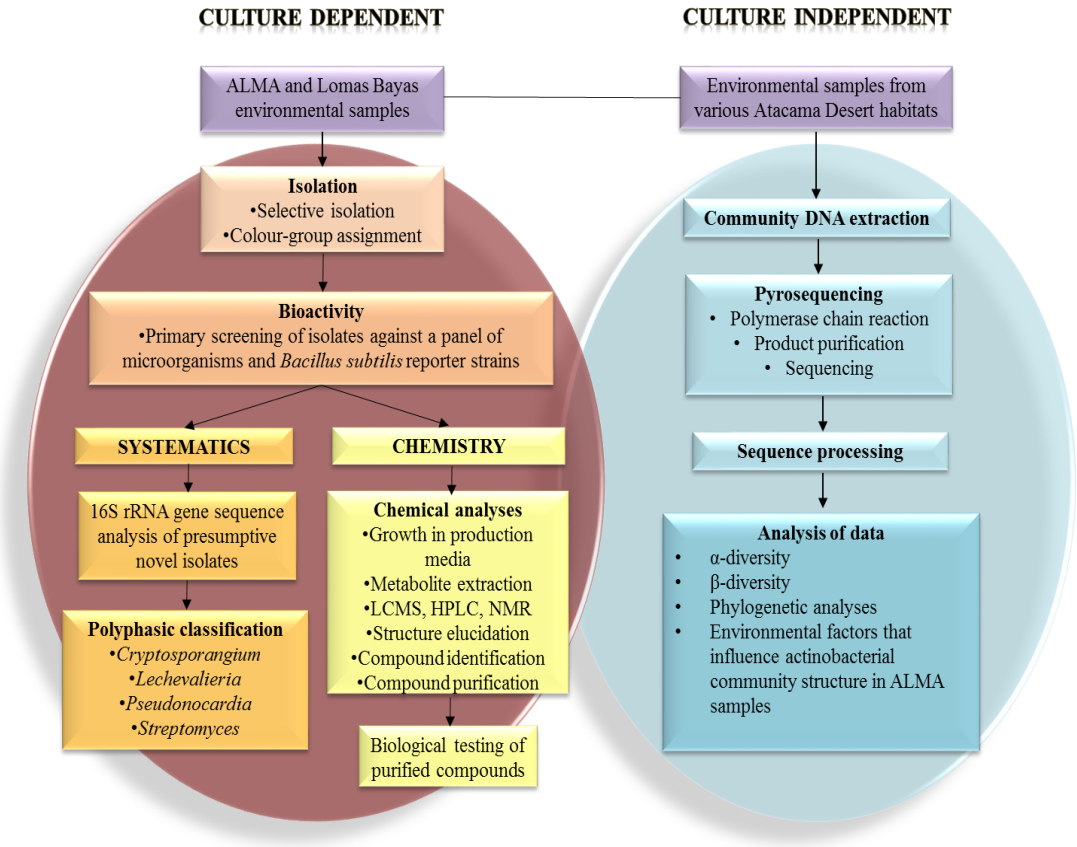

Figure 1.2 The biphasic strategy used to determine actinobacterial diversity in Atacama Desert environmental samples.

Outline of key contents included in each chapters of this thesis:

1. General Introduction
    - Background
    - Objectives and content of thesis
    - Biodiversity, bioprospecting and biogeography
    - Unexplored actinobacterial diversity as a taxonomic road map to drug discovery
    - Novel actinobacteria from desert ecosystems as a source of new drug leads
    - Culture-independent approaches to determine the extent of actinobacterial diversity in natural habitats

2. Materials and Methods
    - Sampling locations
    - Selective isolation of actinobacteria from ALMA and extreme hyper-arid Lomas Bayas environmental samples
    - Selection, maintenance and colour-group assignment
    - Comparative 16S rRNA gene sequencing studies
    - Classification of presumptively novel actinobacteria
    - Screening for bioactivity
    - Culture-independent study of actinobacterial diversity in Atacama Desert environmental samples

3. Biosystematic studies on actinobacterial strains isolated from the Cerro Chajnantor and Lomas Bayas environmental samples
    - Selective isolation of actinobacteria from the Cerro Chajnantor and Lomas Bayas environmental samples
    - Dereplication of presumptive actinobacteria
    - Screening of isolates for bioactivity
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- Classification of putatively novel *Streptomyces* isolates

5. New specialized metabolites synthesized by novel actinobacteria isolated from soils of the Chajnantor Plateau
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    - Actinobacterial community composition
    - Actinobacterial community variation between ALMA environmental samples

8. Major outcomes and perspectives for future work

1.3 Biodiversity, bioprospecting and biogeography

Biological diversity or biodiversity includes *genetic diversity*, the variation of genes and genomes within a species (intraspecific diversity); *species diversity*, the number of species within a community, and *ecological diversity*, the number of communities in an ecosystem (Bull 2004; Harper and Hawksworth 1995). For most practical purposes the basic unit used in biodiversity studies is the species (Heywood and Baste 1995; Duelli and Obrist 2003). Microbial diversity deals with studies of archaea, bacteria, fungi, microalgae and protozoa. The simplest measure of diversity is species or α-diversity, that is the number of species within a community or habitat, in turn, β-diversity is a measure of between area diversity and γ-diversity is the extent of within-area diversity at biome

and bioregional levels (Whittaker 1972). Other ecological measures include *species abundance*, the number of individuals per species (McGill et al. 2007) and *functional diversity*, the activity of organisms in communities and ecosystems (Petchey and Gaston 2006).

Biodiversity underpins biotechnology as new products and processes depend upon the discovery, evolution and exploitation of organisms (Bull et al. 1992; Bull et al. 2000; Bull et al. 2016). The number of published prokaryotic species, which currently stands at around 13,000, is very small compared to the number of animal and plant species, somewhat less in comparison to the number of fungi and protozoa (Mora et al. 2011). Establishing the total number of prokaryotic species remains subjective and is dependent upon the matrix used to define species (Yarza et al. 2014). Yarza and his colleagues estimated the total number of prokaryotic species to be around 4×10^5, a value much lower than an earlier estimate of 2×10^6 (Curtis et al. 2002) which was just for those present in oceanic waters. These values seem low as there is evidence that all microbiomes seem to harbour significant numbers of unique prokaryotic taxa, a recent example for the human microbiome identified more than 60 prospective new species (Browne et al. 2016). It seems plausible, therefore, that each of the >1 million species of animals will support at least one novel prokaryotic species, not to mention those associated with the quarter of a million plant species.

It is now common knowledge that the vast majority of prokaryotes in nature are either uncultivable or remain to be cultivated (Staley and Konopka 1985; Torsvik et al. 2002; Ward 1998; Amann et al. 1995; Rappé and Giovannoni 2003; Oren 2004) hence an astonishing genetic and metabolic diversity is available for bioprospecting, a term introduced to cover the systematic search in nature for organisms that produce novel bioactive molecules. Such studies are increasingly focused on the isolation and screening of extremophilic and extremotolerant microorganisms isolated from biomes under extreme environmental pressures (Bull 2011; Bull et al. 2016).

Recent culture-independent data derived from 16S rRNA gene sequencing surveys show that prokaryotic diversity can be divided into two categories (de Pascale et al. 2012). On one hand, there are abundant phylotypes which form a major fraction of the total 16S rRNA gene pool, but a tiny fraction of all of the phylotypes. In contrast, there is an overwhelming number of low abundance phylotypes, the "rare biosphere" (Sogin et al. 2006; Bent and Forney 2008; Pedrós-Alió 2012; Lynch and Neufeld 2015) or "dark matter" (Rinke et al. 2013; Hedlund et al. 2014), which, despite their diversity, constitute a minor fraction of the total ribosomal gene pool. The species richness of "rare

biospheres" has still to be unravelled (Kirchman et al. 2010; Will et al. 2010; Bartram et al. 2011; Yang et al. 2012), as is its significance for bioprospecting campaigns.

Although only a tiny fraction of actinobacterial diversity from natural habitats has been cultivated and screened for bioactivity it is clear that filamentous actinobacteria are an invaluable source of medically useful antibiotics, as exemplified by the synthesis of erythromycin from *Saccharopolyspora erythraea* NRRL 4338^{T} (Oliynyk et al. 2007); gentamicin from "*Micromonospora purpurea"* NRRL 2953 (Weinstein et al. 1963; Wagman 1980), which is now a synonym of *Micmonospora echinospora* NBRC 13149^{T} (Kasai et al. 2000); teichoplanin from *Actinoplanes teichomyceticus* AB 8327^{T} (Somma et al. 1984)and vancomycin from *Amycolatopsis orientalis* NRRL 2430^{T} (Lechevalier et al. 1986; Pittenger and Brigham 1956)

Filamentous actinobacteria, especially streptomycetes, are also unusual as they are able to synthesize many bioactive metabolites, as exemplified in Table 1.1. Since chemical diversity often follows biological diversity it can be anticipated that screening novel actinobacteria for bioactivity will lead to a continuing source of new antibiotics, especially since whole-genome sequences of representatives of diverse taxa, such as the genera *Amycolatopsis, Salinispora, Saccharopolyspora, Saccharothrix* and *Streptomyces* have shown that they are rich in biosynthetic gene clusters that code for the production of unknown compounds that are expected to be bioactive (Bentley et al. 2002; Oliynyk et al. 2007; Udwary et al. 2011) (Strobel et al. 2012; Tang et al. 2015). Consequently, it can be expected that the taxonomic approach to drug discovery (Figure 1.1) will lead to the detection of new antibiotic drug leads. In addition, understanding how such organisms adapt to harsh environmental conditions, as shown by analyses of the genome of *Modestobacter caceserii* KNN 45-2b^{T}, a strain isolated from an extreme hyper-arid Atacama Desert soil, is an important challenge (Busarakam et al. 2016a).

Biogeography, the study of the global distribution of species across space and time, is important for understanding biodiversity, notably for establishing whether prokaryotic species are found in more than one geographical area or are restricted to a defined area, in other words whether they are cosmopolitan or endemic species (Staley and Gosink 1999; Ramette and Tiedje 2007). This subject clearly has implications for the taxonomic approach to the discovery of natural products from actinobacteria. The obligate marine genus *Salinispora* has proved to be an interesting model by which to study bacterial biogeography (Jensen et al. 2007; Freel et al. 2012; Jensen and Mafnas 2006). In wide ranging genetical and phylogenetic studies evidence was found for a cosmopolitan distribution of *Salinispora arenicola* and regional endemism for the two

remaining species, *Salinispora pacifica* and *Salinispora tropica*. The co-occurance of *S. arenicola* with the other two *Salinispora* species was seen to be evidence of ecological differentiation while comparatively high levels of intraspecific diversity were apparent in *S. pacifica*. Antony-Babu et al. (2008) provided genetic and phenotypic evidence for *Streptomyces griseus* ecovars across a beach and dune sand system thereby showing that endemism can occur within an actinobacterial species where spore dispersal is not constrained. Coupling between taxonomic and chemical diversity may occur at the level of ecovars as well as at higher taxonomic ranks (Ward and Goodfellow 2004).

Table 1.1 Approximate number of bioactive microbial metabolites in periods from 1940 to 2010 according to their producers.

Periods	*1940–1974 Early years*	*%*	*1975–2000 Mid-era*	*%*	*2001–2010 New age*	*%*	*Total*
Species							
Actinobacteria	3400	62	7200	42	3100	28.5	13700
Streptomyces Ssp.	2900		5100		2400		10400
Other actinobacteria	500		2100		700		3300
All microscopic bacteria	800	15	2300	13	1100	10	4200
Myxobacteriales	25		400		210		635
Cyanobacteria	10		30		1250!		1290!
All Fungi	1300	23	7700	45	6600	61	15600
Microscopic fungi	950		5400		4900		11250
Basidiomycetes	300		1800		1500		3600
Other fungi	20		200		160		380!
Total per year	5500/180		17000/690		10800/1100		33500

Taken from Bérdy (2012).

1.4 Unexplored actinobacterial diversity as a taxonomic roadmap to drug discovery

1.4.1 Habitat selection

The first step in taxonomic approaches to bioprospecting involves the selection of the ecosystems to be sampled. Innumerable actinobacteria have been isolated and screened for bioactivity since the momentous discovery that *Streptomyces griseus* produced streptomycin (Schatz et al. 1944), the first antibiotic to be used for anti-tuberculosis therapy. Early bioprospecting campaigns built upon this discovery as they were focused mainly on the isolation and screening of *Streptomyces* strains from a range of soil types. Initially, this pragmatic approach to bioprospecting led to the discovery of many new antibiotics, including ones of therapeutic value, such as chloramphenicol from *Streptomyces venezuelae* ATCC 10712^{T} (Ehrlich et al. 1948) and neomycin from *Streptomyces fradiae* ATCC 10745^{T} (Waksman and Lechevalier 1949). This approach fell out of favour when common soil streptomycetes were repeatedly found to produce well known antibiotics.

Williams and his colleagues were amongst the first to realize that the presence, distribution, numbers and kinds of actinobacteria in natural habitats were influenced by environmental factors such as aeration, pH, temperature and the availability of organic matter and water (Williams and Mayfield 1971; Williams et al. 1972; Goodfellow and Williams 1983). It subsequently become clear that the prospect of isolating novel actinobacteria was a product of the biological properties of environmental samples, as exemplified by the presence of large numbers of novel or putatively novel acidophilic and acidotolerant actinobacteria in acid forest soils (Khan and Williams 1975; Kim et al. 2003; Golinska et al. 2015b; Golinska et al. 2013b; Golinska et al. 2013a) and their alkaliphilic counterparts in a beach and dune sand system (Antony-Babu and Goodfellow 2008; Antony-Babu et al. 2008).

The realization that novel actinobacteria were a feature of previously overlooked natural habitats promoted a shift towards the isolation and screening of novel actinobacteria from many different sources (Tiwari and Gupta 2013), notably extreme habitats (Ward and Goodfellow 2004; Bull 2011; Goodfellow et al. 2013). In practice, this strategy led to comprehensive studies on the biosynthetic potential of novel actinobacterial strains isolated from previously unexplored sources, including insects (Carr et al. 2012; Madden et al. 2013; Guo et al. 2012; Kim et al. 2014a; Bai et al. 2016;

Beemelmanns et al. 2016), roots and leaves of medicinal and tropical plants (Duangmal et al. 2008; Qin et al. 2009; Janso and Carter 2010; Golinska et al. 2015a; Wardecki et al. 2015) and, in particular, from diverse marine habitats (Bull et al. 2005; Jensen 2010; Zotchev et al. 2012; Manivasagan et al. 2014). An interesting feature of such studies is that rare actinobacteria representing taxonomically diverse genera were seen to be a rich source of new antibiotics though streptomycetes still remain supreme in this respect (Tiwari and Gupta 2012).

1.4.2 Selective isolation of actinobacteria from natural habitats

Most actinobacteria in natural habitats are saprophytes which tend to be overgrown by fungi and other bacteria on standard nutrient media hence the need to use selective procedures to isolate them from environmental samples in dilution plate experiments. It is now established practice to add antifungal antibiotics, such as cycloheximide, nystatin and pimaricin, to actinobacterial isolation media to control or eliminate the growth of fungal colonies (Porter 1960; Gregory and Lacey 1962; Williams and Davies 1965). Similarly, penicillin G and polymixin B select actinobacteria from fast-growing bacteria (Williams and Davies 1965; Nonomura and Ohara 1969), as do nalidixic acid and trimethoprim (Hayakawa et al. 1996).

In order to isolate representative strains from actinobacterial communities present in natural habitats it is necessary to use several taxon-specific isolation procedures as individual populations that constitute communities have different biological needs, including growth and incubation requirements. Specific selective isolation procedures used to isolate actinobacteria from environmental samples have been the subject of comprehensive reviews (Nolan and Cross 1988; Labeda and Shearer 1990; Goodfellow and Fiedler 2010; Tiwari and Gupta 2012). The choice of selective media for bioprospecting campaigns is somewhat subjective but is influenced by the biome to be studied, as exemplified by the need to use acidified selective media for the isolation of acidophilic actinobacteria (Khan and Williams 1975; Busti et al. 2006; Golinska et al. 2013b; Golinska et al. 2013a; Golinska et al. 2015b). Nevertheless, the selection of both macro- and micro-habitats as a source of novel metabolically active actinobacteria remains a matter of experience and judgement.

The procedures used to selectively isolate actinobacteria from environmental samples while many and varied tend to follow a number of common steps, namely

pretreatment of samples, detachment of propagules (hyphal fragments and spores) from particulate matter, inoculation and incubation of selective isolation plates and selection of representative colonies or choice of target strains for preservation and further study. The selective experimental procedures highlighted in the following sections are ones considered to be most relevant to the isolation of taxonomically diverse actinobacteria from arid desert soils, additional methods can be found in the review articles cited above.

1.4.3 Pretreatment of environmental samples

Selection of actinobacteria can be enhanced by chemical and physical pretreatment of either environmental samples or propagules in suspensions before inoculating onto selective media (Cross 1982; Goodfellow and Fiedler 2010). It is particularly important to detach actinobacterial propagules from particulate substrates and to dispense soil aggregates as microorganisms may be bound within them. The resultant suspensions are serially diluted, plated onto selective media and incubated (Williams et al. 1984). However, the physico-chemical interactions of microbial propagules with organic matter and soil particles affect their recovery from environmental samples.

Several cell dispersion procedures have been used to detach microbial propagules from particulate material (Hopkins et al. 1991; Hayakawa et al. 2000), including the use of buffered diluents (Niepold et al. 1979) and ultrasonication (Ramsay 1984). Procedures such as these address the problem of representative sampling but involve a trade-off between the extent of propagule release and cell death. The dispersion and differential centrifugation (DDC) method, a multistage procedure introduced by Hopkins et al. (1991), combines several physico-chemical treatments and has been shown to be especially effective in increasing the yield of actinobacterial propagules from samples taken from natural habitats (Hopkins et al. 1991; Macnaughton and O'Donnell 1994; Atalan et al. 2000; Sembiring et al. 2000; Maldonado et al. 2005). There is also some evidence that different S*treptomyces* species may be preferentially isolated at different stages of the DDC procedure (Atalan et al. 2000).

The effectiveness of physical pretreatment techniques depends to some extent on the biological properties of the target bacteria. In general, actinobacterial spores tend to be more resistant to desiccation than vegetative cells of bacteria hence simply air-drying soil samples greatly increases the prospect of isolating spore-forming strains (Williams et al. 1972; Hayakawa et al. 1991b; Hayakawa et al. 1991a; Whitham et al. 1993).

Resistance to desiccation is usually associated to some degree with resistance to heat, drying soil samples, for instance, held at or above 100^{o}C for an hour reduces the number of unwanted bacteria thereby facilitating the recovery of actinobacteria (Nonomura and Ohara 1969). Actinobacterial propagules are more sensitive to dry than to wet heat which means that relatively low heat treatment regimes can be used to treat soil suspensions (Rowbotham and Cross 1977; Manfio et al. 2003). It is, however, important to recognize that while pretreatment procedures improve the ratio of actinobacterial to bacterial counts on isolation plates, the number of actinobacteria may be decreased (Williams et al. 1972).

1.4.4 Choice of selective isolation media

Selective media are designed to support the growth of target microorganisms at the expense of unwanted ones. Media selectivity is an expression of nutrient competition, pH, presence of inhibiting compounds and incubation conditions. The choice of selective isolation media can be critical, especially in bioprospecting campaigns designed to isolate representative samples of actinobacterial communities from natural habitats. Many 'non-selective' media have been formulated for the isolation of broad ranges of actinobacterial taxa (Williams et al. 1984; Goodfellow and Fiedler 2010). Surprisingly, many such media were designed without reference to the biological properties of actinobacteria *per se*, as illustrated by the use of colloidal chitin (Lingappa and Lockwood 1962; Hsu and Lockwood 1975) and starch-casein agars (Küster and Williams 1964). These media are now known to select for a relatively narrow range of *Streptomyces* species (Vickers et al. 1984; Williams et al. 1984), but are still widely used to detect the presence of streptomycetes in extreme habitats where these organisms may be the major component of prokaryotic communities (Pathom-Aree et al. 2006a; Okoro et al. 2009). In contrast, arginine-vitamin (Nonomura and Ohara 1969) and humic acid agars (Hayakawa and Nonomura 1987) support the recovery of taxonomically diverse actinobacteria from poorly studied biomes (Whitham et al. 1993; Busarakam 2014; Wang et al. 2015; Busarakam et al. 2016a).

The most effective taxon-specific selective isolation strategies are based on the biological properties of the target actinobacteria, notably using information on nutrient and antibiotic sensitivity profiles drawn from phenotypic databases (Goodfellow and Haynes 1984; Goodfellow and O'Donnell 1994; Goodfellow and Fiedler 2010; Williams and Vickers 1988). Selective media have been recommended for the isolation of many

actinobacterial genera (Goodfellow and Fiedler 2010; Tiwari and Gupta 2012), including ones found to support the isolation of novel or rare species of *Actinomadura* (Athalye et al. 1981; Busarakam 2014), *Amycolatopsis* (Tan 2002; Busarakam et al. 2016a), *Dactylosporangium* (Kim et al. 2011), *Nocardia* (Orchard and Goodfellow 1974, 1980) and *Streptomyces* (Vickers et al. 1984; Busarakam 2014). The effectiveness of such selective isolation media is often enhanced by the use of appropriate pretreatment regimes (Labeda and Shearer 1990; Goodfellow and Fiedler 2010).

It is obvious that incubation conditions will contribute to selectivity as the growth of actinobacteria is influenced by gaseous and temperature requirements and by the length of incubation. Inoculation of selective isolation plates at 4ºC, 25ºC and 45ºC favours the isolation of psychrophilic, mesophilic and thermophilic actinobacteria, respectively. Incubation for up to five weeks may be required to isolate members of the families *Micromonosporaceae* and *Streptosporangiceae* whereas *Nocardiaceae* and *Streptomycetaceae* strains generally grow well on selective isolation media after 7 to 10 days (Goodfellow and Fiedler 2010; Labeda and Shearer 1990).

1.4.5 Colony selection

The selection of actinobacterial colonies growing on selective isolation plates is one of the most time-consuming and subjective stages of the culture-dependent bioprospecting strategy. Colonies can be selected randomly if a broad-range of actinobacteria are sought or with a degree of objectivity where target-specific isolation media are used. Actinobacteria can be assigned to target taxa on the basis of characteristic colonial features and by examining colonies directly on selective isolation media using a long working distance objective to detect diagnostic morphological features, such as the presence of spore vesicles or spore chain morphology. Streptomycetes, for instance, produce characteristic filamentous leathery colonies covered by an abundant aerial spore mass which may be pigmented whereas *Amycolatopsis* strains form branched filamentous colonies covered by powdery white sterile aerial hyphae (Atalan et al. 2000; Tan et al. 2006b; Busarakam et al. 2016a). However, it is rarely possible to distinguish between species of actinobacterial genera growing on selective isolation plates. In such instances a sample of the various colony types need to be subcultured and preserved for further study. It is also good practice to select unusual colonies for further study as they may prove to be members of novel taxa.

1.4.6 Dereplication of isolates

Dereplication, a critical stage in the culture-dependent bioprospecting pipeline, is designed to rapidly assign actinobacteria with similar phenotypic properties to taxonomically meaningful groups so that representative isolates can be selected for screening assays. An increasing number of dereplication procedures are available for this purpose, including chemotaxonomic and molecular fingerprinting techniques (Schumann and Maier 2014; Maldonado et al. 2008; Ferguson et al. 1997). An effective way of dereplicating *Streptomyces* strains was introduced by (Williams et al. 1969) who assigned large numbers of these organisms to groups based on aerial spore mass, substrate mycelial and diffusible pigment colours formed on oatmeal agar and on their ability to produce melanin pigments on yeast extract- malt extract- iron agar. Colour-groups were later shown to be a measure of streptomycete diversity in rhizosphere and non-rhizosphere soils (Williams and Vickers 1988; Atalan et al. 2000; Sembiring et al. 2000), as colour-group representatives were found to belong either to validly published or novel *Streptomyces* species based on computer-assisted identification (Williams and Vickers 1988; Atalan et al. 2000) and polyphasic taxonomic procedures (Manfio et al. 2003; Goodfellow et al. 2007). It is encouraging that a linear correlation has been found between streptomycete colour-groups and corresponding *rep*-PCR data (Antony-Babu et al. 2010).

The assignment of streptomycetes to colour-groups has provided an insight into their taxonomic diversity in a beach and dune sand ecosystem (Antony-Babu and Goodfellow 2008), in desert soils (Okoro et al. 2009; Busarakam 2014) and marine sediments (Goodfellow and Haynes 1984; Pathom-Aree et al. 2006a) thereby facilitating the choice of representative isolates for bioactivity assays (Goodfellow and Fiedler 2010; Busarakam 2014) . It is particularly significant that this approach has led to a marked increase in hit rates in screening assays (Goodfellow and Fiedler 2010) .

1.4.7 Screening for bioactivity

Primary screening of dereplicated actinobacteria can be achieved using standard “kill” assays in order to detect compounds active against panels of Gram-positive and Gram-negative bacteria and in some cases yeasts (Fiedler 2004; Baltz 2007). In practice, this often simply involves taking agar plugs from actinobacterial lawns and placing them onto

corresponding lawns of wild type strains, incubating overnight then recording zones of inhibition. Variants of this approach include spotting culture filtrates or organic extracts directly onto wild type strains and examining for inhibition of growth either by eye or spectrophometerically (2008; Bredholdt et al. 2007; Hong et al. 2009). Hits can be prioritized according to the taxonomic novelty of the actinobacterial strains using rDNA sequence comparisons to known actinobacteria. Further prioritization can be achieved by detecting the modes of action (MOA) of bioactive agents in secondary screens (Fiedler 2004) .

An array of genetic constructs are available to detect the MOA of different classes of antibiotics (2004b); Hutter et al. (2004a). Mutant strains can be designed to carry reporters, such as β-galactosidase and luciferase genes, which are fixed to promoters that respond to specific antibiotics that trigger the expression of the reporter gene (Hutter et al. 2004b; Urban et al. 2007). The production of β-galactosidase, for instance, leads to the colourless 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) being cleaved into β-D-galactose and the blue-coloured 5-bromo-4-chloro-3-hydroxyindole (Figure 1.3). The formation of the blue pigment shows that the bioactive compound is repressing the target pathway. *Bacillus subtilis* reporter strains have been used to detect inhibitors of cell wall, DNA, fatty acid and protein biosynthesis (Hutter et al. 2004a; Busarakam 2014).

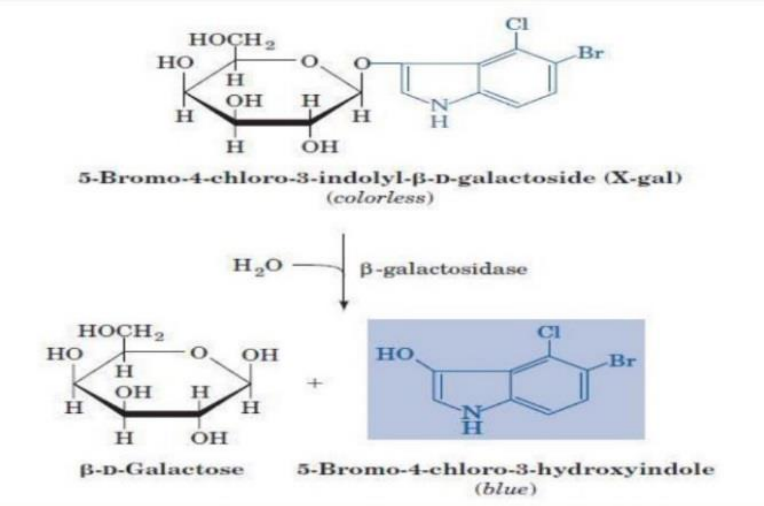

Figure 1.3 Cleavage of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) to β-D-galactose and 5-bromo-4-chloro-3-hydroxyindole (Blue compound). (Adapted from (Voet and Voet 2011).

1.4.8 Detection and classification of novel actinobacterial species

The quality of actinobacterial isolates examined in bioprospecting campaigns is a function of two parameters, diversity and dereplication, each of which is much more important than the overall number of strains. Diversity in this context is measured in its taxonomic sense, namely how many different species, genera and families make up the strain selection. The more taxonomically diverse the collection the greater the chance of finding novel specialized metabolites. Dereplication, as stated earlier, is the assignment of isolates to taxonomically predictive groups. Bioprospecting studies still tend to be based on large numbers of strains which include multiple repeats of clonal or closely related isolates which makes for inefficient screens.

The taxonomic approach to culture-dependent bioprospecting may take one of several forms depending on the nature of the exercise. For instance, dereplicated isolates shown to belong to novel taxa can be screened for bioactivity or representative strains selected from isolation media can be screened and those considered interesting checked for novelty. Either way diversity and dereplication can be achieved using various taxonomic measures. Presently the method of choice is 16S ribosomal RNA gene sequencing (Goodfellow and Fiedler 2010; Yarza et al. 2014; Yarza and Munoz 2014), though this can be expected to change as whole-genome sequencing becomes more prevalent (Klenk and Göker 2010; Mende et al. 2013; Sangal et al. 2014; Thompson et al. 2014). Nevertheless, it is still a requirement to describe novel species of prokaryotes using a combination of genotypic and phenotypic features (Wayne et al. 1987; Kämpfer and Glaeser 2012; Vandamme et al. 1996; Gillis et al. 2005) and to follow the rules embodied in the *International Code of Nomenclature for Bacteria* (Lapage et al. 1975; Lapage et al. 1992).

The current strategy used to classify prokaryotes is based on the integrated use of taxonomic information generated from the application of chemotaxonomic, molecular systematic and phenotypic methods (Vandamme et al. 1996; Schleifer 2009; Goodfellow 2000). This polyphasic approach has led to major improvements in the classification of archaea and bacteria, not least in establishing phylogenetic relationships within and between actinobacterial taxa (Ludwig et al. 2012; Girard et al. 2013). The choice of genotype and phenotypic methods for any particular study is critical as they need to reflect the rank and biological properties of taxa and the use of new procedures that provide improved resolution between taxa (Tindall et al. 2010; Cody et al. 2014; Vandamme and

Peeters 2014; Schumann and Maier 2014; Kim et al. 2014b). Methods found to be useful in the classification of actinobacteria are shown in Table 1.2.

The evolutionary conserved nature, ubiquitous distribution and size of 16S rRNA genes was instrumental in them becoming the phylogenetic marker of choice following the pioneering studies on the universal tree of life by Karl Woese and his colleagues (Woese and Fox 1977; Woese 1987). In essence, the use of such sequences in the classification and identification of prokaryotes rests on comparisons of new sequences against comprehensive databases of known sequences, notably those of type strains, as illustrated by Chun and his colleagues (Chun et al. 2007; Kim et al. 2012b; Kim and Chun 2014). Extensive comperative 16S rRNA gene sequencing studies have revolutionized our understanding of phylogenetic relationships among prokaryotes (Yarza and Munoz 2014; 2010; Yarza et al. 2008). Indeed, the framework for the classification of archaea and bacteria in the current edition of *Bergey's Manual of Systematic Bacteriology* is based on comparative 16S rRNA gene sequence data, as shown by the assignment of actinobacteria to 6 classes, 23 orders, 53 families and 222 genera (Goodfellow et al. 2012a) . The procedures used to generate, align and analyse high quality 16S rRNA gene sequences are not considered here as they have been covered in recent reviews (Rosselló-Móra et al. 2011; Ludwig et al. 2011).

Strain prioritization based on comparative 16S rRNA gene sequences is an important stage in the culture-dependent approach to natural product discovery at a time when actinobacterial strain culture collections are rapidly expanding in light of improved selective isolation procedures. Isolates representing dereplicated taxa, such as *Streptomyces* colour-groups, found to form distinct lineages in the actinobacterial 16S rRNA gene tree can be prioritized for screening, especially for low throughput screens. Alternatively, isolates found to produce new metabolites in more broadly-based screening programmes can be checked for novel sequences using digital databases such as the EzTaxon platform (Kim and Chun 2014). Either way putatively novel isolates should be examined further to establish whether they merit publication as new taxa. The importance of publishing new internationally recognised names for commercially significant prokaryotes is often overlooked even though an organism's name is a key to all that is known about it (Goodfellow and Fiedler 2010).

Table 1.2 Sources of taxonomic information for the classification of actinobacteria.

Cell component		Taxonomic rank		
		Genus or above	**Species**	**Subspecies or below**
Chemical markers	Fatty acids	/	/	
	Isoprenoid quinones	/	/	
	Mycolic acids	/	/	
	Peptidoglycans	/		
	Polar lipids	/	/	
	Polysaccharides	/	/	
Chromosomal DNA	Base composition (mol % G+C)	/	/	
	DNA:DNA hybridisation		/	/
	Restriction patterns (AFLP, PFGE, RFLP, ribotyping)		/	/
	Whole-genome analyses	/	/	/
DNA segments	DNA probes	/	/	/
	16S rRNA gene sequencing	/	/	/
	Multi-locus sequence analyses		/	/
	PCR-based DNA fingerprinting (PCR-RFLP, ITS, RAPD, Repetitive-PCR		/	/
Expressed features	Metabolism		/	
	Morphology	/	/	
	Physiology	/	/	
Proteins	Amino acid sequence analyses	/	/	
	Multilocus sequence typing	/	/	/
Ribosomal RNA	Restriction patterns (ARDRA)		/	/
	t-RNA fingerprinting		/	/
Whole organism	MALDI-TOF mass spectrometry		/	/

Abbreviations: AFLP, amplified fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analyses; ITS, intergenic 16S-23S rRNA transcribed spacer sequences; PFGE, pulse field gel electrophoresis; RFLP, restriction fragment length polymorphism; RAPD, randomly amplified polymorphic DNA fingerprinting; PCR, polymerase chain reaction.

In general, the discontinuous distribution of chemotaxonomic and morphological markers underpin actinobacterial phylogenies based on 16S rRNA gene sequence data (Goodfellow 2012a; Ludwig et al. 2012). *Streptomyces* strains, for example, form an extensively branched substrate mycelium, aerial hyphae that typically differentiate into chains of spores, have LL-diaminopimelic acid (LL-A_2pm) but no diagnostic sugars in the peptidoglycan, contain major proportions of saturated, *iso*- and branched chain fatty acids, usually have either hexa- or octahydrogenated menaquinones (MK-9[H_6], MK9[H_8]) as the predominant isoprenologue and complex polar lipid patterns rich in diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (Kämpfer 2012). In contrast, *Pseudonocardia* strains form substrate and aerial mycelia with spore chains produced by acropetal budding or fragmentation, have *meso*-A_2pm, arabinose and galactose in the peptidoglycan, tetrahydrogenated menaquinones with eight isoprene units (MK8[H_4]) as the major isoprenologue, *iso*-branched hexadecanoic acid as the predominant fatty acid and either phosphatidylcholine or phosphatidylethanolamine as diagnostic polar lipids (Huang and Goodfellow 2015).

In short, discontinuously distributed markers such as those outlined above provide an effective means of evaluating actinobacterial phylogenies, especially in instancies where phylogenetic relationships do not allow groups to be recognised with confidence (Labeda et al. 2011) or where the use of different treeing algorithms yield different phylogenies (Ludwig and Klenk 2005; Ludwig et al. 2012; Kämpfer and Glaeser 2012). Standard procedures are widely used to detect chemical markers found to be of value in actinobacterial systematics (Table 1.3), notably protocols for the detection of diaminopimelic acid isomers (Staneck and Roberts 1974; Hasegawa et al. 1983), fatty acids (Kroppenstedt 1985), including mycolic acids (Minnikin et al. 1980), and menaquinones and polar lipids (Minnikin et al. 1984).

Several molecular systematic methods are available to assign closely related bacterial strains to species where insufficient variation is present in 16S rRNA gene sequences (Wayne et al. 1987; Stackebrandt et al. 2002). The genus *Streptomyces* is a good case in point as many multimembered lineages recognised in an extensive comparison of 16S rRNA gene sequences of type strains were not supported by high bootstrap values (Labeda 2011). In such instances, DNA:DNA hydridisation (DDH) studies allow closely related strains to be clustered into genomic species, that is, species that encompass strains that have approximately 70% or more DNA relatedness with $<5^{o}$C stability in pairwise heteroduplices (Wayne et al. 1987). DDH assays are extensively used

to delineate actinobacterial species, including *Streptomyces* species (Labeda and Lyons 1991a, b; Labeda 1998; Rong and Huang 2012) even though they are tedious to carry out and subject to experimental error (Gevers et al. 2005; Rosselló-Móra et al. 2011), the reliability of the different DNA:DNA reassociation methods has been evaluated by (Rosselló-Mora 2006). It is now good practise to carry out DDH experiments only when strains share high 16S rRNA gene sequence similarities, recommended thresholds have risen from 97.0% (Stackebrandt and Goebel 1994) to 99.0% (Meier-Kolthoff et al. 2013) then back to 98.7% (Kim et al. 2014b) though cut off values vary depending on the taxa under study (Stackebrandt and Ebers 2006; Meier-Kolthoff et al. 2013).

Stackebrandt et al. (2002) recommended the application of DNA fingerprinting and multilocus sequence analyses (MLSA) to distinguish between closely related bacterial species. Comparative MLSA is a product of multilocus sequence typing that has been widely used in epidemiological surveys of medically significant taxa (Cody et al. 2014) and shown to be of value in unravelling species diversty between actinobacteria that share very high 16S rRNA gene sequences (Ludwig et al. 2012). The underlying rationale and steps involved in MLSA analyses have been described in detail (Cody et al. 2014; Rong and Huang 2012). In essence, MLSA is based on the generation and analyses of 400-500 base pair sequences of fragments of five to seven housekeeping genes and the presentation of the resultant data in concatenated gene trees. Such trees show greater resolution between closely related actinobacteria than corresponding 16S rRNA gene trees, as evidenced by comparative studies on the genera *Kribbella* (Curtis and Meyers 2012), *Micromonospora* (Carro et al. 2012) and *Salinispora* (Freel et al. 2012), and extensive investigations on phytopathogenic (Labeda et al. 2014; Labeda 2011) and commercially significant (Guo *et al.*, 2008; Rong & Huang, 2010, 2012; Rong *et al.*, 2010, 2012) streptomycetes. A strong correlation between MLSA, DDH and phenotype data was highlighted by (Rong and Huang 2014) who also noted that the DDH 70% threshold corresponded to a five gene sequence of 0.007. MLSA data can also be used to resolve the taxonomic status of isolates that form distinct branches in unstable regions of the 16S rRNA ene tree, as exemplified by the classification of the type strain of *Streptomyces leeuwenhoekii* (Busarakam et al. 2014).

The advent of rapid and relatively inexpensive whole-organism sequencing (WGS) technologies and associated bioinformatic tools are leading to a step change in the way prokaryotic systematics is being conducted (Klenk and Göker 2010; Chun and Rainey 2014; Harris and Okoro 2014), not least the introduction of new parameters for establishing species boundaries (Table 1.3). The average nucleotide identity (ANI) is a

particularly attractive metric for the delineation of species with a 94% ANI value corresponding to the 70% DDH cut-off (Richter and Rosselló-Móra 2009; Arahal 2014), WGS data also provide useful insights into an organism's ecological, metabolic and biotechnological potential (Wu et al. 2012; Doroghazi and Metcalf 2013; Sangal et al. 2014) and into phenomena such as gene duplication and horizontal gene transfer (Kamneva and Ward 2014). However, it is important that taxonomies based on WGS data follow sound taxonomic practices, such as the use of the nomenclatural type concept and the requirement to deposit type strains in two service culture collections in different countries (Lapage et al. 1992; Stackebrandt et al. 2014). A case has also been made for the continued use of phenotypic data in prokaryotic systematics (Kämpfer et al. 2014). In contrast, strong arguments have been presented to revise the principles and practices of prokaryotic systematics in light of the new development, not least the urgent need to streamline procedures for the publication of new taxa (Sutcliffe *et al.*, 2012 (Sutcliffe et al. 2012; Vandamme and Peeters 2014; Thompson et al. 2014; Whitman 2014).

Comparative genome-based analyses have been used to unravel actinobacterial relationships that proved difficult to resolve using established taxonomic procedures. It has, for instance, been shown that the genus *Kitasatospora* merits recognition as a sister genus to *Streptomyces* in the family *Streptomycetaceae (Girard et al. 2014; Girard et al. 2013)* and that the genera *Amycolatopsis* and *Rhodococcus* encompass multiple lineages thereby providing a framework for their taxonomic revision (Creason et al. 2014; Sangal et al. 2014; Tang et al. 2015). Whole- genome sequences are rich in genes that provide an understanding of the ecophysiology of individual strains, as in the case of *Micromonospora lupini* Lupac 08 and *Modestobacter caceresii* KNN44-2b^{T}, strains isolated from nitrogen-fixing nodules of *Lupidus angustifolious* and extreme hyper-arid Atacama Desert soil, respectively (Trujillo et al. 2007; Busarakam et al. 2016b). Similarly, comparative genomics has revealed evidence of marine adaptation in representatives of *Salinispora* species (Penn and Jensen 2012). An innovative approach to natural product discovery based on diverse actinobacterial WGS data showed a correlation between phylogeny and the discontinuous distribution of families of biosynthetic gene clusters (Doroghazi et al. 2014), an observation that opens up the prospect of a focused taxonomic approach to genome mining for natural products (Challis 2014).

Table 1.3 Bioinformatic tools and resources used to calculate genome-based tresholds for the circumscription of prokaryotic species.

Genome relatedness index	Threshold for delineation of species	Gene finding/annotation required?	Bioinformatic tools	Description	URL
ANI	95-96%	No	Jspecies	Java-based standardize software that calculates ANIb and ANIm (Richter & Roselló-Móra, 2009)	http://imedea.uib-csic.es/jspecies/
			EzGenome	Web-based resource for ANIb calculation	http://www.ezbiocloud.net/ezgenome
GBDP	0.258	No	Genome to Genome Database Calculator	Web-based resource for calculation of pairwise, GDP distance using NCBI-BLAST, BLAT, BLASTZ and MUMner (Meier-Kolthoff *et al.*, 2013)	http://www.gqdc.dsmz.de
MUMi	None available	No	MUMi	Web-based resource for calculation of MUMi (Deloger *et al.*, 2009)	http://genome.jouy.inra.fr/mumi/
Nucleotide identity	96.5%	Yes	specI	Web-based resource and Linux standardize program for identification using 40 universal, single-copy marker genes (Mende *et al.*, 2013)	http://vm-lux.embl.de/~mende/specI//

Abbreviations: ANI, average nucleotide identity (see Arahal, 2014); GBDP, genome BLAST distance phylogeny (Henz et al. 2005); MUMi, another distance type index (see Deloger *et al.*, 2009). Modified from Chun and Rainey (2014).

Several chemical procedures can be used to detect patterns of specialized metabolites in extracts of dereplicated strains (Goodfellow and Fiedler 2010). A critical step, dereplication of extracts can be achieved using LC-MS and chemoinformatic-based approaches. The laboratory information management system (NAPIS) is now used extensively to select the most active and novel extracts for downstream processing (Jaspars, pers. comm.). HPLC-MS analyses are generally used to confirm the novelty of key specialized metabolites. The final steps in the process, scale-up fermentation and structural elucidation of pure compounds vary depending on the nature of the strains and bioactive agents, as exemplified by the discovery of the chaxamycins (Rateb et al. 2011a) and chaxapeptin (Elsayed et al. 2015).

1.5 Novel actinobacteria from desert ecosystems as a source of new drug leads

Non-polar deserts. The likelihood of isolating novel actinobacteria that produced new specialized metabolites is enhanced when bioprospecting strategies are focused on neglected and unexplored ecosystems (Goodfellow and Fiedler 2010; Tiwari and Gupta 2012), such as those found in desert regions. Deserts cover nearly 20% of the Earth's land surface but have received little attention from microbiologists until recently (Bhatnagar and Bhatnagar 2005; Bull et al. 2016). However, it is well known that living conditions in desert habitats are challenging for microorganisms due to the lack of liquid water, low organic carbon, high UV radiation and large fluctuations in temperature. Such harsh environmental conditions can be expected to favour the evolution of novel actinobacteria with new chemistries (Okoro et al. 2009; Goodfellow et al. 2013).

Small numbers of taxonomically diverse actinobacteria have been isolated from non-polar deserts, including the Mongolian (Zenova et al. 2009; Kurapova et al. 2012), Namibia (Wink et al. 2003a), Sahara (Meklat et al. 2011; Saker et al. 2015; Saker et al. 2014) , Taklamakan (Luo et al. 2012b) and Thar (Tiwari et al. 2015; Harwani 2013) deserts, notably rare actinobacteria belonging to genera classified in the families *Geodermatophilaceae (Blastococcus, Geodermatophilus* and *Modestobacter*), *Pseudonocardiaceae (Dactylosporangium, Prauserella, Saccharopolyspora* and *Saccharothrix), Streptosporangiaceae (Microbispora, Microtetraspora, Nonomuraea* and *Streptosporangium)* and *Thermomonosporaceae (Actinomadura* and *Sprillospora*). Isolates from desert soils validly published as new genera are few, but include *Geodermatophilus* (Luedemann 1968), *Jiangella* (Song et al. 2005) and *Yuhushiella* (Mao et al. 2011). In contrast, the number of desert isolates validly published as new species is rising fast, as illustrated by *Amycolatopsis deserti*

from an arid Australian composite soil (Busarakam et al. 2016a), *Mycetocola manganoxydans* and *Nocardioides deserti* from the Taklamakan Desert, in Xinjiang Province, China (Luo et al. 2012b; Tuo et al. 2015) and *Prauserella isguenensis*, *Saccharothrix algeriensis* and *Saccharothrix saharensis* from the Algerian Sahara (Zitouni et al. 2004b; Saker et al. 2015; Boubetra et al. 2013). Specialized metabolites produced by some of these strains include the new anthracycline antibiotic, mutactimycin PR (Zitouni et al. 2004a) and novel dithiolopyroline analogues (Bouras et al. 2008).

The Atacama Desert. The most extensive surveys of culturable actinobacterial diversity in desert biomes have been concentrated on sites in the Atacama Desert in Northern Chile (Okoro et al. 2009; Busarakam 2014). The location and abiotic conditions associated with this desert have been considered in several reviews (Gómez-Silva et al. 2008; Azua-Bustos et al. 2012; Bull et al. 2016; Bull and Asenjo 2013) and will not be considered here. Bull and his colleagues explained that the uniqueness of the Atacama Desert residues "*in its amazing diversity of ecological niches, its geology and geochemistry, its elevation and topography and its radiation intensity*". Until recently, the significance of such features was lost on biologists as the prevailing view held sway that the harsh conditions in much of the desert, notably extreme dryness, very low organic carbon, presence of organic oxidants and intense UV-radiation made any form of life virtually impossible (McKay et al. 2004; Navarro-González et al. 2003). Subsequently, pioneering studies carried out by Chilean microbiologists showed that microorganisms were able to colonise several Atacama Desert habitats (Gómez-Silva et al. 1990; Prado et al. 1993; Campos et al. 2009). It is now clear that different kinds of microorganisms have become adapted to the extreme conditions that prevail in diverse habitats within the desert (Azua-Bustos et al. 2012; DiRuggiero et al. 2013; Paulino-Lima et al. 2013; Ziolkowski et al. 2013).

Microbial surveys of Atacama Desert soils and regoliths have been focused mainly on the isolation of strains from hyper-arid and extreme hyper-arid regions, that is, where the mean annual rainfall to mean annual evaporation is 0.05 and 0.002% respectively (Houston 2006). Very low counts of heterotrophic bacteria have been recorded from such soils, notably from the Yungay region, one of the driest areas on Earth (Navarro-González et al. 2003). Actinobacteria were first isolated from Atacama Desert soils around fifty years ago (Cameron et al. 1966; Opfell and Zebal 1967) but despite these early leads they rarely featured in subsequent broadly-based culture-dependent surveys, exceptions include the isolation of *Arthrobacter, Kocuria, Cellulomonas* and *Geodermatophilus* strains from the Yungay region

(Gómez-Silva et al. 2008), putatively novel *Geodermatophilus* and *Streptomyces* species from one of the driest areas in the Atacama Desert at Maria Elena South 275 km northeast of the Yungay region (Azua-Bustos et al. 2015) and *Nocardia, Micromonospora, Prauserella* and *Streptomyces* strains from the Tarapaci and Antofagasta regions of the desert (Bull et al. 2016).

Recently, extensive bioprospecting campaigns employing selective isolation, screening and polyphasic taxonomic procedures have shown that low counts of taxonomically diverse actinobacteria that show bioactivity can be recovered from hyper-arid and extreme hyper-arid soil taken from the Salar de Atacama and Yungay regions of the Atacama Desert, respectively (Okoro et al. 2009; Busarakam 2014). Dereplicated isolates were mainly found to be putatively novel *Streptomyces* species though some strains were shown to form distinct phyletic lines within the evolutionary radiation occupied by rare genera, including *Actinomadura, Couchiplanes, Kribbella, Lechevalieria, Modestobacter, Nonomuraea* and *Saccharothrix.* Subsequently, some of these isolates were classified as new species of *Modestobacter* (Busarakam et al. 2016b), *Lechevalieria* (Okoro et al. 2010)and *Streptomyces* (Santhanam *et al.,* 2012a, b, 2013), notably *Streptomyces leeuwenhoekii* C34^{T} (Busarakam et al. 2014). In contrast, representatives of thermophilic actinobacteria isolated from Salar de Atacama soil were found to be *bona fide* members of two rare species, *Amycolatopsis ruanii* and *Amycolatopsis thermalba* (Busarakam et al. 2016a).

Initial studies on the natural product chemistry of taxonomically novel strains isolated from Atacama Desert soils are encouraging given the discovery of novel chemical entities with biological activity (Table 1.4). It is particularly interesting that *S. leeuwenhoekii* strains are to the fore in this respect as the high quality non-fragmented genomic sequence of the type strain of this species contains 35 clusters that apparently encode for the biosynthesis of many novel and undescribed specialized metabolites (Gomez-Escribano et al. 2015). In contrast, the whole-genome sequence of *Modestobacter caceserii* KNN45-2b^{T} contains few biosynthetic gene clusters (Busarakam et al. 2016b) hence members of this taxon are unlikely to be a source of new antibiotics though they may produce melanin compounds that may act as sunscreens. The putatively novel *Streptomyces* strain isolated from a Salar de Tara high altitude soil (4,500m) in the Chilean highlands by Schulz et al. (2011) shows inhibitory activity against type 4 phosphodieterase, a discovery that might prove useful in the treatment of inflammatory diseases such as asthma.

Table 1.4 Novel specialized metabolites produced by actinobacteria isolated from Atacama Desert soils.

Organisms	Products	Structures	Bioactivity	References
Modestobacter sp. strain KNN45-2b	Peptide	Known compounds such as anthranilic acid ethyl ester and 3-β- indolacrylic acid	None	Busarakam *et al.*, unpublished
Streptomyces leeuwenhoekii C58	Atacamycins	22-membered macrolactone polyketides	Antibacteria, antitumor, inhibits activity against phosphodiesterase types 2 and 4	(Nachtigall et al. 2011; Elsayed et al. 2015)
Streptomyces leeuwenhoekii C34^T	Chaxalactones	22-membered macrolactone polyketides	Antibacteria	(Rateb et al. 2011b)
Streptomyces leeuwenhoekii C34^T	Chaxamycins	Ansamycin polyketide	Antibacteria, anticancer	(Rateb et al. 2011a)
Streptomyces leeuwenhoekii C58	Chaxapeptin	Lasso peptide	Anticancer	(Elsayed et al. 2015)
Streptomyces leeuwenhoekii C79	Four specialized metabolites	ND	Antibacteria	Fiedler *et al*., unpublished
Streptomyces sp. strain DB634	Ataquinones	ND	Antimicrobial, inhibits activity against phosphodiesterase type 4	(Schulz et al. 2011)

ND, not determined.

1.6 Culture-independent approach to determine the extent of actinobacterial diversity

1.6.1 Detecting the unseen majority

A strong case can be made for the application of effective procedures for the selective isolation, dereplication and classification of actinobacteria from environmental samples in order to select high quality strains for biotechnological purposes. Culture-dependent strategies have shown that actinobacterial taxa once seen to be rare in natural habitats are an integral part of actinobacterial communities, as shown by the isolation and characterization of novel *Actinospica, Amycolatopsis* and *Dactylosporangium* species (Tan et al. 2006a; Kim et al. 2011; Golinska et al. 2015b). It has also been found that representatives of novel actinobacterial species are a rich source of bioactive compounds (Goodfellow and Fiedler 2010; Jensen 2010; Becerril-Espinosa et al. 2013).

A dramatically different approach to estimating the extent of prokaryotic diversity in natural habitats arose through the application of culture-independent molecular methods, including fluorescent *in situ* hybridisation (Moter and Göbel 2000; Amann and Fuchs 2008) and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), but most notably by the recognition of previously unknown taxa through the use of oligonucleotide primers designed to amplify 16S rRNA gene sequences present in extracted community DNA (Olsen et al. 1986; Pace et al. 1986) . The strengths and weaknesses of the early culture-independent molecular-based methods will not be considered here as they are covered in detail in several comprehensive reviews (Head et al. 1998; Bull et al. 2000; O'Donnell et al. 1993). Culture-independent studies clearly show that standard culture-dependent strategies grossly underestimated the extent of prokaryotic diversity in environmental samples (Ward et al. 1990; Amann et al. 1995; Suzuki et al. 1997; Vartoukian et al. 2010). Culture-dependent procedures have also seriously underestimated the degree of actinobacterial diversity in samples taken from marine and terrestrial habitats (2003b; Stach et al. 2003a).

In more recent times high throughput sequencing platforms and associated analytical tools are being used to generate bacterial genomes (Harris and Okoro 2014; Sangal et al. 2014). The strengths and weaknesses of the 454 GS FLX+ (Roche), 550xI SOLID (Life Technologies), HiSeq 2000/2500 (Illumina) and PacBio RS (Pacific

Biosciences) platforms and associated sample preparation and template amplification technologies have been considered in detail (Claesson et al. 2010; 2012b; Loman et al. 2012a; Liu et al. 2012; Luo et al. 2012a). The size of sequencer reads and coverage are platform specific. The Illumina platform is the most economic and pair-end and mate-end reads yield, highly readable assemblies with high coverage while the PacBio platform gives much larger reads that can generate genomes without any gaps. All of the platforms can be used to generate data requited for assembling draft genome sequences. Similarly, targeted sequencing of 16S rRNA genes for taxonomic and metagenomics studies can be carried out on any of the sequencing platforms.

Roche 454 sequencing, now referred to as 454-pyrosequencing, is especially useful for sequencing short DNA fragments as it allows high coverage per sample (Claesson *et al.*, 2009) and many additional samples per run (Sogin et al. 2006; Hamady et al. 2008; Rothberg and Leamon 2008). This method has been shown to be accurate, flexible and amendable to automation and has the added advantages of not requiring the generation of clone libraries, the need for labelled primers or gel electrophoresis. The principles underpinning pyrosequencing can be found elsewhere (Ronaghi 2001; Fakruddin et al. 2013). In short, pyrosequencing is based on the luminescence detection of pyrophosphate released during DNA synthesis. A schematic outline of the procedure is shown in Figure 1.4.

High throughput 16S rRNA pyrosequencing technologies have given an insight into the relative abundance and diversity of prokaryotes in several natural habitats, including Brazilian savanna soils (Rampelotto et al. 2013), desert sand (Neilson et al. 2012; An et al. 2013; An et al. 2015), glacial surfaces (Liu et al. 2015), permafrost soils (Yang et al. 2012), surface and subsurface peat layers (Serkebaeva et al. 2013) and cold seeps in the Red Sea (Yang et al. 2015). Such studies have not only highlighted bacterial diversity, including that of many unknown taxa, but have shown that microbial community structure is influenced by environmental factors, temporal differences and land use procedures. In a more restricted study, 454 pyrosequencing revealed the relative abundance and diversity of bacterial predators, namely *Bdellovibrio* and related organisms, in fresh and salt water (Li and Williams 2015).

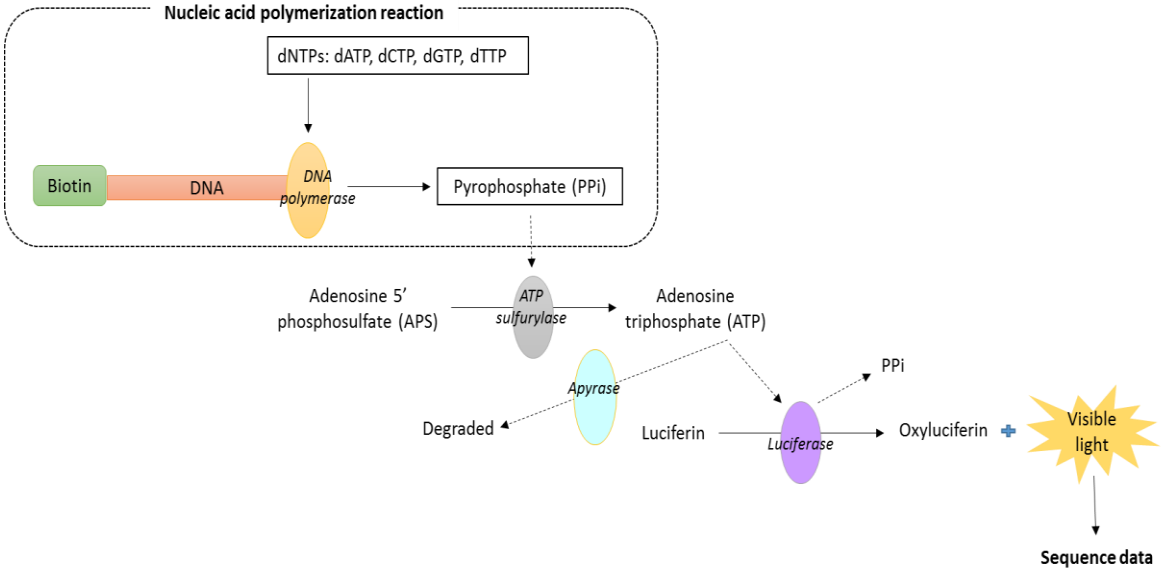

Figure 1.4 Schematic representation of pyrosequencing (Modified from Fakruddin et al., 2013).

1.6.2 The twin-track approach

Metagenomic analyses are being increasingly favoured over culture-dependent methods in surveys of microbial communities as they reveal the amazing levels of prokaryotic diversity, much of it novel, present in natural habitats. Indeed, culture-based methods are seen, with considerable justification, to be limited in scope as they are labour intensive, time-consuming, underestimate prokaryotic diversity and tend to overplay the significance of fast-growing and spore-forming taxa (Bull et al. 2000). However, it is also important to recognize that culture-independent data can be skewed by factors that include choice of PCR primers, differential cell lysis, rRNA copy numbers and the dominance of individual taxa at the time of sampling (O'Donnell et al. 1993; Bull et al. 2000; Engelbrektson et al. 2010) and by sequencing errors and data handling problems (Claesson et al. 2010; Kunin et al. 2010; Nakamura et al. 2011; Liu et al. 2012; Roh et al. 2010).

Given constraints such as those outlined above a case can be made for using both culture-dependent and culture-independent methods for determining the extent of prokaryotic diversity in natural bacterial communities. In such twin-track approaches the metagenomic element provides an invaluable perspective into the extent of prokaryotic diversity in environmental samples while corresponding culture-based analyses allow the selection and growth of dereplicated isolates for biotechnological and bioprospecting purposes. Within a broader context there is a need to devise innovative procedures to isolate culturable representatives of uncultured taxa for such purposes (Stewart 2012).

The twin-track approach though not widely applied has been used to analyse bacterial communities in anoxic rice paddy soil (Hengstmann et al. 1999), arid soils (Dunbar et al. 1999), the human gut (Wilson and Blitchington 1996; Suau et al. 1999), marine sediment (Chandler et al. 1997), seawater (Suzuki et al. 1997), in cold desert soils (Babalola et al. 2009), high temperature petroleum reservoirs (Orphan et al. 2000; Kaster et al. 2009) and more recently on glacier surfaces (Liu et al. 2015), in river and tapwater (Vaz-Moreira et al. 2011, 2013), and commercial salad leaf vegetables (Jackson et al. 2013). The outcome of such studies, while mixed, tends to show similar relationships between culture-dependent and culture-independent data though significant differences are also apparent. There are reports that culture-dependent and culture-independent methods target different bacteria (Vaz-Moreira et al. 2011) and that some taxa, including members of the rare biosphere, are detected only using culture-dependent procedures (Wilson and Blitchington 1996; Suau et al. 1999; Shade et al. 2012) .

1.6.3 Back to the Atacama Desert

Culture-independent methods have been used to establish the extent of prokaryotic diversity in Atacama Desert habitats and in some instances have been designed to gain an insight into the functional ecology of taxa (Azua-Bustos et al. 2012; Bull and Asenjo 2013). The outcome of such studies have been mixed as actinobacteria have been shown to be dominant in some habitats (Bull et al. 2016) but are absent or barely feature in others (Demergasso et al. 2010; Drees et al. 2004; de los Rios et al. 2010; DiRuggiero et al. 2013). The twin-track approach to establishing actinobacterial structure in Atacama Desert habitats has still to be realised.

Actinobacteria seem to predominate in soil samples taken from in and around the hyper-arid core region of the Atacama Desert, namely the Yungay region (Connon et al. 2007; Neilson et al. 2012; Crits-Christoph et al. 2013). Connon and her colleagues found that actinobacteria accounted for 94% of 16S rRNA genes cloned from Yungay soil samples, the majority of which were most closely, albeit very loosely related to the genera *Frankia* and *Rubrobacter* as well as to uncultured taxa. This dominance was underpinned in a 454-pyrosequencing study carried out by (Neilson et al. 2012) who found that members of the families *Conexibacteriaceae, Nutriliruptoraceae, Patulibacteriaceae* and *Solirubrobacteriaceae* and the subclass *Rubrobacteridae* were abundant in various Yungay samples, these taxa have rarely been isolated but are known to form deep-seated lineages in the actinobacterial 16S rRNA gene tree (Ludwig et al. 2012). Similarly, Crits-Christoph et al. (2013) analysed microbial communities at several geographical sites in

the Yungay region based on pyrosequencing of bar-coded 16S rRNA gene amplification products, generated using archeal and bacterial primer sets, and found communities dominated by actinobacteria belonging to the orders *Acidimicrobiales* and *Rubrobacteriales* and to representatives of the thermophilic families *Patulibacteriaceae* and *Solirubrobacteriaceae.* Sizeable actinobacterial populations have been found in hyper-arid, high altitude, non-fumerolic soil from Socompa Volcano on the Chilean-Argentine border (Costello et al. 2009) and in sediment samples from the Salar de Gorbea, south-east of Antofagasta (Bull and Asenjo 2013).

Functional ecological analyses of actinobacterial taxa found in Atacama Desert habitats are in their infancy. However, in one such study the metagenome generated from a high altitude debris field on Llullaillaco Volcano near the Chilean-Argentine border was almost solely composed of actinobacterial lineages, notably by the genome belonging to the genus *Pseudonocardia* (Lynch et al. 2012). Metabolic pathways encoded by this genome included an ability to use trace gases (CO, H_2) and C1 compounds, traits exhibited by several *Pseudonocardia* species (Huang and Goodfellow 2015)). It is also interesting that phylogenetic data from one of the sites studied by (Neilson et al. 2012) suggested the presence of chemoautotrophic taxa able to acquire energy through the oxidation of carbon monoxide, nitrate, iron and/or sulphur.

Chapter 2. Materials and Methods

2.1 Sampling locations

Twenty three environmental samples (Table 2.1) were collected from seven locations in the Atacama Desert by Professors Michael Goodfellow (School of Biology, Newcastle University) and Alan T. Bull (School of Biosciences, University of Kent, Canterbury) between October 2004 and November 2012; the environmental samples from Lomas Bayas were collected by Professor Luis Cáceras (University of Antofagasta, Antofagasta, Chile) in 2012 and 2014. Samples were collected aseptically with implements sterilised in the field with ethanol and placed into sterile polycarbonate bottles. Following transport to the UK all samples were stored at 4ºC.

Location descriptions

1. Cerro Chajnantor: this mountain is located within the volcanic Puricó Complex, a pyroclastic shield of ignimbrite. The Atacama Large Millimeter Array (ALMA) Observatory, constructed on the Chajnantor plateau (*ca.* 5000 masl), is accessed *via* an unpaved road from which sampling sites were determined at various altitudes. All but one of the sites lacked vegetation and all were given ALMA codings (Figure 2.1).

2. Laguna Chaxa, Salar de Atacama: the Salar is the largest salt flat in Chile at *ca.* 2300 masl and within which the Laguna is an area of open water bounded by highly crystalline salt encrusted soils. Samples (CHX) were collected from the hyper-arid halite soils, at the lake margin, one of the soil samples was colonised by cyanobacteria.

3. Yungay: the Yungay area is described as the extreme hyper-arid core of the Atacama Desert and as the closest analogue of Martian soils on Earth. A large proportion of the Yungay is super-rich in nitrates, while some of the slopes on the Cerros Aguas Blancas present evidence of flash flooding episodes in geological time. Two sites were sampled: nitrate rich soil from a tamarisk oasis (code Y2), and transect samples from the Cerros Aguas Blancas (CAB codings and Y6).

4. Valle de la Luna: an extreme hyper-arid area in the Cordillera de la Sal; the sample (VDL) was collected at one of the sand formation sites.

5. Paranal/Paposo: the sampling site was to the west of the optical-infrared Paranal Observatory adjacent to Route 5 linking Antofagasta and the coastal village of Paposo.

6. Lomas Bayas: another extreme hyper-arid area and a centre of copper mining located north-east of Antofagasta. Samples (LB) were collected at non-mining sites.

7. Laguna Miñiques: a high altitude brackish water lake located in the altiplano south-east of San Pedro de Atacama. Sediment samples (LM) were collected from the lake margin.

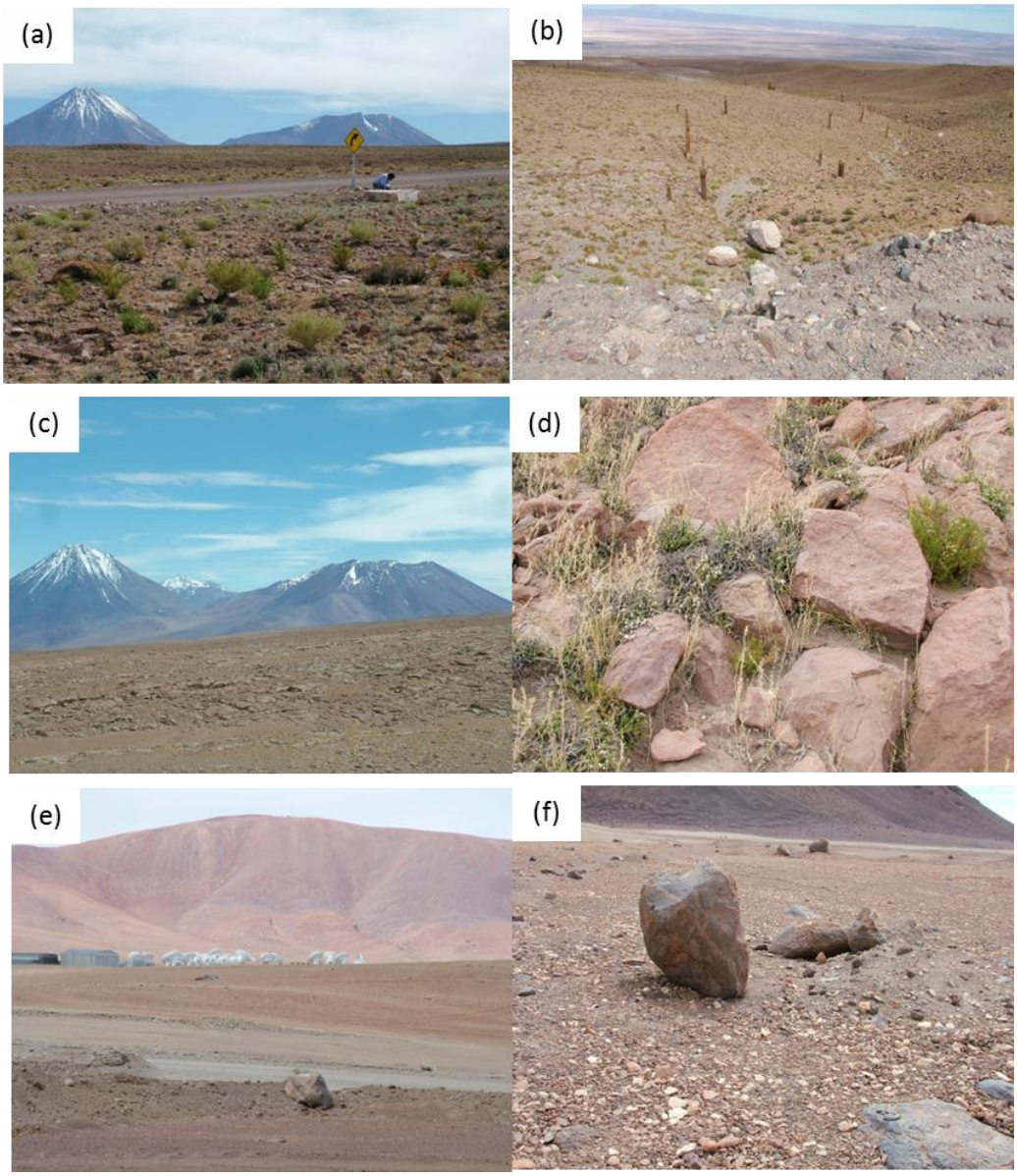

Figure 2.1 Cerro Chajnantor sampling sites at; 3018 masl (a, b), 4000 masl (c, d) and 5046 masl (e,f).

Table 2.1 Location, habitat and dates of collection of the environmental samples from the Atacama sampling locations and sites.

Location	Environmental sample		Collection date	Latitude (°S)	Longitude (°W)	Altitude (msl)	Habitat
Cerro Chajnantor	ALMA	1[1,2]	26.10.2012	23°04'39''	67°57'43''	3018	Surface soil (2cm)
		2[1,2]					Subsurface soil (30cm)
		3[1,2]		23°03'31''	67°52'27''	4000	Surface soil (2cm)
		4[1,2]					Subsurface soil (30cm)
		5[1,2]		23°00'49''	67°45'31''	5046	Surface soil (2cm)
		6[1,2]					Subsurface soil (30cm)
		8					Regolith
Lomas Bayas	LB	1[1]	26.10.2012	23°24'27.36''	69°31'3.8''	1500	Extreme hyper-arid soil
		3	27.2.2014				
Paranal/ Paposo	POP 2		31.10.2011	22°75'	68°42'	1945	Coarse gritty hyper-arid soil
Laguna Chaxa, Salar de Atacama	CHX	1	26.10.2012	23°17'33''	68°10'99''	2219	Hyper-arid halite surface soil (2cm)
		2		23°17'33"	68°10'99"	2219	Hyper-arid halite subsurface soil (30cm)
		3		23°17'36"	68°10'83"	2222	Hyper-arid halite soil enriched with cyanobacteria
Laguna Miñiques	LM	1	28.10.2012	23°45'55''	67°47'65''	3944	Sediment from lake margin

		2					Lake sediment
1.Yungay : Cerros Aguas Blancas	CAB	2		24º05’24.9’’	69º58’31.9’’	1016	Extreme hyper-arid soil (2cm)
		3		24º06’40.3’’	70º02’03.5’’		Extreme hyper-arid soil (2cm)
	Y6	1	13.11.2010	24º06’18.4’’	70º01’15.4’’	1047	Extreme hyper-arid surface soil (2cm)
		2		24º06’18.6’’	70º01’55.6’’	1033	Extreme hyper-arid subsurface soil (30cm)
		3		24º06’18.6’’	70º01’55.6’’		Extreme hyper-arid subsurface soil (100cm)
2. Tamarisk oasis	Y2		11.11.2010	24º04’50.5’’	69º55’08.3’’	949	Degraded tamarisk leaves and extreme hyper-arid nitrate soil
Valle de la Luna	VDL		5.10.2004	23º02’	68º20’	2450	Extreme hyper-arid sand (2cm)

All samples were included in the culture-independent study; [1] : Samples examined in the culture-dependent study; [2]: Samples used to acquire physico-chemical data.

2.2 Selective isolation of actinobacteria from ALMA environmental samples

Pilot experiment 1. $10^{-1/2}$ suspensions of environmental samples (1g) collected from the surface and subsurface ALMA sites on Cerro Chajnantor (Table 2.2) were prepared using ¼ strength Ringer's solution (Oxoid, UK). The resultant suspensions were blended using an Ultra-Turrax T25 homogenizer, shaken at level 4 on a tumble shaker (TMI Tumbler, Luckham Ltd., Sussex, UK) overnight and 10^{-1} to 10^{-4} dilutions prepared in ¼ strength Ringer's solution. Aliquots of each dilution (0.1ml) were spread, in triplicate, onto the isolation media listed in Table 2.2. All of the plates were left to dry for 30 minutes in a laminar flow hood prior to incubation, as recommended by Vickers and Williams (1987). The inoculated plates were incubated at 28°C for up to one month, during which representatives of the various actinobacterial colony types growing on the selective isolation plates were subcultured onto yeast extract-malt extract agar plates (Shirling and Gottlieb 1966), using sterile toothpicks, and incubated at 28°C for 14 days prior to preparing stock cultures.

Table 2.2 Selective media used in pilot experiments for the isolation of actinobacteria from the ALMA environmental samples.

Media	Selective agents	Target organisms
Arginine-vitamin agar*[2] (Nonomura and Ohara 1969)	Arginine (1g/L) and vitamins	Family *Streptosporangiaceae*
Gauze's No. 1 agar*[1,3,4] (Zakharova et al. 2003)	High carbon to nitrogen ratio	Rare or uncommon actinobacteria and *Streptomyces*
Gauze's No. 1 agar*[2] (Zakharova et al. 2003)	Nalidixic acid (25 µg/ml)	Rare or uncommon actinobacteria and *Streptomyces*
Glucose-yeast extract agar *[1] (Athalye et al. 1981)	High carbon to nitrogen ratio	*Actinomadura* spp.
Glucose-yeast extract agar *[2] (Athalye et al. 1981)	Rifampicin (25 µg/ml)	*Actinomadura* spp.
Humic acid-vitamin agar*[2] (Hayakawa and Nonomura 1987)	Humic acid (1g/L)	Family *Streptosporangiaceae*
Oligotrophic agar*[1,2] (Senechkin et al. 2010)	Low carbon and nitrogen sources	Rare or uncommon actinobacteria

* All of the media were supplemented with cycloheximide (25 µg/ml) and nystatin (25 µg/ml).

Key: [1,2,3,4] , Media used in pilot experiments 1, 2, 3 and 4, respectively.

Pilot experiment 2. The ALMA 2 environmental sample was chosen for further study based on the pilot 1 results. To this end, replicated environmental samples were heat pretreated at 100°C for 15 minutes and 120°C for 15 minutes, respectively. The heat pretreated environmental samples were then subject to a modification of the dispersion and differentiation centrifugation procedure (DDC., Hopkins et al. (1991)), as shown in Figure 2.2. $10^{-1/2}$ and 10^{-1} dilutions of the heat pretreated samples were prepared in ¼ strength Ringer's solution. Fractions derived from the modified DDC procedure and aliquots from the dilutions were used to inoculate the selective isolation media listed in Table 2.2, in each case three replicate plates were inoculated with 0.1ml aliquots of the various preparations. In addition, approximately 0.1g of each of the heat pretreated environmental samples were sprinkled directly onto each of the isolation media. All of the plates were left to dry for 30 minutes in a laminar flow hood prior to incubation, as recommended by Vickers and Williams (1987). The inoculated plates were incubated at 28°C for up to one month during which representatives of the various actinobacterial colony types growing on the selective isolation plates were subcultured and stock cultures prepared, as described for pilot experiment 1.

Pilot experiment 3. An environmental sample from the ALMA 2 site was heat pretreated at 120°C for 15 minutes and used to prepare $10^{-1/2}$ and 10^{-1} dilutions in ¼ strength Ringer's solution. In addition, the environmental sample was treated using the modified DDC procedure (Figure 2.2). Aliquots (0.1 ml) of the soil dilutions and DDC fractions were plated onto Gauze's No. 1 agar (Zakharova et al. 2003) supplemented with cycloheximide (25 µg/ml) and nystatin (25 µg/ml) and different combinations of antibiotics, as shown in Table 2.3. Three plates were prepared for each dilution and for the DDC fractions and, as before, were left to dry for 30 minutes in a laminar flow hood prior to incubation. In addition, approximately 0.1g of the heat pretreated sample was sprinkled directly onto each of the selective media. The inoculated plates were incubated at 28°C and 40°C for up to a month over the incubation period. The number of actinobacteria and total number of bacteria growing on some of the isolation plates expressed as the mean number of colony forming units (cfu's) per gram dry weight soil. Representatives of the various actinobacterial colony types growing on some of the selective isolation plates were subcultured and stock cultures prepared, as described for pilot experiment 1.

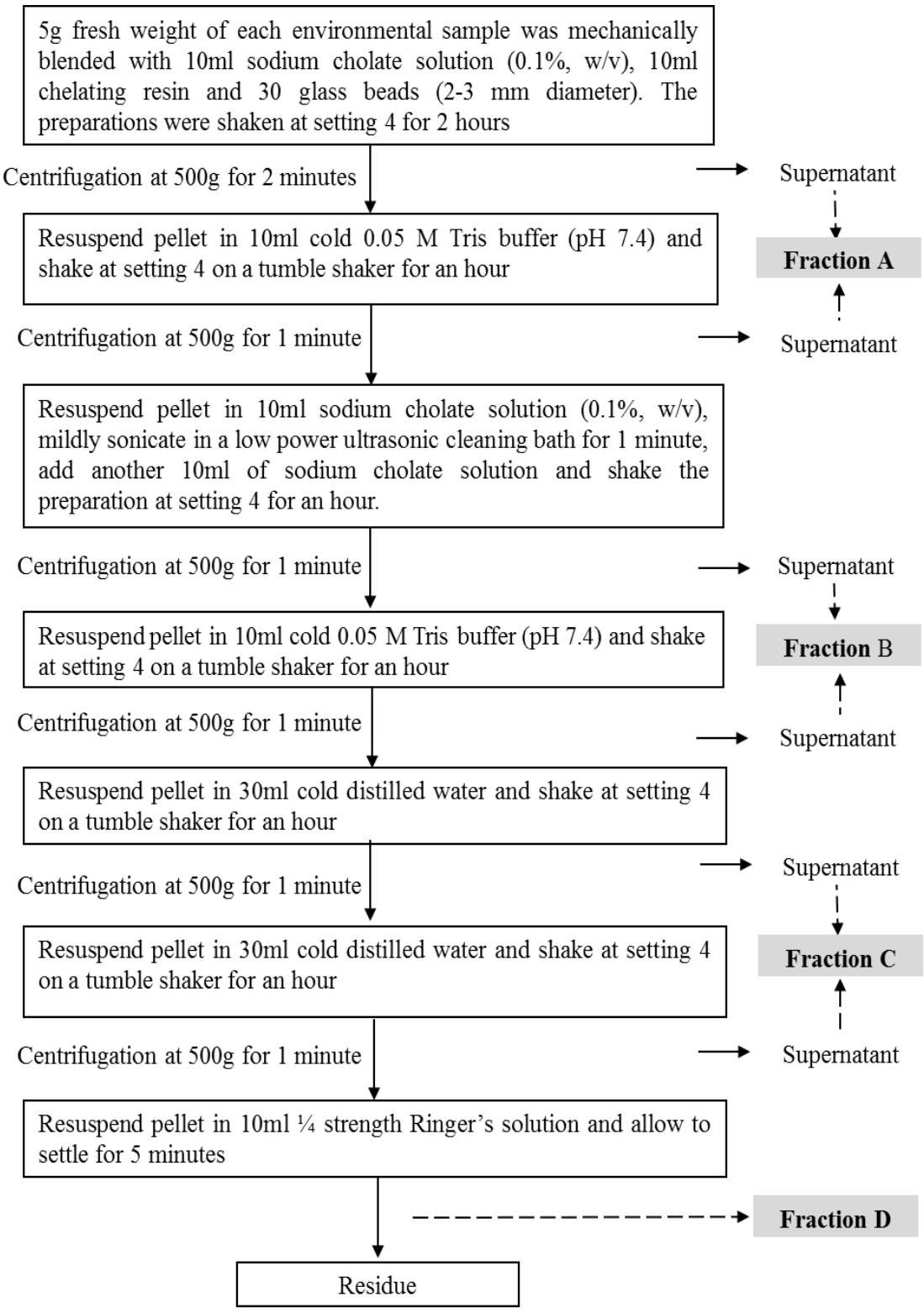

Figure 2.2 Dispersion and differential centrifugation procedure (modified from Hopkins *et al.*, 1991).

Table 2.3 Antibiotics used to supplement Gauze's No. 1 agar for the selective isolation of actinobacteria in pilot experiment 3.

Antibiotics	Target sites	References
Rifampicin (25 µg/ml)	DNA-dependant RNA polymerase	(Williams and Piddock 1998)
Rifampicin (25µg/ml) and nalidixic acid (25µg/ml)	DNA-dependant RNA polymerase DNA gyrase subunit	Williams and Piddock (1998) Sugino et al. (1977)
Rifampicin (25µg/ml) , nalidixic acid (25µg/ml) and gentamicin (10µg/ml)	DNA-dependant RNA polymerase DNA gyrase subunit and ribosome	Williams and Piddock (1998); Sugino et al. (1977) Tangy et al. (1985)

Pilot experiment 4. The selective isolation procedure used in this experiment was based on the results of the pilot 3 experiment. Environmental samples (1g) collected from ALMA 1 to 6 sites were heat pretreated at 120ºC for 15 minutes; $10^{-1/2}$ and 10^{-1} dilutions were then prepared in ¼ strength Ringer's solution, and aliquots (0.1ml) of the dilutions plated on Gauze No. 1 agar in each case supplemented with cycloheximide (25µg/ml) and nystatin (25µg/ml). One batch of the isolation medium was supplemented with rifampicin (25µg/ml) and nalidixic acid (25µg/ml) and the second one with rifampicin (25µg/ml) and nalidixic acid (25µg/ml) and gentamicin (10µg/ml). Three plates were prepared for each dilution and for each of the isolation media and the plates were left to dry for 30 minutes in a laminar flow hood, as before. All of the inoculated plates were incubated at 28ºC for 28 days. After incubation, the number of actinobacteria and total number of growing bacteria on the isolation plates were expressed as the mean number of cfu's per gram dry weight environmental sample. Representatives of the various actinobacterial colony types growing on the isolation plates were subcultured and stock cultures prepared, as described for pilot experiment 1.

Final selective isolation from ALMA surface samples. Actinobacteria were isolated from heat pretreated (50ºC for 40 minutes) and non-heat-pretreated environmental samples collected from the ALMA surface samples (ALMA 1, ALMA 3 and ALMA 5). Aliquots (0.1ml) of the $10^{-1/2}$ and 10^{-1} dilutions prepared in ¼ strength Ringer's solution were used to inoculate isolation plates supplemented with cycloheximide (25 µg/ml) and nystatin (25 µg/ml) and in some cases with antibacterial antibiotics as shown below:

1. Heat pretreated environmental samples:
    - Gauze's No. 1 agar (Zakharova et al. 2003) supplemented with nalidixic acid (25 µg/ml) and rifampicin (25 µg/ml)
    - Gauze's No. 1 agar (Zakharova et al. 2003) supplemented with gentamicin (10µg/ml) and nalidixic acid (25 µg/ml)
2. Non-heat pretreated environmental samples:
    - Gauze's No. 1 agar (Zakharova et al. 2003)
    - Glucose-yeast extract agar (Athalye et al. 1981)
    - Oligotrophic agar (Senechkin et al. 2010)

In addition, the isolation plates were inoculated by directly sprinkling soil particles (*ca*. 0.1g) from the environmental samples onto them. In all cases, three replica plates were prepared; all of the plates had been left to dry for 30 minutes in a laminar flow hood prior to incubation. The inoculated plates were incubated at 28^{o}C for up to a month. The number of actinobacteria and total number of bacteria growing on the isolation plates were expressed as the mean number of cfu's per gram dry weight environmental sample. Representatives of the various actinobacterial colony types growing on the isolation plates were subcultured and stock cultures prepared, as described for pilot experiment 1.

2.3 Selective isolation of actinobacteria from extreme hyper-arid Lomas Bayas soil sample

Environmental samples from Lomas Bayas (1g dry weight) were added to 4.5ml and to 9ml of ¼ strength Ringer's solution to give $10^{-1/2}$ and 10^{-1} dilutions which were blended using the Ultra-Turrax T25 homogenizer and then shaken overnight on the tumble shaker at level 4. Aliquots (0.1ml) of each dilution were spread over the isolation media listed in Table 2.4. In addition, soil particles (~0.1g) were sprinkled directly over the isolation media. In all cases triplicate plates that had been left to dry for about 30 minutes in a laminar flow hood were prepared, as described earlier. The inoculated plates were incubated at 28^{o}C for up to a month. The number of actinobacteria and the total number of bacteria growing on the isolation plates were expressed as the mean number of cfu's per gram dry weight environmental sample. Representatives of the various actinobacterial colony types growing on the isolation plates were subcultured and stock cultures prepared, as described for pilot experiment 1.

Table 2.4 Media used for the selective isolation of actinobacteria from the Lomas Bayas environmental samples.

Media	Selective agents	Target organism(s)
Arginine-vitamin agar* (Nonomura and Ohara 1969)	Arginine (1g/L) and vitamins	Family *Streptosporangiaceae*
Diagnostic Sensitivity agar (Orchard et al. 1977)	Tetracycline hydrochloride (32 µg/ml)	*Nocardia* spp.
Gauze's No. 1 agar* (Zakharova et al. 2003)	High carbon to nitrogen ratio	Rare or uncommon actinobacteria
Glucose-yeast extract agar * (Athalye et al. 1981)	High carbon to nitrogen ratio	*Actinomadura* spp.
Humic acid-vitamin agar* (Hayakawa and Nonomura 1987)	Humic acid (1g/L)	Family *Streptosporangiaceae*
Oligotrophic agar* (Senechkin et al. 2010)	Low carbon to nitrogen ratio	Rare or uncommon actinobacteria
Peptone-yeast-extract agar* (Goodfellow and Dawson 1978)	None, general purpose medium	Bacteria
Raffinose-histidine agar* (Vickers et al. 1984)	Raffinose and histidine	Rare *Streptomyces* spp.
SM1 agar* (Tan et al. 2006b)	Neomycin sulphate, D-sorbitol (4 µg/ml)	*Amycolatopsis* spp.
Starch-casein agar* (Küster and Williams 1964)	High carbon to nitrogen ratio	*Streptomyces* spp.
Tap water agar*	Nutrient deficient	Rare actinobacteria

* All of the media were supplemented with cycloheximide (25 µg/ml) and nystatin (25 µg/ml).

2.4 Selection, maintenance and colour-group assignment

Representatives of actinobacterial colony types were taken from the selective isolation plates and subcultured onto yeast extract-malt extract agar (ISP medium 2; Shirling & Gottlieb, 1966), using sterile toothpicks, and incubated at 28^{o}C for two weeks. Two hundred and nineteen colonies were taken from plates prepared from the ALMA environmental samples and 66 from the Lomas Bayas environmental samples. All of the subcultured strains produced leathery colonies covered by aerial hyphae; some formed diffusible pigments. Biomass of the isolates grown on ISP 2 agar (Shirling and Gottlieb 1966) for two weeks at 28^{o}C was used to prepare triplicate glycerol (20% w/v) stocks in screw-capped cryotubes. One of the glycerol stocks, the working culture, was stored at -20^{o}C; the remaining two were kept at -80^{o}C for long-term preservation.

Two hundred and eighty four actinobacterial strains were subcultured onto oatmeal (ISP medium 3; Shirling & Gottlieb, 1966) and peptone-yeast extract-iron extract agar plates (PYEA, 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 the production of melanin pigments on the peptone-yeast extract-iron agar plates.

2.5 Comparative 16S rRNA gene sequencing studies

DNA extraction. Genomic DNA was extracted from 48 representatives of colour-groups (Table 2.5) using a bead beating method, as described by Fujimoto et al. (2004). The isolates were grown on ISP 2 agar (Shirling & Gottlieb, 1966) for 2 weeks at 28^{o}C after which a loopful of biomass was scrapped from each of the cultures and transferred to Eppendorf tubes containing 3g of acid washed sterile glass beads (0.1 mm diameter; Sigma, UK) in 500µL sterile water. Following bead beating (Thermoscientific, UK) at 5.5 m/s for 30 seconds the preparations were kept on ice for a minute. This process was repeated twice followed by centrifugation for 2 minutes at 13000 rpm. The supernatants were combined then transferred to fresh tubes and the concentration of each DNA extract determined using a Nanodrop spectrophotometer (Thermoscientific, UK) and the preparations kept at -20^{o}C until required.

Gel electrophoresis. The quality of the DNA preparations was checked by agarose gel electrophoresis (1%, w/v agarose in 0.5x TBE, 40 minutes at 110V stained with 0.5 µg/ml ethidium bromide). The gels were loaded with 4µl preparations of crude DNA from each of the isolates, mixed with 2µl of loading dye (0.5 µg/ml, Sigma) and a Lambda DNA/HindIII marker (Gene Ruler ^{TM} MBI Fermentas, Vilnius, Lithuania); which was used to determine the sizes of DNA fragments. The gels were visualised and photographs taken on a BioRad^{TM} Multimager UV transilluminator.

PCR amplification. Amplification of the PCR products was achieved using a procedure modified from the one described by Busarakam (2014). To this end, each of the crude DNA preparations were used as DNA templates in a 25µl polymerase chain reaction (PCR). PCR preparations were set up using 2x MyFi Mix (Bioline, UK), which contained DNA polymerase, dNTPs, $MgCl_2$ (at optimized concentrations), and 20 µM of each forward and reverse primer (27f: AGAGTTTGATCCTGGCTCAG and 1525r: AAGGAGGTGATCCAGCC, respectively (Lane 1991), 1µ of 200ng DNA and sterile distilled water. The negative control was distilled water and the positive control DNA extracted from *Verrucosispora maris* DSM 45365^{T}. The PCR reactions were achieved as follows: initial denaturation at 95^{o}C for a minute, 30 cycles of 95^{o}C for a minute, 55^{o}C for a minute and 72^{o}C for a minute, and finally 72^{o}C for 5 minutes. The concentration of the PCR products were checked using the Nanodrop spectrophotometer and the quality of amplified 16S rRNA genes checked by gel electrophoresis using a 1kb DNA ladder (Gene Ruler ^{TM} MBI Fermentas, Vilnius, Lithuania) and the protocol mentioned above. The PCR products were stored in -20^{o}C until required.

Purification of PCR products. The PCR products were purified using ExoSAP-IT kits (USB, Corporation, Ohio, USA) by following the manufacturer's protocol. Each PCR product (5µl) was mixed with 2µl of ExoSAP-IT and the resultant preparations incubated first at 37^{o}C for 15 minutes and then at 80^{o}C for 15 minutes to degrade any remaining primers and nucleotides. The purified products were stored at -20^{o}C before being sent to GENEIUS, a Newcastle University spin out company, for sequencing.

Phylogenetic analyses. The standard 'in house' procedure was followed. To this end, each of the 16S rRNA gene sequences were edited using ABI format files in BioEdit version 7.2.5 (Hall 1999) and converted to FASTA format files. The chromatograms were checked and poor quality nucleotide sequences at the beginning and end of sequences

were deleted and unresolved nucleotides edited to 'N' manually. The nearest match of each of the edited sequences was established using the Basic Local Alignment Search Tool (BLAST) and pairwise sequence similarities calculated using the global alignment algorithm implemented using the EzTaxon-e web server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012b). Phylogenetic trees showing relationships between isolates and closely related marker strains were generated using the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms from MEGA6 software version 5.0 (Tamura et al. 2013). Bootstrap values based on 1000 repeats (Felsenstein 1985) were generated using the evolutionary distance model of Jukes and Cantor (1969).

2.6 Classification of presumptively novel actinobacteria

Selection and maintenance of strains. Twenty five strains known to form distinct branches in 16S rRNA gene trees were selected for further study to determine whether they merited recognition as novel species. Eleven of the strains were from the present study, the remainder consisted of twelve *Streptomyces* strains isolated by Busarakam (2014) and 3 strains isolated by Professor Martha Trujillo (University of Salamanca, Spain). The isolates, their source and associated marker type strains are shown in Table 2.5.

Table 2.5 Isolates and associated type strains included in the polyphasic taxonomic analyses.

Strains	Source/ strain histories
A. Genus *Actinomadura*	
Isolate:	H59[1,2,3,4]; ALMA 2 environmental sample
A.napierensis Cook et al. (2005)	DSM 44846 [T(3)]; A.E. Cook, B60; soil from South Africa
A.yumaensis Labeda et al. (1985)	DSM 43931 [T(3)]; D.P. Labeda, BK147; soil from Yuma County, Arizona, USA
B. Genus *Amycolatopsis*	
Isolates:	H5[1,2,3,4], H6[1,2,3,4]; ALMA 4 environmental sample
A.balhimycina Wink et al. (2003b)	DSM 44591 [T(3)]; J.M. Wink, FH 1894; soil from India

A.lexingtonensis Labeda et al. (2003)	DSM 44653 T(3); D.P. Labeda, LDDC 12275-99; equine placenta from Lexington, Kentucky, USA
A.kentuckyensis Labeda et al. (2003)	DSM 44652 T(3); D.P. Labeda, LDDC 9447-99; equine placenta from Kentucky, USA
A.mediterranei (Margalith and Beretta 1960) Lechevalier et al. (1986)	DSM 43304 T(3) ; K. Kieslich, ETH 28579; soil from Mediterranean area
A.pretoriensis Labeda et al. (2003)	DSM 44654 T(3) ; D.P. Labeda, ARC OV1 0181; equine placenta from Pretoria, South Africa
A.tolypomycina Wink et al. (2003b)	DSM 44544 T(3); J. Wink, FH6113; soil from Tokyo, Japan

C. Genus *Cryptosporangium*

Isolate:	H7(1,2,3,4); ALMA 4 environmental sample
C. arvum Tamura et al. (1998)	NBRC 15965 T(3); Tamura & Hatano, YU 629-21; soil from vegetable field, Japan
C. aurantiacum (Ruan et al. 1985) Tamura and Hatano (2001)	DSM 46144T(3); IMET 9261 T; KCC A-0241 T; IMAS, 4.1052 T; J.S. Ruan, 71-C38; lake mud, China
C. japonicum Tamura et al. (1998)	NBRC 15966 T(3); M. Hayakawa, YU 636-3; soil from sugar cane field, Okinawa Prefecture, Japan
C. minutisporangium (Ruan et al. 1985) Tamura and Hatano (2001)	NBRC 15962 T(3); ATCC 49415 T; IMRU LL-A-60; J.S. Ruan, A-60 ; soil, China
C. mongoliense Ara et al. (2012)	NBRC 105887 T(1,3); I. Ara, MN08-A0264; soil, Mongolia

C. Genus *Lentzea*

Isolate:	H45(1,2,3,4); ALMA 6 environmental sample
L. kentuckyensis Labeda et al. (2007)	DSM 44909T(3); NRRL B-24416 T; J.M. Donahue, LDDC 2876-05; equine placenta, USA

D.* Genus *Pseudonocardia

Isolates:	ATK01 MT (1,2,3,4), ATK03 MT (1,2,3,4), ATK17 MT (1,2,3,4); CAB3 environmental sample H57(1,2,3,4), H58(1,2,3,4), H69(1,2,3,4) ; ALMA 2 environmental sample H96(1,2,3,4); ALMA 3 environmental sample H99(1,2,3,4); ALMA 4 environmental sample
P. adelaidensis Kaewkla and Franco (2010)	DSM 45352T(3); Kaewkla & Franco, EUM221; grey box tree (*Eucalyptus microcarpa*)

P. bannensis Zhao et al. (2011a)	DSM 45300 $^{T(3)}$; G.Z. Zhao, YIM 63101; surface-sterilized roots of *Artemisia annua*
P. khuvsgulensis Ara et al. (2011)	NBRC 105886 $^{T(3)}$; I. Ara, MN08-A0297; soil of Khuvsgul Lake, Mongolia
P. kunmingensis Zhao et al. (2011b)	DSM 45301$^{T(3)}$; G.Z. Zhao, YIM 63158; surface-sterilized roots of *Artemisia annua*
P. petroleophila (Hirsch and Engel 1956) Warwick et al. (1994)	DSM 43193$^{T(3)}$; NCIMB 9438^{T}; P. Hirsch, 102; soil, Germany
P. rhizophila Li et al. (2010)	DSM 45381$^{T(3)}$; J. Li, YIM 67013; rhizosphere soil, China
P. xinjiangensis (Xu et al. 1999) Huang et al. (2002)	DSM 44661 $^{T(3)}$; CIP 107366^{T}; CCTCC AA 97020 ^{T}L.H. Xu, XJ-45; soil, China
P. zijingensis Huang et al. (2002)	DSM 44774$^{T(3)}$; AS 4.1545 ^{T}; Y. Huang, 6330; soil, China
E. Genus *Streptomyces*	
Isolate:	H9$^{(1,2,3,4)}$; ALMA 4 environmental sample
S. melanogenes Suguwara and Onuma (1957)	DSM 40192 $^{T(2,3)}$; Suguwara & Onuma; soil, Japan
S. noboritoensis (Isono et al. 1957)	NRRL B- 12152^{T} $^{(2,3)}$; soil from Inada-noborito, Kawasaki City, Kanagawa Prefacture, Japan
S. polyantibioticus le Roes-Hill and Meyers (2009)	DSM 44925$^{T(3)}$; le Roes-Hill & Meyers, SPR; river bank, South Africa
Isolates:	C59 $^{KM\ (2,3)}$, KNN 26.b $^{KM\ (3)}$, KNN 38.1b $^{KM\ (2,3)}$, KNN 64.5b$^{KM\ (2,3)}$; Salar de Atacama environmental sample
Streptomyces fimbriatus (Millard and Burr 1926) (Waksman 1953)	NRRL B-3175 $^{T(2,3)}$; Millard & Burr, AS 4.1598; isolated from common potato scab
Isolates:	KNN 6.11a $^{KB\ (2,3)}$, KNN 35.1b $^{KB\ (2,3)}$, KNN 35.2b $^{KB\ (2,3)}$, KNN 42.f $^{KB\ (3)}$, KNN 48.3 $^{KB(3)}$, KNN 83.e $^{KB\ (3)}$; Salar de Atacama environmental sample
Streptomyces ghanaensis (Wallhäusser et al. 1966)	NRRL B-12104 $^{T(2,3)}$; A.G. Hoechst, FH 1290; soil, Ghana

Isolates examined for: [1], cultural properties; [2], chemotaxonomic markers; [3], phenotypic tests; [4], spore chain arrangement and spore surface ornamentation.

KB, and MT, strains isolated by Kanungnid Busarakam and Martha Trujillo, respectively.

2.7 Acquisition of phenotypic properties

2.7.1 Test strains and data acquisition

A broad range of standard phenotypic tests (Table 2.6) were carried out on the 25 strains isolated from the ALMA, Salar de Atacama and Yungay environmental samples together with associated marker strains. Details of the test media can be found in Appendix 2, the underlying rationale of the biochemical and degradation tests was spelt out by Busarakam (2014). The balance of the tests was carried out using API ZYM kits (Biomeriuex Co., Hampshire, UK) and GEN III MicroPlates (BIOLOG Inc., Hayward, CA, USA).

2.7.2 Inoculation of test media

The biochemical, degradation and tolerance tests were carried out in Replidishes (Sterilin Ltd., Staffordshire, UK) at 28ºC unless stated otherwise, and the results recorded after the 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 suspensions of spores and hyphae of each strain. Inocula were prepared by scraping spores/hyphal fragments from ISP 2 agar plates (Shirling and Gottlieb 1966), which had been incubated at 28ºC for 14 days, into 1 ml of ¼ strength Ringer's solution held in bijou tubes. The preparations were ground using sterile micro-pestles in Eppendorf tubes and resuspended in 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 following gentle agitation to achieve an even suspension. An automatic multipoint inoculator (Denley-Tech; Denley Instrument Ltd., Sussex, UK) was used to inoculate the Replidishes. All of the tests were carried out in duplicate. Details of the composition of the test media are given in Appendix 2.

Table 2.6 Phenotypic tests.

Type of test	Tests
A. Biochemical tests (w/v):	Aesculin hydrolysis (0.1%) [r], Allantoin hydrolysis (0.1%) [b], Arbutin hydrolysis (0.1%) [r], Hydrogen sulphide production [b], Nitrate reduction [b], Urease production [r]
B. Degradation tests (%, w/v):	Adenine (0.5) [r], Casein (1) [r], Cellulose (1%) [r], Chitin (0.4%) [r], Elastin (0.3), Guanine (0.3) [r], Hypoxanthine (0.4), Starch (0.1) [r], Tributyrin (0.1%) [r], Tween 20 (1% v/v) [r], Tween 40 (1% v/v), Tween 60 (1% v/v) [r], Tween 80 (1% v/v) [r], L-Tyrosine (0.4) [r], Uric acid (0.4) [r], Xanthine (0.4) [r], Xylan (0.4%) [r]
A. Tolerance tests	
1. pH	4.0 [r], 5.0 [r], 6.0 [r], 8.0 [r], 9.0 [r] and 10.0 [r]
2. Temperature	4 [r], 10 [r], 20 [r], 30 [r], 40 [r], 45 [r] and 50°C [r]

Carried out in [b], bijou tubes or [r], Replidishes.

2.7.3 Biochemical, degradation and tolerance tests

The allantoin and urea hydrolysis tests were carried out using the procedures described by Gordon *et al.* (1974) and aesculin and arbutin hydrolysis after Kutzner (1976) and Williams *et al.* (1983), respectively; in all cases results were recorded after 7 and 14 days. The ability of the strains to produce hydrogen sulphide was recorded after 7 days following the procedure described by Küster and Williams (1964) and nitrate and nitrite reduction after 14 days using the medium and method used by Gordon and Mihm (1962).

Most of the degradation tests were carried out using modified Bennett's agar (Jones 1949) as the basal medium. Adenine, casein, elastin, guanine, hypoxanthine, L-tyrosine, uric acid, xanthine and xylan were added to the basal medium at the appropriate concentrations (Table 2.6) with care taken to ensure an even distribution of these insoluble compounds. The tests were read weekly up to 21 days and clearing of the insoluble compounds from under and around areas of growth recorded as positive results. Cellulose degradation was recorded after 14 days following the addition of Congo red solution (0.1%, w/v) (Appendix 3) to Replidish wells, pale yellow to straw coloured zones of clearing from under and around areas of growth within 15 minutes were recorded as

positive results, undigested carboxymethylcellulose stains pinky red once excess reagent is discarded. Similarly, the basal medium supplemented with soluble starch (1%, w/v) was used to detect starch degradation, positive results were recorded when zones of clearing from around areas of growth were apparent following the addition of Lugol's iodine (Sigma, UK) to 14 day colonies, undigested starch stains blue black.

The balance of the degradation tests was based on well-established procedures. Colloidal chitin agar was used to detect chitinolytic activity (Hsu & Lockwood, 1975), zones of clearing around test strain growth within 4 weeks were recorded as positive results. Sierra's medium (1957) was used to test for the ability of the strains to produce esterases that metabolize Tweens 20, 40, 60 and 80, positive results were recorded by the appearance of insoluble calcium salt precipitates in the agar media. The degradation of glycerol tributyrin (Sigma T3688), due to the production of esterases, was sought after 14 days, zones of clearing around test strain growth due to the release of water soluble butyric acid are recorded as positive results.

Tolerance tests were done using ISP 2 agar (Shirling & Gottlieb, 1966) as the basal medium, apart from the *Pseudonocardia* strains, which were examined on modified Bennett's agar (Agrawal, unpublished). The ability of the isolates and marker strains to grow at several pH values and sodium chloride concentrations (Table 2.6) was examined; the pH values were adjusted using relevant buffers (Appendix 3). Growth after incubation for 21 days was recorded as a positive result. The ability of strains to grow at a range of temperatures was tested and inoculated Replidishes incubated and read weekly for up to 6 weeks at 4^{o}C and 10^{o}C and for 2 weeks at the remaining temperatures. The Replidishes incubated at 40^{o}C, 45^{o}C and 50^{o}C were placed into plastic bags to prevent the media drying out.

2.8 Data acquired using API kits and BIOLOG microplates

API ZYM kits. The isolates and marker strains were grown on ISP 2 agar plates (Shirling and Gottlieb 1966) for 7 days at 28^{o}C. Biomass scrapped from the inoculated plates was transferred to sterile disposal bijoux bottles containing 2 ml of sterile ¼ strength Ringer's solution, the resultant preparations were emulsified until turbidity readings were obtained that were equivalent to McFarland scale 5.0 (Murray *et al*., 1999). Aliquots (65µl) of the spore/mycelial suspensions were added to the cupules of each test strip using pipettes and the latter incubated at 28^{o}C overnight when single drops of reagents ZYM A (Tris-HCl and sodium lauryl sulphate in water) and ZYM B (diazonium salt Fast Blue BB in 2-

methoxyethanol) were added to each cupule. The strips were developed in strong light for 5 minutes to remove any yellow colour in the cupules due to excess of unreacted Fast Blue BB. Enzymatic activity was detected by the colour changes shown in Table 2.7, as recommended by the manufacturer. To this end, 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 recorded as negative (0) and positive (1), respectively.

Table 2.7 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-Naphthyl butyrate	6.5	Violet	
4	Esterase lipase (C8)	2-Naphthyl caprylate	7.5	Violet	
5	Lipase	2-Naphthyl 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	
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	
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	

GEN III MicroPlate^{TM} BIOLOG tests. The ability of the test strains to metabolize a broad range of carbon compounds, to grow in the presence of antibiotics, heavy metals, dyes, over a range of pH values and in the presence of several concentrations of sodium chloride were determined using GEN III Microplates which were incubated in an Omnilog instrument (BIOLOG Inc., Hayward, CA, USA). The *Pseudonocardia* strains were grown on modified Bennett's agar (Agrawal, unpublished) and the remaining strains on ISP 2 agar (Shirling and Gottlieb 1966). Cell suspensions of the test strains were prepared using a "gelling" inoculation fluid and the cell densities adjusted to 98% transmittance. The microplates were then inoculated with the cell suspensions and run in Phenotype Microarray mode at 28°C for 7 to 14 days depending on the speed by which each tested strain gave positive results. The exported data were analyzed with the *opm* package for R (Vaas *et al.,* 2012; 2013) v.1.0.6. Carbon source utilization and chemical sensitivity assays (Table 2.8) were determined as described by Mohammadipanah et al. (2014).

2.9 Cultural and morphological properties

Growth on ISP media. Fourteen isolates (Table 2.5) and their associated marker strains were examined for cultural properties following growth on tryptone-yeast extract, yeast extract malt-extract, oatmeal, tryptone-yeast extract, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1 to 7; Shirling and Gottlieb (1966)) for 2 weeks at 28^{o}C. Aerial spore mass, substrate mycelial and diffusible pigment colours were recorded using National Bureau of Standards (NBS) Colour Name Charts (Kelly 1958) and the ability to form melanin pigments was recorded from the peptone-yeast extract-iron agar plates.

Scanning electron microscopy. Isolates H5, H5, H7, H9, H45 and H59 (Table 2.8) were grown on oatmeal agar (Shirling and Gottlieb 1966) at 28^{o}C for 21 days and then examined for spore chain arrangement and spore surface ornamentation. Preparations were observed by examining gold-coated dehydrated specimens using a scanning electron microscope (Cambridge Stereoscan 240 instrument), as described by O'Donnell et al. (1993).

Table 2.8 Colourimetric carbon utilization and tolerance tests included in GEN III Microplates.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
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 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
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
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	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
H1 Tween 40	H2 γ-Amino-Butryric Acid	H3 α-Hydroxy-Butyric 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 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

2.10 Chemotaxonomy

Test strains. Twenty isolates and 4 type strains (Table 2.5) were selected and examined for key chemical markers known to be of value in actinobacterial systematics (Goodfellow et al. 2012a).

Chemotaxonomic markers. The detection of the isomers of diaminopimelic acid, predominant menaquinones, whole-organism sugars and diagnostic polar lipids were based on standard procedures recommended, respectively by Staneck and Roberts (1974), Collins et al. (1985), Lechevalier and Lechevalier (1970) and Minnikin et al. (1984), as modified by Kroppenstedt and Goodfellow (2006). In all cases in house protocols were followed, as shown in Appendix 4.

Cellular fatty acids of isolates H7, H9, H45 KNN35-1b and KNN35-2b and the type strains of *S. fimbriatus, S. ghanaensis, S. melanogenes* and *S. noboritoensis* were determined by the identification service at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Fatty acid methyl esters (FAMES) were obtained from biomass samples (40mg) by saponification, methylation and extraction following the procedure described by (Miller 1982) with minor modifications from Kyukendall *et al.* (1998). The FAMES were separated by gas chromatography (Agilent 6890N instrument) and the resultant peaks automatically integrated; fatty acid names and percentages were determined using the standard Microbial Identification (MIDI) system, version 5 (Sasser, 1990). Similarly, the cellular fatty acids of *Streptomyces* isolates KNN35-1b, KNN35-2b and KNN42f were extracted, methylated and determined by gas chromatography (Hewlett Packard Instrument 6890) and analysed using the MIDI system, version 5, these analyses were carried out by Dr. Byung-Yang Kim (ChunLab, Seoul, South Korea).

2.11 Screening for bioactivity

Test strains. The 284 isolates from the ALMA and Lomas Bayas environmental samples were screened against a panel of wild type microorganisms and *Bacillus subtilis* reporter strains as shown below.

Plug assays. The isolates were grown on both ISP 2 and ISP 3 agar (Shirling and Gottlieb 1966) for 14 days at 28^{o}C. Antimicrobial screening was achieved using a

standard agar plug assay (Fiedler 2004) against wild type strains of *Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Saccharomyces cerevisiae* and *Staphylococcus aureus*. Agar plugs were taken from the cultures using the back of sterile blue tips and arranged in square Petri dishes (23cm x 23cm, Sigma-Aldrich, UK). Bacterial and yeast lawns were prepared by inoculating 100µL of overnight cultures grown in Luria Bertani broth into a medium containing 100mL of Luria Bertani broth and nutrient agar, mixing well and pouring into the square Petri dishes that contained the plugs prepared from the ISP agar plates. The plates were incubated at 37ºC overnight and examined for the presence of inhibition zones around the agar plugs, the diameters of the inhibition zones were recorded.

***Bacillus subtilis* reporter strains.** Isolates were grown on ISP 2 agar (Shirling and Gottlieb 1966) for 14 days at 28ºC. Screening against six *Bacillus subtilis* reporter strains (Table 2.9) was achieved using the standard agar plug assay, as outlined above. The test media were supplemented with 100µL of X-gal to give a final concentration of 50µg/mL. For each of the erythromycin resistant reporter strains, 100µL erythromycin (100µg/mL) was added to the prepared media while chloramphenicol (100µg/mL) was added to the media for the *B. subtilis phi*105^{CH} reporter strain. The assays were carried out in square Petri dishes (23cm x 23cm) which were incubated at 37ºC overnight. 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 37ºC and examined for blue rings around the filter paper discs (Brunstein 2010). The plates were examined for the presence of blue halos around inhibition zones; the blue halos are produced when 5-bromo-4-chloro-3-hydroxyindole is formed as a result of X-gal cleavage due to induction of bioactive substances by the reporter genes.

Table 2.9 *Bacillus subtilis* reporter strains and positive controls used in the plug assays to determine the modes of action of unknown antimicrobial compounds.

Reporter genes	Positive controls	Targets
*yvq*I^{ER}	Bacitracin	Cell wall synthesis
*yvg*S^{ER}	Rifampicin	RNA synthesis
*ypu*A^{ER}	Cefoxitin	Cell envelope synthesis
*Phi*105^{CH}	Nalidixic acid	DNA synthesis
*yja*X^{ER}	Triclosan	Fatty acid synthesis
*yhe*H	Tetracycline	Sporulation

^{ER}: erythromycin resistant, ^{CH}: chloramphenicol resistant.

2.12 Culture-independent study of actinobacterial diversity in Atacama Desert environmental samples

2.12.1 Preparation of environmental samples for pyrosequencing

Community DNA extraction. Community DNA was extracted from all of the environmental samples (Table 2.1). Each sample (0.5 grams) was ground in a sterile pestle and mortar, DNA extracted using an UltraClean Soil DNA extraction kit (MO BIO Laboratories, Inc., USA), using the manufacturer's protocol, and stored at -20ºC. The quality of the community DNA preparations was checked using agarose gel electrophoresis (1%, w/v, agarose in 0.5x TBE, 40 minutes at 110 V), as described earlier.

Assignment of barcodes. Actinobacterial specific regions in the community DNA preparations were amplified using specific actinobacterial primers, namely Com2xf (5'-AAA CTC AAA GGA ATT GAC GG-3') and Ac1186r (5'- CTT CCT CCG AGT TGA CCC-3') according to the protocol of Schäfer et al. (2010). A sample-specific 10-bp barcode was attached to the Com2xf primer. Four replicate samples were prepared from each of the environmental samples, the latter were assigned individual barcodes.

PCR conditions. Polymerase chain reaction preparations were set up using 2x MyFi Mix (Bioline, UK) which contained DNA polymerase, dNTPs, $MgCl_2$ at optimized concentrations, 20 µM of each forward and reverse primer Com2xf and Ac1186r, respectively, (Schäfer et al. 2010), DNA extracted from *Verrucosispora maris* DSM 45365^T was used as the positive control and sterile distilled water as the negative control. The controls were included in each PCR reaction, the latter were carried out under the following conditions: initial denaturation at 94ºC for a minute, 30 cycles of 94ºC for a minute, 60ºC for 30 seconds and 72ºC for 30 seconds, and finally 72ºC for 5 minutes. The quality of the amplified products was established using agarose gel electrophoresis (1%, w/v, agarose in 0.5x TBE, 40 minutes at 110 V) with a 100kb DNA ladder (Gene Ruler ^{TM} MBI Fermentas, Vilnius, Lithuania) as marker, as described earlier. The preparations were kept at -20ºC prior to use.

Purification of PCR products and quality check. The PCR products were purified using the Agencourt AMPure XP PCR Purification System (Beckman Coulter, U.S.A.)

and MagnaRack (Thermo Fisher Scientific Inc., U.S.A), according to the protocols of the manufacturers (Figure 2.3). The concentration of each sample was quantified using a Qubit® Fluorometer (Thermo Fisher Scientific Inc., U.S.A) to match the minimum concentration of 5 µg/mL. Samples were pooled using an equal DNA concentration of each sample to give a final concentration of 100µg/mL. The quality of the samples were checked using an Agilent bioanalyzer at Newgene Ltd. (International Centre for Life, Newcastle upon Tyne).

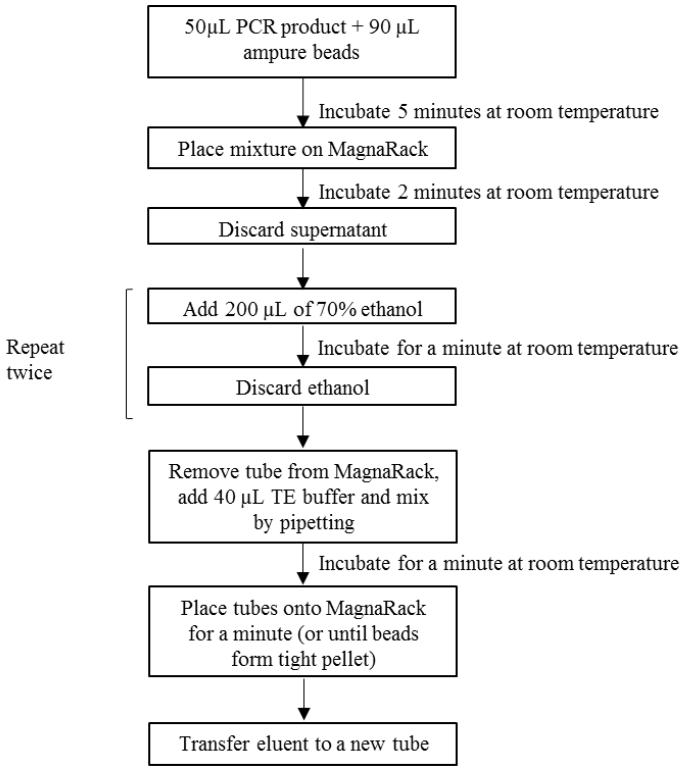

Figure 2.3 Purification of PCR products using AMPure beads and MagnaRack.

Roche 454 sequencing. The prepared amplicon pool of PCR products was sent to the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois in Urbana-Champaign. The pool was quantified using a Qubit fluorometer (Invitrogen, CA) and average fragment sizes were determined by analyzing 1 µl of the library on a Bioanalyzer (Agilent, CA) using a High-Sensitivity DNA LabChip. Gel-size selection was performed on the pool to remove chimeras seen in the DNA LabChip trace file and QC repeated to ensure proper size selection.

Libraries were diluted to $1x10^6$ molecules/µl for sequencing. Emulsion-based clonal amplification and sequencing on the Roche 454 Genome Sequencer FLX+ system was performed according to the manufacturer's instructions (454 Life Sciences, Branford, CT, USA). The library was sequenced at designated regions of a 70x75 PicoTiter Plate

using flow pattern A with the Roche XL+ sequencing kit (454 Life Sciences) and software version 2.9. Signal processing and base calling were performed using the bundled 454 Data Analysis Software version 2.9 for amplicons.

2.12.2 Processing of pyrosequencing data

Bioinformatic analyses. The raw sequences generated from the pyrosequencing step were sent to ChunLab, Seoul, Korea (www.chunlab.com) for processing using a series of bioinformatic and statistical pipelines (Figure 2.4), as described by Jeon et al. (2013). Barcodes and primer sequences present in each raw dataset were trimmed followed by quality filtering and denoising where low quality sequences and those with read length <270bp were removed. Each read was then identified using a robust global pairwise sequencing alignment and BLASTN against the EzTaxon-e database (Kim *et al.*, 2012a; www.ezbiocloud.net/eztaxon), the taxonomic classification cut-off points were: ≥ 94% similarity for generic assignments, ≥ 90% for family assignments, cut-off values below these levels were designated as 'unclassified' while sequences that did not match 97% similarity to the ExTaxon-e database were the subject of chimera checks. Chimera sequences formed during the PCR step were detected and removed from the dataset by UCHIME (Edgar et al. 2011). To determine operational taxonomic units (OTUs) the 97% cut-off value was used and diversity indices were calculated, as described in Edgar et al. (2011). Data files were obtained for each sample designated as clc (*.clc) files which were then analysed using the CLCommunity v3.30 program (ChunLab 2014).

2.12.3 Data analyses

Alpha diversity indices. Alpha diversity indices calculated in the bioinformatics pipeline (Figure 2.3) during sequence processing were included in the clc (*.clc) files which were obtained using the <Alpha-diversity> menu in the CLCommunity program (ChunLab 2014).

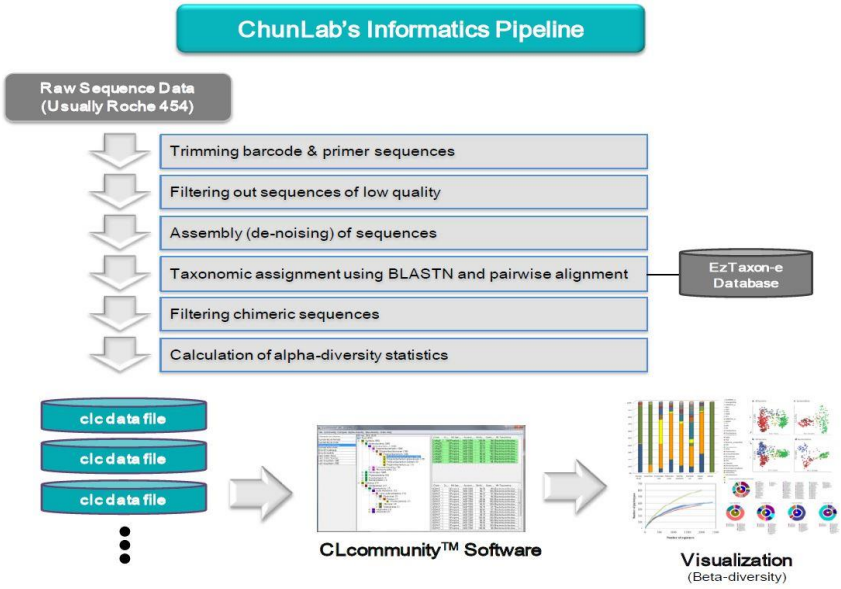

Figure 2.4 ChunLab bioinformatics pipeline (ChunLab 2014).

Taxonomic composition. Analyses were carried out using features available in the CLCommunity program (ChunLab 2014), to determine the actinobacterial diversity and community structure in the Atacama Desert environmental samples. These analyses allowed a comparison of species richness and the number of OTUs in each environmental samples; results were presented as rarefaction and rank abundance curves. The taxonomic composition of actinobacteria detected in each of the environmental samples were analysed and presented in appropriate graphs.

Actinobacterial community structure. Operational taxonomic unit (OTU) tables were generated using the <Taxonomic Composition> menu from the CLCommunity program (ChunLab 2014). The results in the OTU tables provided information on taxonomic affiliations and the ratio and number of reads assigned to each OTU. The OTU tables were saved in comma delimited (.csv) format prior to further analysis. In the case of the ALMA environmental samples, identified genera were checked and identified manually from the OTU table prior to analysis as outlined below.

Venn diagrams. Shared richness in the observed operational taxonomic units (OTUs) were calculated and Venn diagrams generated from the raw sequence data using the venn () command in the Mothur v1.37.4 program (http://www.mothur.org/).

Heat map. The OTUs detected in the ALMA environmental samples were grouped into clusters based on their data values by hierarchical clustering using the Bray-Curtis dissimilarity matrix (Yang et al. 2012), this analysis was performed using *R* package vegan. A heatmap was generated using *R* package heatplus from Bioconductor (Ploner 2015).

Multivariate statistical analyses. These analyses were used to evaluate the β-diversity of actinobacterial community composition in the ALMA 1 to 6 environmental samples based on their taxonomic differences and the influence of environmental variables (Phommasack 2009). The OTU table generated using the CLCommunity program is suitable for analysis using linear method, were standardised to zero mean and unit standard deviation prior to analyses using the *R* package vegan. To observe the actinobacterial community composition in each of the ALMA environmental samples and to find similarities between them, unconstrained ordination, principle component analysis (PCA) was used. In these analyses, complex data from the OTU table were summarized and reduced to a smaller number thereby facilitating the interpretation of data (Ramette 2007; Van den Brink et al. 2003; Paliy and Shankar 2016; Armstrong and Hilton 2011). The results were displayed as biplots which showed relationships between the environmental samples and genera based on the sampling sites and detected actinobacterial genera. Unconstrained ordination, redundancy analysis (RDA) was carried out to determine the effect of environmental factors (altitude, conductivity, organic matter [OM], pH and redox potential) on actinobacterial communities in the ALMA environmental samples. In this analysis, the best fit of the response variables (community structure) to the explanatory variables (environmental parameters) was establish followed by a permutation test to observe the significance of the relationships. Data were represented as a biplot with sample plots and arrows showing the direction and importance of each environmental variable. Positive correlations between the environmental variables were indicated by arrows pointing in the same direction; negative correlations were indicated by arrows pointing in the opposite site and arrows at 90 degrees to one another indicated the absence of any correlation (Ramette 2007; Paliy and Shankar 2016).

Chapter 3. Biosystematic studies of actinobacterial strains isolated from the Cerro Chajnantor and Lomas Bayas environmental samples

3.1 Abstract

Filamentous actinobacteria were isolated from high altitude and extreme hyper-arid Atacama Desert soils using several selective isolation procedures. Representatives of colony types growing on the isolation plates were assigned to numerous multi-and single-membered colour-groups based on their ability to form pigments on oatmeal and peptone-yeast extract –iron agar plates. Phylogenetic analyses showed that most of the strains representing colour-groups formed distinct lineages in *Kineococcus, Microbispora, Nocardiopsis* and *Streptomyces* 16S rRNA gene trees though many of the remaining isolates had colonial characteristic typical of *Streptomyces.* A majority of the isolates examined in standard plug assays inhibited the growth of one or more of a panel of wild type microorganisms while some of those screened against *Bacillus subtilis* reporter strains inhibited sporulation and cell envelope, cell wall, DNA and fatty acid synthesis. It is clear that taxonomically diverse filamentous actinobacteria from high altitude and extreme hyper-arid Atacama Desert soils have the capacity to synthesize a broad range of bioactive compounds.

3.2 Introduction

Until recently, little interest was shown in the microbiology of non-polar deserts even though they account for nearly 20% of the landmass of the planet. Nevertheless, small numbers of taxonomically diverse actinobacteria have been recovered from geographically separated non-polar desert soils, as exemplified by studies on the Amargosa (Luedemann 1968), Mojave (Garrity et al. 1996), Sahara (Meklat et al. 2011), Taklamaken (Luo et al. 2012b) and Thar (Tiwari et al. 2015) deserts. In addition to the ever present streptomycetes studies such as those have reported the presence of poorly studied genera classified in the families *Geodermatophilaceae, Pseudonocardiaceae, Streptosporangiaceae* and *Thermomonosporaceae,* as cited in Chapter 1. In a particularly interesting study Meklat et al. (2011) found that 26 out of 52 halophilic actinobacteria from Saharan soils showed activity against one or more of a panel of pathogenic

microorganisms. The greatest activity was shown by *Actinopolyspora, Nocardiopsis, Saccharomonospora* and *Streptomonospora* strains, some of which contained genes encoding for polyketide and nonribosomal peptide synthases. Members of other neglected genera, such as *Saccharothrix*, have been found to synthesize novel specialized metabolites following their isolation from non-polar desert soil (Zitouni *et al.*, 2004a, 2004b).

Although actinobacteria featured in the earliest studies on the microbiota of Atacama Desert soils it is only within the past decade that they became the focus of substantial studies, initially on their numbers and taxonomic provenance in hyper- and extreme hyper-arid soils (2010; Okoro et al. 2009) and later on their ability to synthesize new bioactive compounds (Bull and Asenjo 2013; Bull et al. 2016). The most extensive biosystematic studies on Atacama Desert actinobacteria were undertaken by Busarakam (2014) who, in addition to streptomycetes, detected putatively novel species of rare and poorly studied genera, such as *Actinomadura, Amycolatopsis, Couchiplanes, Kribbella, Modestobacter, Pseudonocardia* and *Saccharothrix*, based on extensive 16S rRNA sequencing studies. Polyphasic studies on several of these isolates led to valid or emended publications of *Amycolatopsis, Modestobacter* and *Streptomyces* species (2016b; Busarakam et al. 2014; 2016a). Representative strains isolated by Busarakam (2014) were shown to inhibit the growth of wild type microorganisms while selected streptomycetes were found to inhibit cell envelope, cell wall, fatty acid and RNA synthesis in plug assays based on the use of *Bacillus subtilis* reporter strains.

The aim of the present study was to build upon and extend the aforementioned pioneering work on Atacama Desert actinobacteria (Busarakam 2014; Okoro et al. 2009). To this end, actinobacteria isolated from high altitude and extreme hyper-arid Atacama Desert soils were characterized and screened for bioactive compounds using the taxonomic approach to drug discovery recommended by Goodfellow and Fiedler (2010).

3.3 Materials and Methods

3.3.1 Enumeration, selective isolation and preservation of actinobacteria isolated from environmental samples collected from the Cerro Chajnantor and Lomas Bayas sampling site

Representative actinobacteria were isolated from the sampling sites using a range of selective isolation procedures, as described in Chapter 2, page 38 to 42. Where practical the numbers of actinobacteria per gram dry weight soil were expressed as a percentage of the total bacterial counts. Representatives of the various colony types of actinobacteria growing on the selective isolation plates were subcultured onto yeast extract-malt extract agar plates (ISP 2 medium; Shirling & Gottlieb, 1966) and incubated for 2 weeks at 28ºC. Biomass taken from the incubated plates was used to prepare triplicate glycerol stock cultures (20%, v/v), as described in Chapter 2.

3.3.2 Assignment of representative *strains* to colour-groups

The 284 representative isolates were assigned to colour-groups based on aerial spore mass, substrate mycelium and diffusible pigment colours on oatmeal agar and the formation of melanin pigments on peptone-yeast extract-iron agar plates, as described in Chapter 2.

3.3.3 *Actinobacterial screening of representative actinobacteria*

All of the representative strains isolated from the Cerro Chajnantor and Lomas Bayas environmental samples were screened against a panel of wild type microorganisms and *Bacillus subtilis* reporter strains using the procedures described in Chapter 2.

3.3.4 *Classification of representative actinobacteria to generic rank*

Phylogenetic analyses. Thirty one isolates were selected to represent selected colour-groups. Genomic DNA was extracted from biomass of the isolates which had been scrapped from yeast-extract malt-extract plates (ISP2 medium; Shirling & Gottlieb, 1966) that had been incubated for 14 days at 28ºC, according to the protocol described by

Fujimoto et al. (2004). PCR amplification of 16S rRNA genes and purification of PCR products were carried out, as described in Chapter 2. The resultant almost complete 16S rRNA gene sequences (~1500 nucleotide [nt]) were edited using the ABI format files in BioEdit version 7.2.5 (Hall 1999) and the nearest match of each of the edited sequences established using the Basic Local Alignment Search Tool (BLAST) and pairwise sequence similarities calculated using the global alignment algorithm implemented using the EzTaxon e web server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Phylogenetic trees showing relationships between isolates and closely related type strains were generated following the procedure described by Cao et al. (2015) using the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms from MEGA6 software version 5.0 (Tamura et al. 2013). Bootstrap values based on 1000 repeats (Felsenstein 1985) were generated using the evolutionary distance model of Jukes and Cantor (1969).

3.4 Results

3.4.1 Selective isolation of actinobacteria from the Cerro Chajnantor environmental samples

Pilot experiment 1. All of the isolation plates were badly contaminated with fast growing bacteria though 54 isolates were retrieved from some of the isolation media, as shown in Table 3.5.

Pilot experiment 2. Most of the selective isolation plates inoculated with suspensions of the heat pre-treated ALMA 2 environmental sample were overrun by fast-growing bacteria though fewer contaminants were present on plates seeded with the suspension pretreated at 120^{o}C for 15 minutes. Fifteen isolates were retrieved from some of the isolation plates, as shown in Table 3.5.

Pilot experiment 3. The results of this experiment are shown in Table 3.1. In general, small numbers but high proportions of actinobacterial colonies were recovered on Gauze's No. 1 agar plates supplemented with either rifampicin or rifampicin with nalidixic acid following incubation at 28^{o}C for 30 days. The highest counts of actinobacteria were recorded when these and corresponding plates supplemented with gentamicin, nalidixic acid and rifampicin were inoculated directly with ALMA 2 soil particles that had been heated at 120^{o}C for 15 minutes. Indeed, only actinobacterial

colonies were detected on selective media inoculated with soil particles irrespective of whether the plates were incubated at 28°C or 40°C though fewer colonies were recorded on the plates incubated at the higher temperature. It is also apparent that slightly higher counts of actinobacterial colonies were recorded from Gauze's No. 1 agar plates supplemented either with rifampicin or with nalidixic acid and rifampicin when inoculated with suspensions of fraction B from the dispersion and differential centrifugation procedure when compared with those seeded with soil suspensions following incubation at 28°C. Nineteen isolates retrieved from some of the isolation plates were studied further, as outlined in Table 3.5.

Pilot experiment 4. Small numbers of actinobacterial colonies were detected on the Gauze's No. 1 agar plates supplemented with nalidixic acid and rifampicin following inoculation with suspensions of ALMA 1-4 and 6 soils, no growth was found on plates seeded with ALMA 5 soil suspensions. The cleanest plates were those prepared from the 3 subsurface soils, namely from ALMA 2, 4 and 6. In general, fewer, if any, actinobacterial colonies were detected on the corresponding Gauze's No. 1 agar plates supplemented with gentamicin and nalidixic acid (Table 3.2). Twenty four isolates were taken from the Gauze's No. 1 supplemented with nalidixic acid and rifampicin for further study, as shown in Table 3.5.

Selective isolation from ALMA surface environmental samples. Small numbers of actinobacteria and much larger numbers of unwanted bacteria grew on almost all of the selective isolation media prepared from the dilution series irrespective of the source of the environmental samples or whether the initial soil suspension had or had not been heat pre-treated (Table 3.3). In general, the highest actinobacterial counts were recorded for the Gauze's No. 1 agar plates supplemented with nalidixic acid and rifampicin though with the exception of the ALMA 1 sample the ratio of actinobacterial to bacterial colony counts were low. Similarly, high actinobacterial counts were recorded from the glucose-yeast extract agar plates though in this case the ratio of actinobacterial to bacterial counts was relatively high. The lowest numbers of actinobacteria were recorded from the oligotrophic agar plates. The plates inoculated with soil particles supported the growth of innumerable actinobacterial colonies. In general, actinobacterial counts fell progressively from the lowest to the highest altitude sampling site, as shown in Table 3.3. One hundred and ten isolates were obtained from the isolation plates for further study, as shown in Table 3.5.

Table 3.1 Number and percentage of actinobacterial to total bacterial colonies (cfu's per gram dry weight soil) from the ALMA 2 environmental sample that had been heated at 120°C for 15 minutes growing on selective isolation plates seeded with suspensions of soil, DDC fractions and soil particles prior to incubation at 28°C and 40°C for 30 days.

Temperature	Colony count / Medium	Dilution plate			DDC (Fraction B)			Soil particles		
		Actinobacteria		Bacterial counts	Actinobacteria		Bacterial counts	Actinobacteria		Bacterial counts
		Number	%		Number	%		Number	%	
28°C	Gauze No. 1 agar + rifampicin (25µg/ml)	0.3×10^1	75	0.4×10^1	0.7×10^1	100	0.7×10^1	1.72×10^1	100	1.72×10^1
	Gauze No. 1 agar + rifampicin (25µg/ml) + nalidixic acid (25µg/ml)	0.3×10^1	75	0.4×10^1	0.9×10^1	90	1.0×10^1	$>30 \times 10^1$	100	$>30 \times 10^1$
	Gauze No. 1 agar + rifampicin (25µg/ml) + nalidixic acid (25µg/ml) + gentamicin (10µg/ml)	-	-	-	-	-	-	1.89×10^1	100	1.89×10^1
40°C	Gauze No. 1 agar + rifampicin (25µg/ml)	-	0	0.5×10^1	-	-	-	$>30 \times 10^1$	100	$>30 \times 10^1$
	Gauze No. 1 agar + rifampicin (25µg/ml) + nalidixic acid (25µg/ml)	-	-	-	0.2×10^1	100	0.2×10^1	-		-
	Gauze No. 1 agar + rifampicin (25µg/ml) + nalidixic acid (25µg/ml) + gentamicin (10µg/ml)	-	-	-	0.5×10^1	100	0.5×10^1	-		-

-, no growth; *Small numbers of actinobacteria were observed on DDC fraction C but not on DDC fractions A, D and the residue.
All media were supplemented with cycloheximide and nystatin (both at 25 µg/ml).

Table 3.2 Actinobacterial and total number of bacterial colonies (cfu's per gram dry weight soil) growing on selective isolation media together with the percentage of actinobacteria to the total number of bacteria after incubation at 28ºC for 30 days following inoculation with suspensions of ALMA soils that had been heated at 120ºC for 15 minutes.

ALMA environmental sample	Medium	Gauze No. 1 agar + nalidixic acid (25µg/ml) + rifampicin (25µg/ml)		Gauze No. 1 agar + gentamicin (10µg/ml) + nalidixic acid (25µg/ml)	
	Colony counts	Number	%	Number	%
1	Actinobacteria	0.9×10^1	15	3.2×10^1	0.005
	Bacteria	6.1×10^1		6.32×10^3	
2	Actinobacteria	7.7×10^1	79	1.3×10^1	<1.0
	Bacteria	9.7×10^1		$>30 \times 10^1$	
3	Actinobacteria	0.1×10^1	9	-	-
	Bacteria	1.1×10^1		-	
4	Actinobacteria	2.8×10^1	100	0.3×10^1	<1.0
	Bacteria	2.8×10^1		$>30 \times 10^1$	
6	Actinobacteria	0.7×10^1	100	-	-
	Bacteria	0.7×10^1		-	

-, no growth; * The ALMA 5 plates did not support the growth of any colonies.
Both media were supplemented with cycloheximide and nystatin (both at 25 µg/ml).

Table 3.3 Number of actinobacterial and bacterial colonies (cfu's per gram dry weight soil) growing on selective isolation media after incubation at 28°C for 30 days following inoculation with suspensions of ALMA 1, 3 and 5 environmental samples together with the % of actinobacterial to the total number of bacteria.

ALMA environmental sample	Medium / Colony count	Heat pretreated at 50°C for 40 minutes				Non-heat pretreated					
		Gauze No. 1 agar + gentamicin (10µg/ml) + nalidixic acid (25µg/ml)		Gauze No. 1 agar + nalidixic acid (25µg/ml) + rifampicin (25µg/ml)		Gauze No. 1 agar		Glucose-yeast extract agar		Oligotrophic agar	
		Number	%	Number	%	Number	%	Number	%	Number	%
1	Actinobacteria	4.37 x 10^2	17	6.43 x 10^2	13	3.81 x 10^3	n/a	2.2 x 10^2	13	1.41 x 10^2	7
	Bacteria	2.51 x 10^3		4.9 x 10^3		>30 x 10^1		1.71 x 10^3		1.96 x 10^3	
3	Actinobacteria	0.5 x 10^1	0.7	9.1 x 10^1	<0.3	4.76 x 10^2	n/a	1.5 x 10^3	12	None	n.a
	Bacteria	7.3 x 10^2		>30 x 10^1		>30 x 10^1		1.21 x 10^4		2.5 x 10^3	
5	Actinobacteria	None	n.a	4.8 x 10^2	0.9	2.3 x 10^2	45	2.75 x 10^2	22	1 x 10^2	0.8
	Bacteria	9.4 x 10^2		5.3 x 10^2		5.1 x 10^2		1.24 x 10^3		1.2 x 10^2	

-, no growth; n/a, not applicable; All media were supplemented with cycloheximide and nystatin (both at 25 µg/ml).

3.4.2 Selective isolation of actinobacteria from the Lomas Bayas environmental sample

Selective isolation. All but one of the selective isolation media inoculated with soil dilutions or with soil particles supported the growth of actinobacteria but not unwanted bacteria though actinobacterial numbers were low in all cases (Table 3.4). Sixty four isolates were taken from the various selective media for further study as shown in Table 3.5.

Table 3.4 Total actinobacterial colony counts (cfu's per gram dry weight soil) growing on selective isolation media after incubation at 28°C for 30 days following inoculation with either soil suspensions or soil particles prepared from the Lomas Bayas environmental sample.

Medium	Colony counts of actinobacteria (cfu/gram dry weight soil)	
	Serial dilution	Soil sprinkle
Arginine-vitamin agar	2.2×10^1	2.2×10^1
Diagnostic agar + tetracycline hydrochloride	0.1×10^1	0.2×10^1
Gauze No. 1 agar	0.1×10^1	0.3×10^1
Glucose-yeast extract agar	1.5×10^1	0.5×10^1
Humic acid-vitamin agar	1.2×10^1	0.6×10^1
Oligotrophic agar	0.2×10^1	0.2×10^1
Raffinose-histidine agar	1.4×10^1	0.5×10^1
SM1 agar	1.2×10^1	1.6×10^1
Starch-casein agar	1.9×10^1	2.0×10^1
Yeast extract- malt extract agar	1.0×10^1	0.5×10^1

Colonies were not detected on tap water agar; All of the media were supplemented with cycloheximide and nystatin (both at 25 µg/ml).

Table 3.5 Strains isolated from selective isolation media inoculated with suspensions prepared from the Cerro Chajnantor and Lomas Bayas environmental samples

Soil sample		Isolation media	No. of isolates	Laboratory codes
A. ALMA environmental samples				
Pilot experiment 1				
ALMA	2	Glucose-yeast extract agar	3	H1*, H2*, H3
	4	Glucose-yeast extract agar	6	H4, H5*, H6*, H7*, H8, H9*
	6	Gauze's No. 1 agar	14	H41, H42, H43*, H44*, H45*, H46*, H47*, H48, H49, H50, H51*, H52, H53*, H54
		Glucose-yeast extract agar	17	H24, H25, H26*, H27, H28*, H29, H30, H31, H32, H33*, H34, H35, H36*, H37, H38, H39, H40*
		Oligotrophic agar	14	H10*, H11, H12*, H13*, H14*, H15, H16, H17, H18, H19, H20, H21, H22, H23
Pilot experiment 2				
ALMA	2	Glucose-yeast extract agar supplemented with rifampicin	12	H55, H56*, H57*, H58*, H59*, H60, H61*, H62*, H63, H64, H65, H66
		Oligotrophic agar	3	H67*, H68, H69*
Pilot experiment 3				
ALMA	2	Gauze's No. 1 agar supplemented with rifampicin	14	H70, H71, H72*, H73, H74, H75, H76, H77, H78, H79, H80, H81, H82, H83, H84
		Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	3	H85, H86, H87
		Gauze's No. 1 agar supplemented with gentamicin, nalidixic acid and rifampicin	2	H88, H89
Pilot experiment 4				
ALMA	1	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	3	H90*, H91, H92
	2	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	2	H93*, H94

	3	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	2	H95*, H96*
	4	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	10	H97*, H98, H99*, H100, H101, H102, H103, H104, H105, H106
	6	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	7	H107, H108, H109, H110, H111, H112, H113
Selective isolation from ALMA surface environmental samples				
ALMA	1	Gauze's No. 1 agar	13	H117, H118, H119, H120, H121, H122, H123, H124, H125, H126, H127, H128, H129,
		Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	7	H132 [S], H133 [S], H134 [S], H135, H136, H137, H138
		Gauze's No. 1 agar supplemented with gentamicin and nalidixic acid	32	H139, H140, H141, H142, H143, H144, H145, H146, H147, H148, H149, H150[1,2], H151, H152, H153, H154, H155, H156, H157, H158, H159, H160, H161, H162, H163, H164, H165, H166, H167, H168, H169, H170
		Glucose-yeast extract agar	3	H114, H115, H116
		Oligotrophic agar	2	H130 [S], H131 [S]
	3	Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	1	H171
		Gauze's No. 1 agar supplemented with gentamicin and nalidixic acid	6	H172, H173, H174, H175, H176 [S], H177 [S]
	5	Gauze's No. 1 agar	6	H193 [S], H194, H195, H196, H197, H198
		Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin	25	H199, H200 [S], H201 [S], H202, H203, H204[S], H205[S*], H206, H207, H208, H209, H210, H211, H212, H213, H214 [S], H215[S*], H216 [S], H217, H218, H219, H220, H221, H222, H223

Glucose-yeast extract agar	15	H178 [S], H179 [S], H180 [S], H181 [S], H182, H183, H184, H185, H186, H187, H188, H189, H190, H191, H192 [S]
B. Lomas Bayas		
Arginine-vitamin agar	16	LB7[‡], LB8[‡*], LB9[‡], LB10[‡], LB11[‡], LB12[‡], LB49, LB50, LB51, LB52, LB53, LB54[*], LB55, LB56, LB57, LB58
Gauze's No. 1 agar	8	LB16[‡], LB17[‡], LB18[‡], LB19[‡], LB20[‡*], LB21[‡], LB22[‡], LB66[‡]
Glucose-yeast extract agar	5	LB13[‡], LB14[‡], LB15[‡], LB62, LB63
Humic acid-vitamin agar	7	LB23[‡], LB24[‡], LB25[‡*], LB26[‡], LB27[‡], LB28[‡], LB29[‡]
Yeast extract-malt extract agar	5	LB1[‡], LB2[‡*], LB3[‡], LB4[‡], LB6[‡]
Raffinose-histidine agar	8	LB30[‡], LB31[‡], LB32[‡], LB33[‡*], LB34, LB35[‡], LB36[‡], LB65
SM1 agar	1	LB64
Starch-casein agar	14	LB37[‡], LB38[‡], LB39[‡], LB40[‡], LB41[‡], LB42[‡], LB43[‡], LB44[‡], LB45[‡], LB46[‡], LB47[‡], LB59, LB60, LB61

All of the media were supplemented with cycloheximide (25 µg/ml) and nystatin (25 µg/ml).
[‡], Isolated by Mr. Owajiogak Hayford, M.Sc. student in the School of Biology; *Isolates included in the 16S rRNA gene sequencing studies; [S], Isolates obtained from sprinkle plates.

3.4.3 Dereplication of presumptive actinobacteria

All of the colonies subcultured from the selective isolation plates were considered to be actinobacteria as they produced leathery colonies were mainly covered by abundant aerial hyphae. Most of these strains were considered to have colonial features characteristic of the genus *Streptomyces.* The 220 strains isolated from the ALMA environmental samples were assigned to 29 multi-membered and 92 single-membered colour-groups based on aerial spore mass, substrate mycelial and diffusible pigment colours on oatmeal agar and the production of melanin pigment on peptone-yeast extract agar (Table 3.6). Similarly, the 64 strains isolated from the Lomas Bayas environmental samples were grouped into 11 multi-membered and 24 single-membered colour-groups (Table 3.6). The detailed properties of the colour-groups given in Appendix 1.

Table 3.6 Representative actinobacteria isolated from the Cerro Chajnantor and Lomas Bayas environmental samples and assigned to multi- and single-membered colour-groups

Colour-group	Codes
Cerro Chajnantor	
Multi-membered colour-groups	H1[1], H36[1], H54[1], H56[1], H57[1], H58[1], H67[1], H69[1], H74[1], H75[1], H76[1], H77[1], H4[4], H73[4], H86[4], H87[4], H153[4], H6[6], H106[6], H7[7], H104[7], H105[7], H9[49], H39[49], H13[12], H21[12], H60[12], H44[12], H89[12], H119[12], H123[12], H135[12], H136[12], H142[12], H143[12], H145[12], H147[12], H148[12], H150[12], H159[12], H161[12], H163[12], H168[12], H171[12], H172[12], H173[12], H174[12], H176[12], H193[12], H199[12], H205[12], H215[12], H213[12], H214[12], H14[26], H181[26], H16[28], H132[28], H25[36], H64[36], H34[45], H146[45], H41[10], H42[10], H59[52], H99[52], H166[52], H61[53], H62[53], H68[9], H126[9], H70[22], H71[22], H85[22], H95[71], H96[71], H101[75], H102[75], H103[75], H111[79], H112[79], H113[81], H125[81], H116[84], H124[84], H127[84], H128[84], H152[84], H154[84], H134[94], H137[94], H139[95], H140[95], H141[95], H155[95], H156[95], H178[108], H179[108], H183[111], H184[111], H185[112], H217[112], H218[112], H203[112], H186[113], H188[113], H202[113], H204[113], H189[115], H190[115], H191[115], H192[115], H194[115], H195[115], H196[115], H198[115], H200[116], H201[116], H209[116], H210[116], H211[116], H219[116], H222[116],H220[114], H223[114]
Single-membered colour-groups	H2[2], H3[3], H5[5], H8[8], H10[23], H11[24], H12[25], H15[27], H17[29], H18[30], H19[31], H20[32], H22[33], H23[34], H24[35], H26[37], H27[38], H28[39], H29[40], H30[41], H31[42]*, H32[43], H33[44], H35[46], H37[47], H38[48], H40[50], H43[11], H45[13], H46[14], H47[15], H48[16], H49[17], H50[18], H51[19], H52[20], H53[21], H55[51], H63[54], H65[55], H66[56], H72[57], H78[58], H79[59], H80[60], H81[61], H82[62], H84[63], H85[64], H88[65], H90[66], H91[67], H92[68], H93[69], H94[70], H97[72], H98[73], H100[74], H107[76], H108[77], H109[78], H110[80], H114[82], H115[83], H117[85], H118[86], H120[87], H121[88], H122[89], H129[90], H130[91], H131[92], H133[93], H138[96], H144[97], H151[98], H157[99], H158[100],

	H160[101], H162[105], H164[103], H165[104], H167[12], H175[106], H177[107], H180[109], H182[110], H206[117], H207[118], H212[119], H216[120], H221[121]
Lomas Bayas	
Multi-membered colour-groups	LB1[7], LB29[7], LB2[4], LB6[4], LB24[4], LB4[10], LB19[10], LB7[14], LB9[5], LB14[5], LB15[5], LB17[5], LB18[5], LB27[5], LB28[5], LB30[5], LB13[1], LB36[1], LB37[1], LB40[1], LB43[1], LB44[1], LB46[1], LB16[3], LB64[3], LB65[3], LB20[14], LB25[14], LB21[6], LB22[6], LB26[6], LB33[13], LB34[13], LB49[17], LB51[17], LB52[17], LB53[17], LB59[24], LB60[24]
Single-membered colour-groups	LB3[35], LB8[15], LB11[33], LB12[34], LB23[31], LB31[30], LB32[9], LB35[16], LB38[29], LB39[8], LB41[12], LB42[11], LB45[2], LB50[18], LB54[19], LB55[20], LB56[21], LB57[22], LB58[23], LB61[25], LB62[26], LB63[27], LB66[28]

[1-121], numbers of colour-groups.

The source and cultural characteristics of the 220 strains isolated from the Cerro Chajnantor sampling sites and assigned to colour-groups are shown in Appendix 1. Isolates H41 and H42 were the only strains that produced melanin pigments on peptone-yeast extract-iron agar, these strains were isolated from the ALMA 2 environmental sample and classified as single-membered colour-groups. Twenty one out of the 47 isolates formed diffusible pigments (21% of the total) were assigned to single-membered colour-groups. The exception, colour-group 95 contained 5 strains isolated from the ALMA 1 environmental sample, these strains produced a dark grey yellow diffusible pigment, a dark yellow brown substrate mycelium and a light grey aerial spore mass.

The largest taxon, colour-group 2, encompassed 31 isolates (14% of total) which produced a yellow white substrate mycelium and a white aerial spore mass. The members of this colour-group were recovered from all of the ALMA environmental samples, apart from the ALMA 4 soil, and were mainly from subsurface soils. Colour-group 1, the next largest group, contained 11 strains isolated from the ALMA 2 environmental sample and a single isolate from ALMA 6 soil; these strains formed a white substrate mycelium and white aerial spore mass. Colour-groups 84, 116 and 95 encompassed 6, 7 and 5 isolates, respectively, all from the ALMA 1 environmental sample. Strains formed a yellow-green substrate mycelium and a dark bluish grey aerial spore mass. In contrast, the colour-group

116 isolates formed a light grey olive substrate mycelium and a medium grey to white aerial spore mass and a dark yellow diffusible pigment. Colour-group 4 also contained 5 isolates, 4 from the ALMA 2 environmental sample and the remaining strain from ALMA 1 soil, these organisms exhibited a yellow brown substrate mycelium and a yellow white aerial spore mass. The remaining 21 multi-membered colour groups included between 2 and 4 isolates. The results of the corresponding studies on the 64 Lomas Bayas are given in Table B, Appendix 1. Ten out of the 64 strains produced diffusible pigments on oatmeal agar. The most populated taxon, colour group LB5, included 8 isolates which exhibited a mild reddish orange substrate mycelium and a light grey aerial spore mass. Colour-group LB1, the next largest group, encompassed 7 isolates which formed a grey yellow substrate mycelium and a pale green yellow aerial spore mass. The balance of the multi-membered groups contained between 2 and 4 strains.

3.4.4 Screening of isolates for bioactivity

Plug assays. The results obtained for the 284 isolates screened for bioactivity against wild type microorganisms are shown in Appendix 5. One hundred and seven of these strains (~74%) inhibited the growth of one or more of the wild type microorganisms though almost half of the Lomas Bayas isolates were completely inactive. Strains showing activity were recovered from all of the ALMA soils; four out of the five strains that inhibited all of the wild type microorganisms, isolates H122, H142, H161 and H165, were isolated from ALMA 1 soil on Gauze's No. 1 agar with or without antibacterial antibiotics; isolates H142 and H161 were from colour-group 12 while the 2 remaining strains formed single-membered colour-groups. The remaining strain that showed activity against all of the wild type microorganisms, isolate H55, was recovered from ALMA 2 soil on glucose-yeast extract agar supplemented with rifampicin; this organism formed a single-membered colour-group. Isolates H150 and H151 from colour-group 12 showed activity against all but one of the wild type strains though other members of this taxon showed no activity.

One hundred and forty one of the isolates showed activity against the *Bacillus subtilis* strain (49%), 124 against the *Saccharomyces cerevisiae* strain (41%), 103 against the *Staphylococcus aureus* strain (36%), 53 against the *Pseudomonas fluorescens* strain (19%) and finally 17 isolates, that is, 7% against the *Escherichia coli* strain. The isolates inhibiting the growth of the *E. coli* strain were isolated from samples taken from ALMA

1 (5 strains), ALMA 2 (7 strains), ALMA 5 (single isolate) and ALMA 6 (4 strains). Nine of the strains (isolates H10, H45, H65, H72, H83, H93, H122, H157 and H165) were from single membered colour-groups (Appendix 5) and with one exception were isolated on Gauze's No. 1 agar with or without antibacterial antibiotics, the remaining strain, isolate H10, was from an oligotrophic agar plate. The balance of the isolates were representatives of colour-group 12 (strains H142 and H161), 22 (strain 71), 45 (strain H34), 49 (strain H39), 53 (isolates H61 and H62) and 112 (strain H185); five of these strains were isolated on glucose-yeast extract agar with or without rifampicin (isolates H34, H39, H61, H62 and H135), the remainder on Gauze's No. 1 agar supplemented with either rifampicin (strain H71) or with gentamicin and rifampicin (strains H142 and H161).

It can be seen from Table 3.1 that some of the isolates showed activity against the wild type strains when grown on one or other of the growth media though many strains gave positive results when cultivated on ISP2 and ISP3 agar. Isolates H55, H161 and H165, for example, showed activity against the *E. coli* strain when cultivated on ISP 2 agar whereas isolates H10, H34, H39, H71, H83, H151 and H185 only showed corresponding activity on ISP 3 agar; the balance of the strains, isolates H45, H61, H62, H93, H122 and H142 exhibited zones of inhibition against *E. coli* on both growth media (Appendix 5).

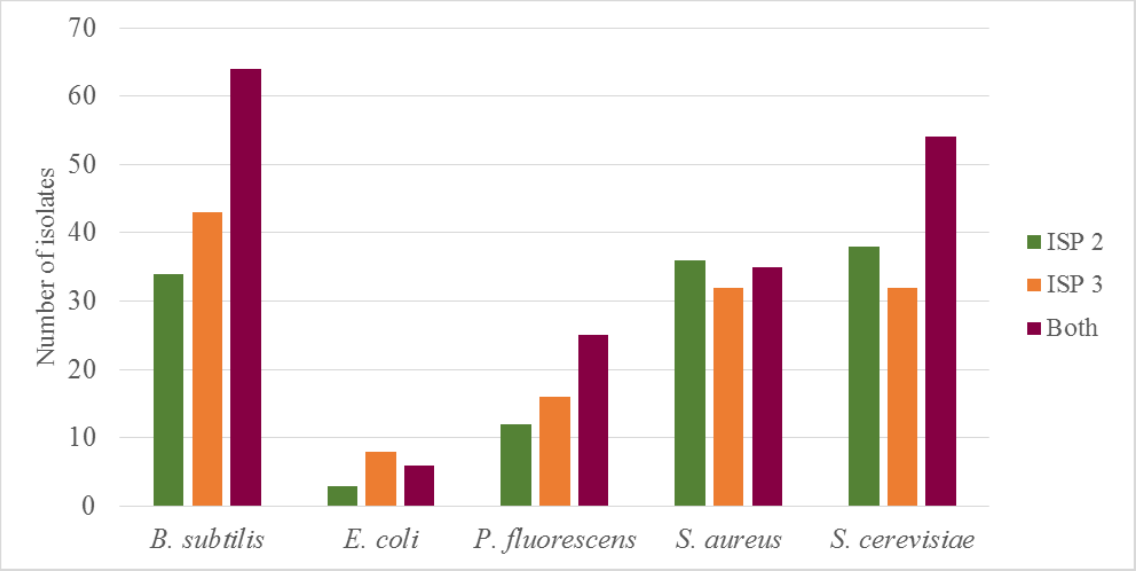

Figure 3.1 Number of isolates grown on ISP 2 and ISP 3 agar that inhibited the growth of a panel of wild type microorganisms following incubation overnight on Luria Bertani agar at 37°C.

***Bacillus subtilis* reporter strains.** The results for the isolates screened against the *B. subtilis* reporter strains are shown in Table 2, Appendix 5. Approximately half of the 422 positive reactions exhibited blue halos indicating activity against the various target sites (Figure 3.2). Fourteen isolates showed broad spectrum activity giving positive reactions against all of the *B. subtilis* reporter strains although relatively few blue halos were observed (Table 3.8 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolates LB3 and LB6 and between them and their nearest phylogenetic neighbours.. Isolates H107 and H108 gave zones of inhibition with blue halos against 5 and 6 of the *B. subtilis* reporter strains, respectively; these isolates formed single membered colour-groups (Appendix 1) and were recovered from ALMA 6 soil on Gauze's No. 1 agar supplemented with nalidixic acid and rifampicin.

The number of isolates inhibiting the growth of the *B. subtilis* reporter strains with and without the formation of blue halos is shown in Figure 3.2. It is apparent from these results that most of the zones of inhibition formed by these strains lacked blue halos. Nevertheless the ability to form inhibition zones with blue halos against five out of the six *B. subtilis* reporter strains was shown by strains isolated from all of the sampling locations (Table 2, Appendix 5). Twelve of the isolates (9%) inhibited DNA synthesis, 26 (20%) fatty acid synthesis, 39 (30%) sporulation, 40 (30%) RNA synthesis and 51 (33%) both cell envelope and cell wall synthesis. Ninety three out of the 136 isolates (68%) that showed activity against the wild type *B.subtilis* in the primary screens showed activity against one or more of the *B. subtilis* reporter strains with 55 (59%) of them producing blue halos (Table 3.8).

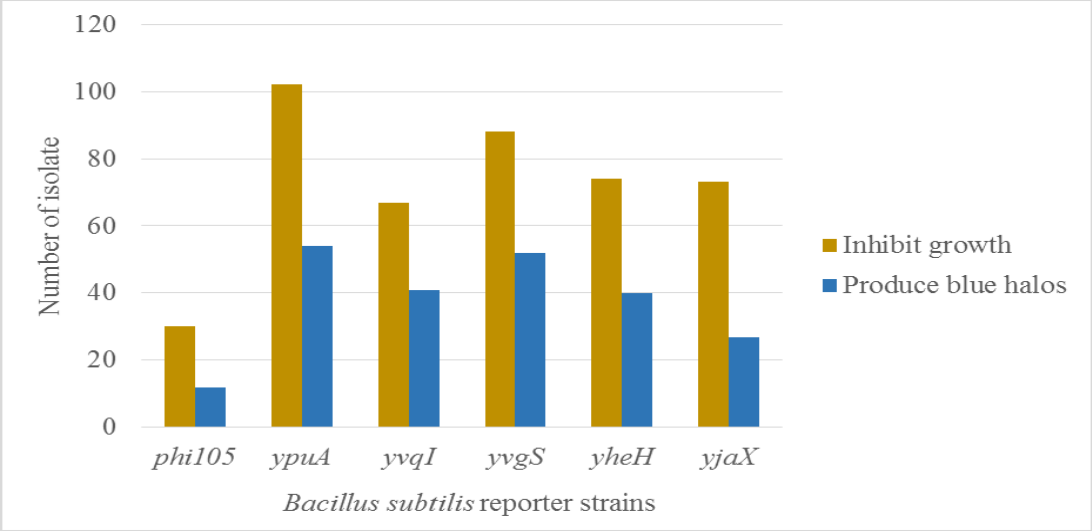

Figure 3.2 Number of isolates inhibiting the growth of *B. subtilis* reporter strains following incubation overnight at 37°C. Key: *phi*105 (inhibits DNA synthesis); *ypu*A (inhibits cell envelope synthesis), *yvq*I (inhibits cell wall synthesis), *yvg*S (inhibits RNA synthesis), *yhe*H (inhibits sporulation) and *yja*X (inhibits fatty acid synthesis).

Table 3.7 Isolates which showed activity against the *B. subtilis* strain in the primary screens and inhibited the growth of *B. subtilis* reporter strains irrespective of whether or not blue halos were produced.

Isolate	Reporter genes					
	phi105	*ypuA*	*yvqI*	*yvgS*	*yheH*	*yjaX*
H4[3]	-	+	+	+	+	+
H5[1]	-	+	+	+	+	+
H9[1]	-	+	+	+	+	+
H11[1]	-	+	+	+	+	+
H13[1]	-	+	+	+	+	+
H14[1]	-	+	+	+	+	+
H15[2]	-	-	+	-	+	+
H16[2]	-	+	-	-	-	-
H17[3]	-	+	+	+	+	-
H25[2]	+	-	-	-	-	-
*H26[2]	+	+	+	+	+	+
H27[1]	-	+	-	+	+	+
H29[3]	-	+	-	+	+	+
H34[1]	-	+	+	+	+	+
H36[2]	-	+	-	+	+	-

H37[1]	-	+	-	+	-	+
H39[1]	-	+	+	+	-	+
H41[2]	-	-	-	-	-	+
H43[2]	-	-	-	-	-	+
H45[3]	-	+	-	+	-	+
H46[2]	-	+	+	+	-	-
H49[2]	-	+	+	+	+	+
H52[1]	-	+	+	+	+	+
H55[1]	-	+	+	+	+	+
*H59[2]	+	+	+	+	+	+
H68[2]	+	-	+	-	+	+
*H69[2]	+	+	+	+	+	+
H70[1]	-	+	+	+	+	+
H72[1]	-	+	+	+	+	+
*H78[2]	+	+	+	+	+	+
*H82[1]	+	+	+	+	+	+
*H84[1]	+	+	+	+	+	+
*H85[1]	+	+	+	+	+	+
H94[1]	-	+	+	+	+	+
*H103[0]	+	+	+	+	+	+
*H107[2]	+	+	+	+	+	+
*H108[3]	+	+	+	+	+	+
*H109[3]	+	+	+	+	+	+
*H112[0]	+	+	+	+	+	+
H115[1]	-	+	+	+	+	+
H116[3]	-	+	+	+	+	+
H117[3]	-	+	+	+	+	+
H118[3]	-	+	-	+	-	-
H120[1]	-	+	+	+	+	+
H121[1]	-	+	+	+	+	+
H122[1]	-	+	+	+	+	+
H124[3]	-	+	+	+	+	+
H127[3]	-	+	-	+	+	-
H128[3]	-	-	-	+	+	-
H134[1]	-	+	+	+	+	+
H137[1]	-	+	+	+	-	+
H139[1]	-	+	+	+	+	+
H140[1]	-	+	-	+	+	+
H141[1]	-	+	+	+	+	+
H142[1]	-	+	+	+	+	+

H146[1]	-	+	+	+	+	+
H150[1]	-	+	+	+	+	+
H151[3]	-	-	-	-	+	-
H152[1]	-	+	+	+	-	+
H153[2]	-	+	+	+	-	+
H154[1]	-	+	+	+	+	+
H157[3]	-	-	-	+	+	-
H158[1]	-	+	+	+	-	-
H159[2]	-	+	+	+	+	+
H160[1]	-	+	-	+	-	-
H161[1]	-	+	+	+	+	+
H162[1]	-	+	-	+	+	-
H164[3]	-	-	-	+	-	-
*H167[1]	+	+	+	+	+	+
H175[2]	+	+	-	+	-	+
H179[3]	-	+	-	-	-	-
H180[1]	-	+	-	-	-	-
H183[3]	-	+	-	-	-	-
H184[3]	-	+	-	-	-	-
H185[1]	+	+	-	+	+	+
H186[3]	-	+	-	-	-	-
H188[1]	-	+	-	+	-	+
H190[3]	-	-	+	-	+	-
H194[3]	-	-	-	-	+	+
H195[1]	+	+	-	+	-	-
H199[1]	-	+	-	+	+	+
H203[3]	-	-	-	-	+	+
*H204[1]	+	+	+	+	+	+
H206[1]	-	+	-	+	+	+
H212[2]	-	+	-	-	-	-
H216[2]	-	+	-	+	-	-
H217[3]	-	-	-	+	-	-
H221[1]	-	+	-	-	-	-
LB2[1]	-	+	-	+	-	-
LB13[3]	+	-	+	-	-	-
LB21[1]	-	+	-	+	-	+
LB22[1]	-	+	-	+	+	+
LB24[1]	-	+	-	+	-	+
LB34[3]	-	+	-	-	-	-
LB62[3]	-	+	-	-	-	-

+, inhibited the growth of *B. subtilis* reporter strains; -, no inhibition; , formation of blue halos.

*, isolate that inhibited the growth of all of the *B. subtilis* reporter strains.

[1], isolates grown on ISP2 and ISP3; [2], isolate grown on ISP2; [3], isolates grown on ISP3 that showed activity against the wild type *B. subtilis* in the primary screens; [0], isolates did not show activity against the wild type *B. subtilis* strain in primary screens.

Isolates that inhibited the growth of the wild type *B. subtilis* strains in primary screens but did not inhibit that of the *B. subtilis* reporter strains: H10, H12, H21, H50, H60, H61, H62, H71, H73, H74, H80, H83, H87, H88, H90, H91, H93, H98, H111, H129, H131, H144, H155, H156, H165, H178, H189, H192, H193, H200, H202, H209, H219, H223, LB1, LB16, LB27, LB36, LB54, LB57, LB59, LB63.

3.4.5 Classification of representative strains isolated from the Cerro Chajnantor and Lomas Bayas environmental samples based on 16S rRNA gene sequences

Phylogenetic analyses. Twenty-five out of 45 strains isolated from the Cerro Chajnantor environmental samples were assigned to the genera *Microbispora* (1 isolate), *Nocardia* (4 isolates), *Nocardiopsis* (2 isolates), and *Streptomyces* (18 isolates). In addition, 7 strains from the Lomas Bayas environmental samples were classified in the genera *Actinomadura* (2 isolates), *Kineococcus* (2 isolates) and *Streptomyces* (4 isolates). Additional isolates from the Cerro Chajnantor environmental samples were the subject of polyphasic studies designed to describe them as putative new species; these strains, were assigned to the genera *Actinomadura* (3 isolate), *Amycolatopsis* (5 isolates), *Cryptosporangium* (1 isolate), *Lentzea* (1 isolate), *Pesudonocardia* (5 isolates) and *Streptomyces* (1 isolate), as described in Chapter 4 and 5.

The genus *Kineococcus.* Isolate LB3, a single-membered taxon, and isolate LB6, a representative of multi-membered colour-group 3 (3 isolates) formed distinct branches in the *Kineococcus* 16S rRNA gene tree Figure 3.3. The isolates formed a subclade together with *Kineococcus glutinatus* YIM 75677^T and *Kineococcus xinjiangensis* S2-20^T, a relationship that was underpinned by all of the tree-making algorithms and by a 100% bootstrap value. The isolates were most closely related to the type strain of *Kineococcus xinjiangensis* sharing a 16S rRNA gene sequence similarity with the latter

of 98.34% and 99.57% respectively, values which correspond 23 and 6 nucleotide (nt) differences at 1382 and 1383 locations Table 3.9. The isolates shared a 16S rRNA gene sequence similarity of only 97.52% which corresponds to 35 nt differences at 1410 sites.

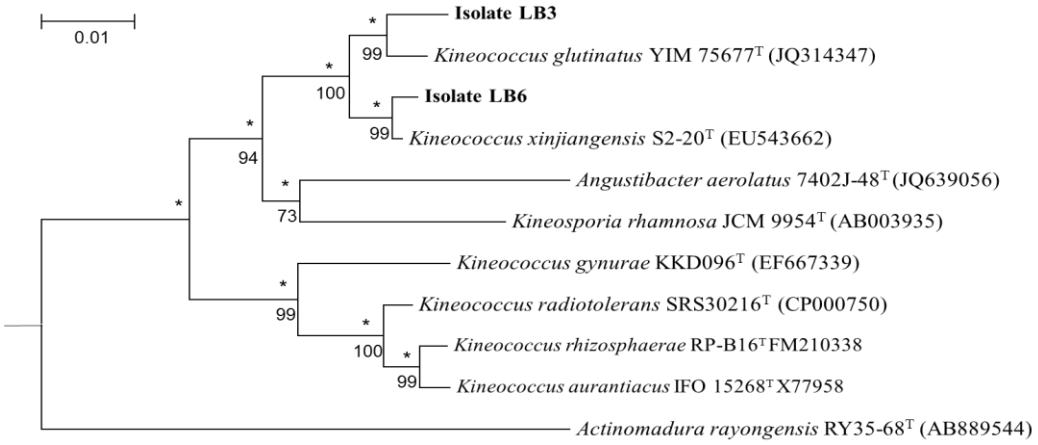

Figure 3.3 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates LB3 and LB6 and between them and the type strains of *Kineococcus* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood 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 0.01 substitutions per nucleotide position.

Table 3.8 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolates LB3 and LB6 and between them and their nearest phylogenetic neighbours.

	1	2	3	4	5	6	7	8	9	10	11
1. Isolate LB3	**---**	**35/1410**	**73/1379**	**71/1318**	**77/1380**	**75/1410**	**26/1419**	**83/1418**	**76/1409**	**23/1382**	**143/1391**
2. Isolate LB6	**97.52**	**---**	**78/1382**	**74/1322**	**77/1384**	**73/1425**	**27/1432**	**74/1431**	**68/1422**	**6/1383**	**137/1396**
3. *Kineococcus rhizosphaerae* RP-B16^T	**94.71**	**94.36**	---	44/1346	11/1385	99/1401	70/1405	14/1406	83/1397	70/1374	136/1394
4. *Kineococcus gynurae* KKD096^T	**94.61**	**94.4**	96.73	---	42/1326	95/1345	71/1349	39/1350	78/1342	66/1314	124/1334
5. *Kineococcus aurantiacus* IFO 15268^T	**94.42**	**94.44**	99.21	96.83	---	102/1385	71/1391	16/1391	85/1382	67/1376	141/1389
6. *Angustibacter aerolatus* 7402J-48^T	**94.68**	**94.88**	92.93	92.94	92.64	---	66/1456	99/1457	72/1451	66/1378	149/1403
7. *Kineococcus glutinatus* YIM 75677^T	**98.17**	**98.11**	95.02	94.74	94.9	95.47	---	72/1473	69/1464	19/1385	141/1409
8. *Kineococcus radiotolerans* SRS30216^T	**94.15**	**94.83**	99	97.11	98.85	93.21	95.11	---	73/1468	61/1384	136/1409
9. *Kineosporia rhamnosa* JCM 9954^T	**94.61**	**95.22**	94.06	94.19	93.85	95.04	95.29	95.03	---	57/1375	138/1400
10. *Kineococcus xinjiangensis* S2-20^T	**98.34**	**99.57**	94.91	94.98	95.13	95.21	98.63	95.59	95.85	---	129/1381
11. *Actinomadura rayongensis* RY35-68^T	**89.72**	**90.19**	90.24	90.7	89.85	89.38	89.99	90.35	90.14	90.66	---

The genus *Microbispora.* Isolate H205, a representative of multi-membered colour-group 12 (which includes 29 isolates), formed a well supported branch in the *Microbispora* 16S rRNA gene tree together with the type strain of *Microbispora bryophytorum* (Figure 3.4). These strains shared a 98.67% 16S rRNA gene sequence similarity which corresponds to 18 nt differences at 1349 locations (Table 3.10).

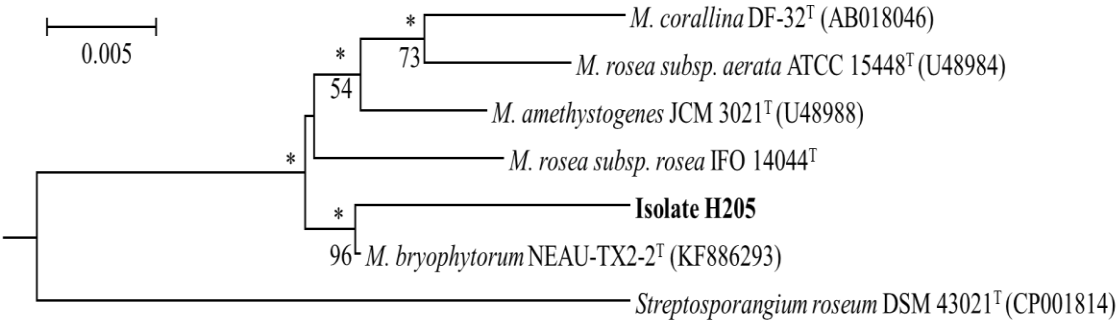

Figure 3.4 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolate H205 and the type strains of closely related *Microbispora* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support, only values above 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 3.9 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate H205 and its nearest phylogenetic neighbours.

	1	2	3	4	5	6	7
1. Isolate H205	**---**	**31/1347**	**18/1349**	**40/1345**	**40/1348**	**31/1343**	**72/1348**
2. *M. amethystogenes* JCM 3021^T	**97.7**	---	13/1412	27/1409	21/1411	28/1405	64/1411
3. *M. bryophytorum* NEAU-TX2-2^T	**98.67**	99.08	---	22/1439	23/1412	18/1451	59/1478
4. *M. corallina* DF-32^T	**97.03**	98.08	98.47	---	22/1410	29/1433	75/1438
5. *M. rosea subsp. aerata* ATCC 15448^T	**97.03**	98.51	98.37	98.44	---	26/1406	72/1411
6. *M. rosea subsp. rosea* IFO 14044^T	**97.69**	98.01	98.76	97.98	98.15	---	67/1450
7. *Streptosporangium roseum* DSM 43021^T	**94.66**	95.46	96.01	94.78	94.9	95.38	---

The genus *Nocardia.* Four strains isolated from Cerro Chajnantor environmental samples, namely isolates H1 and H56 (ALMA 2), H36 (ALMA 6) and H95 (ALMA 3), were assigned to the *Nocardia* 16S rRNA gene tree (Figure 3.5). Strains H1, H36 and

H56 belong to multi-membered colour-group 1 which contains 11 strains and isolate H95 to multi-membered colour-group 71 (2 strains). Isolate H1 formed a subclade together with *Nocardia wallacei* ATCC 49873^T, this taxon was supported by all the tree-making algorithms and by a 99% bootstrap value; these strains shared a 16S rRNA gene sequence similarity of 99.06%, which corresponds to 13 nt differences at 1388 locations (Table 3.11). Isolate H56 formed a distinct branch in a well supported subclade that included the type strains of *Nocardia carnea, Nocardia flavorosea, Nocardia rhamnosiphila, Nocardia sienata* and *Nocardia testacea*. This isolate was found to be most closely related to *Nocardia rhamnosiphila* NRRL B-24637^T; these organisms shared a 16S rRNA sequence similarity of 99.3%, which corresponds to 10 nt differences at 1437 locations (Table 3.11). The remaining strains, isolates H36 and H95, formed a 16S rRNA subclade that was supported by all of the tree-making algorithms and by a 100% bootstrap value; they shared a 16S rRNA gene sequence similarity of 99.2%, which is equivalent to 11 nt differences at 1418 locations (Table 3.11). These isolates were most closely related to *Nocardia tenerifensis* NBRC 101015^T sharing 16S rRNA gene sequence similarities with the latter of 97.83% and 98.87%, respectively, values that correspond to 31 and 16 nt differences at 1430 and 1422 sites.

The genus *Nocardiopsis.* Isolate H13, a representative of multi-membered colour-group 12 (29 isolates), and isolate H67, a representative of multi-membered colour-group 1 (11 isolates), formed distinct branches in the *Nocardiopsis* 16S rRNA gene tree (Figure 3.6). Isolate H13 formed a well supported 16S rRNA subclade together with the type strains of *N. dassonvillei subsp. albirubida, N. dassonvillei subsp. dassonvillei* and *Nocardiopsis synnematoformans.* This isolate was most related to *N. dassonvillei subsp. dassonvillei* sharing a 99.31% 16S rRNA gene sequence similarity with the latter, a value which corresponds to 10 nt differences at 1450 locations (Table 3.12). The second strain, isolate H67, formed a loose 16S rRNA subclade together with *Nocardiopsis trehalosi* VKM Ac-942^T, a relationship which was supported by all of the tree-making algorithms and by a 100% bootstrap value; these strains shared a 96.82% 16S rRNA gene sequence similarity, which corresponds to 45 nt differences at 1413 locations (Table 3.12).

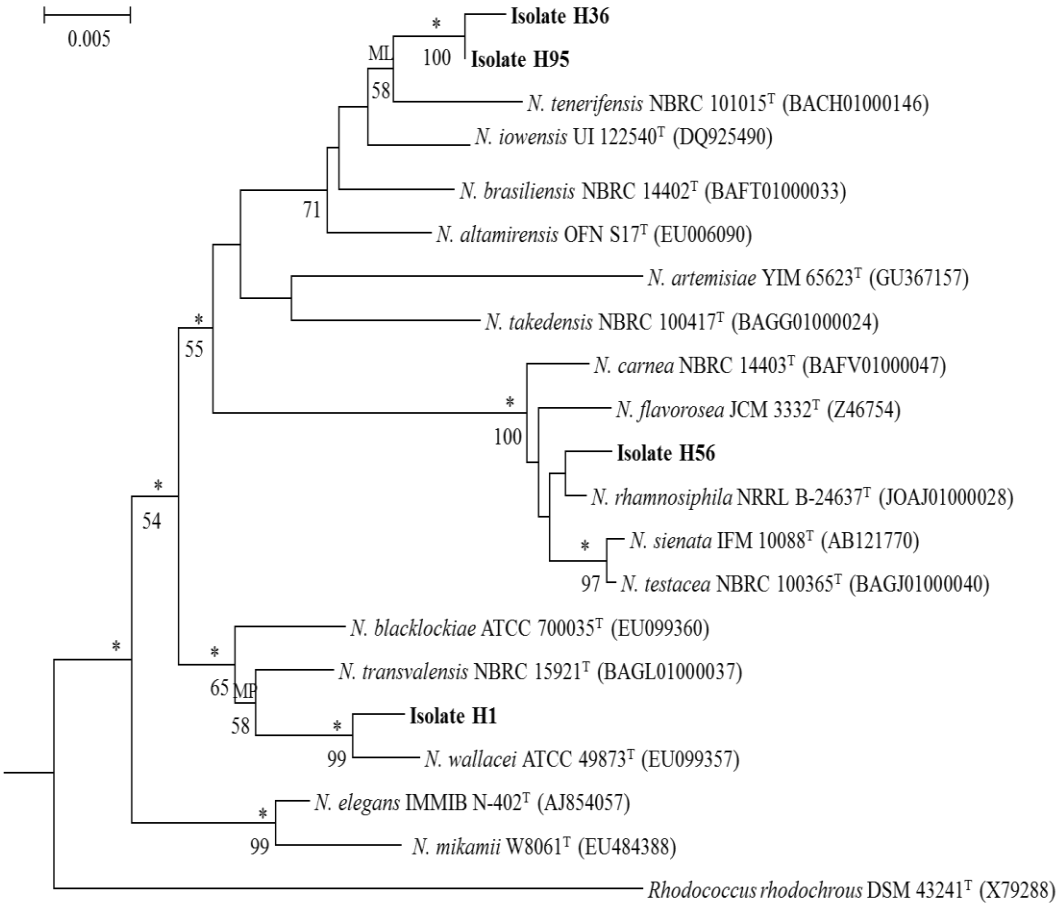

Figure 3.5 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H1, H36, H56 and H95 and between them and the type strains of closely related *Nocardia* species. . *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates a branch of the tree that was recovered using the maximum-likelihood 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.

Table 3.10 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H1, H36, H56 and H95 and between them and their nearest phylogenetic neighbours.

	1	**2**	**3**	**4**	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. Isolate H1	**---**	**55/1418**	**60/1423**	**44/1423**	**32/1320**	**57/1426**	**24/1410**	**45/1428**	**55/1426**	**33/1426**	**55/1426**	**48/1404**	**34/1410**	**56/1426**	**56/1389**	**51/1428**	**48/1426**	**59/1426**	**24/1428**	**13/1388**	**74/1427**
2. Isolate H36	**96.12**	**---**	**68/1427**	**11/1418**	**30/1331**	**68/1430**	**47/1405**	**36/1432**	**70/1430**	**57/1430**	**70/1430**	**34/1412**	**66/1418**	**69/1430**	**69/1398**	**36/1432**	**31/1430**	**70/1430**	**43/1432**	**41/1383**	**94/1431**
3. Isolate H56	**95.78**	**95.23**	**---**	**59/1422**	**49/1333**	**65/1437**	**46/1412**	**52/1437**	**12/1437**	**57/1437**	**15/1437**	**52/1415**	**60/1424**	**10/1437**	**11/1402**	**61/1437**	**60/1435**	**13/1437**	**50/1437**	**55/1386**	**87/1436**
4. Isolate H95	**96.91**	**99.22**	**95.85**	**---**	**23/1319**	**53/1422**	**37/1409**	**21/1424**	**56/1422**	**43/1422**	**56/1422**	**20/1403**	**53/1409**	**55/1422**	**56/1388**	**21/1424**	**16/1422**	**56/1422**	**28/1424**	**32/1387**	**81/1423**
5. *N. altamirensis* OFN S17^{T}	**97.58**	**97.75**	**96.32**	**98.26**	---	53/1348	29/1323	18/1348	46/1348	40/1338	48/1348	23/1343	43/1348	45/1348	46/1346	31/1348	19/1346	46/1348	32/1348	37/1313	74/1347
6. *N. artemisiae* YIM 65623^{T}	**96**	**95.24**	**95.48**	**96.27**	96.07	---	43/1412	52/1475	57/1475	50/1445	60/1455	46/1425	59/1459	57/1475	58/1415	42/1475	54/1464	59/1475	48/1475	61/1386	92/1464
7. *N. blacklockiae* ATCC 700035^{T}	**98.3**	**96.65**	**96.74**	**97.37**	97.81	96.95	---	31/1414	44/1412	31/1412	47/1412	34/1407	34/1412	44/1412	45/1392	42/1414	41/1412	44/1412	15/1414	28/1388	71/1413
8. *N. brasiliensis* NBRC 14402^{T}	**96.85**	**97.49**	**96.38**	**98.53**	98.66	96.47	97.81	---	44/1475	43/1445	48/1455	18/1427	52/1459	43/1475	47/1415	30/1477	27/1466	48/1475	34/1477	38/1388	78/1466
9. *N. carnea* NBRC 14403^{T}	**96.14**	**95.1**	**99.16**	**96.06**	96.59	96.14	96.88	97.02	---	48/1445	12/1455	48/1425	55/1459	8/1475	12/1415	54/1475	53/1464	12/1475	45/1475	53/1386	82/1464
10. *N. elegans* IMMIB N-402^{T}	**97.69**	**96.01**	**96.03**	**96.98**	97.01	96.54	97.8	97.02	96.68	---	48/1445	49/1420	13/1429	49/1445	52/1407	39/1445	50/1443	52/1445	23/1445	32/1386	66/1444
11. *N. flavorosea* JCM 3332^{T}	**96.14**	**95.1**	**98.96**	**96.06**	96.44	95.88	96.67	96.7	99.18	96.68	---	52/1425	55/1439	9/1455	12/1415	56/1455	55/1453	12/1455	45/1455	55/1386	82/1454
12. *N. iowensis* UI 122540^{T}	**96.58**	**97.59**	**96.33**	**98.57**	98.29	96.77	97.58	98.74	96.63	96.55	96.35	---	54/1425	50/1425	47/1412	31/1427	19/1425	50/1425	38/1427	48/1387	79/1426
13. *N. mikamii* W8061^{T}	**97.59**	**95.35**	**95.79**	**96.24**	96.81	95.96	97.59	96.44	96.23	99.09	96.18	96.21	---	57/1459	59/1415	52/1459	54/1448	59/1459	33/1459	41/1386	67/1448
14. *N. rhamnosiphila* NRRL B-24637^{T}	**96.07**	**95.17**	**99.3**	**96.13**	96.66	96.14	96.88	97.08	99.46	96.61	99.38	96.49	96.09	---	9/1415	53/1475	54/1464	9/1475	44/1475	54/1386	77/1464
15. *N. sienata* IFM 10088^{T}	**95.97**	**95.06**	**99.22**	**95.97**	96.58	95.9	96.77	96.68	99.15	96.3	99.15	96.67	95.83	99.36	---	53/1415	57/1413	2/1415	46/1415	57/1382	82/1414
16. *N. takedensis* NBRC 100417^{T}	**96.43**	**97.49**	**95.76**	**98.53**	97.7	97.15	97.03	97.97	96.34	97.3	96.15	97.83	96.44	96.41	96.25	---	29/1466	54/1475	35/1477	43/1388	70/1466
17. *N. tenerifensis* NBRC 101015^{T}	**96.63**	**97.83**	**95.82**	**98.87**	98.59	96.31	97.1	98.16	96.38	96.53	96.21	98.67	96.27	96.31	95.97	98.02	---	58/1464	35/1466	47/1386	76/1464
18. *N. testacea* NBRC 100365^{T}	**95.86**	**95.1**	**99.1**	**96.06**	96.59	96	96.88	96.75	99.19	96.4	99.18	96.49	95.96	99.39	99.86	96.34	96.04	---	45/1475	55/1386	82/1464
19. *N. transvalensis* NBRC 15921^{T}	**98.32**	**97**	**96.52**	**98.03**	97.63	96.75	98.94	97.7	96.95	98.41	96.91	97.34	97.74	97.02	96.75	97.63	97.61	96.95	---	19/1388	71/1466
20. *N. wallacei* ATCC 49873^{T}	**99.06**	**97.04**	**96.03**	**97.69**	97.18	95.6	97.98	97.26	96.18	97.69	96.03	96.54	97.04	96.1	95.88	96.9	96.61	96.03	98.63	---	74/1387
21. *Rhodococcus rhodochrous* DSM 43	**94.81**	**93.43**	**93.94**	**94.31**	94.51	93.72	94.98	94.68	94.4	95.43	94.36	94.46	95.37	94.74	94.2	95.23	94.81	94.4	95.16	94.66	---

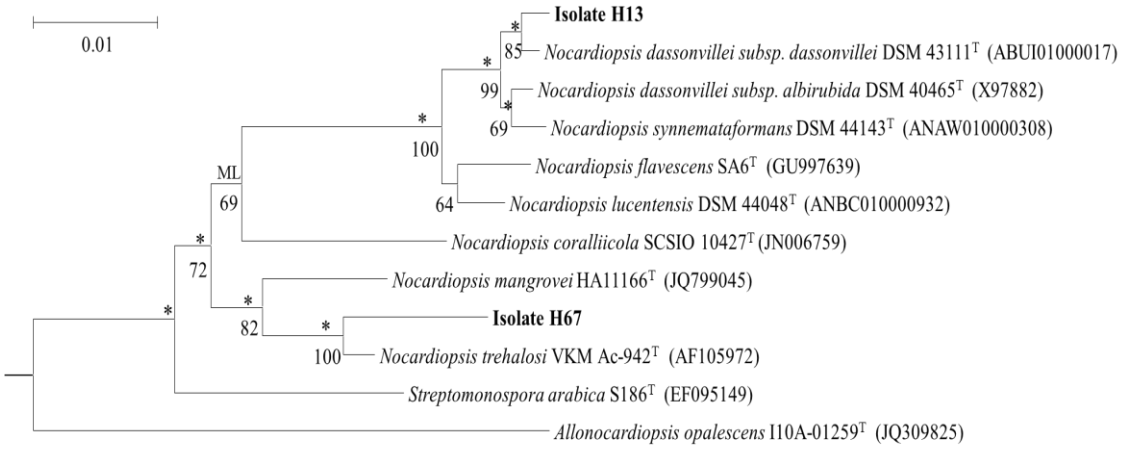

Figure 3.6 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolates H13 and H67 and between them and the type strains of closely related *Nocardiopsis* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates a branch of the tree that was recovered using the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support, only values above 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 3.11 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate H13 and H67 and between them and their closest phylogenetic neighbours.

	1	**2**	3	4	5	6	7	8	9	10	11	12
1. Isolate H13	**---**	**89/1409**	**55/1399**	**14/1449**	**10/1450**	**21/1401**	**22/1450**	**67/1425**	**15/1450**	**59/1444**	**64/1376**	**126/1437**
2. Isolate H67	**93.68**	**---**	**73/1388**	**88/1413**	**91/1413**	**74/1383**	**86/1413**	**61/1406**	**92/1413**	**45/1413**	**63/1367**	**124/1400**
3. *Nocardiopsis coralliicola* SCSIO 10427^{T}	**96.07**	**94.74**	---	53/1399	52/1399	53/1381	50/1399	43/1398	53/1399	51/1399	53/1374	109/1399
4. *Nocardiopsis dassonvillei subsp. albirubida* DSM 40465^{T}	**99.03**	**93.77**	96.21	---	8/1461	20/1401	16/1461	63/1429	6/1461	49/1456	61/1376	125/1440
5. *Nocardiopsis dassonvillei subsp. dassonvillei* DSM 43111^{T}	**99.31**	**93.56**	96.28	99.45	---	21/1401	17/1491	73/1443	9/1491	52/1474	63/1376	126/1444
6. *Nocardiopsis flavescens* SA6^{T}	**98.5**	**94.65**	96.16	98.57	98.5	---	13/1401	53/1399	20/1401	52/1399	59/1358	118/1397
7. *Nocardiopsis lucentensis* DSM 44048^{T}	**98.48**	**93.91**	96.43	98.9	98.86	99.07	---	70/1443	20/1491	50/1474	58/1376	123/1444
8. *Nocardiopsis mangrovei* HA11166^{T}	**95.3**	**95.66**	96.92	95.59	94.94	96.21	95.15	---	72/1443	37/1435	57/1374	118/1422
9. *Nocardiopsis synnemataformans* DSM 44143^{T}	**98.97**	**93.49**	96.21	99.59	99.4	98.57	98.66	95.01	---	51/1474	66/1376	127/1444
10. *Nocardiopsis trehalosi* VKM Ac-942^{T}	**95.91**	**96.82**	96.35	96.63	96.47	96.28	96.61	97.42	96.54	---	46/1375	109/1436
11. *Streptomonospora arabica* S186^{T}	**95.35**	**95.39**	96.14	95.57	95.42	95.66	95.78	95.85	95.2	96.65	---	96/1376
12. *Allonocardiopsis opalescens* I10A-01259^{T}	**91.23**	**91.14**	92.21	91.32	91.27	91.55	91.48	91.7	91.2	92.41	93.02	---

The genus *Streptomyces.* The 24 *Streptomyces* strains included in the 16S rRNA gene sequencing studies showed a range of bioactivity profiles but were mainly chosen to represent single-membered colour-groups (Table 3.12). These isolates were assigned to ten subclades together with their closest phylogenetic neighbours, as shown in Figure 3.7.

Table 3.12 Source, colour-group assignment and bioactivity profiles of Atacama Desert isolates assigned into genus *Streptomyces.*

Isolates	Pilot study	Isolation medium	Colour groups	Bioactivity	
				Wild type strains	*B. subtilis* reporter strains
ALMA 1					
H90	4	Gauze's No. 1	66^{S}	b, c, e	-
ALMA 2					
H61	2	GYEA	53^{M}	a, b, c, d, e	-
H62		GYEA	53^{M}	a, b, c, e	-
H2	1	GYEA	2^{S}	-	-
H72	3	Gauze's No. 1	57^{S}	a, b, c, e	*phi105, ypuA, yvqI, yvgS, yheH, yjaX*
H93	4	Gauze's No. 1	69^{S}	a, b, c, d, e	-
ALMA 6					
H14	1	Oligotrophic	26^{M}	c, e	*ypuA, yvqI, yvgS, yheH, yjaX*
H44		Gauze's No. 1	12^{M}	c, e	-
H10		Oligotrophic	23^{S}	a, b, c, d, e	-
H12		Oligotrophic	25^{S}	d	-
H26		GYEA	37^{S}	c, d, e	*phi105, ypuA, yvqI, yvgS, yheH, yjaX*
H33		GYEA	44^{S}	d	-
H40		GYEA	50^{S}	-	-
H43		Gauze's No. 1	11^{S}	c	*yjaX*
H46		Gauze's No. 1	14^{S}	c, e	*ypuA, yvqI, yvgS*
H47		Gauze's No. 1	15^{S}	-	-
H51		Gauze's No. 1	19^{S}	c, d, e	*ypuA, yvqI, yvgS, yheH*
H53		Gauze's No. 1	21^{S}	-	-
Lomas Bayas					
LB2		ISP 2	4^{M}	c	*ypuA, yvgS*

LB20		Gauze's No. 1	44^{M}	d	*phi105, ypuA, yvgS*
LB33		Raffinose-histidine	13^{M}	-	-
LB8		Arginine-vitamin	15^{S}	-	*yvgS*

Key: GYEA, glucose-yeast extract agar; a, *Escherichia coli*; b, *Pseudomonas fluorescens*; c, *Bacillus subtilis*; d, *Staphylococcus aureus* and e, *Saccharomyces cerevisiae;* ^{M} , multi-membered colour-group; ^{S}, single-membered colour-group; *B. subtilis* reporter strains that produced blue halos are highlighted in blue; *phi*105 (inhibits DNA synthesis); *ypu*A (inhibits cell envelope synthesis), *yvq*I (inhibits cell wall synthesis), *yvg*S (inhibits RNA synthesis), *yhe*H (inhibits sporulation) and *yja*X (inhibits fatty acid synthesis).

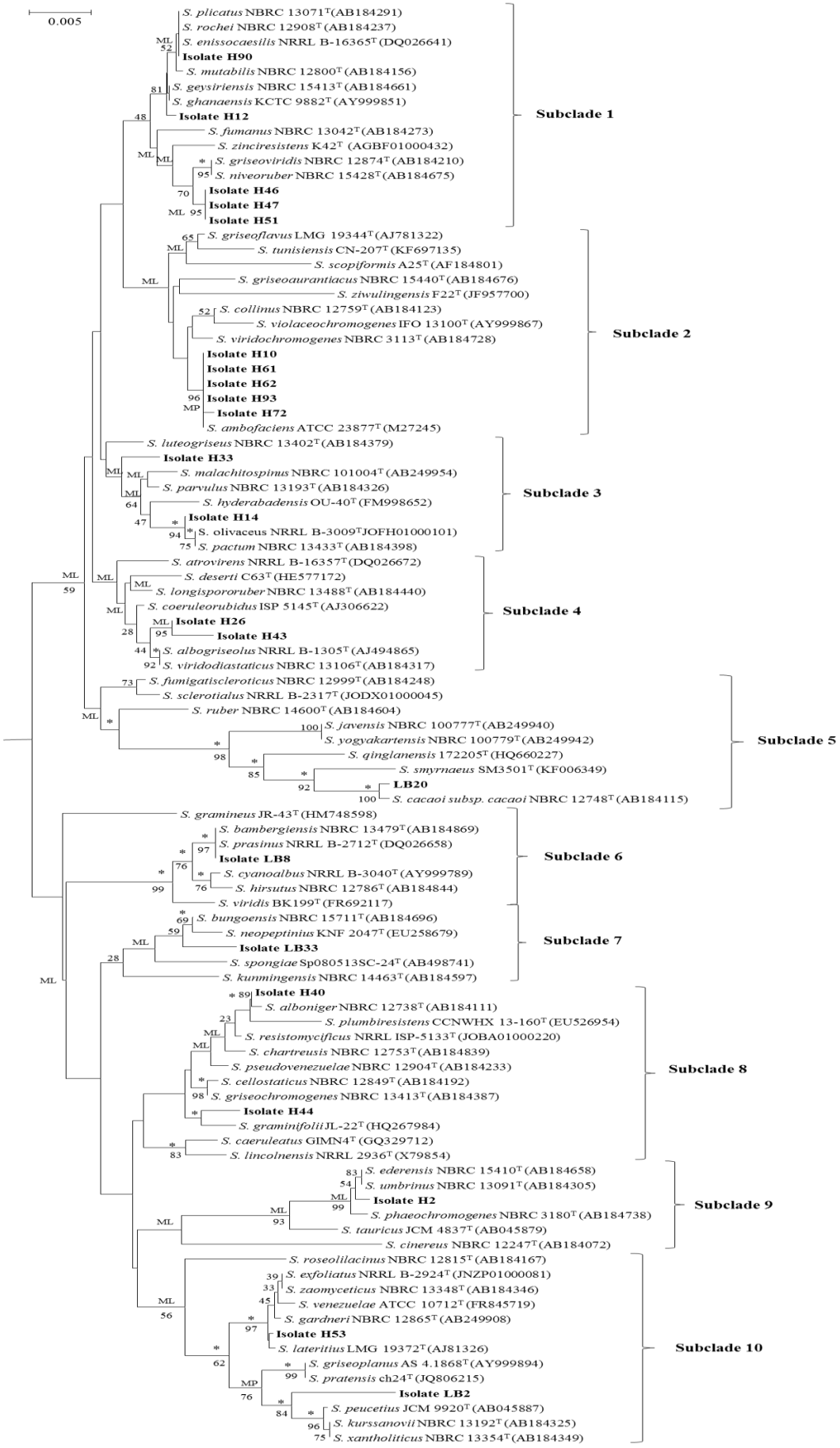
0.005
S. plicatus NBRC 13071T(AB184291)
S. rochei NBRC 12908T(AB184237)
S. enissocaesilis NRRL B-16365T (DQ026641)
Isolate H90
S. mutabilis NBRC 12800T(AB184156)
S. geysiriensis NBRC 15413T(AB184661)
S. ghanaensis KCTC 9882T(AY999851)
Isolate H12
S. fumanus NBRC 13042T(AB184273)
S. zinciresistens K42T (AGBF01000432)
S. griseoviridis NBRC 12874T(AB184210)
S. niveoruber NBRC 15428T(AB184675)
Isolate H46
Isolate H47
Isolate H51
Subclade 1
S. griseoflavus LMG 19344T(AJ781322)
S. tunisiensis CN-207T(KF697135)
S. scopiformis A25T(AF184801)
S. griseoaurantiacus NBRC 15440T(AB184676)
S. ziwulingensis F22T(JF957700)
S. collinus NBRC 12759T(AB184123)
S. violaceochromogenes IFO 13100T(AY999867)
S. viridochromogenes NBRC 3113T(AB184728)
Isolate H10
Isolate H61
Isolate H62
Isolate H93
Isolate H72
S. ambofaciens ATCC 23877T(M27245)
Subclade 2
S. luteogriseus NBRC 13402T(AB184379)
Isolate H33
S. malachitospinus NBRC 101004T(AB249954)
S. parvulus NBRC 13193T(AB184326)
S. hyderabadensis OU-40T(FM998652)
Isolate H14
S. olivaceus NRRL B-3009TJOFH01000101)
S. pactum NBRC 13433T(AB184398)
Subclade 3
S. atrovirens NRRL B-16357T(DQ026672)
S. deserti C63T(HE577172)
S. longispororuber NBRC 13488T(AB184440)
S. coeruleorubidus ISP 5145T(AJ306622)
Isolate H26
Isolate H43
S. albogriseolus NRRL B-1305T(AJ494865)
S. viridodiastaticus NBRC 13106T(AB184317)
Subclade 4
S. fumigatiscleroticus NBRC 12999T(AB184248)
S. sclerotialus NRRL B-2317T(JODX01000045)
S. ruber NBRC 14600T(AB184604)
S. javensis NBRC 100777T(AB249940)
S. yogyakartensis NBRC 100779T(AB249942)
S. qinglanensis 172205T(HQ660227)
S. smyrnaeus SM3501T(KF006349)
LB20
S. cacaoi subsp. cacaoi NBRC 12748T(AB184115)
Subclade 5
S. gramineus JR-43T(HM748598)
S. bambergiensis NBRC 13479T(AB184869)
S. prasinus NRRL B-2712T(DQ026658)
Isolate LB8
S. cyanoalbus NRRL B-3040T(AY999789)
S. hirsutus NBRC 12786T(AB184844)
Subclade 6
S. viridis BK199T(FR692117)
S. bungoensis NBRC 15711T(AB184696)
S. neopeptinius KNF 2047T(EU258679)
Isolate LB33
S. spongiae Sp080513SC-24T(AB498741)
S. kunmingensis NBRC 14463T(AB184597)
Subclade 7
Isolate H40
S. alboniger NBRC 12738T(AB184111)
S. plumbiresistens CCNWHX 13-160T(EU526954)
S. resistomycificus NRRL ISP-5133T(JOBA01000220)
S. chartreusis NBRC 12753T(AB184839)
S. pseudovenezuelae NBRC 12904T(AB184233)
S. cellostaticus NBRC 12849T(AB184192)
S. griseochromogenes NBRC 13413T(AB184387)
Isolate H44
S. graminifolii JL-22T(HQ267984)
S. caeruleatus GIMN4T(GQ329712)
S. lincolnensis NRRL 2936T(X79854)
Subclade 8
S. ederensis NBRC 15410T(AB184658)
S. umbrinus NBRC 13091T(AB184305)
Isolate H2
S. phaeochromogenes NBRC 3180T(AB184738)
S. tauricus JCM 4837T(AB045879)
S. cinereus NBRC 12247T(AB184072)
Subclade 9
S. roseolilacinus NBRC 12815T(AB184167)
S. exfoliatus NRRL B-2924T(JNZP01000081)
S. zaomyceticus NBRC 13348T(AB184346)
S. venezuelae ATCC 10712T(FR845719)
S. gardneri NBRC 12865T(AB249908)
Isolate H53
S. lateritius LMG 19372T(AJ81326)
S. griseoplanus AS 4.1868T(AY999894)
S. pratensis ch24T(JQ806215)
Isolate LB2
S. peucetius JCM 9920T(AB045887)
S. kurssanovii NBRC 13192T(AB184325)
S. xantholiticus NBRC 13354T(AB184349)
Subclade 10

Figure 3.7 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the assignment of *Streptomyces* strains isolated from the Cerro Chajnantor and Lomas Bayas environmental samples to subclades that encompassed type strains of closely related *Streptomyces* species. Numbers at the nodes indicate levels of bootstrap support based on 1000 resampled datasets; only above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Subclade 1. The 5 isolates assigned to this taxon fell into two multimembered groups which were supported by all of the tree-making algorithms and by high bootstrap values (Figure 3.8). Isolates H12 and H90 formed distinct phyletic lines in the larger of the two multimembered groups and shared a 16S rRNA gene similarity of 97.97%, which corresponds to 31 nt differences at 1401 locations (Table 3.14). Isolate H12 was most closely related to *Streptomyces ghanaensis* KCTC 9882^{T}, these strains shared a 16S rRNA gene sequence similarity of 99.5%, which equates to 7 nt differences at 1410 sites. Isolate H90 is also most closely related to the *S. ghanaensis* type strain albeit sharing a low 16S rRNA gene sequence similarity with the latter of 97.85%, this corresponds to 30 nt differences at 1398 locations. Isolates H46, H47 and H51 formed a well supported group (Figure 3.8) but were also closely related to the type strains of *Streptomyces griseoviridis* and *Streptomyces niveoruber* sharing 16S rRNA gene sequence similarities with the latter of 98.95, 99.16 and 97.67% and 98.95, 99.16 and 97.67%, respectively (Table 3.14). Isolates H46 and H47 were also closely related, they shared a 16S rRNA gene sequence similarity of 99.16%, a value equivalent to 12 nt differences at 1429 locations.

Subclade 2. The 5 isolates recovered in this taxon formed a subclade that was supported by all of the tree-making algorithms and by a 98% bootstrap value (Figure 3.9). Isolate H93 was most closely related to isolates H62 and H72 sharing 16S rRNA gene sequence similarities with the latter of 99.62 and 99.39%, values that correspond to 5 and 8 nt differences at 1314 and 1307 locations, respectively. Similarly, isolate H10 was most closely related to isolates H62 and H72 sharing a 98.93% 16S rRNA gene sequence similarity with each of these strains, a value that was equivalent to 14 nt differences at 1311 and 1308 sites, respectively. The final isolate, strain H61, shared its highest 16S rRNA gene sequence similarity with isolate H72, namely 98.63%, a value that corresponds to 18 nt differences at 1313 locations. The 5 isolates were most closely

related to the type strain of *Streptomyces ambofaciens* sharing16S rRNA sequence similarities with the latter that fell within the range 97.11 to 98.17%, which corresponds to between 24 to 41 nt differences at 1312 locations (Table 3.15).

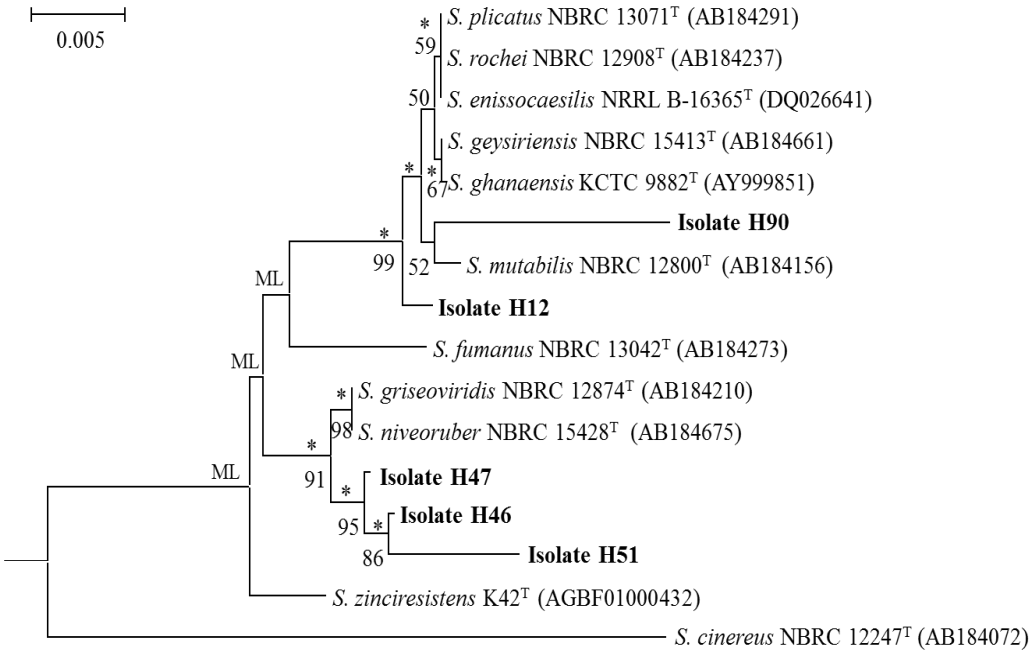

Figure 3.8 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H12, H46, H47, H51 and H90 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates branches of the tree that were recovered using the maximum-likelihood 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.

Table 3.13 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H12, H46, H47, H51 and H90 and between them and their closest phylogenetic neighbours.

	1	**2**	**3**	**4**	**5**	6	7	8	9	10	11	12	13	14	15	16
1. Isolate H12	---	**29/1420**	**24/1424**	**39/1401**	**31/1401**	**10/1427**	**23/1426**	**9/1427**	**7/1410**	**22/1426**	**8/1429**	**22/1426**	**10/1427**	**10/1427**	**25/1425**	**79/1425**
2. Isolate H46	**97.96**	---	**12/1429**	**21/1406**	**53/1402**	**32/1430**	**27/1431**	**31/1430**	**24/1409**	**15/1430**	**34/1430**	**15/1430**	**32/1430**	**31/1429**	**26/1429**	**78/1431**
3. Isolate H47	**98.31**	**99.16**	---	**23/1409**	**54/1406**	**29/1437**	**24/1438**	**28/1437**	**20/1412**	**12/1437**	**31/1437**	**12/1437**	**29/1437**	**29/1436**	**23/1436**	**75/1438**
4. Isolate H51	**97.22**	**98.51**	**98.37**	---	**43/1381**	**50/1415**	**45/1416**	**49/1415**	**36/1393**	**33/1415**	**50/1415**	**33/1415**	**50/1415**	**50/1415**	**44/1414**	**95/1415**
5. Isolate H90	**97.79**	**96.22**	**96.16**	**96.89**	---	**36/1410**	**57/1409**	**37/1410**	**30/1398**	**55/1409**	**36/1410**	**55/1409**	**36/1410**	**34/1408**	**54/1408**	**105/1408**
6. *S. enissocaesilis* NRRL B-16365^T	**99.3**	**97.76**	**97.98**	**96.47**	**97.45**	---	21/1462	1/1463	1/1420	19/1462	4/1458	19/1457	0/1463	0/1461	18/1471	72/1446
7. *S. fumanus* NBRC 13042^T	**98.39**	**98.11**	**98.33**	**96.82**	**95.95**	98.56	---	20/1462	20/1419	20/1462	23/1457	20/1457	21/1462	21/1460	23/1461	70/1447
8. *S. geysiriensis* NBRC 15413^T	**99.37**	**97.83**	**98.05**	**96.54**	**97.38**	99.93	98.63	---	0/1420	18/1462	5/1458	18/1457	1/1463	1/1461	17/1461	72/1446
9. *S. ghanaensis* KCTC 9882^T	**99.5**	**98.3**	**98.58**	**97.42**	**97.85**	99.93	98.59	100	---	18/1419	5/1420	18/1419	1/1420	1/1418	17/1418	72/1415
10. *S. griseoviridis* NBRC 12874^T	**98.46**	**98.95**	**99.16**	**97.67**	**96.1**	98.7	98.63	98.77	98.73	---	21/1457	0/1457	19/1462	19/1460	11/1461	69/1446
11. *S. mutabilis* NBRC 12800^T	**99.44**	**97.62**	**97.84**	**96.47**	**97.45**	99.73	98.42	99.66	99.65	98.56	---	21/1457	4/1458	4/1456	22/1456	76/1446
12. *S. niveoruber* NBRC 15428^T	**98.46**	**98.95**	**99.16**	**97.67**	**96.1**	98.7	98.63	98.76	98.73	100	98.56	---	19/1457	19/1455	11/1456	69/1446
13. *S. plicatus* NBRC 13071^T	**99.3**	**97.76**	**97.98**	**96.47**	**97.45**	100	98.56	99.93	99.93	98.7	99.73	98.7	---	0/1461	18/1461	72/1446
14. *S. rochei* NBRC 12908^T	**99.3**	**97.83**	**97.98**	**96.47**	**97.59**	100	98.56	99.93	99.93	98.7	99.73	98.69	100	---	18/1459	72/1445
15. *S. zinciresistens* K42^T	**98.25**	**98.18**	**98.4**	**96.89**	**96.16**	98.78	98.43	98.84	98.8	99.25	98.49	99.24	98.77	98.77	---	65/1445
16. *S. cinereus* NBRC 12247^T	**94.46**	**94.55**	**94.78**	**93.29**	**92.54**	95.02	95.16	95.02	94.91	95.23	94.74	95.23	95.02	95.02	95.5	---

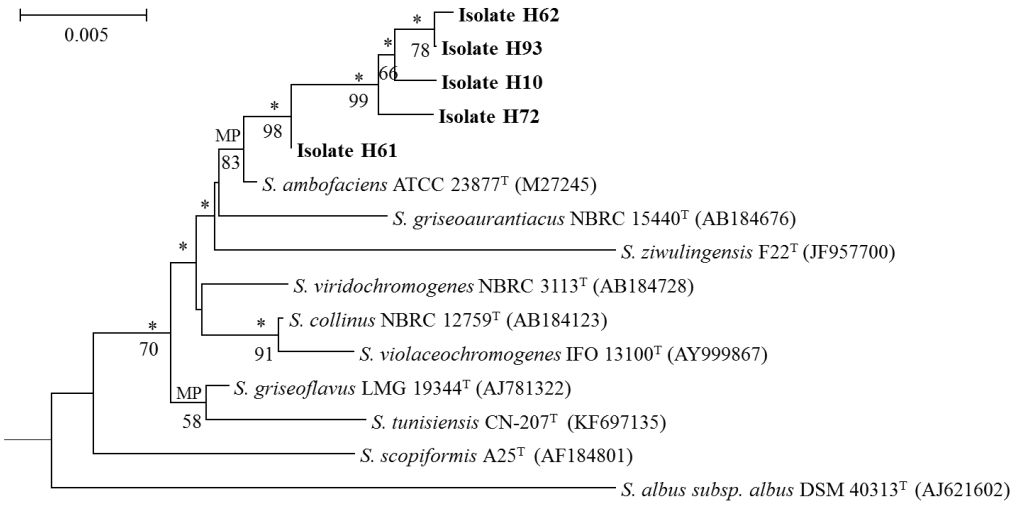

Figure 3.9 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H10, H61, H62, H72 and H93 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods. MP indicates branches of the tree that were recovered using 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.

Table 3.14 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H10, H61, H62, H72 and H93 and between them and their closest phylogenetic neighbours.

	1	**2**	**3**	**4**	**5**	6	7	8	9	10	11	12	13	14	15
1. Isolate H10	---	**22/1336**	**14/1311**	**14/1308**	**22/1332**	**25/1335**	**31/1334**	**36/1327**	**32/1334**	**39/1309**	**35/1321**	**35/1332**	**33/1333**	**48/1329**	**70/1331**
2. Isolate H61	**98.35**	---	**27/1317**	**18/1313**	**46/1392**	**41/1419**	**47/1418**	**55/1410**	**48/1418**	**57/1394**	**53/1404**	**27/1376**	**49/1417**	**47/1388**	**89/1416**
3. Isolate H62	**98.93**	**97.95**	---	**11/1311**	**5/1314**	**35/1317**	**41/1316**	**46/1309**	**42/1316**	**44/1290**	**41/1303**	**45/1314**	**43/1315**	**58/1310**	**77/1313**
4. Isolate H72	**98.93**	**98.63**	**99.16**	---	**8/1309**	**24/1312**	**30/1311**	**35/1304**	**31/1311**	**36/1284**	**30/1298**	**34/1309**	**32/1310**	**49/1306**	**68/1308**
5. Isolate H93	**98.35**	**96.7**	**99.62**	**99.39**	---	**74/1398**	**80/1397**	**85/1389**	**81/1397**	**85/1370**	**78/1379**	**55/1353**	**82/1396**	**77/1362**	**119/1394**
6. *S. ambofaciens* ATCC 23877^T	**98.13**	**97.11**	**97.34**	**98.17**	**94.71**	---	8/1454	18/1455	9/1464	35/1401	30/1408	13/1391	8/1462	28/1392	49/1456
7. *S. collinus* NBRC 12759^T	**97.68**	**96.69**	**96.88**	**97.71**	**94.27**	99.45	---	20/1445	7/1454	41/1400	30/1408	5/1391	8/1453	31/1391	51/1449
8. *S. griseoaurantiacus* NBRC 15440^T	**97.29**	**96.1**	**96.49**	**97.32**	**93.88**	98.76	98.62	---	22/1454	49/1392	42/1399	21/1383	21/1453	39/1383	52/1453
9. *S. griseoflavus* LMG 19344^T	**97.6**	**96.61**	**96.81**	**97.64**	**94.2**	99.39	99.52	98.49	---	34/1400	23/1408	12/1391	9/1462	31/1391	47/1455
10. *S. scopiformis* A25^T	**97.02**	**95.91**	**96.59**	**97.2**	**93.8**	97.5	97.07	96.48	97.57	---	49/1395	40/1353	38/1399	42/1367	57/1396
11. *S. tunisiensis* CN-207^T	**97.35**	**96.23**	**96.85**	**97.69**	**94.34**	97.87	97.87	97	98.37	96.49	---	21/1365	28/1407	45/1379	66/1404
12. *S. violaceochromogenes* IFO 13100^T	**97.37**	**98.04**	**96.58**	**97.4**	**95.93**	99.07	99.64	98.48	99.14	97.04	98.46	---	13/1390	36/1374	52/1386
13. *S. viridochromogenes* NBRC 3113^T	**97.52**	**96.54**	**96.73**	**97.56**	**94.13**	99.45	99.45	98.55	99.38	97.28	98.01	99.06	---	31/1390	51/1454
14. *S. ziwulingensis* F22^T	**96.39**	**96.61**	**95.57**	**96.25**	**94.35**	97.99	97.77	97.18	97.77	96.93	96.74	97.38	97.77	---	62/1387
15. *S. albus subsp. albus* DSM 40313^T	**94.74**	**93.71**	**94.14**	**94.8**	**91.46**	96.63	96.48	96.42	96.77	95.92	95.3	96.25	96.49	95.53	---

Subclade 3. It is evident from Figure 3.10 that the two isolates assigned to this taxon are not closely related. Isolate H14 forms a well supported group with the type strains of *Streptomyces olivaceus* and *Streptomyces pactum* sharing a 99.37% 16S rRNA gene sequence similarity with each of these organisms, a value which corresponds in each case to 9 nt differences at 1483 locations (Table 3.16). Isolate H33 was most closely related to *Streptomyces luteogriseous* NBRC 13402^T sharing a 98.39% 16S rRNA gene sequence similarity with this strain, a value which equates to 23 nt differences at 1429 sites.

Subclade 4. The two isolates assigned to this group were only distantly related as they shared a 16S rRNA gene sequence similarity of only 96.93%, a value equivalent to 43 nt differences at 1401 locations (Table 3.17). In contrast, isolate H26 was closely related to several *Streptomyces* species, notably to the type strains of *Streptomyces albogriseolus and Streptomyces atrovirens* (Figure 3.11). The 16S rRNA gene sequence similarity between isolate H26 and these two type strains was 99.44% in each case, a value that corresponds in both instances to 8 nt differences at 1434 sites. In contrast, isolate H43 was loosely, albeit most closely, related to *Streptomyces longispororuber* NBRC 13106^T; these organisms share a 16S rRNA gene sequence similarity of 99.3%, a value that corresponds to 47 nt differences at 1393 locations (Table 3.17).

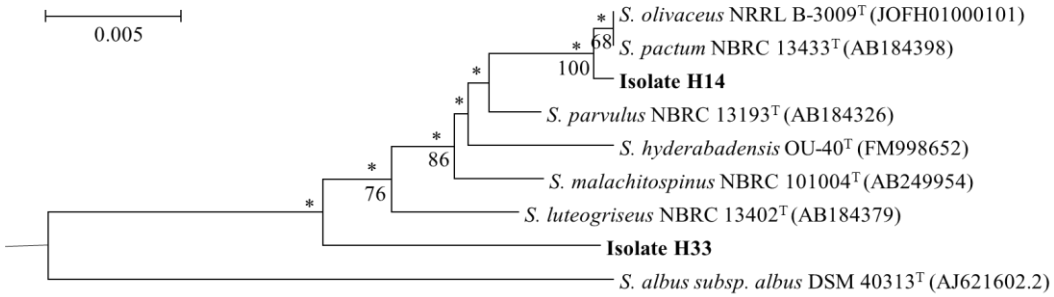

Figure 3.10 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H14 and H33 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the 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.

Table 3.15 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H14 and H33 and between them and their closest phylogenetic neighbours.

	1	**2**	3	4	5	6	7	8	9
1. Isolate H14	---	**36/1423**	**14/1402**	**23/1423**	**20/1429**	**9/1433**	**9/1433**	**17/1430**	**66/1432**
2. Isolate H33	**97.47**	---	**30/1396**	**23/1429**	**32/1435**	**39/1436**	**39/1436**	**30/1436**	**66/1437**
3. *S. hyderabadensis* OU-40^T	**99**	**97.85**	---	18/1399	13/1399	14/1402	14/1402	12/1399	54/1400
4. *S. luteogriseus* NBRC 13402^T	**98.38**	**98.39**	98.71	---	13/1434	21/1435	21/1435	12/1435	57/1434
5. *S. malachitospinus* NBRC 101004^T	**98.6**	**97.77**	99.07	99.09	---	13/1463	13/1457	9/1462	59/1458
6. *S. olivaceus* NRRL B-3009^T	**99.37**	**97.28**	99	98.54	99.11	---	0/1461	10/1463	59/1461
7. *S. pactum* NBRC 13433^T	**99.37**	**97.28**	99	98.54	99.11	100	---	10/1458	59/1459
8. *S. parvulus* NBRC 13193^T	**98.81**	**97.91**	99.14	99.16	99.38	99.32	99.31	---	59/1459
9. *S. albus subsp. albus* DSM 40313^T	**95.39**	**95.41**	96.14	96.03	95.95	95.96	95.96	95.96	---

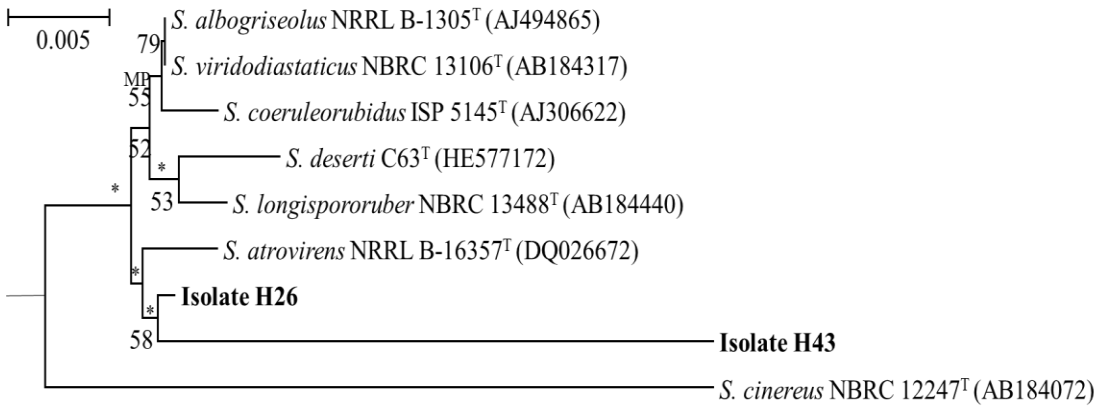

Figure 3.11 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H26 and H43 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods. MP indicates a branch of the tree that was recovered using 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.

Table 3.16 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H26 and H43 and between them and their closest phylogenetic neighbours.

	1	**2**	3	4	5	6	7	8	9
1. Isolate H26	**---**	**43/1401**	**8/1434**	**8/1434**	**11/1420**	**19/1425**	**10/1425**	**8/1434**	**59/1428**
2. Isolate H43	**96.93**	**---**	**56/1415**	**56/1415**	**56/1402**	**59/1399**	**47/1393**	**56/1415**	**98/1406**
3. *S. albogriseolus* NRRL B-1305^T	**99.44**	**96.04**	---	11/1484	6/1429	19/1434	7/1427	0/1463	58/1442
4. *S. atrovirens* NRRL B-16357^T	**99.44**	**96.04**	99.26	---	13/1429	19/1434	13/1427	10/1463	61/1442
5. *S. coeruleorubidus* ISP 5145^T	**99.23**	**96.01**	99.58	99.09	---	19/1426	9/1419	6/1429	58/1424
6. *S. deserti* C63^T	**98.67**	**95.78**	98.68	98.68	98.67	---	16/1425	19/1434	62/1427
7. *S. longispororuber* NBRC 13488^T	**99.3**	**96.63**	99.51	99.09	99.37	98.88	---	7/1427	62/1423
8. *S. viridodiastaticus* NBRC 13106^T	**99.44**	**96.04**	100	99.32	99.58	98.68	99.51	---	58/1442
9. *S. cinereus* NBRC 12247^T	**95.87**	**93.03**	95.98	95.77	95.93	95.66	95.64	95.98	---

Subclade 5. Isolate LB20 was recovered at the periphery of a taxon that was supported by the maximum-likelihood and neighbour-joining tree-making algorithms and by a 84% bootstrap value (Figure 3.12). It formed a well supported group together with the type strain of *Streptomyces cacaoi subspecies cacaoi*, these strains share a 99.85% 16S rRNA gene sequence similarity, which corresponds to 2 nt differences at 1324 locations (Table 3.18).

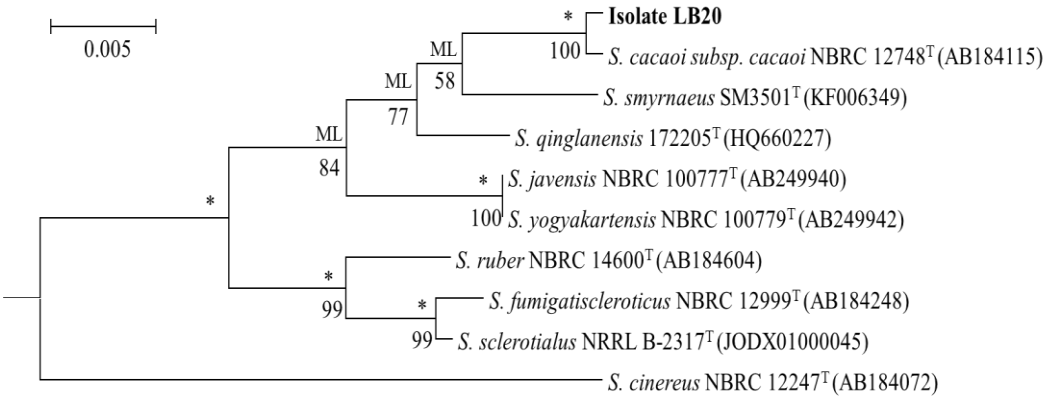

Figure 3.12 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolate LB20 and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates branches of the tree that were recovered using the maximum-likelihood 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.

Table 3.17 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate LB20 and its closest phylogenetic neighbours.

	1	2	3	4	5	6	7	8	9	10
1. Isolate LB20	**---**	**2/1324**	**40/1323**	**22/1322**	**20/1322**	**35/1315**	**38/1320**	**19/1322**	**22/1322**	**81/1323**
2. *S. cacaoi subsp. cacaoi* NBRC 12748^T	**99.85**	---	46/1462	24/1464	26/1464	40/1409	43/1460	23/1464	24/1464	89/1449
3. *S. fumigatiscleroticus* NBRC 12999^T	**96.98**	96.85	---	38/1460	46/1460	16/1409	5/1459	52/1460	38/1460	71/1446
4. *S. javensis* NBRC 100777^T	**98.34**	98.36	97.4	---	31/1463	30/1407	35/1460	40/1463	0/1465	76/1447
5. *S. qinglanensis* 172205^T	**98.49**	98.22	96.85	97.88	---	41/1407	43/1478	24/1470	31/1463	69/1447
6. *S. ruber* NBRC 14600^T	**97.34**	97.16	98.86	97.87	97.09	---	13/1406	48/1407	30/1407	66/1408
7. *S. sclerotialus* NRRL B-2317^T	**97.12**	97.05	99.66	97.6	97.09	99.08	---	49/1464	35/1460	68/1443
8. *S. smyrnaeus* SM3501^T	**98.56**	98.43	96.44	97.27	98.37	96.59	96.65	---	40/1463	76/1447
9. *S. yogyakartensis* NBRC 100779^T	**98.34**	98.36	97.4	100	97.88	97.87	97.6	97.27	---	76/1447
10. *S. cinereus* NBRC 12247^T	**93.88**	93.86	95.09	94.75	95.23	95.31	95.29	94.75	94.75	---

Subclade 6. The sole strain assigned to this taxon, isolate LB8, formed a distinct group together with the type strains of *Streptomyces bambergiensis* and *Streptomyces prasinus*, a relationship which is supported by a 97% bootstrap value and by all of the tree-making algorithms (Figure 3.13). The isolate is most closely related to *S. bambergiensis* NBRC 13479^T; these strains share a 99.42% 16S rRNA gene sequence similarity which equates to 8 nt differences at 1383 locations (Table 3.19).

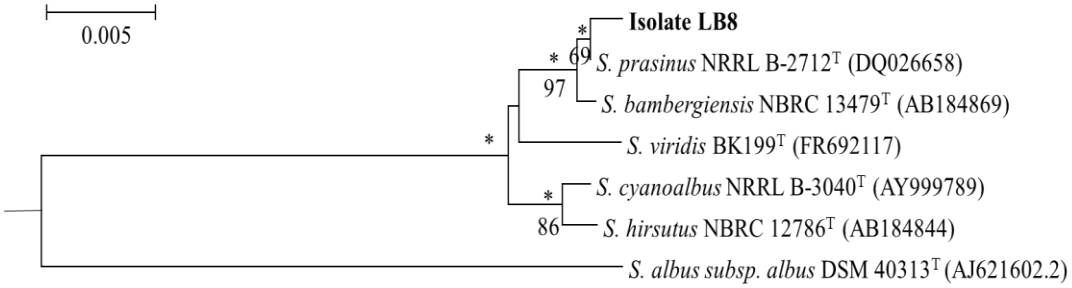

Figure 3.13 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolate LB8 and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony 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.

Table 3.18 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate LB8 and its closest phylogenetic neighbours.

	1	2	3	4	5	6	7
1. Isolate LB8	**---**	**8/1383**	**16/1383**	**16/1383**	**6/1383**	**13/1376**	**75/1383**
2. *S. bambergiensis* NBRC 13479^T	**99.42**	---	16/1460	15/1460	2/1460	11/1430	71/1458
3. *S. cyanoalbus* NRRL B-3040^T	**98.84**	98.9	---	5/1465	16/1475	19/1445	73/1460
4. *S. hirsutus* NBRC 12786^T	**98.84**	98.97	99.66	---	15/1465	18/1435	74/1461
5. *S. prasinus* NRRL B-2712^T	**99.57**	99.86	98.92	98.98	---	12/1454	70/1460
6. *S. viridis* BK199^T	**99.06**	99.23	98.69	98.75	99.17	---	71/1430
7. *S. albus subsp. albus* DSM 40313^T	**94.58**	95.13	95	94.93	95.21	95.03	---

Subclade 7. The single isolate in this taxon formed a loose but well supported 16S rRNA subclade together with *Streptomyces kunmingensis* NBRC 15711^T (Figure 3.14); these organisms shared a 98.78% gene sequence similarity, which corresponds to 17 nt differences at 1390 sites. However, the isolate was marginally more closely related to the type strain of *Streptomyces neopeptinus*, these organisms shared a 99.05% 16S rRNA gene sequence similarity, a value that is equivalent to 13 nt differences at 1371 locations (Table 3.20).

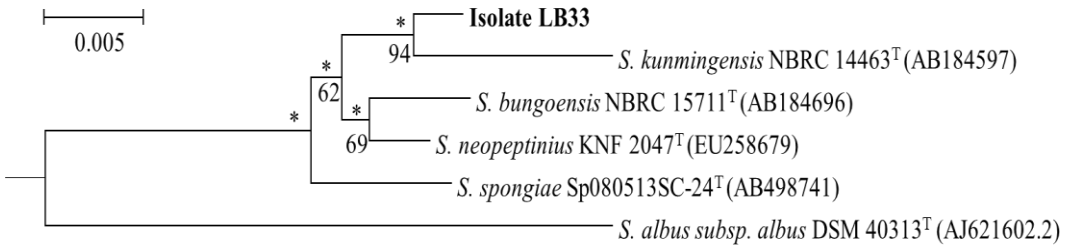

Figure 3.14 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolate LB33 and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony 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.

Table 3.19 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate LB33 and its closest phylogenetic neighbours.

	1	2	3	4	5	6
1. Isolate LB33	**---**	**18/1391**	**17/1390**	**13/1371**	**19/1391**	**69/1391**
2. *S. bungoensis* NBRC 15711^T	**98.71**	---	31/1451	11/1398	25/1463	67/1460
3. *S. kunmingensis* NBRC 14463^T	**98.78**	97.86	---	27/1397	29/1451	76/1451
4. *S. neopeptinius* KNF 2047^T	**99.05**	99.21	98.07	---	19/1398	67/1398
5. *S. spongiae* Sp080513SC-24^T	**98.63**	98.29	98	98.64	---	67/1460
6. *S. albus subsp. albus* DSM 40313^T	**95.04**	95.41	94.76	95.21	95.41	---

Subclade 8. Isolates H40 and H44 were well separated in a 16S rRNA subclade that was supported by all of the tree-making algorithms and by a 85% bootstrap value (Figure 3.15). Isolate H40 and *Streptomyces alboniger* NBRC 12738^{T} formed a well supported taxon, they shared a 98.53% 16S rRNA gene sequence similarity which corresponds to 21 nt differences at 1430 locations (Table 3.21). However, isolate H40 is slightly more closely related to the type strain of *Streptomyces pseudovenezuelae*, these organisms shared a 16S rRNA gene similarity of 98.6%, a value equivalent to 20 nt differences at 1422 sites. In turn, isolate H44 formed a loose 16S rRNA subclade together with the type strains of *Streptomyces cellostaticus* and *Streptomyces griseochromogenes*, but was most closely related to *Streptomyces graminifolii* JL-22^{T}, these strains had a 16S rRNA gene sequence similarity of 98.6%, which is equivalent to 18 nt differences at 1286 locations.

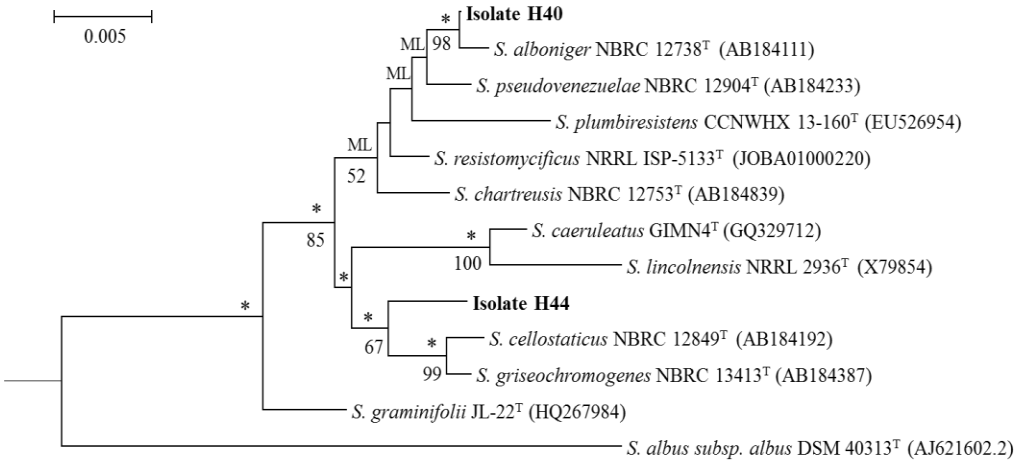

Figure 3.15 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H40 and H44 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates branches of the tree that were recovered using the maximum-likelihood 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.

Table 3.20 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H40 and H44 and between them and their closest phylogenetic neighbours.

	1	**2**	3	4	5	6	7	8	9	10	11	12	13
1. Isolate H40	**---**	**43/1329**	**21/1430**	**22/1394**	**39/1430**	**25/1430**	**21/1388**	**37/1430**	**41/1431**	**22/1419**	**20/1432**	**22/1430**	**83/1430**
2. Isolate H44	**96.76**	**---**	**39/1326**	**21/1297**	**28/1326**	**34/1326**	**18/1286**	**28/1326**	**44/1327**	**35/1318**	**33/1328**	**31/1326**	**81/1326**
3. *S. alboniger* NBRC 12738^T	**98.53**	**97.06**	---	26/1397	23/1436	16/1436	24/1389	21/1436	30/1435	18/1418	11/1436	13/1436	72/1436
4. *S. caeruleatus* GIMN4^T	**98.42**	**98.38**	98.14	---	21/1397	25/1397	27/1383	20/1397	11/1396	26/1394	16/1397	17/1397	71/1397
5. *S. cellostaticus* NBRC 12849^T	**97.27**	**97.89**	98.4	98.5	---	24/1463	28/1408	4/1460	31/1462	29/1427	21/1461	20/1463	68/1460
6. *S. chartreusis* NBRC 12753^T	**98.25**	**97.44**	98.89	98.21	98.36	---	14/1408	24/1460	31/1462	15/1427	13/1461	8/1463	64/1460
7. *S. graminifolii* JL-22^T	**98.49**	**98.6**	98.27	98.05	98.01	99.01	---	26/1405	33/1417	26/1397	20/1406	18/1418	56/1405
8. *S. griseochromogenes* NBRC 13413^T	**97.41**	**97.89**	98.54	98.57	99.73	98.36	98.15	---	30/1459	29/1427	19/1460	20/1460	69/1460
9. *S. lincolnensis* NRRL 2936^T	**97.13**	**96.68**	97.91	99.21	97.88	97.88	97.67	97.94	---	33/1428	22/1462	26/1483	71/1459
10. *S. plumbiresistens* CCNWHX 13-160^T	**98.45**	**97.34**	98.73	98.13	97.97	98.95	98.14	97.97	97.69	---	13/1429	13/1427	74/1427
11. *S. pseudovenezuelae* NBRC 12904^T	**98.6**	**97.52**	99.23	98.85	98.56	99.11	98.58	98.7	98.5	99.09	---	8/1461	65/1460
12. *S. resistomycificus* NRRL ISP-5133^T	**98.46**	**97.66**	99.09	98.78	98.63	99.45	98.73	98.63	98.25	99.09	99.45	---	66/1460
13. *S. albus subsp. albus* DSM 40313^T	**94.2**	**93.89**	94.99	94.92	95.34	95.62	96.01	95.27	95.13	94.81	95.55	95.48	---

Subclade 9. Isolate H2 was rcovered at the periphery of a well supported 16S rRNA subclade that included the type strains of *Streptomyces ederensis*, *Streptomyces phaeochromogenes* and *Streptomyces umbrinus* (Figure 3.16). The isolate was most closely related to *Streptomyces ederensis* NBRC 15410^T sharing a 16S rRNA gene sequence similarity with the latter of 97.46%, this corresponds to 36 nt differences at 1416 sites (Table 3.22).

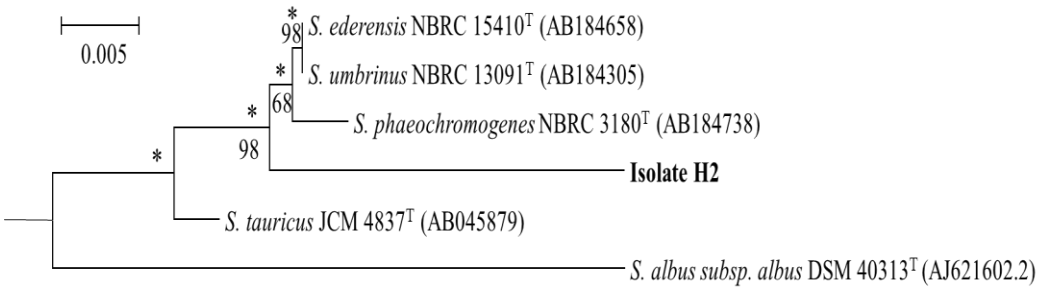

Figure 3.16 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolate H2 and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony 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.

Table 3.21 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate H2 and its closest phylogenetic neighbours.

	1	2	3	4	5	6
1. Isolate H2	**---**	**36/1416**	**47/1422**	**46/1422**	**37/1420**	**102/1422**
2. *S. ederensis* NBRC 15410^T	**97.46**	---	6/1433	17/1433	0/1432	76/1431
3. *S. phaeochromogenes* NBRC 3180^T	**96.69**	99.58	---	24/1465	9/1463	80/1460
4. *S. tauricus* JCM 4837^T	**96.77**	98.81	98.36	---	17/1463	68/1460
5. *S. umbrinus* NBRC 13091^T	**97.39**	100	99.38	98.84	---	76/1458
6. *S. albus subsp. albus* DSM 40313^T	**92.83**	94.69	94.52	95.34	94.79	---

Subclade 10. Isolates H53 and LB2 were recovered at the periphery of two distinct taxa, as shown in Figure 3.17. The first isolate was most closely related to the type strain of *Streptomyces lateritus* sharing a 16S rRNA gene sequence similarity of 99.51% with the latter, a value that corresponds to 7 nt differences at 1430 locations (Table 3.23). Similarly, isolate LB2 was most closely associated with *Streptomyces xantholiticus* NBRC 13354^{T}, these organisms shared a 16S rRNA gene sequence similarity of 98.77% which equates to 17 nt differences at 1377 sites.

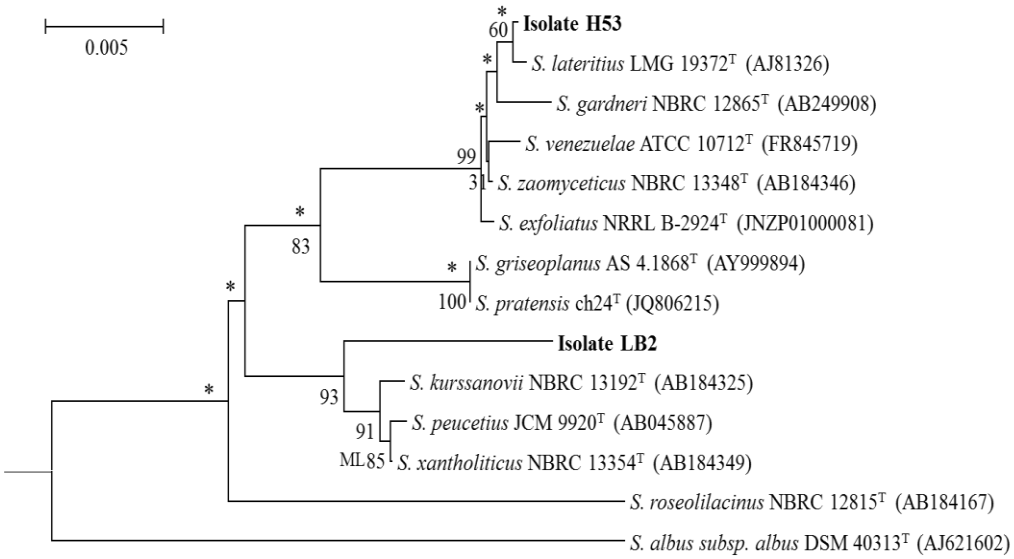

Figure 3.17 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H53 and LB2 and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered using the 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.

Table 3.22 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates H53 and LB2 and their closest phylogenetic neighbours.

	1	**2**	3	4	5	6	7	8	9	10	11	12	13	14
1. Isolate H53	**---**	**35/1371**	**9/1430**	**9/1424**	**22/1393**	**32/1430**	**7/1430**	**33/1431**	**20/1370**	**43/1430**	**10/1430**	**32/1431**	**8/1430**	**68/1431**
2. Isolate LB2	**97.45**	**---**	**34/1375**	**36/1375**	**28/1374**	**19/1375**	**37/1375**	**18/1377**	**26/1359**	**46/1375**	**37/1375**	**17/1377**	**35/1375**	**66/1377**
3. *S. exfoliatus* NRRL B-2924^T	**99.37**	**97.53**	---	5/1451	17/1401	26/1460	4/1464	26/1470	17/1370	41/1452	3/1484	25/1450	1/1457	61/1460
4. *S. gardneri* NBRC 12865^T	**99.37**	**97.38**	99.66	---	22/1401	26/1447	5/1451	26/1451	22/1370	40/1444	6/1451	25/1437	4/1444	61/1447
5. *S. griseoplanus* AS 4.1868^T	**98.42**	**97.96**	98.79	98.43	---	21/1401	21/1401	21/1401	0/1370	38/1401	20/1401	20/1401	18/1401	61/1401
6. *S. kurssanovii* NBRC 13192^T	**97.76**	**98.62**	98.22	98.2	98.5	---	25/1460	3/1460	21/1370	30/1452	25/1460	2/1450	25/1457	53/1460
7. *S. lateritius* LMG 19372^T	**99.51**	**97.31**	99.73	99.66	98.5	98.29	---	25/1464	21/1370	37/1452	3/1464	24/1450	3/1457	60/1460
8. *S. peucetius* JCM 9920^T	**97.69**	**98.69**	98.23	98.21	98.5	99.79	98.29	---	21/1370	33/1452	25/1470	1/1452	25/1457	53/1462
9. *S. pratensis* ch24^T	**98.54**	**98.09**	98.76	98.39	100	98.47	98.47	98.47	---	38/1370	20/1370	20/1370	18/1370	60/1370
10. *S. roseolilacinus* NBRC 12815^T	**96.99**	**96.65**	97.18	97.23	97.29	97.93	97.45	97.73	97.23	---	40/1452	31/1445	40/1452	68/1452
11. *S. venezuelae* ATCC 10712^T	**99.3**	**97.31**	99.8	99.59	98.57	98.29	99.8	98.3	98.54	97.25	---	24/1450	2/1457	60/1460
12. *S. xantholiticus* NBRC 13354^T	**97.76**	**98.77**	98.28	98.26	98.57	99.86	98.34	99.93	98.54	97.85	98.34	---	24/1450	54/1452
13. *S. zaomyceticus* NBRC 13348^T	**99.44**	**97.45**	99.93	99.72	98.72	98.28	99.79	98.28	98.69	97.25	99.86	98.34	---	60/1457
14. *S. albus subsp. albus* DSM 40313^T	**95.25**	**95.21**	95.82	95.78	95.65	96.37	95.89	96.37	95.62	95.32	95.89	96.28	95.88	---

3.5 Discussion

The results of the present study add to a growing weight of evidence which indicates that Atacama Desert habitats contain small numbers of taxonomically diverse, filamentous actinobacteria which have the capacity to produce bioactive compounds (Bull and Asenjo 2013; Bull et al. 2016). In general, the actinobacterial counts recorded from both the Cerro Chajnantor and Lomas Bayas sites chimed with corresponding results reported from arid Atacama Desert soils (Okoro et al. 2009; Busarakam 2014). These results when taken together with those from earlier culture-dependent studies (Cameron et al. 1966; Opfell and Zebal 1967) and from culture-independent surveys (Navarro-González et al. 2003; Connon et al. 2007; Costello et al. 2009; Crits-Christoph et al. 2013; Neilson et al. 2012) provide strong evidence that actinobacteria are components of microbial communities in a broad swathe of Atacama Desert habitats.

The surprising discovery that isolation plates from the initial selective isolation experiments were overrun by fast-growing bacteria necessitated the application of more robust selective isolation protocols, notably ones involving the use of heat-pretreated soil or soil suspensions. Plating heat-pretreated preparations from ALMA soils onto Gauze's No. 1 agar supplemented with cocktails of antibacterial antibiotics allowed the growth of actinobacterial colonies in some cases on plates relatively free of unwanted bacterial colonies. The highest count in these experiments, 6.43×10^2 colony forming units per gram dry weight soil, was recorded on Gauze's No. 1 agar plates supplemented with nalidixic acid and rifampicin which had been inoculated with ALMA 1 soil suspensions that had been heat-pretreated at 50^{o}C for 40 minutes. Relatively high actinobacterial counts were found when non- heat pre-treated suspensions of the ALMA soils were plated onto Gauze's No. 1, glucose-yeast extract and oligotrophic agar plates though in most cases the actinobacterial counts were low compared with those of the bacteria.

The results outlined above are not easy to interpret, but they do underline a critical point that is completely overlooked in studies designed to selectively isolate actinobacteria from environmental samples, namely that standard isolation protocols may need to be tailored to control the growth of fast-growing bacteria. Indeed, in this content a particularly interesting finding was that selective isolation plates inoculated with ALMA soil particles supported the growth of many actinobacterial colonies on isolation media free of unwanted bacteria. The potential value of this approach to the selective isolation of actinobacteria from environmental samples requires further study though in the case of the Lomas Bayas soil similar numbers of actinobacterial colonies were

recorded on a range of selective isolation media irrespective of whether they were inoculated with soil particles or soil suspensions. However, this apparently anomalous result can be attributed to the predominance of actinobacteria in extreme hyper-arid Atacama Desert soils, a point underlined by culture-independent studies (Neilson et al. 2012; Crits-Christoph et al. 2013). The low numbers of actinobacteria recovered on a range of selective isolation media inoculated with suspensions of Lomas Bayas soil is in good agreement with corresponding results recorded from extreme hyper-arid soil from the Yungay core region (Busarakam 2014).

All of the strains taken from the selective isolation plates were considered to be actinobacteria as they produced leathery substrate mycelia which mainly carried abundant aerial hyphae. It is also apparent that the selected isolates encompassed a considerable degree of taxonomic variation as they were assigned to 40 multi- and 92 single-membered colour-groups, a result in accord with those represented for hyper-arid and extreme hyper-arid Atacama Desert soils (Busarakam 2014; Okoro et al. 2009). It is particularly interesting that several strains isolated from the Cerro Chajnantor sampling sites were discontinuously distributed, members of colour-groups 85, 85, 112, 113, 115 and 116, for instance, were only isolated from ALMA 1 soil.

It seems likely that a clear majority of the isolates belong to the genus *Streptomyces* given their ability to form extensively branched substrate mycelia covered by an abundant aerial spore mass, a point underlined by associated 16S rRNA data generated on selected isolates. These findings are critical as there are a strong grounds for considering such colour-groups to be reliable indicators of species diversity within the genus *Streptomyces* (Atalan et al. 2000; Pathom-aree et al. 2006b; Antony-Babu and Goodfellow 2008; Busarakam 2014). It is clear from this and earlier studies (Busarakam 2014; Okoro et al. 2009) that Atacama Desert soils are a rich source of taxonomically diverse streptomycetes.

It is also apparent from the phylogenetic analyses that most of selected isolates formed distinct branches in *Amycolatopsis*, *Kineococcus*, *Microbispora*, *Nocardiopsis* and *Streptomyces* 16S rRNA gene trees consistent with their classification as novel species supporting chemotaxonomic, morphological and phenotypic data underpin this. The acquisition of such corroborating data for putatively novel strains isolated from arid Atacama Desert soils led to the valid publication of novel *Modestobacter* and *Streptomyces* species (Busarakam et al. 2016b; Busarakam et al. 2014). In general, members of non-streptomycete taxa do not produce distinctive substrate mycelial pigments or aerial spore mass colour on oatmeal agar, a situation which may lead to their

assignment to the same colour-groups, as exemplified in the present study by the grouping together of *Nocardia* and *Nocardiopsis* strains in colour-group 1. It can be concluded from the present and previous studies (Okoro *et al.*, 2009; 2010; Santhanam *et al.*, 2012a; 2012b; 2013; Busarakam, 2014; Busarakam *et al.*, 2016a; 2016b) that the Atacama Desert landscape is a rich reservoir of rare and novel filamentous actinobacteria suitable for bioprospecting campaigns.

The results of this study provide further evidence of the effectiveness of the taxonomic approach to drug discovery as nearly 74% out of the 218 strains isolated from Cerro Chajnantor soils and around 50% of the 64 Lomas Bayas strains inhibited the growth of one or more of the panel of wild type microorganisms; in corresponding studies of actinobacteria isolated from hyper-arid and extreme hyper-arid Atacama Desert soils nearly 68%, that is, 92 out of 126 dereplicated strains inhibited the same group of wild type microorganisms (Busarakam 2014). It can be concluded from these and other bioprospecting campaigns that high hit rates can be achieved when representative actinobacteria are examined in primary screens (Eccleston et al. 2008; Guo et al. 2015).

It was particularly interesting that 70 of the strains isolated from Cerro Chajnantor soils inhibited the growth of the *E. coli* wild type strain and/or that of the *P. fluorescens* strain, as Gram-negative bacteria are difficult to kill (Butler and Cooper 2011). Indeed, there is a pressing need to find new natural products to control the spread of Gram-negative pathogens (Boucher et al. 2009; Donadio et al. 2010; Genilloud 2014). The present study also demonstrated the importance of growing isolates for standard plug assay on more than one growth medium, as exemplified by the finding that 7 of the isolates that inhibited the growth of the *E. coli* strain only did so when grown on ISP 3 agar. In studies restricted to one growth medium 10 isolates from arid Atacama Desert soils were found to inhibit the growth of both *E. coli* and *P. fluorescens* wild type strains (Busarakam 2014).

It is especially encouraging that so many of the strains isolated from the Cerro Chajnantor and Lomas Bayas soils produced blue halos when screened against the *B. subtilis* reporter strains thereby indicating an overall ability to inhibit cell envelope, cell wall, DNA and fatty acid synthesis, as well as the capacity in inhibit sporulation. Such datasets are important as they allow future studies to be focused on one or more cellular targets. However, it is also important not to overlook isolates that inhibit *B. subtilis* reporter strains without the formation of blue halos as such strains may synthesize bioactive compounds with distinctive modes of action. It was also interesting that 14 isolates from Cerro Chajnantor soils inhibited the growth of all of the *B. subtilis* reporter

strains, such strains as they may produce bioactive compounds that have broad spectrum activity.

It is apparent from this study that small numbers of taxonomically diverse filamentous actinobacteria are present in high altitude and extreme hyper-arid Atacama Desert soils, an outcome in line with previous surveys albeit of different sampling sites (Bull and Asenjo 2013; Busarakam 2014; Bull et al. 2016). The isolation of members of the genera *Kineococcus, Lentzea, Microbispora* and *Nocardia* is of particular interest as members of these taxa have not been recovered previously from Atacama Desert soils. Sixty one of strains isolated from the Cerro Chajnantor and Lomas Bayas soils were selected for chemical screening, as described in Chapter 5. In addition, complementary culture-independent studies were designed to establish whether the taxa highlighted in this culture-dependent study are representative of actinobacterial communities present in the ALMA and Lomas Bayas soils, the results of these studies presented in Chapters 6 and 7.

Acknowledgements

The selective isolation of strains LB1 to LB47 was done by Mr. Owajiogak Hayford (M.Sc. student, School of Biology, Newcastle University), 16S rRNA gene sequencing of 5 isolates obtained from Lomas Bayas sample were carried out by Ms. Yvonne Wu and Ms. Arailym Ziyat (M.Sc. students, School of Biology, Newcastle University).

Chapter 4. Polyphasic taxonomic studies on putatively novel actinobacteria isolated from Atacama Desert habitats

4.1 Abstract

Polyphasic studies were undertaken to establish whether selected filamentous actinobacteria isolated from Atacama Desert soils which formed distinct branches in 16S rRNA gene trees belonged to novel species. Strains isolated from sampling sites on Cerro Chajnantor were found to form new centres of taxonomic variation within the genera *Actinomadura*, *Amycolatopsis*, *Cryptosporangium* and *Pseudonocardia* while ones recovered from arid soils from the Salar de Atacama and Yungay core regions of the desert belonged to new *Pseudonocardia* and *Streptomyces* species. A wealth of genotype and phenotype data showed that three strains from the Yungay core region and six isolates from hyper- and extreme hyper-arid soils could be separated readily from the type strains of their nearest phylogenetic neighbours and thereby merited recognition as novel species of *Pseudonocardia* and *Streptomyces*, respectively. It is proposed that these strains should be recognized as *Pseudonocardia yungayensis* sp. nov. and *Streptomyces asenjonii* sp. nov..

4.2 Introduction

A major attraction of filamentous actinobacteria, notably streptomycetes, is their unique ability to synthesize new bioactive natural products with a wide spectrum of activity (Hopwood 2007; Bérdy 2012; Barka et al. 2016). A practical approach to the detection of such specialized metabolites, notably antibiotics, is based on the premise that extreme habitats support populations of taxonomically novel actinobacteria that have the capacity to produce new natural products that can be developed as drug leads (Bull 2011; Goodfellow et al. 2013). However, innovative approaches are needed to selectively isolate, dereplicate and screen novel filamentous actinobacteria as processing large numbers of common strains leads to the wasteful rediscovery of known natural products (Baltz 2008; Genilloud 2014; Bérdy 2012).

The taxonomic approach to drug discovery recommended by Goodfellow and Fiedler (2010) has been found to especially effective in the isolation of novel filamentous actinobacteria from marine habitats (Bull and Stach 2007) and hyper- and extreme hyper-

arid Atacama Desert soils (Okoro et al. 2009; Bull and Asenjo 2013; Bull et al. 2016). Indeed, polyphasic taxonomic studies on dereplicated actinobacteria have resulted in the valid publication of novel species of *Amycolatopsis* (Busarakam et al. 2016a), *Lechevalieria* (Okoro et al. 2010) and *Modestobacter* (Busarakam et al. 2016b). In addition, several new *Streptomyces* species have been recognized (2013; 2012b; Santhanam et al. 2012a), one of which, *Streptomyces leeuwenhokeii,* encompasses strains that synthesize new antibiotics (2011b; Rateb et al. 2011a) and chaxapeptin, a new lasso peptide (Elsayed et al. 2015).

The present study was designed to establish the taxonomic provenance of putatively novel filamentous actinobacteria isolated from high altitude Atacama Desert soils using the taxonomic approach to drug discovery. The selected organisms were the subject of polyphasic taxonomic studies, as were several streptomycetes previously isolated from hyper-arid and extreme hyper-arid Atacama Desert soils. The resultant data showed that most of the isolates belonged to novel species of *Actinomadura, Amycolatopsis, Cryptosporangium, Pseudonocardia* and *Streptomyces.*

4.3 Materials and Methods

4.3.1 Selection and maintenance of strain

Twenty one strains known to form distinct branches in 16S rRNA gene trees were examined to determine whether they merited recognition as novel species. The source of the isolates and the type strains of their nearest phylogenetic neighbours together with details of the taxonomic tests carried out on them are shown in Table 4.1.

Table 4.1 Source of isolates and their associated type strains included in the polyphasic taxonomic analyses.

Genus	Isolate / source/ isolation medium	Associated type strain
Strains isolated from Cerro Chajnantor soil samples:		
Actinomadura	H59 [(1,2,3,4)]; ALMA 2; Gauze No. 1 agar + nalidixic acid and rifampicin (each at 25µg/ml)	*A.napierensis* DSM 44846 [T (3)] *A.yumaensis* DSM 43931 [T (3)]
Amycolatopsis	H5 [(1,2,3,4)], H6 [(1,2,3,4)]; ALMA 4 ; glucose-yeast extract agar	*A.balhimycina* DSM 44591 [T (3)] *A.lexingtonensis* DSM 44653 [T (3)]

		A.kentuckyensis DSM 44652 T (3) *A.mediterranei* DSM 43304 T (3) *A.pretoriensis* DSM 44654 T (3) *A.tolypomycina* DSM 44544 T (3)
Cryptosporangium	H7 (1,2,3,4) ; ALMA 4; Gauze No. 1 agar + nalidixic acid and rifampicin (each at 25µg/ml)	*C. arvum* NBRC 15965 T (3) *C. aurantiacum* DSM 46144T (3) *C. japonicum* NBRC 15966 T (3) *C. minutisporangium* NBRC 15962 T (3) *C. mongoliense* NBRC 105887 T (3)
Pseudonocardia	H57 (1,2,3,4), H58 (1,2,3,4) ; ALMA 2; glucose-yeast extract agar + rifampicin (25µg/ml) H69(1,2,3,4) ; ALMA 2; Gauze No. 1 agar + nalidixic acid and rifampicin (each at 25µg/ml) H96 (1,2,3,4); ALMA 3; Gauze No. 1 agar + nalidixic acid and rifampicin (each at 25µg/ml) H99 (1,2,3,4) ; ALMA 3; Gauze No. 1 agar + nalidixic acid and rifampicin (each at 25µg/ml)	*P. adelaidensis* DSM 45352T (3) *P. zijingensis* DSM 44774T (3) *P. kunmingensis* DSM 45301T (3) *P. petroleophila* DSM 43193T (3) *P. khuvsgulensis* NBRC 105886 T (3) *P. rhizophila* DSM 45381T(3)
Strains isolated from extreme hyper-arid soil by Professor Martha Trujillo:		
Pseudonocardia	ATK01 (1,2,3,4), ATK03 (1,2,3,4), ATK17 (1,2,3,4); CAB3	*P. bannensis* DSM 45300 T (3) *P. xinjiangensis* DSM 44661T (3)
Strains isolated from hyper- and extreme-hyper arid Atacama Desert soil by Busarakam (2014):		
Streptomyces	C59 (2,3), KNN 26.b (3), KNN 38.1b (2,3), KNN 64.5b (3) KNN 6.11a (3), KNN 35.1b (2,3), KNN 35.2b (2,3), KNN 42.f(3), KNN48.3(3), KNN 83.e (3)	*S. fimbriatus* NRRL B-3175 T (2,3) *S. ghanaensis* NRRL B-12104T (2,3)

*All selective media were supplemented with cycloheximide and nystatin (each at 25µg/ml).

Strains examined for: [1], cultural properties; [2], chemotaxonomic markers; [3], phenotypic tests; [4], spore chain arrangement and spore surface ornamentation.

Full histories of the marker strains can be found in Table 2.5, Chapter 2.

Phylogenetic analyses.. The test strains were grown on yeast-extract malt-extract plates (ISP2 medium, Shirling & Gottlieb, 1966) for 14 days at 28°C, according to the protocol described by (Fujimoto et al. 2004). Genomic DNA of the strains was extracted from biomass scrapped from the plates. PCR amplification of 16S rRNA genes and purification of the PCR products were carried out, as described in Chapter 2. The resultant almost complete 16S rRNA gene sequences (~1450 nucleotides [nt]) were edited using the ABI format files in BioEdit version 7.2.5 (Hall 1999) and the nearest match of each of the edited sequences was established using the Basic Local Alignment Search Tool (BLAST) and pairwise sequence similarities determined using the global alignment algorithm implemented with the EzTaxon e web server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Phylogenetic trees showing relationships between the isolates and between them and closely related marker strains were generated using the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms from MEGA6 version 5.0 software (Tamura et al. 2013). Bootstrap values based on 1000 repeats (Felsenstein 1985) were generated using the evolutionary distance model of Jukes and Cantor (1969).

Multilocus sequence analyses. The experimental and data handling procedures used in these analyses were based upon modifications of described procedures (Labeda *et al.*, 2014; Labeda, 2016). Biomass for extraction of DNA was prepared following cultivation of isolates KNN 6.11a, KNN 35.1b, KNN 35.2b, KNN 42.f, KNN 48.3 and KNN 83.e in yeast extract-malt extract broth (ISP2 medium, Shirling & Gottlieb, 1966) at 28°C. Genomic DNA was isolated from all of the isolates using UltraClean® Microbial DNA isolation kits (MoBio Labs, Carlsbad, CA) by following the manufacturer's instructions. Partial sequences of the house-keeping genes *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase beta subunit), *recA* (recombinase A) and *trpB* (tryptophan synthetase, beta subunit) were amplified and sequenced using the primers and protocols, as described previously by Labeda *et al.* (2014). The amplified products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA), sequenced using BigDye 3.1 on an ABI model 3730 sequencer and assembled using Sequencher version 5.2 (Gene Codes, Ann Arbor, MI).

The gene sequences for the 5 house-keeping loci for each of the strains sequenced were deposited in GenBank and were also organized using the Bacterial Isolate Genomic Sequence Database (BIGSdb) version 1.12.3 (Jolley and Maiden 2010) on the ARS Microbial Genomic Sequence Database server (http://199.133.98.43). Genome

sequences, where available, were up loaded into the sequence bin for the respective isolates in the BIGSdb isolate database. The genome sequences were scanned within BIGSdb for house-keeping loci, the sequences of which were then tagged and the allele sequences and respective allele designations added to the sequence database when new alleles were found. The strain record was then updated with the matching allele identification for each locus held in the strain database. The sequences for the alleles of the loci for all of the strains in the study were individually aligned with MAFFT (Katoh and Standley 2013), subsequently concatenated head to tail in-frame, and exported in FASTA format, providing a dataset of 706 *Streptomyces* strains and 2622 positions (Labeda 2016).

Phylogenetic relationships were constructed in IQ-Tree version 1.41 (Nguyen et al. 2015) using the maximum-likelihood algorithm based on the general time reversible model (Nei and Kumar 2000) with invariable sites plus a discrete Gamma model based on 4 rate categories (Gu et al. 1995) which had been shown to be the optimal model for such data using jModelTest 2 (Darriba et al. 2012). The individual trees were the subject of 1000 ultrafast bootstrap replications (Minh et al. 2013) followed by 1000 replications of assessment of branch supports with single branch tests using the SH-like approximate likelihood ratio test (Guindon et al. 2010). MLSA evolutionary distances were determined using MEGA 6.0 by calculating the Kimura 2-parameter distances (Kimura, 1980). Strain pairs having ≤ 0.007 MLSA evolutionary distances were considered conspecific based on the guideline empirically determined by Rong and Huang (2012), namely that this MLSA distance (Kimura 2-paramenter distance), computed from the partial sequences of these house-keeping loci, equates to the 70% DNA:DNA cut-off point recommended foe the delineation of species by Wayne et al. (1987).

Chemotaxonomy. Biomass for the chemotaxonomic studies carried out on most of the test strains shown in Table 4.1 were prepared in shake flasks containing 1 litre of ISP 2 broth (Shirling & Gottlieb, 1966), the flasks were shaken at 180 rpm for 14 days at 28^{o}C and the biomass collected by centrifugation at 4000 rpm for 10 minutes, washed in distilled water and freeze dried. Biomass of the *Pseudonocardia* strains was prepared in the same way albeit in modified Bennett's broth (Agrawal, unpublished). The cultivated strains were examined for the presence of diaminopimelic acid isomers, fatty acids, polar lipids, menaquinones and sugars using the 'in house' protocols, as described in Appendix 4.

Cultural and morphological properties. All of the strains (Table 4.1) were examined for cultural properties following growth on tryptone-yeast extract, yeast extract-malt extract, oatmeal, tryptone-yeast extract, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1 to 7; Shirling and Gottlieb (1966)) for 2 weeks at 28ºC, as described in Chapter 2. Spore chain arrangement and spore surface ornamentation were determined using cultures grown on ISP 3 agar (Shirling and Gottlieb 1966) by scanning electron microscopy, as described by O'Donnell et al. (1993).

Phenotypic tests. All of the strains were examined for a broad range of phenotypic properties. Inocula of most of the strains were prepared by scrapping spores/hyphal fragments from ISP 2 agar plates (Shirling and Gottlieb 1966) that had been incubated at 28ºC for 14 days. Similarly, inocula were prepared from the *Pseudonocardia* strains which were grown on modified Bennett's agar (Agrawal, unpublished). In both cases, biomass preparations were ground using sterile micropestles in Eppendorf tubes and then added to Bijou tubes containing ¼ strength Ringer's solution to give a turbidity of 5 on the McFarland scale (Murray et al. 1999). Biochemical and degradation tests and the ability of the strains to grow over a range of temperatures were performed out using standard microbiological procedures, as described in Chapter 2. The remaining tests were carried out using API ZYM kits (Biomeriuex Co., Hampshire, UK) and GEN III MicroPlates (BIOLOG Inc., Hayward, CA, USA), as detailed in Chapter 2.

4.4 Results

4.4.1 Classification of Actinomadura strains

Phylogenetic analyses. Three isolates, strains H59, LB25 and LB54, were found to form distinct phyletic lines within the evolutionary radiation occupied by the genus *Actinomadura* (Figure 4.1). Strain H59 was isolated from the ALMA 2 environmental sample on glucose-yeast extract agar and is one of three representatives of multi-membered colour-group 52 (Appendix 1). Isolates LB25 and LB54 were isolated from a Lomas Bayas environmental sample and represent multi-membered colour-groups 14 and 10, which encompass 3 and 2 isolates, respectively (Appendix 1).

Isolate H59 is closely related to *Actinomadura yumaensis* JCM 3369^T, this relationship is supported by all the tree-making algorithms and by a 99% bootstrap value;

these organisms share a 16S rRNA gene sequence similarity of 97.0%, a value which corresponds to 44 nt differences at 1441 locations (Table 4.2). However, isolate H59 is marginally more closely related to *Actinomadura napierensis* B60^{T}; these organisms share a 97.3% 16S rRNA gene sequence similarity which equates to 36 nt differences albeit at only 1339 locations. In turn, isolate LB25 forms a loose subclade together with *Actinomadura geliboluensis* A8036^{T}, an association that is supported by all the tree-making algorithms and by a 100% bootstrap value; these strains share a 16S rRNA gene sequence similarity of 97.8%, a value that corresponds to 30 nt differences at 1364 locations (Table 4.2). The remaining strain, isolate LB54, lies towards the periphery of a 16S rRNA gene subclade that includes *Actinomadura citrea* IFO 14678^{T}, *Actinomadura coerulea* IFO 14679^{T}, *Actinomadura glauciflava* AS 4.1202^{T}, *Actinomadura luteofluorescens* IFO 13057^{T}, *Actinomadura mexicana* A290^{T} and *Actinomadura verrucisospora* NBRC 14100^{T}; the status of this taxon is supported by all of the tree-making algorithms and by a 95% bootstrap value. Isolate LB54 was found to be most closely related to the type strains of *Actinomadura coerulea* and *Actinomadura verrucosospora*, it shares a 16S rRNA gene similarity of 99.2% with these strains, a value that equates to 11 nt differences at 1369 and 1368 sites, respectively.

Characterization of strain H59. This isolate was studied further as it clearly forms a distinct branch in the *Actinomadura* 16S rRNA gene tree and hence may be the nucleus of a novel *Actinomadura* species.

Cultural and morphological properties. The isolate grew moderately well on most of the ISP media but poorly on ISP 5 and 7 (Table 4.3). It is also evident from this Table that the aerial hyphae were predominantly white and the substrate mycelia mainly yellow white; colonies were leathery, irregular, umbonate with undulate margins. The strain formed an extensively branched substrate mycelium that bore aerial hyphae on oatmeal agar, some of the aerial hyphae differentiated into chains of smooth spores (Figure 4.2).

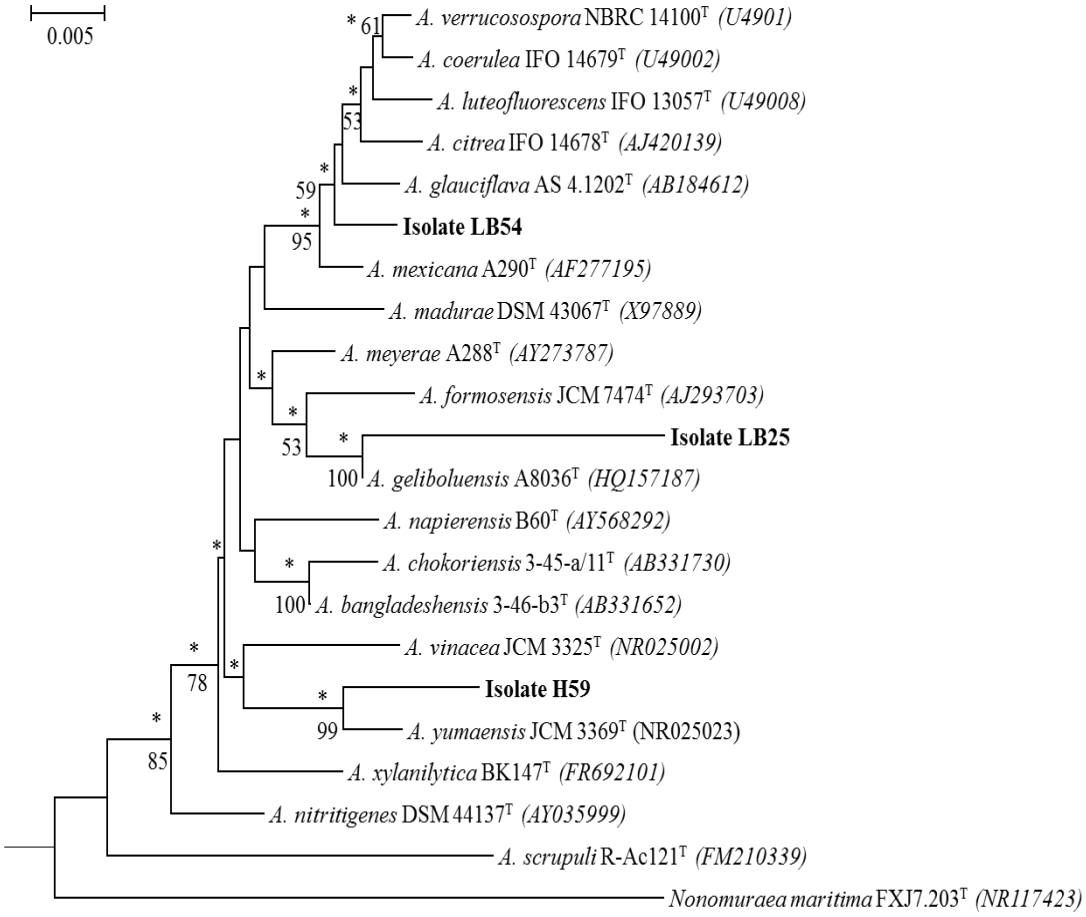

Figure 4.1 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H59, LB25 and LB54 and between them and the type strains of closely related *Actinomadura* species. *Asterisks* indicate branches of the tree that were also recovered using 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.

Table 4.2 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolates H59, LB25 and LB54 and between them and their nearest phylogenetic neighbours.

	1	**2**	**3**	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Isolate H59	**---**	**67/1358**	**43/1367**	**44/1441**	**53/1428**	**49/1403**	**75/1393**	**57/1428**	**51/1420**	**65/1436**	**36/1339**	**67/1443**	**64/1444**	**57/1429**	**67/1437**	**60/1445**	**49/1403**	**48/1413**	**49/1404**	**50/1413**	**50/1404**	**119/1435**
2. Isolate LB25	**95.1**	**---**	**56/1360**	**58/1363**	**60/1362**	**56/1358**	**84/1360**	**52/1362**	**53/1363**	**50/1354**	**53/1342**	**46/1364**	**30/1364**	**42/1361**	**53/1361**	**44/1363**	**58/1358**	**54/1359**	**61/1361**	**57/1359**	**58/1359**	**107/1359**
3. Isolate LB54	**96.9**	**95.9**	**---**	**33/1372**	**29/1371**	**11/1368**	**60/1358**	**32/1368**	**29/1369**	**30/1363**	**32/1342**	**21/1372**	**28/1369**	**23/1366**	**29/1368**	**23/1372**	**17/1368**	**15/1366**	**16/1368**	**12/1369**	**11/1369**	**85/1362**
4. *A. yumaensis* JCM 3369^T	**97.0**	**95.7**	**97.6**	---	30/1430	35/1408	61/1399	33/1434	32/1421	36/1438	29/1342	43/1444	33/1445	29/1437	35/1440	30/1447	35/1408	28/1420	38/1408	36/1420	36/1409	89/1437
5. *A. vinacea* JCM 3325^T	**96.3**	**95.6**	**97.9**	97.9	---	30/1407	60/1396	33/1417	29/1429	29/1432	32/1342	30/1440	32/1438	29/1421	31/1431	22/1441	32/1407	29/1419	35/1408	34/1418	32/1408	90/1429
6. *A. verrucosispora* NBRC 14100^T	**96.5**	**95.9**	**99.2**	97.5	97.9	---	65/1381	32/1404	29/1397	30/1399	35/1338	25/1408	28/1405	24/1403	29/1404	23/1408	9/1409	18/1402	13/1406	18/1403	6/1407	91/1397
7. *A. scrupuli* R-Ac121^T	**94.6**	**93.8**	**95.6**	95.6	95.7	95.3	---	53/1394	62/1399	63/1389	53/1342	66/1399	60/1399	61/1396	65/1396	57/1398	71/1381	64/1394	73/1383	69/1395	68/1382	94/1394
8. *A. nitritigenes* DSM 44137^T	**96.0**	**96.2**	**97.7**	97.7	97.7	97.7	96.2	---	25/1409	33/1425	30/1344	34/1432	26/1434	27/1428	32/1431	27/1434	36/1404	28/1417	37/1406	37/1413	33/1405	78/1426
9. *A. xylanilytica* BK147^T	**96.4**	**96.1**	**97.9**	97.8	98.0	97.9	95.6	98.2	---	29/1440	25/1343	32/1431	26/1441	24/1414	29/1422	23/1431	30/1397	24/1410	29/1399	30/1411	30/1398	83/1443
10. *A. chokoriensis* 3-45-a/11^T	**95.5**	**96.3**	**97.8**	97.5	98.0	97.9	95.5	97.7	98.0	---	24/1334	31/1444	26/1455	20/1427	27/1435	7/1449	32/1399	31/1412	35/1400	34/1411	32/1400	89/1455
11. *A. napierensis* B60^T	**97.3**	**96.1**	**97.6**	97.8	97.6	97.4	96.1	97.8	98.1	98.2	---	30/1344	27/1344	25/1340	29/1343	20/1343	37/1338	30/1341	36/1341	34/1339	36/1339	84/1338
12. *A. formosensis* JCM 7474^T	**95.4**	**96.6**	**98.5**	97.0	97.9	98.2	95.3	97.6	97.8	97.9	97.8	---	18/1452	26/1435	31/1445	25/1453	29/1408	22/1421	25/1410	27/1420	25/1409	93/1444
13. *A. geliboluensis* A8036^T	**95.6**	**97.8**	**98.0**	97.7	97.8	98.0	95.7	98.2	98.2	98.2	98.0	98.8	---	11/1437	25/1443	16/1455	30/1405	26/1419	33/1408	32/1417	30/1406	89/1457
14. *A. meyerae* A288^T	**96.0**	**96.9**	**98.3**	98.0	98.0	98.3	95.6	98.1	98.3	98.6	98.1	98.2	99.2	---	22/1432	13/1436	26/1403	23/1416	30/1405	29/1414	26/1404	86/1429
15. *A. madurae* DSM 43067^T	**95.3**	**96.1**	**97.9**	97.6	97.8	97.9	95.3	97.8	98.0	98.1	97.8	97.9	98.3	98.5	---	20/1444	31/1404	21/1421	30/1405	33/1416	31/1405	85/1435
16. *A. bangladeshensis* 3-46-b3^T	**95.9**	**96.8**	**98.3**	97.9	98.5	98.4	95.9	98.1	98.4	99.5	98.5	98.3	98.9	99.1	98.6	---	25/1408	24/1421	29/1409	27/1420	25/1409	83/1446
17. *A. luteofluorescens* IFO 13057^T	**96.5**	**95.7**	**98.8**	97.5	97.7	99.4	94.9	97.4	97.9	97.7	97.2	97.9	97.9	98.2	97.8	98.2	---	18/1402	13/1406	16/1403	10/1407	93/1397
18. *A. mexicana* A290^T	**96.6**	**96.0**	**98.9**	98.0	98.0	98.7	95.4	98.0	98.3	97.8	97.8	98.5	98.2	98.4	98.5	98.3	98.7	---	14/1403	16/1414	16/1403	88/1411
19. *A. citrea* IFO 14678^T	**96.5**	**95.5**	**98.8**	97.3	97.5	99.1	94.7	97.4	97.9	97.5	97.3	98.2	97.7	97.9	97.9	97.9	99.1	99.0	---	19/1403	11/1407	93/1399
20. *A. glauciflava* AS 4.1202^T	**96.5**	**95.8**	**99.1**	97.5	97.6	98.7	95.1	97.4	97.9	97.6	97.5	98.1	97.7	98.0	97.7	98.1	98.9	98.9	98.7	---	16/1404	94/1408
21. *A. coerulea* IFO 14679^T	**96.4**	**95.7**	**99.2**	97.4	97.7	99.6	95.1	97.7	97.9	97.7	97.3	98.2	97.9	98.2	97.8	98.2	99.3	98.9	99.2	98.9	---	92/1397
22. *Nonomuraea maritima* FXJ7.203^T	**91.7**	**92.1**	**93.8**	93.8	93.7	93.5	93.3	94.5	94.3	93.9	93.7	93.6	93.9	94.0	94.1	94.3	93.3	93.8	93.4	93.3	93.4	---

Table 4.3 Growth and cultural characteristics of isolate H59 on ISP media after incubation for 14 days at 28°C.

ISP media	Characteristics		
	Growth	Aerial hyphae	Substrate mycelium
Glycerol-asparagine agar (ISP medium 5)	+	None	Yellow white
Inorganic salts-starch agar (ISP medium 4)	++	Light olive gray	Yellow white
Oatmeal agar (ISP medium 3)	++	White	Yellow white
Peptone-yeast extract-iron agar (ISP medium 6)	++	White	Light brown
Tryptone-yeast extract agar (ISP medium 1)	++	White	Gray yellow
Tyrosine agar (ISP medium 7)	+	None	Yellow white
Yeast extract-malt extract agar (ISP medium 2)	++	None	Light yellow brown

++ moderate growth; + good growth; + poor growth. Diffusible pigments were not produced on any of the media.

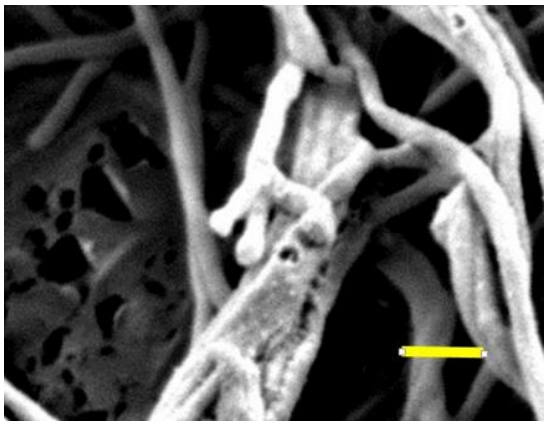

Figure 4.2 Scanning electron micrograph of *Actinomadura* isolate H59 showing chains of smooth spores following growth on oatmeal agar after incubation at 28°C for 10 days. Bar 1µm.

Chemotaxonomy. The whole-organism hydrolysates of isolate H59 were found to contain *meso*-A_2pm and galactose, glucose, mannose and ribose. The polar lipid pattern consisted of diphosphatidylglycerol, methyl- phosphatidylethanolamine, phosphatidylglycerol, aminoglycolipid, aminolipid, aminophospholipid and phospholipids (Figure 4.3) and the major menaquinones was MK9(H_8) (52%), other

components were MK10(H_6) (15%), MK9(H_6) (13%) together with a small proportion of MK9(H_4) (4%).

Phenotypic properties. Identical results were obtained for the duplicated phenotypic tests carried out on isolate H59, *A. napierensis* DSM 44846^T and *A.yumaensis* DSM 43931^T, apart for some of the BIOLOG tests. It is evident from Table 4.4 that the isolate can be differentiated readily from its closest phylogenetic neighbours using a range of phenotypic properties though it is also apparent that all three strains have many properties in common. The isolate, unlike the type strain of *A. napierensis*, produces α-chymotrypsin and metabolizes D-fructose, D-fucose, β-gentiobiose, D-mannose, glycerol, L-rhamnose, *myo*-inositol and a number of other compounds. In contrast, the *A. napierensis* strain, unlike isolate H59, produces α-glucuronidase and naphthol-AS-BI-phosphohydrolase (API tests), degrades starch and uric acid and utilizes D-maltose, L-alanine, L-arginine, L-histidine and L-serine and a number of other compounds and was resistant to aztreonam, lincomycin, minocycline and troleandomycin. In turn, isolate H59 can be differentiated from the type strain of *A. yumaensis* by its ability to produce β-glucosidase, utilize D-fructose-6-phosphate, D-glucose-6-phosphate, glycl-L-proline, D- and L-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-β-D-mannosamine and a number of other compounds. In contrast, only the *A.yumaensis* strain metabolized, for example, D-maltose, D-mannitol, L-alanine, D-turanose, is resistant to aztreonam, fusidic acid, guanidine hydrochloride, minocycline and vancomycin and grows in the presence of up to 8% w/v, sodium chloride and at 45^{o}C.

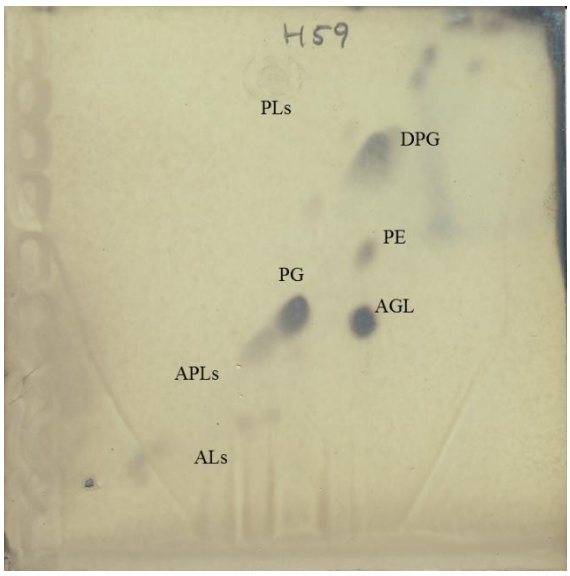

Figure 4.3 Two-dimensional thin-layer chromatography of polar lipids of isolate H7 stained with molybdenum blue spray (Sigma). Chloroform : methanol : water (32.5:12.5:2.0, v/v) was used in the first direction, followed by chloroform : acetic acid : methanol : water (40.0:7.5:6.0:2.0, v/v) in the second direction. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatodylglycerol, PL, phospholipids; AL, aminolipid; AGL, aminoglycolipid and APL, aminophospholipid.

Table 4.4 Phenotypic characteristics that differentiate isolate H59 from *Actinomadura napierensis* DSM 44846^T and *Actinomadura yumaensis* DSM 43931^T

Characteristics	Isolate H59	*A. napierensis* DSM 44846^T	*A.yumaensis* DSM 43931^T
API ZYM tests:			
α-Chymotripsin	+	-	+
β-Glucosidase	+	+	-
α-Glucuronidase, naphthol-AS-BI-phosphohydrolase	-	+	-
Lipase (C14)	-	+	-
GEN III BIOLOG microplate tests			
(a) Utilization of sugars:			
N-Acetyl-neuraminic acid	+	-	-
N-Acetyl-D-galactosamine, *N*-acetyl-β-D-mannosamine, D-fructose-6-phosphate, D-glucose-6-phosphate	+	+	-
L-Alanine, D-maltose	-	+	+
L-Histidine	-	+	-
Dextrin	+	+	+
D-Fructose, glycerol, *myo*-inositol, L-rhamnose	+	-	+

D-Fucose	+	-	-
L-Fucose	+	+	-
β-Gentiobiose	+	-	+
D-Mannitol	-	-	+
(b) Utilization of amino acids:			
D-Aspartic acid, glycyl-L-proline	+	+	-
(c) Utilization of organic acids:			
Acetoacetic acid, bromo-succinic acid	-	+	-
Acetic acid, *p*-hydroxy-phenylacetic acid	-	+	+
Butyric acid, citric acid	-	-	+
D-Gluconic acid, D-glucuronic acid, D-lactic acid methyl ester, quinic acid	+	-	-
Methyl pyruvate, L-malic acid, α-*keto*-glutaric acid	+	+	-
(d) Utilization of other compounds:			
Gelatin	+	+	-
Pectin	+	-	-
(e) Growth in the presence of:			
Aztreonam, minocycline	-	+	+
Fusidic acid, guanidine hydrochloride, vancomycin	-	-	+
Sodium bromate	-	+	+
Sodium lactate (1%)	-	+	+
Sodium chloride (8%)	-	-	+
Troleandomycin	-	+	+
Other phenotypic tests			
Degradation tests:			
Elastin	+	+	-
Starch	-	+	-
Uric acid	-	+	-
Growth at 45ºC	-	-	+

+, positive result; -, negative result.

Positive results recorded for isolate H59, *A. napierensis* DSM 44846^T and *A. yumaensis* NBRC 14689^T:

- API ZYM tests: acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase and valine arylamidase.
- GEN III BIOLOG microplate tests: utilization of *N*-acetyl-D-glucosamine, D-cellobiose, D-glucose, L-glutamic acid, β-hydroxy-butyric acid, D-malic acid, β-methyl-D-glucoside, L-pyroglutamic acid, sucrose, stachyose and D-trehalose and growth in the presence of 1% NaCl, potassium tellurite, at pH5 and pH6, but not in the presence of nalidixic acid or rifamycin SV.

- Phenotypic tests: Aesculin and arbutin hydrolysis, degradation of casein, hypoxanthine, Tweens 40, 60 and 80 and growth at 20, 30 and 40ºC.

Negative results were recorded for isolate H59, *A. napierensis* DSM 44846^T and *A. yumaensis* NBRC 14689^T:

- API ZYM tests: N-acetyl-β-glucosaminidase, α-fucosidase, α- and β-galactosidase, β-glucuronidase and α-mannosidase.
- GEN III BIOLOG microplate tests: utilization of γ-amino-n-butyric acid, D-arabitol, L-aspartic acid, α-*keto*-butyric acid, α-hydroxy-butyric acid, L-galactonic acid-γ-lactone, D-galacturonic acid, glucuronamide, inosine, D-serine #1, L-lactic acid, α-D-lactose, 3-*O*-methyl-D-glucose, D-melibiose, mucic acid, propionic acid, D-serine #2 and D-sorbitol and resistant to lithium chloride, niaproof, sodium formate and tetrazolium violet.
- Phenotypic tests: Allantoin, H_2S production, nitrate reduction, urea hydrolysis, degradation of adenine, cellulose, chitin, guanine, tributyrin, L-tyrosine, xanthine and xylan and growth at 4, 10 and 50ºC.

Contrasting BIOLOG results obtained for:

(a) isolate H59: utilization of D-raffinose.

(b) *A. napierensis* DSM 44846^T: utilization of D-saccharic acid, D-turanose and grew in the presence of sodium chloride (4%, w/v).

(c) *A. yumaensis* DSM 43931^T: utilization of L-arginine, D-galactose, D-mannose, L-serine and resistance to lincomycin and tetrazolium blue.

4.4.2 Classification of Amycolatopsis strains

Phylogenetic analyses. Isolates H5 and H6 were isolated on glucose-yeast extract agar and isolates H97 and H101 on Gauze's No. 1 agar, in all cases from the ALMA 4 environmental sample. All of the strains formed distinct branches in the *Amycolatopsis* 16S rRNA gene tree (Figure 4.4Isolate H5 (single-membered colour-group 5) formed a subclade together with *Amycolatopsis mediterranei* IMSNU 20056^T, a relationship that was supported by all of the tree-making algorithms and by a 83% bootstrap value; these strains share a 16S rRNA gene sequence similarity of 98.6%, which corresponds to 20 nt differences at 1431 locations (Figure 4.5). However, this isolate shares a slightly higher 16S rRNA gene sequence similarity (98.69%) with *Amycolatopsis pretoriensis* NRRL B-24133^T though in this case the comparison was based on only 1371 sites. Isolate H6

(multi-membered colour-group 6 which encompasses 2 isolates) was also most closely related to the type strain of *Amycolatopsis pretoriensis*; these organisms share a 16S rRNA gene similarity of 99.34%, a value which corresponds to 9 nt differences at 1369 locations. Similarly, strain H97 (single-membered colour-group 72) was most closely related to isolate H6 and to the *A. pretoriensis* strain; it shares a 16S rRNA gene similarity of 98.8% (16 nt differences at 1423 locations) with the former and 98.84% (16 nt differences at 1376 sites) with the latter. The final isolate, strain H101, a representative of multi-membered colour-group 75, which contains 3 strains, forms a loose 16S rRNA subclade together with the type strain of *Amycolatopsis bullii*, a relationship that is supported by all the tree-making algorithms, but not by a high bootstrap value; these organisms share a 16S rRNA gene sequence similarity of 98.36%, which equates to 23 nt differences at 1405 sites. However, isolate H101 is slightly more closely related to *A. pretoriensis* NRRL B-24133^{T}; these strains share a 16S rRNA gene sequence similarity of 98.39%, a value equivalent to 22 nt differences at 1365 locations.

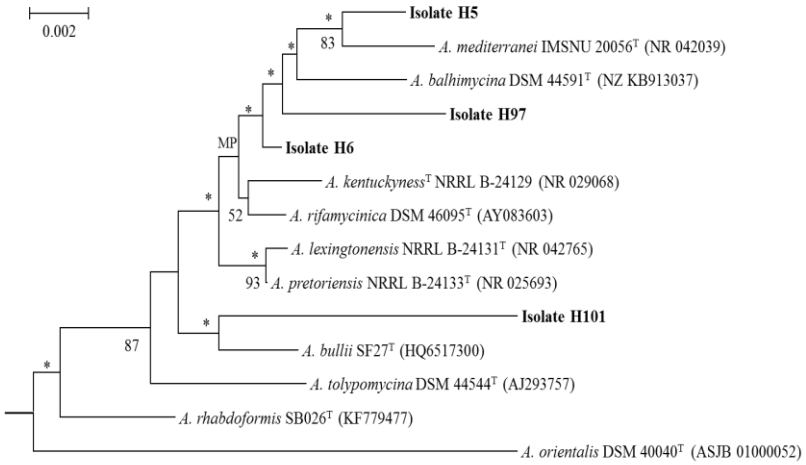

Figure 4.4 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between isolates H5, H6, H97 and H101 and between them and the type strains of closely related *Amycolatopsis* species. *Asterisks* indicate branches of the tree that were recovered using the maximum-likelihood and maximum-parsimony tree-making methods; MP indicates a branch of the tree that was recovered using the maximum-parsimony method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysies of 1000 resampled datasets, only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Table 4.5 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolates H5, H6, H97 and H101 and between them and their nearest phylogenetic neighbours.

	1	**2**	**3**	**4**	5	6	7	8	9	10	11	12	13	14
1. Isolate H5	---	**23/1423**	**31/1426**	**40/1421**	**24/1420**	**24/1411**	**25/1430**	**21/1398**	**20/1431**	**18/1371**	**22/1347**	**27/1429**	**22/1373**	**49/1429**
2. Isolate H6	**98.38**	---	**16/1422**	**32/1424**	**12/1417**	**13/1409**	**11/1428**	**12/1396**	**15/1425**	**9/1369**	**20/1349**	**13/1427**	**16/1371**	**44/1423**
3. Isolate H97	**97.83**	**98.87**	---	**28/1419**	**20/1425**	**21/1416**	**22/1429**	**19/1403**	**24/1426**	**16/1376**	**26/1350**	**24/1428**	**23/1378**	**53/1424**
4. Isolate H101	**97.19**	**97.75**	**98.03**	---	**34/1414**	**23/1405**	**39/1424**	**25/1392**	**43/1421**	**22/1365**	**30/1346**	**41/1423**	**30/1367**	**61/1419**
5. *A. balhimycina* DSM 44591^T	**98.31**	**99.15**	**98.6**	**97.6**	---	19/1438	16/1447	16/1425	16/1440	11/1397	22/1349	18/1446	19/1401	43/1442
6. *A. bullii* SF27^T	**98.3**	**99.08**	**98.52**	**98.36**	98.68	---	13/1441	15/1429	18/1431	12/1397	14/1349	15/1440	13/1404	37/1436
7. *A. kentuckyness* NRRL B-24129^T	**98.25**	**99.23**	**98.46**	**97.26**	98.89	99.1	---	10/1440	14/1454	7/1396	19/1348	8/1462	14/1406	40/1472
8. *A. lexingtonensis* NRRL B-24131^T	**98.5**	**99.14**	**98.65**	**98.2**	98.88	98.95	99.31	---	17/1418	1/1396	17/1348	9/1430	13/1406	36/1436
9. *A. mediterranei* IMSNU 20056^T	**98.6**	**98.95**	**98.32**	**96.97**	98.89	98.74	99.04	98.8	---	14/1391	22/1346	15/1450	18/1393	37/1453
10. *A. pretoriensis* NRRL B-24133^T	**98.69**	**99.34**	**98.84**	**98.39**	99.21	99.14	99.5	99.93	98.99	---	16/1347	4/1397	12/1396	36/1393
11. *A. rhabdoformis* SB026^T	**98.37**	**98.52**	**98.07**	**97.77**	98.37	98.96	98.59	98.74	98.37	98.81	---	16/1349	17/1348	29/1346
12. *A. rifamycinica* DSM 46095^T	**98.11**	**99.09**	**98.32**	**97.12**	98.76	98.96	99.45	99.37	98.97	99.71	98.81	---	13/1406	39/1458
13. *A. tolypomycina* DSM 44544^T	**98.4**	**98.83**	**98.33**	**97.81**	98.64	99.07	99	99.08	98.71	99.14	98.74	99.08	---	36/1403
14. *A. orientalis* DSM 40040^T	**96.57**	**96.91**	**96.28**	**95.7**	97.02	97.42	97.28	97.49	97.45	97.42	97.85	97.33	97.43	---

Characterization of isolates H5 and H6. These isolates were selected for further study in order to establish whether they belong to new *Amycolatopsis* species.

Cultural and morphological properties. Isolate H5 grew well on all of the ISP media, apart from ISP 6, producing predominantly white aerial hyphae and yellow white substrate mycelia (Table 4.6). In contrast, isolate H6 grew moderately well on ISP media 1. 3 and 4 and poorly on ISP 6; aerial hyphae, when produced, were mainly yellow white to pale yellow pink and substrate mycelia predominantly slightly yellow. Colonies of both strains were rough, irregular, umbonate with undulate margins. The substrate hyphae of the isolates fragmented into rod-shaped elements after growth on oatmeal agar for 10 days at 28^{o}C (Figure 4.5).

Table 4.6 Growth and cultural characteristics of isolates H5 and H6 on ISP media after incubation for 14 days at 28^{o}C.

Isolate	ISP media	Characteristics		
		Growth	Aerial hyphae	Substrate mycelium
H5	Glycerol-asparagine agar (ISP medium 5)	+++	White	Yellow white
	Inorganic salts-starch agar (ISP medium 4)	+++	White	Yellow white
	Oatmeal agar (ISP medium 3)	++	White	Yellow white
	Peptone-yeast extract-iron agar (ISP medium 6)	+	None	Mild yellow
	Tryptone-yeast extract agar (ISP medium 1)	+++	White	Mild yellow
	Tyrosine agar (ISP medium 7)	+++	Light yellow pink	Yellow white
	Yeast extract-malt extract agar (ISP medium 2)	+++	Pink white	Slightly orange
H6	Glycerol-asparagine agar (ISP medium 5)	+	Yellow white	Slightly yellow
	Inorganic salts-starch agar (ISP medium 4)	++	White	Yellow white
	Oatmeal agar (ISP medium 3)	++	Yellow white	Pale yellow
	Peptone-yeast extract-iron agar (ISP medium 6)	+-	None	Slightly yellow
	Tryptone-yeast extract agar (ISP medium 1)	++	Pale yellow pink	Mild orange

Tyrosine agar (ISP medium 7)	+	None	Yellow white
Yeast extract-malt extract agar (ISP medium 2)	+	Light yellow pink	Slightly yellow

+++, abundant growth; ++ moderate growth; + sparse growth. Diffusible pigments were not produced on any of the ISP media.

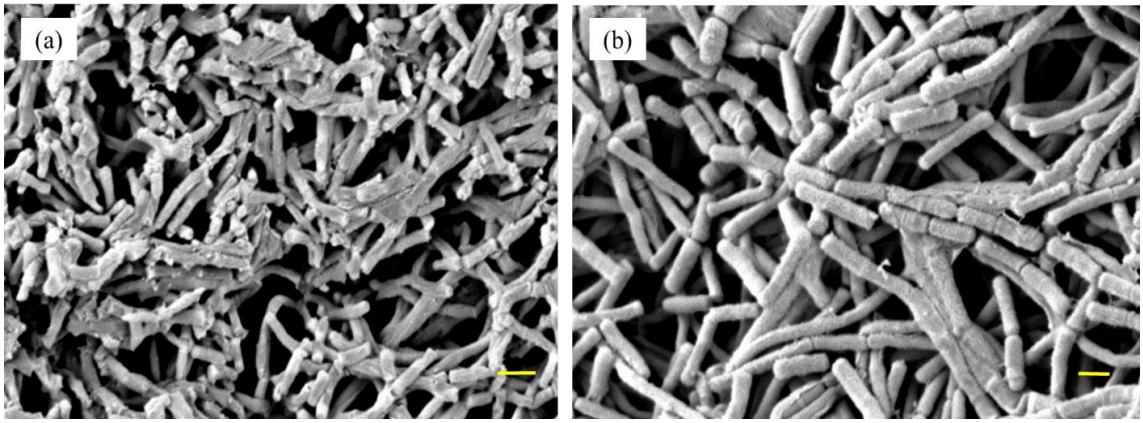

Figure 4.5 Scanning electron micrographs of *Amycolatopsis* isolates (a) H5 and (b) H6 showing fragmentation of substrate hyphae after growth on oatmeal agar after incubation at 28^{o}C for 10 days. Bar 1µm.

Chemotaxonomy. Whole-organism hydrolysates of isolates H5 and H6 were found to contain *meso*-A_2pm, arabinose, galactose, glucose and traces of rhamnose, ribose and xylose. The polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, aminolipid and unknown lipids, glycolipids was detected in isolate H5 while aminophospholipid, phosphoglycolipid and phospholipids were found in isolate H6 (Figure 4.5). The predominant menaquinone in isolates H5 and H6 was MK9(H_6) with 75 and 56%, respectively; isolate H6 also contained MK9(H_4) (10%) and small amount of MK9(H_2) (3%). It can be seen from Table 4.7 that isolates H5 and H6 contain major proportions of *iso*-$C_{15:0,}$ *iso*-$C_{16:0,}$ $C_{16:0}$ and *anteiso*-$C_{17:0,}$ fatty acids though quantitative differences in some components were found between both isolates.

Table 4.7 Fatty acid profiles (%) of isolates H5 and H6.

Fatty acid	H5	H6
Iso-$C_{14:0}$	0.95	3.37
$C_{14:0}$	1.60	0.79
Iso-$C_{15:0}$	10.10	20.52
Anteiso-$C_{15:0}$	6.35	4.69
$C_{15:0}$	0.97	1.80
Iso-$C_{16:0}$ H	0.66	1.02
Iso-$C_{16:0}$	14.44	24.08
Anteiso-$C_{16:0}$	0.90	2.44
$C_{16:0}$	20.52	10.45
Summed feature 3	5.55	4.00
10 methyl $C_{16:0}$	3.92	4.55
Anteiso $C_{17:1}$ w9c	1.30	0.70
Iso-$C_{17:0}$	3.29	3.14
Anteiso-$C_{17:0}$	21.72	10.91
$C_{17:1}$ w8c	0.71	1.08
$C_{17:1}$ w6c	1.98	0.52
$C_{17:0}$	1.38	2.16

Trace proportions (< 0.9 %) are only cited for strains where other fatty acids were found beyond this cut-off point.

Summed feature 3: *Iso*-$C_{16:1}$ w7c/15 2OH

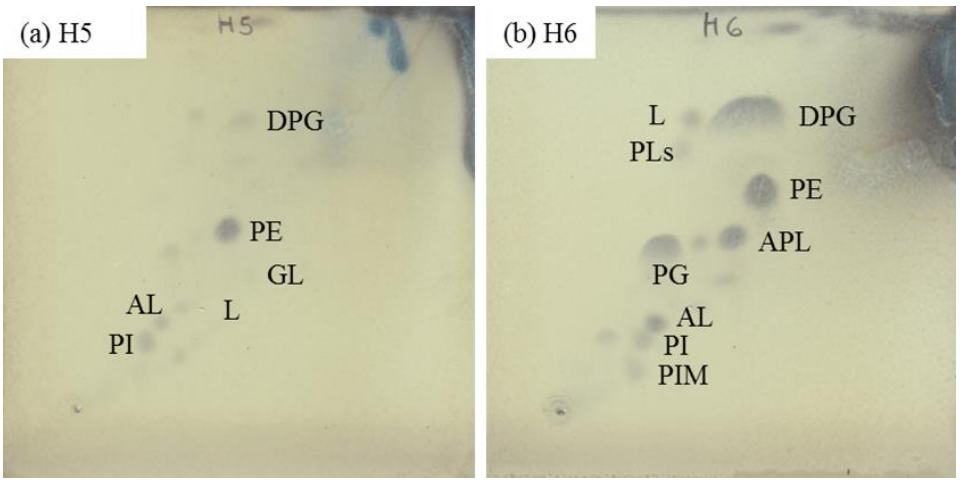

Figure 4.6 Two-dimensional thin-layer chromatography of polar lipids of isolate H7 stained with molybdenum blue spray (Sigma), using the solvents cited in the legend to Figure 4.3. Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatodylglycerol; PL, phospholipids; PI, phosphatidylinositol; PIM, phosphoinositol mannosides; AL, aminolipid; APL. aminophospholipid; GL, glycolipids and L, unknown lipids.

Isolate H5 can be distinguished from the type strain of *A. pretoriensis*, its closest phylogenetic neighbour, by its ability to produce α-fucosidase, β-galactosidase and β-glucosidase, grew at 40^{o}C, hydrolize urea, utilize dextrin, pectin, mucic acid and D-saccharic acid and growth in the presence of nalidixic acid, vancomycin and 4% w/v, sodium chloride. In contrast, *A. pretoriensis* NRRL B-24133^{T}, unlike isolate H5, was found to produce esterase (C4), esterase lipase (C8) and trypsin, degrade hypoxanthine, starch, Tween 60, uric acid and xylan, utilize D-arabitol, D-fructose, D-maltose and L-arginine and growth at of pH5. Similarly, isolate H6 can be differentiated from the type strain of *A. pretoriensis* by its ability to grow at 10 and 40^{o}C, produce cystine arylamidase, α-fucosidase, α-glucuronidase, β-galactosidase and β-glucosidase, utilize butyric acid, D-salicin, D-malic acid and D-turanose and growth in the presence of nalidixic acid, sodium bromide, sodium chloride (8%, w/v), tetrazolium blue, troleandomycin and vancomycin. Unlike isolate H6, *A. pretoriensis* NRRL B-24133^{T} was found to produce esterase (C4) and esterase lipase (C8), degrade starch, Tweens 60 and 80, uric acid and xylan, utilize α-hydroxy-butyric acid, α-*keto*- butyric acid, β-hydroxy-butyric acid, acetoacetic acid, bromo-succinic acid, D-glucose, D-fructose, D-maltose, D-mannose, gelatin, glycl-L-proline, L-histidine and sucrose.

Table 4.8 Phenotypic characteristics that differentiate isolates H5 and H6 from one another and from the type strains of closely related *Amycolatopsis* species.

Characteristics	Isolate H5	Isolate H6	*A. balhimycina* DSM 44591^T	*A. kentuckyensis* DSM 44652^T	*A. lexingtonensis* DSM 4465 ^{T}	*A. mediterranei* DSM 43304 ^{T}	*A. pretoriensis* DSM 44654^T	*A. tolypomycini* DSM 44544^T
API ZYM tests:								
Cystine arylamidase	-	-	+	+	+	+	+	+
Esterase (C4)	-	+	+	-	-	-	-	+
Esterase lipase (C8)	-	+	+	-	-	-	-	+
α-Fucosidase	+	-	+	+	+	+	+	-
β-Galactosidase	+	-	+	+	+	+	+	+
α-Glucuronidase	-	+	+	-	-	-	+	-
β-Glucosidase	+	-	+	-	-	-	+	-
Trypsin	-	+	-	+	+	+	+	+
Valine arylamidase	-	-	+	+	+	+	+	-
GEN III BIOLOG microplate tests								
(a) Utilization of sugars:								
D-Arabitol	-	+	+	+	-	-	+	+
D-Fructose	-	+	+	-	+	+	-	+
D-Glucose	+	+	+	+	+	+	-	+

α-D-Lactose	-	-	-	-	-	+	-	-
β-Methyl-D-glucoside	+	+	-	-	+	+	+	+
myo-Inositol	+	+	+	+	+	+	-	+
(b) Utilization of amino acids:								
L-Arginine	-	+	-	+	+	+	+	+
Glycyl-L-proline	+	+	+	-	+	+	-	+
(c) Utilization of organic acids:								
Bromo-succinic acid	+	+	+	+	+	-	-	+
Butyric acid	-	-	-	-	-	-	+	-
α-*keto*-Butyric acid	+	+	+	+	+	+	-	+
D-Galacturonic acid	-	-	-	-	-	-	-	-
L-Glutamic acid	+	+	+	-	+	+	+	+
β-Hydroxy-butyric acid	+	+	+	+	+	+	-	+
Mucic acid	+	-	-	+	-	-	-	-
Quinic acid	-	-	-	+	+	-	-	-
D-Saccharic acid	+	-	-	+	-	-	-	-
(d) **Utilization of:**								
Gelatin	+	+	-	+	+	+	-	+
(e) Growth in the presence of:								
Fusidic acid, guanidine hydrochloride, lincomycin, tetrazolium blue, troleandomycin	-	-	-	-	-	-	+	-
Minocycline	-	-	-	+	-	-	+	-
Nalidixic acid	+	-	-	+	+	+	+	-
Potassium tellurite	-	+	-	+	+	-	+	+
Sodium chloride (1%, w/v)	+	-	-	+	+	+	+	-
Sodium chloride (8%, w/v)	-	-	-	-	-	-	+	-
Other phenotypic tests:								
(a) Biochemical tests:								

Allantoin	-	-	-	-	-	-	-	+
Arbutin	-	-	+	-	+	-	-	-
Urea	+	-	-	-	-	-	-	-
(b) Degradation tests:								
Elastin	+	+	-	+	+	+	+	+
Hypoxanthine	-	+	+	+	+	+	+	+
Starch	-	+	+	+	-	+	-	-
Uric acid	-	+	-	-	-	-	+	-
Xylan	-	+	-	-	-	-	-	+
Tween 60	-	+	+	+	+	+	-	+
Tween 80	+	+	+	+	+	+	-	+
(c) Growth at:								
10 ºC	-	-	-	-	-	+	+	-
40 ºC	+	-	-	+	+	+	+	+

+, positive result; -, negative result.

Positive results recorded for isolates H5 and H6 and for all of the marker strains:

- API ZYM tests: Acid phosphatase, alkaline phosphatase, α-chymotrypsin, α-*keto*-glutaric acid, leucine arylamidase and α-mannosidase.
- GEN III BIOLOG microplate tests: utilization of acetic acid, *N*-acetyl-D-glucosamine, N-acetyl-β-glucosaminidase, γ-amino-n-butyric acid, D-cellobiose, D-galactose, β-gentiobiose, D-mannitol, propionic acid, D-trehalose, resistance to aztreonam and growth at pH 6.0.
- Phenotypic tests: aesculin hydrolysis, degradation of casein, L-tyrosine, Tween 40 and growth at 20 and 30ºC.

Negative results recorded for isolates H5 and H6 and for all of the marker strains:

- API ZYM tests: α-galactosidase, β-glucuronidase, lipase (C14) and naphthol-AS-BI-phosphohydrolase.

- GEN III BIOLOG microplate tests: utilization of *N*-acetyl-β-D-mannosamine, *N*-acetyl-neuraminic acid, D-aspartic acid, D-fructose-6-phosphate, D-glucose-6-phosphate, inosine, methyl pyruvate, 3-*O*-methyl-D-glucose, D-raffinose, D-serine #1, stachyose and resistant to lithium chloride, niaproof, sodium formate and tetrazolium violet.
- Phenotypic tests: degradation of adenine, cellulose, chitin, guanine, tributyrin and xanthine, nitrate reduction, H_2S production and growth at 4, 45 and 50^{o}C.

Contrasting BIOLOG results were obtained for:

(a) isolate H5: utilization of *N*-acetyl-D-glucosamine, L-aspartic acid, D-fucose, L-histidine, L-lactic acid, D-malic acid, D-mannose, D-salicin, D-serine #2, L-rhamnose, L-serine and D-turanose and growth in the presence of sodium chloride (4%, w/v) and sodium lactate (1%, w/v).

(b) isolate H6: utilization of glucuronamide, L-lactic acid, L-pyroglutamic acid and L-serine and growth in the presence of rifamycin SV.

(c) *A. balhimycina* DSM 44591^{T}: utilization of acetoacetic acid, *N*-acetyl-D-glucosamine, L-lactic acid and D-turanose and growth in the presence of sodium bromate and at pH5.

(d) *A. kentuckyensis* DSM 44652^{T}: utilization of dextrin, D-fucose, glucuronamide, D-mannose, pectin, D-serine #2 and growth in the presence of sodium chloride (4%, w/v) and at pH5.

(e) *A. lexingtonensis* DSM 44653^{T}: utilization of dextrin, D-malic acid and resistance to sodium lactate (1%, w/v) and vancomycin.

(f) *A. mediterranei* DSM 43304 ^{T}: utilization of acetoacetic acid, L-alanine, D-maltose, D-mannose, pectin, D-sorbitol and growth in the presence of sodium lactate (1%, w/v) and vancomycin.

(g) *A. pretoriensis* DSM 44654^{T}: utilization of L-alanine, L-aspartic acid, L-fucose, D-melibiose, L-serine and D-sorbitol.

4.4.3 Classification of Cryptosporangium isolate H7

Phylogenetic analyses. Isolate H7 was recovered from the ALMA 4 environmental sample on glucose-yeast extract agar and assigned to colour-group 7 which contained 3 members. The isolate formed a distinct branch in the *Cryptosporangium* 16S rRNA gene tree (Figure 4.6) and was most closely related to the type strains of *C. cibodasense* and *C. minutisporangium* sharing 98.29% 16S rRNA gene similarity with each of these strains, a value that corresponds to 24 nt differences at 1405 and 1403 locations, respectively (Table 4.9).

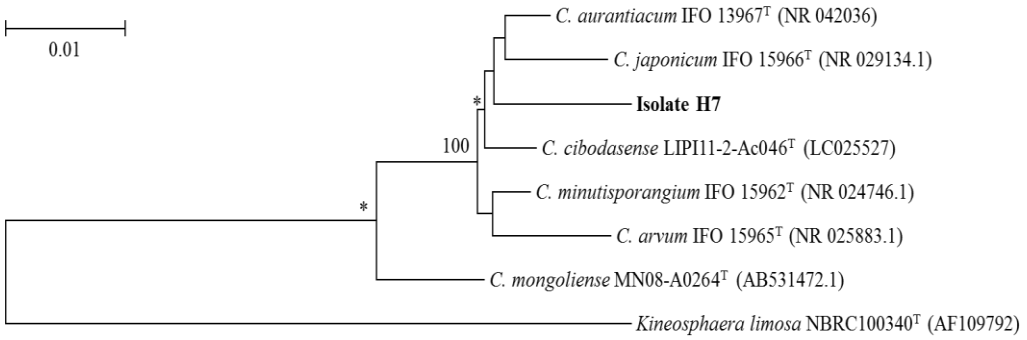

Figure 4.7 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolate H7 and the type strains of *Cryptosporangium* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making methods. The number at one of the nodes indicates a level of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets, this is the only value above 50%. The scale bar indicates 0.001 substitutions per nucleotide position.

Table 4.9 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate H7 and the type strains of *Cryptosporangium* species.

	1	2	3	4	5	6	7	8
1. Isolate H7	**---**	**24/1403**	**27/1405**	**27/1399**	**32/1397**	**43/1403**	**24/1405**	**138/1402**
2. *C. minutisporangium* IFO 15962^T	**98.29**	---	13/1453	21/1449	19/1446	32/1443	16/1453	127/1452
3. *C. aurantiacum* IFO 13967^T	**98.08**	99.11	---	19/1449	30/1446	29/1443	19/1459	131/1452
4. *C. japonicum* IFO 15966^T	**98.07**	98.55	98.69	---	30/1444	44/1439	19/1449	138/1448
5. *C. arvum* IFO 15965^T	**97.71**	98.69	97.93	97.92	---	43/1436	21/1446	136/1445
6. *C. mongoliense* MN08-A0264^T	**96.94**	97.78	97.99	96.94	97.01	---	33/1443	124/1442
7. *C. cibodasense* LIPI11-2-Ac046^T	**98.29**	98.9	98.7	98.69	98.55	97.71	---	127/1452
8. *Kineosphaera limosa* NBRC 100340^T	**90.16**	91.25	90.98	90.47	90.59	91.4	91.25	---

Cultural and morphological properties. This isolate grew well on ISP media 1 and 2 but moderately or poorly on the remaining ISP media (Table 4.10). It can also be seen from the Table that the substrate mycelia were predominantly black and the diffusible pigments pale to dark purple. Colonies were round, crateriform and smooth with entire margins. The strain formed branched substrate hyphae and round or irregularly shaped sporangia most of which were 0.5-1 µm in diameter (Figure 4.7).

Table 4.10 Growth and cultural characteristics of isolate H7 on ISP media after incubation for 14 days at 28ºC.

ISP media	Characteristics		
	Growth	Substrate mycelium	Diffusible pigment
Glycerol-asparagine agar (ISP medium 5)	+	Black	Pale purple
Inorganic salts-starch agar (ISP medium 4)	+	Olive gray	Dark purple
Oatmeal agar (ISP medium 3)	+	Black	Pale purple
Peptone-yeast extract-iron agar (ISP medium 6)	+-	Black	Pale purple
Tryptone-yeast extract agar (ISP medium 1)	++	Black	Pale purple
Tyrosine agar (ISP medium 7)	+	Black	Pale purple
Yeast extract-malt extract agar (ISP medium 2)	+++	Black	Dark purple

+++ abundant growth; ++ moderate growth; + sparse growth; +- trace of growth. Aerial hyphae were not produced on any of the ISP media.

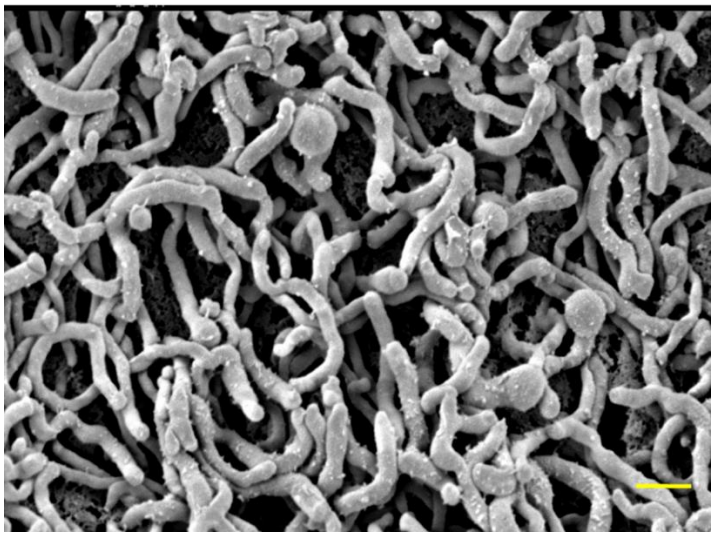

Figure 4.8 Scanning electron micrograph of *Cryptosporangium* isolate H7 showing fragmented substrate hyphae and sporangia following growth on oatmeal agar at 28^{o}C for 10 days. Scale bar, 1µm.

Chemotaxonomy. Whole-organism hydrolysates of isolate H7 contained *meso*-A_2pm and galactose, glucose, mannose, rhamnose, ribose and xylose. The predominant menaquinones were MK9(H_6) (31%), MK9(H_8) (22%) and MK9(H_4) (14%) and the polar lipid pattern consisted of hydroxyphosphatidylethanolamine, methyl-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides and some uncharacterised components (Figure 4.8). The fatty acid profile of isolate H7 was shown to be composed of a predominant proportion of (>10%) of *iso*-$C_{16:0}$ (55.52%), lower proportions (>2.8%) of $C_{17:0}$ (3.18%), *anteiso*-$C_{17:0}$ (5.97%), $C_{17:1}$ w8c (9.32%), $C_{17:0}$ 10 methyl (4.16%) and $C_{18:0}$ w9c (7.0%) and trace amounts of (<0.5%) *iso*-$C_{12:0}$ (0.13%), *iso*-$C_{14:0}$ (1.33%), *iso*-$C_{15:0}$ (0.21%), $C_{14:0}$ (0.12%), *anteiso*-$C_{15:0}$ (0.27%), $C_{15:0}$ (0.52%), *iso*- H $C_{16:1}$ (1.18%), *iso*-$C_{16:1}$ w7c/15 iso H (0.57%), $C_{16:0}$ (2.20%), *anteiso*-$C_{17:1}$ w9c (0.32%), *iso*-$C_{17:0}$ (0.55%), *iso*- H $C_{18:1}$ (0.11%), $C_{18:3}$ w6c (0.13%), *iso*-$C_{18:0}$ (1.53%), $C_{18:0}$ w7c (0.2%), $C_{18:0}$ (1.99%), $C_{19:0}$ (0.22%), $C_{19:1}$ w11c / $C_{19:1}$ w9c (0.92%), un 18.846/$C_{19:1}$ w6c (0.92%) and $C_{19:0}$ (0.22%).

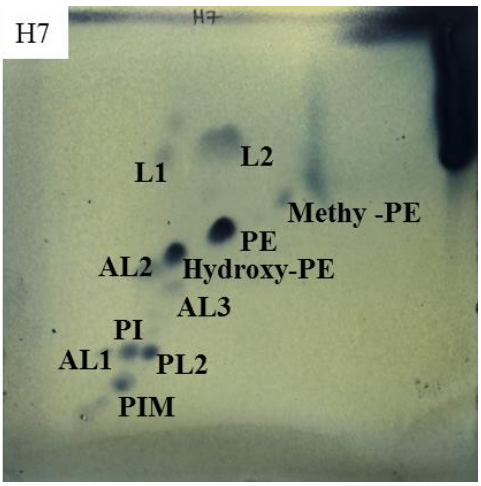

Figure 4.9 Two-dimensional thin-layer chromatography of polar lipids of isolate H7 stained with molybdenum blue spray (Sigma) using the solvents cited in the legend to Figure 4.3. Key: hydroxy-PE, hydroxyphosphatidylethanolamine; PE, phosphatidylethanolamine; methyl-PE, methyl-phosphatidylethanolamine; PIM, phosphatidylinositol mannosides; AL, aminolipids and L, unknown lipids.

Phenotypic properties. Identical results were obtained for the duplicated phenotypic tests carried out on isolate H7 and on the type strains of *Cryptosporangium* species, apart from some of the BIOLOG tests. All of the strains were found to have many phenotypic properties in common (Table 4.11). Isolate H7 grew from 10 to 30^{o}C, from pH 6 to 9, but less well at pH 9.0 and 10.0; it also grew in the presence of 1% w/v, sodium chloride, but not at 2.5% w/v. The isolate can be distinguished from the type strain of *C. minutisporangium*, its closest phylogenetic neighbour, by its ability to produce α- and β-galactosidase, β-glucosidase, leucine arylamidase and trypsin, to utilize dextrin, gelatin D-gluconic acid, L-lactic acid, quinic acid and L-rhamnose, and to grow in the presence of sodium lactate (1%), degrade Tweens 40 and 60 and grow at 10^{o}C (Table 4.11). In contrast, *C. minutisporangium* NBRC 15962 ^{T}, unlike the isolate, produced naphthol-AS-BI-phosphohydrolase and α-mannosidase (API tests), degraded starch, utilized inosine, glycl-L-proline, L-arginine and L-galactonic acid-γ-lactone, was resistant to aztreonam and grew in the presence of sodium chloride (1%, w/v).

Table 4.11 Phenotypic properties that separate isolate H7 from the type strains of *Cryptosporangium* species.

Characterization	Isolate H7	*C. aurantiacum* DSM 46144^{T}	*C. arvum* NBRC 15965^{T}	*C. japonicum* NBRC 15966^{T}	*C. minutisporangium* NBRC 15962^{T}	*C. mongoliense* NBRC 105887^{T}
API ZYM tests:						
N-Acetyl-β-glucosaminidase	-	-	+	-	-	-
α-Chymotrypsin, cystine arylamidase, valine arylamidase	-	-	+	+	-	+
Esterase (C4)	-	-	-	+	-	+
α and β-Galactosidase, β-glucosidase, leucine arylamidase, trypsin	+	+	+	+	-	+
β-Glucuronidase	-	+	+	-	-	-
α-Mannosidase	-	-	-	-	+	-
Naphthol-AS-BI-phosphohydrolase	-	+	+	+	+	+
GEN III Biolog Microplate tests						
(a) Utilization of sugars:						
N-Acetyl-D-glucosamine	+	+	+	+	+	-
N-Acetyl-β-D-mannosamine	+	+	-	-	+	-

D-Cellobiose, D-galactose, β-gentiobiose, α-D-lactose, D-trehalose	+	-	+	+	+	+
D-Fucose	+	+	+	+	+	-
L-Galactonic acid-γ-lactone	-	-	-	-	+	-
D-Glucose-6-phosphate	+	+	+	+	+	+
3-*O*-Methyl-D-glucose	+	-	-	-	+	+
L-Rhamnose	+	+	+	+	-	+
(b) Utilization of amino acids:						
L-Alanine	+	+	+	+	+	+
L-Arginine	-	-	-	-	+	-
D-Serine #2	-	+	-	-	-	-
(c) Utilization of organic acids:						
Bromo-succinic acid	-	-	+	+	-	-
D-Gluconic acid	+	-	+	+	-	-
L-Lactic acid	+	-	+	+	-	-
L-Malic acid	-	+	+	+	-	+
(d) Other compounds:						
Gelatin	+	+	-	-	-	-
(e) Growth in the presence of:						
Aztreonam	-	+	+	+	+	+
Lincomycin	-	+	-	-	-	-
Minocycline, tetrazolium blue	-	-	-	-	-	+
Nalidixic acid	+/-	+	+/-	+	+	-
Rifamycin SV	+	+	-	-	+	+/-

Sodium chloride (1%)	-	+	-	+	+	-
Sodium lactate (1%)	+	-	-	+	-	-
Tetrazolium violet	-	-	-	-	-	+/-
Other phenotypic tests						
(a) Degradation tests:						
Casein	-	+	-	-	-	+
Hypoxanthine	-	+	+	+	-	+
Starch	-	+	+	-	+	+
Tween 40	+	-	+	-	-	-
Tween 60	+	+	+	+	-	+
Tween 80	-	-	+	-	-	-
(b) Growth at:						
10^{o}C	+	-	+	+	-	+
40 ^{o}C	-	-	-	+	-	-

+, positive result; -, negative result.

Positive results recorded for isolate H7 and the type strains of *Cryptosporangium* species:

- API ZYM tests: acid phosphatase, alkaline phosphatase, esterase lipase (C8) and α-glucuronidase.
- GEN III BIOLOG microplate tests: utilization of acetic acid, acetoacetic acid, γ-amino-n-butyric acid, D-arabitol, β-methyl-D-glucoside, D-fructose, D-fucose, D-glucose, D-maltose, D-mannitol, D-mannose, D-melibiose, glycerol, *myo*-inositol, sucrose, stachyose and D-turanose.
- Phenotypic tests: aesculin and arbutin hydrolysis and growth at 20 and 30^{o}C.

Negative results recorded for isolate H7 and the type strains of *Cryptosporangium* species:

- API ZYM tests: α-fucosidase and lipase (C14).
- GEN III BIOLOG microplate tests: utilization of *N*-acetyl-neuraminic acid, D-aspartic acid, α-*keto*-butyric acid, citric acid, glucuronamide, L-histidine, β-hydroxy-butyric acid, *p*-hydroxy-phenylacetic acid, mucic acid, D-saccharic acid, D-serine #1, L-serine, and growth in the

presence of lithium chloride, fusidic acid, guanidine hydrochloride, niaproof, sodium chloride (4% and 8%, w/v), sodium formate, troleandomycin and vancomycin and at pH 5.

- Phenotypic tests: allantoin hydrolysis, nitrate reduction, production of H_2S, urea hydrolysis, degradation of adenine, cellulose, chitin, elastin, guanine, L-tyrosine, tributyrin, uric acid, xanthine and xylan and growth at 4, 45 and 50^{o}C.

Contrasting BIOLOG results was obtained for:

(a) Isolate H7: utilization of L-alanine, D-glucose-6-phosphate, D-glucuronic acid, α-hydroxy-butyric acid, D-lactic acid methyl ester, propionic acid, L-pyroglutamic acid and resistance to nalidixic acid and potassium tellurite.

(b) *C. aurantiacum* DSM 46144^{T}: utilization of α-*keto*-glutaric acid, D-lactic acid methyl ester, L-pyroglutamic acid, quinic acid and D-sorbitol.

(c) *C. arvum* NBRC 15965 ^{T}: utilization of dextrin, D-fructose-6-phosphate, D-raffinose, gly-pro, inosine, pectin and quinic acid, resistance to nalidixic acid and growth at pH6.

(d) *C. japonicum* NBRC 15966 ^{T}: utilization of butyric acid, dextrin, D-malic acid, D-raffinose, D-sorbitol, glycl-L-proline, L-alanine, L-aspartic acid and L-glutamic acid.

(e) *C. minutisporangium* NBRC 15962 ^{T}: utilization of α-*keto*-glutaric acid, D-galacturonic acid, D-glucose-6-phosphate and D-salicin.

(f) *C. mongoliense* NBRC 105887 ^{T}: utilization of D-malic acid, L-pyroglutamic acid, methyl pyruvate, propionic acid, resistant to rifamycin SV, and tetrazolium violet, and growth in the presence of sodium bromate and potassium tellurite.

4.4.4 Classification of Pseudonocardia isolates

Phylogenetic analyses. The phylogenetic relationships between 9 strains isolated from Atacama Desert soils and between them and closely related *Pseudonocardia* type strains are shown in Figure 4.8 where it can be seen that the isolates and associated marker strains fell into 4 subclades. Several of the isolates were assigned to multi-membered subclades but others formed single phyletic lines. Strains ATK01, ATK03 and ATK17, isolated from an extreme hyper-arid soil sample (CAB3), were found to have identical 16S rRNA gene sequences (Table 4.11). These strains formed subclade 4 in the 16S rRNA gene tree together with the type strains of *Pseudonocardia bannaensis* and *Pseudonocardia xinjiangensis*, a relationship that was supported by all of the tree-making algorithms but not by a high bootstrap value. Isolates ATK01, ATK03 and ATK17 share a 16S rRNA gene sequence similarity with the *P. xinjiangensis* strain of 98.26%, a value that corresponds to 23 nt differences at 1325 locations. In turn, they share 16S gene sequence similarities with the *P. bannaensis* strain of 97.87% (30 nt differences at 1409 locations).

Isolates H57, H58 and H69 together with 8 other isolates formed colour-group 1 (see Appendix 1). The first two of these isolates were recovered in 16S rRNA subclade 1 together with the type strains of *Pseudonocardia cypriaca* and *Pseudonocardia hierapolitana*, a taxon that is supported by a 90% bootstrap value and by all of the tree-making algorithms; the type strain of *Pseudonocardia zijiangensis* is loosely associated with this group (Figure 4.9). Isolates H57 and H58 share a 16S rRNA gene similarity of 98.94%, a value that equates to 15 nt differences at 1413 locations. The first of these isolates was closely related to the type strains of *P. adeleidensis*, *P. cypriaca*, *P. hierapolitana* and *P. zijiangensis* sharing 16S rRNA gene similarities with the latter within the range of 98.64 to 98.87%, values that correspond to between 9 and 16 nt differences at between 1395 and 1420 locations (Table 4.12). Similarly, isolate H58 was most closely related to *P. cypriaca* KT2142^{T}, these strains share a 16S rRNA gene similarity of 99.58%, a value that corresponds to 6 nt differences at 1422 locations. The final representative of colour-group 1, isolate H69, formed a distinct phyletic line in 16S rRNA subclade 2 (Figure 4.9), this organism was most closely related to the type strain of *Pseudonocardia kunmingensis;* these organisms share a 98.54% 16S rRNA gene similarity, a value that is equivalent to 37 nt differences at 1417 sites.

The remaining isolates, strains H96, H99 and H215, were assigned to colour-groups 71, 72 and 12; taxa that encompass 2, 1 and 32 strains, respectively. Two of these isolates, strains H96 and H99, formed subclade 3 together with the type strains of

Pseudonocardia khuvsgulensis and *Pseudonocardia rhizophila*, a taxon that is underpinned by a 99% bootstrap value and by all of the tree-making algorithms (Figure 4.9). Isolates H96 and H99 are closely related sharing a 99.14% 16S rRNA sequence similarity, a value that corresponds to 12 nt differences at 1399 locations. These isolates are most closely related to *P. khuvsgulensis* MN08-A0297^{T} sharing 16S rRNA gene sequence similarities with the latter of 98.87 and 99.01%, values equivalent to 16 and 14 nt differences at 1416 and 1409 locations, respectively (Table 4.12). The final isolate, strain H215, is loosely associated with the type strain of *Pseudonocardia adeleidensis* (Figure 4.9), but is most closely related to *P. kunmingensis* YIM 63158^{T}, these strains share a 16S rRNA gene similarity of 98.7%, a value which corresponds to 18 nt differences at 1384 sites.

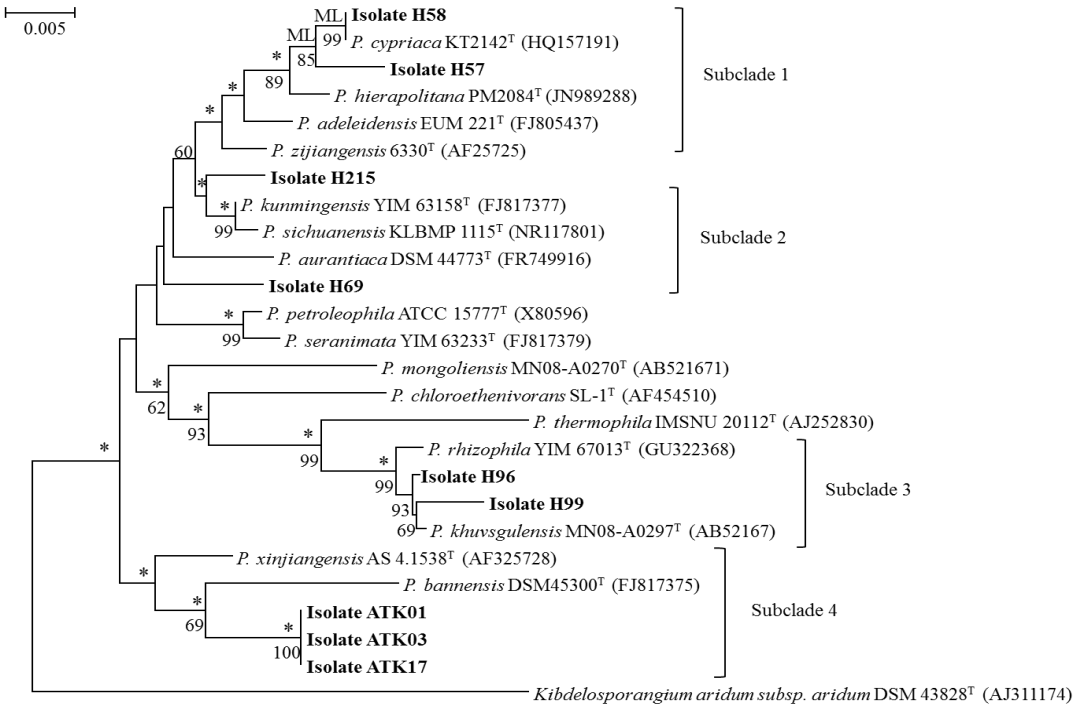

Figure 4.10Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolates ATK01, ATK03, ATK17, H57, H58, H69, H96, H99 and H215 and between them and the type strains of closely related *Pseudonocardia* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood (ML) and maximum-parsimony (MP) 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.

Table 4.12 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates ATK01, ATK03, ATK17, H57, H58, H69, H96, H99 and H215 and between them and the type strains of closely related *Pseudonocardia* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. Isolate ATK01	---	**0/1456**	**0/1448**	**57/1405**	**56/1411**	**47/1402**	**73/1400**	**72/1392**	**42/1401**	**44/1418**	**30/1409**	**39/1411**	**30/1388**	**33/1364**	**23/1325**	**32/1430**	**69/1416**	**70/1452**	**34/1445**	**55/1453**	**46/1441**	**48/1434**	**33/1454**	**75/1452**	**48/1435**	**80/1441**
2. Isolate ATK03	**100**	---	**0/1448**	**57/1405**	**56/1411**	**47/1402**	**73/1400**	**72/1392**	**42/1401**	**44/1418**	**30/1409**	**39/1411**	**30/1388**	**33/1364**	**23/1325**	**32/1430**	**69/1416**	**70/1451**	**34/1445**	**55/1452**	**46/1441**	**48/1434**	**33/1453**	**75/1451**	**48/1435**	**80/1441**
3. Isolate ATK17	**100**	**100**	---	**59/1414**	**57/1419**	**47/1412**	**73/1409**	**72/1402**	**47/1408**	**44/1418**	**31/1413**	**39/1411**	**30/1388**	**33/1364**	**23/1325**	**32/1430**	**69/1426**	**70/1453**	**34/1449**	**55/1454**	**46/1451**	**48/1444**	**33/1455**	**75/1453**	**48/1441**	**80/1451**
4. Isolate H57	**95.94**	**95.94**	**95.83**	---	**15/1413**	**44/1403**	**68/1401**	**69/1396**	**42/1396**	**17/1401**	**62/1399**	**19/1395**	**28/1376**	**37/1353**	**36/1315**	**43/1391**	**66/1421**	**66/1422**	**40/1418**	**16/1419**	**17/1420**	**59/1422**	**37/1424**	**76/1422**	**57/1410**	**83/1421**
5. Isolate H58	**96.03**	**96.03**	**95.98**	**98.94**	---	**38/1409**	**65/1413**	**64/1406**	**40/1407**	**15/1406**	**65/1411**	**17/1400**	**27/1388**	**36/1362**	**37/1323**	**41/1396**	**61/1428**	**62/1428**	**38/1424**	**6/1422**	**18/1423**	**56/1428**	**37/1430**	**74/1428**	**53/1415**	**82/1427**
6. Isolate H69	**96.65**	**96.65**	**96.67**	**96.86**	**97.3**	---	**54/1404**	**54/1395**	**36/1395**	**28/1397**	**49/1398**	**33/1391**	**20/1373**	**25/1347**	**31/1310**	**31/1387**	**54/1418**	**51/1418**	**35/1412**	**37/1416**	**37/1417**	**50/1416**	**29/1419**	**65/1416**	**44/1408**	**80/1417**
7. Isolate H96	**94.79**	**94.79**	**94.82**	**95.15**	**95.4**	**96.15**	---	**12/1399**	**60/1399**	**58/1396**	**77/1401**	**57/1390**	**49/1378**	**51/1353**	**48/1316**	**58/1386**	**16/1416**	**21/1416**	**60/1409**	**62/1412**	**65/1413**	**59/1414**	**62/1416**	**55/1413**	**52/1404**	**107/1414**
8. Isolate H99	**94.83**	**94.83**	**94.86**	**95.06**	**95.45**	**96.13**	**99.14**	---	**65/1395**	**56/1387**	**80/1397**	**55/1381**	**52/1377**	**54/1349**	**54/1322**	**58/1377**	**14/1409**	**19/1409**	**57/1402**	**60/1406**	**63/1407**	**58/1407**	**59/1408**	**54/1406**	**49/1397**	**106/1407**
9. Isolate H215	**97**	**97**	**96.66**	**96.99**	**97.16**	**97.42**	**95.71**	**95.34**	---	**25/1396**	**57/1407**	**22/1391**	**18/1384**	**28/1357**	**30/1320**	**34/1387**	**62/1407**	**64/1407**	**36/1403**	**38/1409**	**44/1410**	**53/1407**	**29/1409**	**75/1407**	**57/1407**	**84/1406**
10. *P. zijiangensis* 6330^{T}	**96.9**	**96.9**	**96.9**	**98.79**	**98.93**	**98**	**95.85**	**95.96**	**98.21**	---	52/1403	16/1412	17/1388	27/1363	26/1323	31/1409	51/1411	51/1415	22/1411	12/1417	14/1419	49/1416	20/1417	57/1416	41/1417	67/1415
11. *P. bannaensis* DSM45300^{T}	**97.87**	**97.87**	**97.81**	**95.57**	**95.39**	**96.49**	**94.5**	**94.27**	**95.95**	96.29	---	48/1397	39/1388	44/1365	31/1325	47/1395	75/1410	75/1410	50/1406	64/1412	53/1414	58/1411	43/1412	75/1416	56/1414	77/1410
12. *P. adeleidensis* EUM 221^{T}	**97.24**	**97.24**	**97.24**	**98.64**	**98.79**	**97.63**	**95.9**	**96.02**	**98.42**	98.87	96.56	---	13/1388	31/1363	34/1323	35/1408	52/1405	51/1408	31/1404	16/1410	18/1412	41/1409	15/1410	62/1409	44/1410	78/1408
13. *P. kunmingensis* YIM 6315$8$	**97.84**	**97.84**	**97.84**	**97.97**	**98.05**	**98.54**	**96.44**	**96.22**	**98.7**	98.78	97.19	99.06	---	22/1358	25/1322	26/1387	48/1385	47/1385	23/1381	27/1387	25/1388	40/1385	2/1387	52/1385	33/1386	69/1384
14. *P. petroleophila* ATCC 157$?$	**97.58**	**97.58**	**97.58**	**97.27**	**97.36**	**98.14**	**96.23**	**96**	**97.94**	98.02	96.78	97.73	98.38	---	25/1317	6/1365	49/1360	44/1360	26/1355	34/1361	33/1363	39/1363	24/1361	57/1360	44/1363	68/1359
15. *P. xinjiangensis* AS 4.1538^{T}	**98.26**	**98.26**	**98.26**	**97.26**	**97.2**	**97.63**	**96.35**	**95.92**	**97.73**	98.03	97.66	97.43	98.11	98.1	---	28/1325	50/1323	50/1323	18/1316	36/1323	26/1323	39/1323	27/1322	49/1320	41/1323	69/1321
16. *P. seranimata* YIM 63233^{T}	**97.76**	**97.76**	**97.76**	**96.91**	**97.06**	**97.76**	**95.82**	**95.79**	**97.55**	97.8	96.63	97.51	98.13	99.56	97.89	---	53/1401	48/1425	26/1421	38/1426	37/1427	40/1420	28/1427	63/1426	42/1421	73/1425
17. *P. khuvsgulensis* MN08-A0$?$	**95.13**	**95.13**	**95.16**	**95.36**	**95.73**	**96.19**	**98.87**	**99.01**	**95.59**	96.39	94.68	96.3	96.53	96.4	96.22	96.22	---	7/1440	47/1433	52/1431	55/1432	48/1438	51/1439	42/1437	45/1422	96/1438
18. *P. rhizophila* YIM 67013^{T}	**95.18**	**95.18**	**95.18**	**95.36**	**95.66**	**96.4**	**98.52**	**98.65**	**95.45**	96.4	94.68	96.38	96.61	96.76	96.22	96.63	99.51	---	50/1466	54/1466	55/1454	45/1456	50/1475	44/1473	49/1438	91/1464
19. *P. aurantiaca* DSM 44773^{T}	**97.65**	**97.65**	**97.65**	**97.18**	**97.33**	**97.52**	**95.74**	**95.93**	**97.43**	98.44	96.44	97.79	98.33	98.08	98.63	98.17	96.72	96.59	---	30/1459	22/1450	43/1451	25/1468	64/1466	38/1434	74/1458
20. *P. cypriaca* KT2142^{T}	**96.21**	**96.21**	**96.22**	**98.87**	**99.58**	**97.39**	**95.61**	**95.73**	**97.3**	99.15	95.47	98.87	98.05	97.5	97.28	97.34	96.37	96.32	97.94	---	12/1456	47/1449	31/1468	68/1466	51/1440	73/1455
21. *P. hierapolitana* PM2084^{T}	**96.81**	**96.81**	**96.83**	**98.8**	**98.74**	**97.39**	**95.4**	**95.52**	**96.88**	99.01	96.25	98.73	98.2	97.58	98.03	97.41	96.16	96.22	98.48	99.18	---	49/1451	28/1456	67/1455	51/1442	77/1454
22. *P. mongoliensis* MN08-A02	**96.65**	**96.65**	**96.68**	**95.85**	**96.08**	**96.47**	**95.83**	**95.88**	**96.23**	96.54	95.89	97.09	97.11	97.14	97.05	97.18	96.66	96.91	97.04	96.76	96.62	---	42/1457	67/1456	47/1439	80/1455
23. *P. sichuanensis* KLBMP 11$1$	**97.73**	**97.73**	**97.73**	**97.4**	**97.41**	**97.96**	**95.62**	**95.81**	**97.94**	98.59	96.95	98.94	99.86	98.24	97.96	98.04	96.46	96.61	98.3	97.89	98.08	97.12	---	57/1475	35/1440	71/1464
24. *P. thermophila* IMSNU 201$?$	**94.83**	**94.83**	**94.84**	**94.66**	**94.82**	**95.41**	**96.11**	**96.16**	**94.67**	95.97	94.7	95.6	96.25	95.81	96.29	95.58	97.08	97.01	95.63	95.36	95.4	95.4	96.14	---	56/1439	105/1463
25.*P. chloroethenivorans* SL-1^{T}	**96.66**	**96.66**	**96.67**	**95.96**	**96.25**	**96.88**	**96.3**	**96.49**	**95.95**	97.11	96.04	96.88	97.62	96.77	96.9	97.04	96.84	96.59	97.35	96.46	96.46	96.73	97.57	96.11	---	82/1438
26. *Kibdelosporangium aridum s*	**94.45**	**94.45**	**94.49**	**94.16**	**94.25**	**94.35**	**92.43**	**92.47**	**94.03**	95.27	94.54	94.46	95.01	95	94.78	94.88	93.32	93.78	94.92	94.98	94.7	94.5	95.15	92.82	94.3	---

Characterization of isolates. All but one of the isolates assigned to the genus *Pseudonocardia* were selected for further study to determine whether they merited recognition as novel species, the exception was isolate H215.

Cultural and morphological properties. In general, the isolates grew well on ISP media 1, 2, 3 and 7, relatively poorly on ISP 6 while growth on ISP 4 and 5 varied between the isolates (Table 4.13). The substrate mycelia of the isolates tended to be various shades of yellow on all of the ISP media, while the aerial mycelia, when produced, were white. All of the isolates formed white aerial hyphae on ISP 3. It can be seen from the scanning electron micrographs that all but one of the isolates formed chains of smooth surfaced spores, the exception, isolate ATK03 produced spores in chains and in vesicles (Figure 4.10).

Chemotaxonomy. All of the isolates had peptidoglycan rich in A_2pm. In addition, the whole-organism hydrolysates of the strains and the type strains of *P. khuvsgulensis* and *P. zijingensis* contained arabinose, galactose, glucose, mannose and ribose; isolate ATK19 and *P. xinjingensis* also contained rhamnose and isolate H57 and *P. xinjiangensis* DSM 44661^T ribose. The polar lipid patterns of all of the strains consisted of diphosphatidylglycerol, phosphatodylglycerol and phosphatidylinositol in all 8 isolates, phisphatidylethanolamine was detected in isolates ATK01, ATK03, ATK17, H57, H69, H96 and H99, phospholipids in isolates ATK01 and H58, glycophosphoinositide in isolates ATK01, ATK03, ATK17, H58 and H69, aminophospholipid in isolates ATK03 and H58, methyl-phosphatidylethanolamine in isolate ATK17, glycolipid in isolates ATK17, H57, H69, H96 and H99 and phosphatidylinositol mannoside in isolates H57, H96 and H99 (Figure 4.11). All of the strains contained MK8(H_4) (41 – 78%) as the predominant menaquinone. It can be seen from Table 4.14 that all of the isolates contained predominant proportions of *iso*-$C_{16:0}$ (19.5-36.4%). The presence of large proportions of *anteiso*-$C_{17:0}$ in isolates classified in subclades 2 and 3 (22.3-25.7%) distinguishes them from those assigned to subclades 1 and 4 (3.7-15.0%). All of the strains contained minor/moderate proportions of *iso*-$C_{15:0}$ (2.9-19.8%), *iso*-$C_{16:1}$H (1.9-5.0%), $C_{16:0}$ (2.8-8.0%), *iso*-$C_{17:0}$ (1.8-13.5%), $C_{17:1}$ w8c (2.5-10.7%), $C_{17:0}$ (1.4-6.1%), 10-methyl $C_{17:0}$ (1.0-3.5%), summed feature 3 (4.4-10.9%) and summed feature 9 (4.9-16.3%) and small proportions of $C_{15:1}$ w6c (0.3-1.1%), *iso*-$C_{18:0}$ (0.2-1.2%), $C_{18:1}$ w9c (0.3-1.5%) and $C_{18:0}$ (0.3-2,1%).

Table 4.13 Growth and cultural characteristics of *Pseudonocardia* isolates on ISP media after incubation for 14 days at 28°C.

Isolate	Characteristic	ISP media						
		1	2	3	4	5	6	7
ATK01	Growth	+++	+++	+++	+++	++	++	++
	Aerial mycelium	None	None	White	White	None	None	None
	Substrate mycelium	Mild yellow	Slightly yellow	Yellow white	Yellow white	Mild yellow	Dark yellow	Dark yellow
ATK03	Growth	+++	+++	+++	+	+	+	+++
	Aerial mycelium	None	None	White	None	None	Nome	None
	Substrate mycelium	Mild yellow	Slightly yellow	Yellow white	Yellow gray	Brilliant yellow	Dark yellow	Dark gray yellow brown
ATK17	Growth	+++	+++	+++	+++	+	+	++
	Aerial mycelium	None	None	White	None	None	None	Yellow white
	Substrate mycelium	Gray greenish yellow	Olive gray	Yellow white	Yellow gray	Pale gray yellow	Mild yellow	Mild yellow
H57	Growth	+++	+++	++	++	+++	+	+++
	Aerial mycelium	None	None	White	White	White	None	None
	Substrate mycelium	Pale yellow	Mild yellow	Yellow white	White	Yellow gray	Gray yellow	Gray yellow

H58	Growth	+++	+++	+++	++	+++	++	+++
	Aerial mycelium	Yellow white	None	White	White	White	None	White
	Substrate mycelium	Pale yellow	Mild yellow	Yellow white	White	Yellow white	Mild yellow	Mild yellow
H69	Growth	+++	+++	++	++	++	+-	++
	Aerial mycelium	Yellow white	White	White	White	White	None	None
	Substrate mycelium	Mild yellow	Dark yellow	Yellow white	Yellow white	Yellow white	Brilliant yellow	Dark gray yellow
H96	Growth	+++	+++	+++	+	+++	++	+++
	Aerial mycelium	None	White	White	White	White	None	None
	Substrate mycelium	Mild yellow	Light olive brown	Yellow white	Yellow white	Yellow white	Light olive brown	Mild yellow
H99	Growth	+++	+++	+++	++	+++	++	+++
	Aerial mycelium	Yellow white	White	White	White	White	None	None
	Substrate mycelium	Mild yellow	Light olive brown	Yellow white	Yellow white	Yellow white	Mild tallow	Mild yellow

+++ abundant growth; ++ moderate growth; + growth; +- sparse growth. Diffusible pigments were not produced on any of the ISP media.

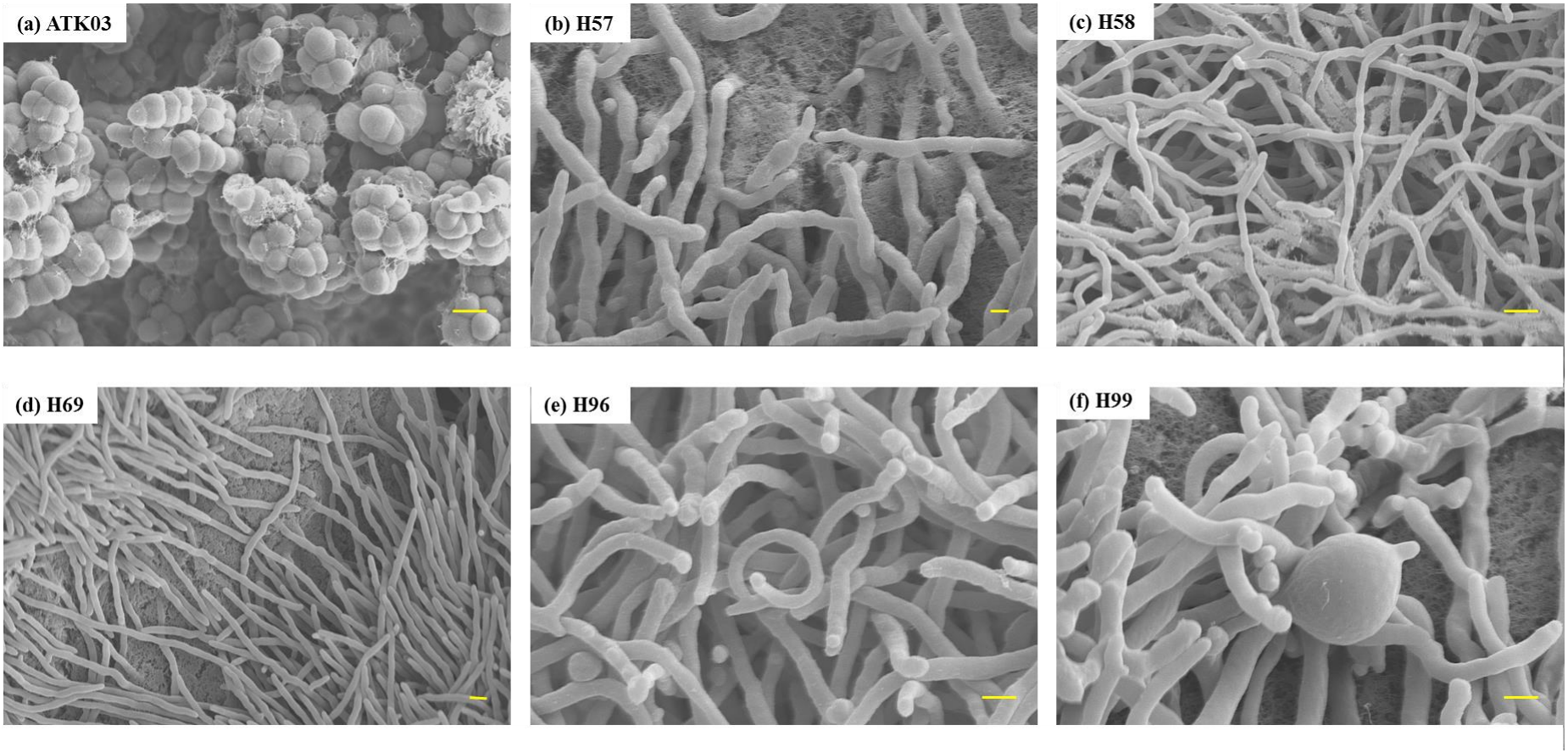

Figure 4.11 Scanning electron micrographs of isolates grown on oatmeal agar for 14 days at 28ºC showing (a) isolate ATK03 with smooth surfaced spores in chains and spore vesicles; (b, c and d) isolates H57, H58 and H69 with long chains of smooth surfaced spores; (e) isolate H96 with smooth surfaced spores in short chains and open loops and (f) isolate H99 with short chains of smooth surfaced spores and a globose body. Scale bars, 1µm.

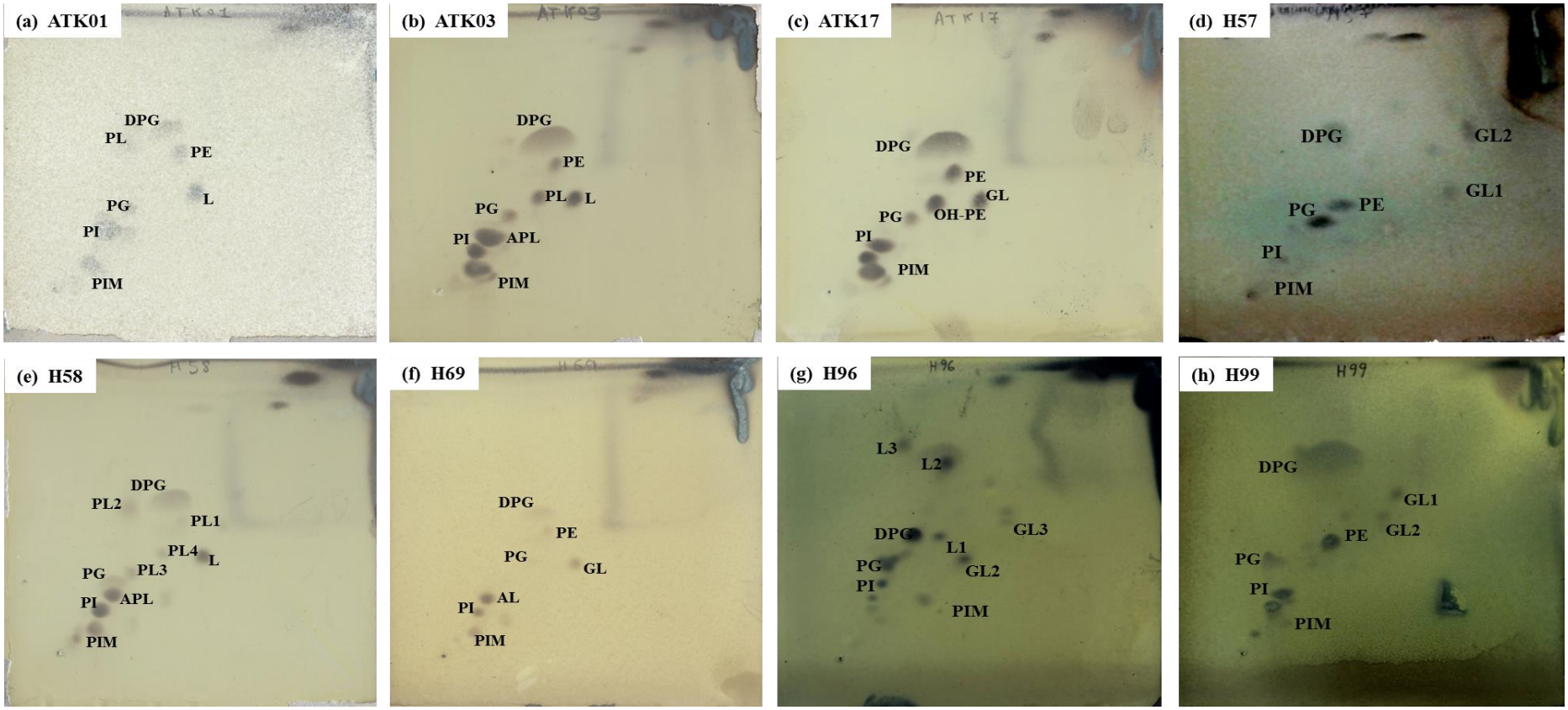

Figure 4.12 Two-dimensional thin-layer chromatography of polar lipids of *Pseudonocardia* isolates stained with molybdenum blue spray (Sigma) using the solvent systems cited in the legend to Figure 4.3. Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; methyl-PE, OH-PE, hydroxy-phosphatidylethanolamine; methyl-PE, phosphatidylethanolamine; PG, phosphatodylglycerol, PI, phosphatidylinositol; PIM, phosphatodylinositol mannosides; PL, phospholipids; AL, aminolipid; APL, aminophospholipid; PIM, phosphatodylinositol mannosides;; GL, glycolipids and L, unknown lipids.

Table 4.14 Fatty acid profiles (%) of *Pseudonocardia* isolates ATK01, ATK03, ATK17, H57, H58, H69, H96 and H99.

Fatty acid	Isolates							
	16S rRNA subclades							
	1		2	3		4		
	H57	H58	H69	H96	H99	ATK01	ATK03	ATK17
Iso-$C_{15:0}$	11.9	5.5	6.7	3.0	2.9	15.4	4.8	19.8
Anteiso-$C_{15:0}$	1.4	1.1	5.8	1.0	0.9	0.5	0.5	0.6
$C_{15:1}$ ω6c	1.1	1.0	0.9	1.1	0.8	0.56	1.0	0.3
Iso-$C_{16:1}$ H	3.4	4.4	3.1	3.2	1.9	2.7	3.0	5.0
Iso-$C_{16:0}$	31.0	24.4	17.4	24.4	26.5	25.0	36.4	19.5
$C_{16:0}$	4.1	5.0	5.6	2.8	4.7	3.9	8.0	5.1
Anteiso-$C_{17:1}$ ω9c	0.9	2.7	5.1	3.6	1. 7	0.5	0.4	1.1
Iso-$C_{17:0}$	4.2	4.7	1.8	4.5	5.0	13.5	2.5	11.0
Anteiso-$C_{17:0}$	8.8	15.0	25.7	22.5	22.3	4.8	3.7	5.1
$C_{17:1}$ ω8c	6.9	7.7	2.5	7.4	7.5	6.9	10.7	3.2
$C_{17:0}$	2.1	3.1	1.4	2.7	5.3	3.5	6.1	2.3
10-Methyl $C_{17:0}$	3.0	2. 6	2.2	2.4	3.5	1.3	1. 6	1.0
Iso-$C_{18:0}$	0.4	0.7	0.2	1.2	1.0	0.9	0.9	0.5
$C_{18:1}$ ω9c	0.5	0.4	0.4	1.5	0.9	0.3	1.3	0.5
$C_{18:0}$	0.5	0.9	1.1	2.0	2.1	0.3	1.0	0.9
Summed feature 3	7.2	8.9	6.9	5.6	5.7	4.5	10.9	4.4
Summed feature 9	11.1	10.0	11.3	8.2	5.8	13.4	4.9	16.3

Trace proportions (< 0.9 %) are only cited for strains where other fatty acids were found in proportions above this cut-off point.
Summed feature 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c and/or $C_{16:1}$ ω6c/$C_{16:1}$ ω7c.
Summed feature 9: 10- methyl $C_{16:0}$ and/or *Iso*-$C_{17:1}$ ω9c.

Phenotypic properties. Identical phenotypic results were obtained for the duplicated test strains, apart from some of the GEN III BIOLOG Microplate tests, as shown in Table 4.15. All of the isolates and associated type strains were found to produce alkaline phosphatase, β-galactosidase, β-glucosidase, α-glucuronidase, leucine arylamidase and valine arylamidase, degrade Tween 60 and produce hydrogen sulphide. The isolates grew well at 20 to 30^{o}C and some isolates showed growth up to 45^{o}C and grew in the presence up to 5% w/v, sodium chloride. In contrast, all of the strains were found to give negative results for allantoin hydrolysis, urea hydrolysis, degradation of adenine, casein, cellulose, chitin, elastin, guanine, tributyrin, uric acid, xanthine and xylan, utilization of L-arginine and citric acid, and growth at 4^{o}C and 50^{o}C

None of the phenotypic properties can be weighted with confidence to distinguish between strains assigned to the 16S rRNA subclades though only subclade 1 strains produced α-fucosidase while subclades 2 and 3 can be distinguished by the ability of members of the former taxon to utilize α-hydroxy-butyric acid (Table 4.15). However, it is also apparent from the Table that members of the individual subclades have many properties in common: subclade 4 strains, for instance, produce α-mannoside and naphthol-AS-BI phosphohydrolase, degrade starch, utilize D-fucose, D-glucose, D-maltose, D-trehalose and propionic acid, and grow at pH6 and in the presence of potassium tellurite. Similarly, strains assigned to subclade 3 produce acid phosphatase, cystine arylamidase, esterase (C4) and esterase lipase (C8), degrade hypoxanthine, hydrolyse aesculin, utilize a broad range of sugars, including L-fucose, β-gentiobiose, D-mannose, D-raffinose and D-turanose and grow at pH 6 and in the presence of potassium tellurite.

The three isolates classified in subclade 4 have many phenotypic properties in common, some of which separate them from their nearest phylogenetic neighbours, that is, from the type strains of *P. bannaensis* and *P. xinjiangensis*. The isolates, unlike *P. xinjiangensis* DSM 44174^{T}; its nearest phylogenetic neighbour, utilize acetic acid, acetoacetic acid and D-raffinose and grow in the presence of aztreonam, lithium chloride and sodium chloride (1%). In contrast, the type strain of *P. xinjiangensis*, unlike isolates ATK01, ATK03 and ATK17, produces α-galactosidase, degrades gelatin, utilizes D-serine #1 and α-*keto*-butyric acid and grows at pH5. Similarly, a broad range of phenotypic properties separate the isolates from *P. bannaensis* DSM 45300^{T}.

Isolates H57 and H58 of subclade 1 have many phenotypic properties in common but can be distinguished by a range of features (Table 4.15). Isolate H57, unlike isolate H58, degrades Tween 40, utilizes L-aspartic acid, D-glucose, α-hydroxy-butyric acid and

α-D-lactose and grows in the presence of sodium lactate and troleandomycin. In contrast, isolate H58 differs from isolate H57 as it produces a range of enzymes, as exemplified by α-chymotrypsin, α-fucosidase, α-mannosidase and naphthol-AS-BI phosphohydrolase, utilizes N-acetyl-D-galactosamine, bromo-succinic acid, D-fucose, α-*keto*-glutaric acid and methyl pyruvate and grows at 40ºC. Isolate H57 can also be distinguished from the type strains of *P. adeleidensis* and *P. zijingensis*, representatives of its closest phylogenetic neighbours (Table 4.12). This isolate, unlike *P. zijingensis* DSM44174^{T}, produces N-acetyl-β-glucosidase, degrades Tween 40, utilizes acetoacetic acid, butyric acid, D-fructose-6-phosphate and D-serine #2, is resistant to aztreonam, lincomycin, minocycline, nalidixic acid, rifampicin, troleandomycin and vancomycin, grows at pH 6 and in the presence of several inhibitory compounds, including lithium chloride, potassium tellurite, sodium chloride (1% w/v), tetrazolium blue and tetrazolium violet (Table 4.15). The *P. zijingensis* strain differs, from isolate H57 given its ability to produce esterase (C4), α-chymotrypsin, cystine arylamidase, α-fucosidase, β-glucuronidase, α-mannosidase and trypsin, and metabolizes a broad range of sugars and organic acids, including D-arabitol, D-fucose, D-galactose, glycerol, D-mannose, D-melibiose, bromo-succinic acid, L- glutamic acid and methyl pyruvate. Similarly, isolate H58 can be distinguished from the *P. zijingensis* strain, one of its closest phylogenetic neighbours, by its ability to produce N- acetyl- β- glucosamidase, utilize acetoacetic acid, butyric acid, α-*keto*-glutaric acid and D-serine #2 and grow at pH 5 and in the presence of a wide range of inhibitory compounds, including fusidic acid, lincomycin, minocycline, nalidixic acid, tetrazolium blue and tetrazolium violet. In contrast, the *P. zijingensis* strain, unlike isolate H58, utilized a broad range of sugars and organic acids that included D-arabitol, D-galactose, β-gentiobiose, glycerol, L-aspartic acid, β-hydroxy-butyric acid, D-galacturonic acid and L-galactonic acid-γ-lactone (Table 4.15).

A combination of phenotypic properties can be weighted to distinguished between the three strains assigned to subclade 2 (Table 4.15). Isolate H69 and the type strain of *P. kunmingensis* have many properties in common, but can be distinguished by the capacity of the former to produce esterase (C4) and to metabolize acetic acid, acetoacetic acid, dextrin, D-fructose-6-phosphate, p-hydroxy-phenylacetic acid, α-*keto*-butyric acid and propionic acid and to grow in the presence of sodium formate and troleandomycin. In contrast, only the *P. kunmingensis* strain produces α-chymotrypsin, β-glucosidase, β-galactosidase, α-mannosidase and trypsin, hydrolizes aesculin and arbutin, degrades hypoxanthine, utilizes D-arabitol, bromo-succinic acid, D-fucose, L-lactic acid, D-malic acid, D-mannitol, methyl pyruvate, stachyose and quinic acid and grows at 10ºC. Similar

combinations of properties can be highlighted to separate isolate H69 and the type strain of *P. petroleophila* from one another.

A wealth of phenotypic features can be highlighted to separate the 4 strains assigned to subclade 3 (Table 4.15). Isolates H96 and H99 have many properties in common, but only the former hydrolyzes arbutin, produces β-glucuronidase and α-naphthol-AS-BI-phosphohydrolase, utilizes L-galactonic acid –γ-lactone, L-malic acid, D-mannitol and β-methyl-D-glucosidase and grows in the presence of lincomycin and sodium lactate. In contrast, only isolate H96 utilizes D-glucose, gelatin and glycerol. Similarly, isolate H99 can be separated from *P. khuvsgulensis* NBRC 105886^{T}, its nearest phylogenetic neighbour, by its ability to produce β-glucuronidase and α-mannosidase,degrade starch, utilize γ-amino-butyric acid, α-*keto*-butyric acid, L-galactonic acid –γ-lactone, α-*keto*-glutaric acid, L-malic acid, D-mannitol, N-acetyl-neuraminic acid, D-saccharic acid and quinic acid and grow in the presence of lincomycin, rifamycin SV and vancomycin. Similarly, the *P. khuvsgulensis* strain, unlike isolate H96, produces lipase (C14) and naphthol-AS-BI-phosphohydrolase, hydrolyzes arbutin, degrades L-tyrosine, metabolizes acetic acid, L-alanine, butyric acid, D-glucose, glycerol, pectin and D-serine #2 and grows in the presence of lithium chloride, sodium chloride (4%) and sodium lactate. Isolate H96 can also be distinguished from the type strain of *P. rhizophila* using a similar battery of phenotypic properties (Table 4.15).

Table 4.15 Phenotypic properties that distinguish isolates ATK01, ATK03, ATK17, H57, H58, H69, H96 and H99 from one another and from the type strains of closely related species.

Characterization	**Subclade 1**				**Subclade 2**			**Subclade 3**				**Subclade 4**				
	Isolate H57	Isolate H58	*P. adeleidensis* DSM 45352^T	*P. zijingensis* DSM 44774^T	Isolate H69	*P. kummingensis* DSM 45301^T	*P. petroleophila* DSM43193^T	Isolate H96	Isolate H99	*P. khuvsgulensis* NBRC 105886^T	*P. rhizophila* DSM 45381^T	Isolate ATK01	Isolate ATK03	Isolate ATK17	*P. banaensis* DSM 45300^T	*P. xinjiangensis* DSM 44661^T
API ZYM tests:																
N-Acetyl-β-glucosaminidase	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+
α-Chymotrypsin, trypsin	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-
Cystine arylamidase	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+
Esterase (C4)	-	+	+	+	+	-	+	+	+	+	+	-	-	+	-	+
Esterase lipase (C8)	+	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+
α-Fucosidase	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
α-Galactosidase	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	+
β-Glucuronidase	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-

Lipase (C14)	-	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-
α-Mannosidase	+	+	+	+	-	+	-	+	+	-	-	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
GEN III BIOLOG Microplate tests																
(a) Utilization of sugars:																
N-Acetyl-D-galactosamine	-	+	-	+/-	-	-	-	+	+	+	-	-	+	-	-	-
N-Acetyl-D-glucosamine	-	-	+/-	+	-	-	-	+	+	+	+	+	-	-	-	+
N-Acetyl-β-D-mannosamine	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
N-Acetyl-neuraminic acid	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
D-Arabitol	-	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-
D-Cellobiose	+	+	+	+	+/-	-	+	+	+	+	+	+	+	+	+	+
Dextrin	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
D-Fructose	+	+	+	+	-	-	+	-	-	+	+	+	-	+	+/-	+/-
D-Fructose-6-phosphate	+	+/-	+/-	-	+	-	-	-	-	-	-	-	-	-	+	-
D-Fucose	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
L-Fucose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+/-
L-Galactonic acid-γ-lactone	-	-	-	+	-	-	-	+	-	-	+	+	+	-	+/-	+
D-Galactose	-	-	+	+	-	-	+	+	+	+	+	+	+	+	-	+/-
D-Galacturonic acid	-	-	+/-	+	-	-	-	-	-	-	-	+	+	-	-	+
β-Gentiobiose	-	+/-	+/-	+	-	-	+	+	+	+	+	+	+	+	-	+
D-Glucose	+	-	+/-	+	+	+	+	-	+	+	+	+	+	+	+	+
D-Glucose-6-phosphate	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-
Glycerol	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+
Glycyl-L-proline	-	+/-	-	+	-	-	-	+	+	+	+	-	-	-	-	+
α-D-Lactose	+	-	-	+	-	-	-	+	+	+	+	+	+	-	+/-	+/-

D-Maltose	-	+/-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	+
D-Mannose	-	+/-	-	+	-	-	+	+	+	+	+	+	+	+	+	+
D-Melibiose	-	-	+	+	-	-	-	+	+	+	+	+	+	+	-	+/-
Methyl pyruvate	-	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+
3-*O*-Methyl-D-glucose	+/-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	+
β-Methyl-D-glucoside	-	+/-	+/-	+	-	+/-	+	+	-	+	+	+	+	-	-	+/-
myo-Inositol	-	-	-	+	-	+/-	+	+	+	+	+	+	+	-	+/-	+/-
D-Raffinose	+/-	-	+/-	+	+	+	+	+	+	+	-	+	+	+	+	-
L-Rhamnose	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+
D-Salicin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+
D-Serine #1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D-Serine #2	+	+	+	-	+	+	-	-	-	+	-	-	-	+	+	-
L-Serine	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+
D-Sorbitol	+/-	-	+	+	+	+	+	-	-	-	-	+	+	-	+	+
Stachyose	+/-	-	+/-	+	-	+	-	+	+	+	-	-	-	+	-	-
Sucrose	-	-	+/-	+	-	+/-	+	+	+	+	+	+	+	+	-	+
D-Trehalose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
(b) Utilization of amino acids:																
L-Alanine	+/-	-	+	+	-	-	+	-	-	+	+	-	+	+	+/-	-
D-Aspartic acid	-	-	+	+/-	-	-	-	-	-	-	-	-	-	-	-	-
L-Aspartic acid	+	-	+	+	-	+/-	+	+	+	-	+/-	+/-	+	-	-	+
L-Glutamic acid	-	+/-	+	+	-	+/-	+	+	+	+	+	+	+	-	-	+
L-Histidine	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
L-Pyroglutamic acid	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+

(c) Utilization of polymers:																
Acetic acid	+	+/-	-	+/-	+	-	+	-	-	+	+	+	+	+	+	-
Acetoacetic acid	+	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-
γ-Amino-*n*-butyric acid	-	+/-	+/-	+	+	+	+	+	+	-	+	-	-	-	+/-	+
Bromo-succinic acid	-	+	+/-	+	-	+	+	-	-	-	+	-	-	+	-	+
Butyric acid	+	+	+	-	+	+	-	-	-	+	-	-	-	+	+	-
α-Hydroxy-butyric acid	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+
β-Hydroxy-butyric acid	-	-	+/-	+	-	+	+	-	-	-	-	-	+	-	-	+
α-*keto*-Butyric acid	-	-	-	-	+	-	+/-	+	+	-	-	-	-	-	-	+
D-Gluconic acid	-	+/-	+	-	-	-	+	+	+	+	+	+/-	+	-	+/-	+
D-Glucuronic acid	+	+	+	+	-	-	-	-	-	-	+	+	+	-	+/-	+
Glucuronamide	+/-	+	-	+/-	+	+/-	-	-	-	-	+	-	-	+	+	-
α-*keto*-Glutaric acid	-	+	+/-	-	+	+	+	+	+	-	+	+	+	+	+	+
p-Hydroxy-phenylacetic acid	-	-	-	-	+	-	-	+	+	-	+/-	-	-	-	-	-
D-Lactic acid methyl ester	+	+	+	+	-	-	-	-	-	-	-	+/-	-	+	-	+
L-Lactic acid	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+
D-Malic acid	-	-	+/-	+	-	+	+	-	-	-	+	-	-	-	-	-
L-Malic acid	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+/-
Mucic acid	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
Propionic acid	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+
Quinic acid	+/-	+/-	+	-	-	+	-	+	+	-	-	+	+	-	-	+
D-saccharic acid	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
(d) Utilization of other compounds:																
Gelatin	-	+/-	+/-	+	+	+	-	-	+	+/-	-	-	-	-	+/-	+
Inosine	-	+/-	-	+	-	-	+	+	+	+	+	+	+	-	+/-	+/-
Pectin	-	-	-	-	+	+	+	-	-	+	-	-	-	+	+/-	+

(e) Growth in the presence of:																
Aztreonam	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-
Fusidic acid	+	+	+/-	-	+	+	-	-	-	-	-	-	-	-	+	-
Guanidine hydrochloride	+	+	-	-	+	+	-	-	-	-	-	-	-	-	+	-
Lincomycin	+	+	-	-	+	+	-	+	-	-	-	-	-	-	+	-
Lithium chloride	+	-	-	-	+	+	+	-	-	+	-	+	+	+	+	-
Minocycline	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	-
1% NaCl	+	+	+/-	-	+	+	+	+	+	+	+	+	+	+	+	-
4% NaCl	+	+	+	-	+	+	-	-	-	+	-	+	+	+	+	-
8% NaCl	+	+	+/-	-	+	+	-	-	-	+	-	-	-	+	+	-
Nalidixic acid	+	+	+	-	+	+	-	+	+	+	+	-	-	-	+	-
Niaproof	+	+	+	-	+	+	-	-	-	-	-	-	-	-	+	-
Potassium tellurite	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Rifamycin SV	+	+	+	-	+	+	-	+	+	-	+/-	-	-	-	+	-
Sodium bromate	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	-
Sodium formate	+	+/-	-	-	+	-	+	-	-	-	+	-	-	-	+	-
Sodium lactate (1%)	+	-	-	-	+	+	+	+	-	+	-	-	-	+	+	+
Tetrazolium violet	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	-
Tetrazolium blue	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	-
Troleandomycin	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-
Vancomycin	+	+	+/-	-	+	+	-	+	+	-	-	-	-	-	+	-
(f) Growth at:																
pH 5	+	+	+/-	-	+	+	-	-	-	-	-	-	-	-	+	+
pH 6	+	+	+/-	-	+	+	+	+	+	+	+	+	+	+	+	+
Other phenotypic tests:																
(a) Biochemical tests:																

Aesculin hydrolysis	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+
Arbutin hydrolysis	+	+	-	+	-	+	-	+	-	+	-	+	+	-	+	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
(b) Degradation tests:																
Hypoxanthine	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Starch	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+
Tween 40	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	+
(c) Growth at:																
10^{o}C	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
40 ^{o}C	-	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-
45 ^{o}C	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-

+ positive results; - negative results; +/-, variable results.

4.4.5 *Classification of putatively novel Streptomyces strains*

Phylogenetic analyses. The 10 *Streptomyces* isolates fell into two 16S rRNA subclades that were supported by all of the tree-making algorithms and very high bootstrap values (Figure 4.12). The 6 isolates assigned to the novel subclade had 16S rRNA gene sequences that were identical or almost identical (Table 4.16). These organisms were most closely related to the type strains of *Streptomyces carpinensis* (99.35-99.51% similarity, 7-9 nt differences), *Streptomyces cellulosae* (99.35-99.50% similarity, 7-8 nt differences), *Streptomyces gancidicus* (99.49-99.64% similarity, 5-7 nt differences), *Streptomyces levis* (99.49-99.57% similarity, 6-8 nt differences), *Streptomyces pseudogriseolus* (99.30-99.57% similarity, 6-9 nt differences) and *Streptomyces warraensis* (99.20-99.36% similarity, 9-11 nt differences).

The *S. fimbriatus* 16S rRNA subclade encompasses considerable variation, as shown in Figure 4.8. Isolates C59 and KNN26.b formed a branch in the subclade together with the *S. fimbriatus* strain. Isolate C59 and the *S. fimbriatus* strain had identical 16S rRNA gene sequences, the corresponding value between isolate KNN 26.b and *S. fimbriatus* NRBC 15411^{T} is 99.64%, a value that corresponds to 16 nt differences at 1383 locations (Table 4.16). These strains are loosely associated with the isolates assigned to the novel subclade sharing 16S rRNA gene similarities with the latter within the range 98.62-99.11%, values that correspond to 18 and 12 nt differences at 1376 and 1353 sites. Isolates KNN38.1b and KNN64.5b were also most closely related to the *S. fimbriatus* strain sharing 16S rRNA gene similarities with this organism of 97.38% and 98.84%, respectively, values that equate to 51 and 36 nt differences at 1382 and 1376 sites.

It is also clear from the analyses of the partial sequences of the house-keeping genes *atpD, gyrB, recA, rpoB* and *trpB* that isolates KNN 11.a, KNN 35.1b, KNN 35.2b, KNN42.f, KNN 48.3 and KNN 83.e belong to a distinct and homogeneous lineage in the *Streptomyces* MLSA gene tree that is supported by a 100% bootstrap value (Figure 4.13, Table 4.17). Members of this taxon are most closely related to the type strains of *Streptomyces ghanaensis* and *Streptomyces viridosporus* sharing MLSA distances with these organisms that fall within the range 0.037-0.038 and 0.038 and 0.040, respectively. It is also clear from these analyses that the type strains of *S. ghanaensis* and *S. viridosporus* are very closely related as they share an MLSA distance of 0.004.

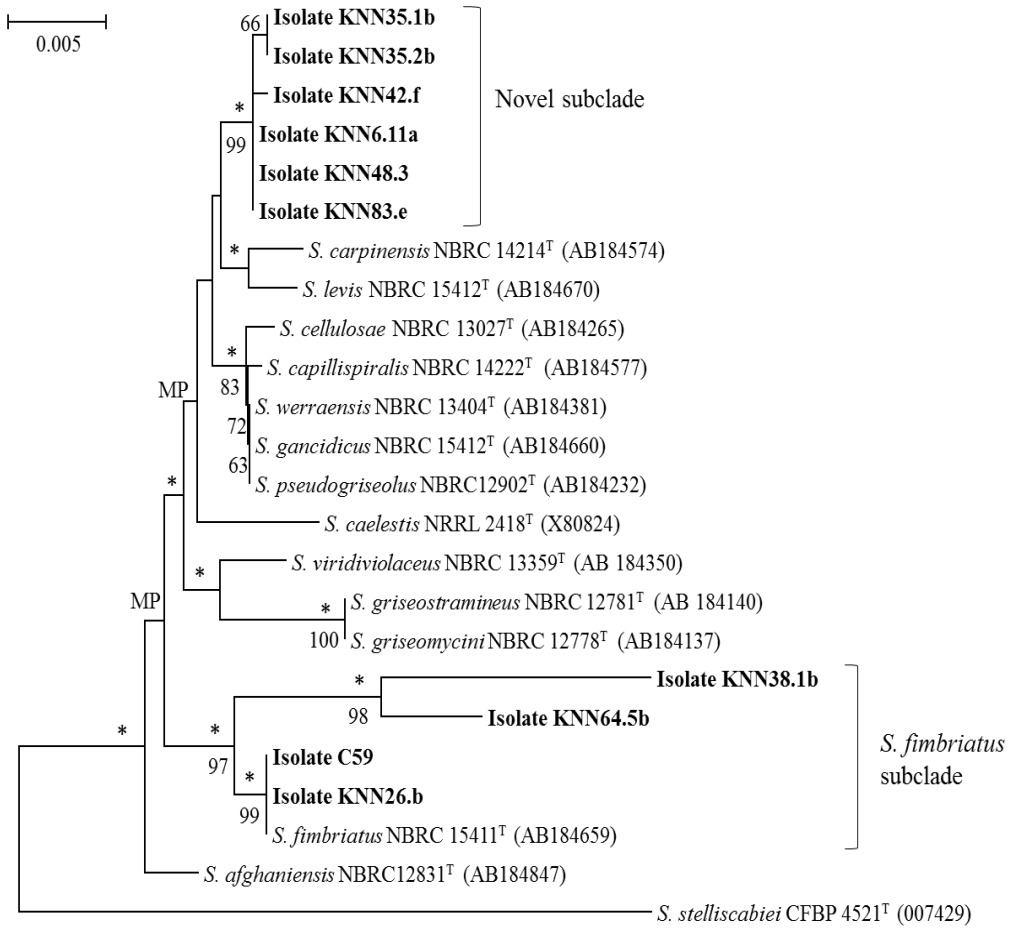

Figure 4.13 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolates C59, KNN6.11a, KNN26.b, KNN 35.1b, KNN 35.2b, KNN 38.1b, KNN 42.f, KNN 48.3, KNN64.5b and KNN83.e and between them and the type strains of closely related *Streptomyces* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood (ML) and maximum-parsimony (MP) tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysies of 1000 resampled datasets, only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Table 4.16 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolates C59, KNN6.11a, KNN26.b, KNN 35.1b, KNN 35.2b, KNN 38.1b, KNN 42.f, KNN64.5b and KNN83.e and between them and the type strains of closely related *Streptomyces* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. Isolate KNN6.11a	---	**2/1381**	**1/1382**	**1/1373**	**0/1407**	**0/1418**	**12/1353**	**17/1375**	**40/1373**	**32/1377**	**16/1405**	**9/1414**	**20/1402**	**20/1414**	**16/1409**	**13/1418**	**5/1403**	**9/1414**	**7/1414**	**7/1414**	**11/1413**	**58/1418**	**6/1409**	**8/1400**
2. Isolate KNN35.1b	**99.86**	---	**1/1382**	**2/1373**	**2/1370**	**2/1382**	**13/1354**	**19/1376**	**41/1373**	**33/1377**	**18/1382**	**11/1382**	**22/1382**	**22/1382**	**18/1382**	**15/1382**	**7/1381**	**7/1382**	**9/1382**	**9/1382**	**13/1382**	**60/1382**	**8/1382**	**10/1382**
3. Isolate KNN35.2b	**99.93**	**99.93**	---	**2/1373**	**1/1371**	**1/1383**	**13/1362**	**18/1376**	**41/1373**	**33/1377**	**17/1391**	**10/1391**	**21/1391**	**21/1391**	**17/1391**	**14/1391**	**6/1390**	**6/1391**	**8/1391**	**8/1391**	**12/1391**	**59/1391**	**7/1391**	**9/1391**
4. Isolate KNN42.f	**99.93**	**99.85**	**99.85**	---	**1/1363**	**1/1373**	**13/1352**	**18/1373**	**41/1372**	**33/1371**	**17/1373**	**10/1373**	**21/1373**	**21/1373**	**17/1373**	**14/1373**	**6/1372**	**6/1373**	**8/1373**	**8/1373**	**12/1373**	**59/1373**	**7/1373**	**9/1373**
5. Isolate KNN48.3	**100**	**99.85**	**99.93**	**99.93**	---	**0/1407**	**12/1342**	**17/1364**	**40/1363**	**32/1367**	**16/1394**	**9/1403**	**20/1391**	**20/1403**	**16/1398**	**13/1410**	**5/1392**	**9/1403**	**7/1403**	**7/1403**	**11/1402**	**57/1410**	**6/1398**	**8/1389**
6. Isolate KNN83.e	**100**	**99.86**	**99.93**	**99.93**	**100**	---	**12/1354**	**17/1376**	**40/1373**	**32/1377**	**16/1406**	**9/1415**	**20/1403**	**20/1415**	**16/1410**	**13/1419**	**5/1404**	**9/1415**	**7/1415**	**7/1415**	**11/1414**	**58/1419**	**6/1410**	**8/1401**
7. Isolate C59	**99.11**	**99.04**	**99.05**	**99.04**	**99.11**	**99.11**	---	**0/1358**	**34/1353**	**15/1355**	**0/1369**	**15/1365**	**16/1366**	**16/1366**	**18/1365**	**15/1365**	**15/1364**	**15/1365**	**15/1365**	**15/1365**	**15/1366**	**59/1367**	**16/1365**	**18/1365**
8. Isolate KNN26.b	**98.76**	**98.62**	**98.69**	**98.69**	**98.75**	**98.76**	**100**	---	**41/1375**	**21/1377**	**5/1382**	**21/1378**	**22/1379**	**22/1379**	**24/1378**	**20/1376**	**20/1375**	**20/1376**	**20/1376**	**20/1376**	**20/1377**	**64/1378**	**21/1376**	**23/1376**
9. Isolate KNN38.1b	**97.09**	**97.01**	**97.01**	**97.01**	**97.07**	**97.09**	**97.49**	**97.02**	---	**26/1375**	**36/1376**	**44/1375**	**45/1376**	**45/1376**	**47/1375**	**51/1373**	**45/1372**	**45/1373**	**45/1373**	**45/1373**	**46/1374**	**91/1374**	**46/1373**	**48/1373**
10. Isolate KNN64.5b	**97.68**	**97.6**	**97.6**	**97.59**	**97.66**	**97.68**	**98.89**	**98.47**	**98.11**	---	**16/1383**	**33/1379**	**34/1380**	**34/1380**	**36/1379**	**35/1377**	**35/1376**	**35/1377**	**35/1377**	**35/1377**	**35/1378**	**79/1379**	**36/1377**	**38/1377**
11. *S. fimbriatus* NBRC 15411^{T}	**98.86**	**98.7**	**98.78**	**98.76**	**98.85**	**98.86**	**100**	**99.64**	**97.38**	**98.84**	---	17/1455	18/1453	18/1456	20/1455	19/1453	19/1451	20/1453	19/1453	19/1453	19/1454	64/1455	20/1453	22/1448
12. *S. werraensis* NBRC 13404^{T}	**99.36**	**99.2**	**99.28**	**99.27**	**99.36**	**99.36**	**98.9**	**98.48**	**96.8**	**97.61**	98.83	---	19/1452	19/1464	11/1459	15/1463	4/1452	8/1463	14/1462	6/1463	16/1461	68/1462	5/1458	13/1448
13. *S. griseostramineus* NBRC 12781^{T}	**98.57**	**98.41**	**98.49**	**98.47**	**98.56**	**98.57**	**98.83**	**98.4**	**96.73**	**97.54**	98.76	98.69	---	0/1453	14/1452	25/1450	22/1449	24/1450	22/1450	22/1450	23/1451	69/1451	24/1450	22/1448
14. *S griseomycini* NBRC 12778^{T}	**98.59**	**98.41**	**98.49**	**98.47**	**98.57**	**98.59**	**98.83**	**98.4**	**96.73**	**97.54**	98.76	98.7	100	---	14/1459	25/1462	22/1451	27/1462	22/1462	22/1462	23/1462	69/1463	24/1457	22/1448
15. *S. viridiviolaceus* NBRC 13359^{T}	**98.86**	**98.7**	**98.78**	**98.76**	**98.86**	**98.87**	**98.68**	**98.26**	**96.58**	**97.39**	98.63	99.25	99.04	99.04	---	20/1457	15/1451	16/1457	17/1457	15/1457	17/1457	73/1457	16/1457	14/1448
16. *S. caelestis* NRRL 2418^{T}	**99.08**	**98.91**	**98.99**	**98.98**	**99.08**	**99.08**	**98.9**	**98.55**	**96.29**	**97.46**	98.69	98.97	98.28	98.29	98.63	---	11/1452	15/1463	18/1462	13/1463	16/1461	68/1474	12/1458	13/1448
17. *S. gancidicus* NBRC 15412^{T}	**99.64**	**99.49**	**99.57**	**99.56**	**99.64**	**99.64**	**98.9**	**98.55**	**96.72**	**97.46**	98.69	99.72	98.48	98.48	98.97	99.24	---	1/1452	10/1451	2/1452	12/1451	63/1451	1/1452	9/1447
18. *S. pseudogriseolus* NBRC12902^{T}	**99.36**	**99.49**	**99.57**	**99.56**	**99.36**	**99.36**	**98.9**	**98.55**	**96.72**	**97.46**	98.62	99.45	98.34	98.15	98.9	98.97	99.93	---	14/1462	6/1463	15/1461	68/1462	2/1458	10/1448
19. *S. carpinensis* NBRC 14214^{T}	**99.5**	**99.35**	**99.42**	**99.42**	**99.5**	**99.51**	**98.9**	**98.55**	**96.72**	**97.46**	98.69	99.04	98.48	98.5	98.83	98.77	99.31	99.04	---	10/1462	16/1461	66/1462	11/1457	7/1448
20. *S. cellulosae* NBRC 13027^{T}	**99.5**	**99.35**	**99.42**	**99.42**	**99.5**	**99.51**	**98.9**	**98.55**	**96.72**	**97.46**	98.69	99.59	98.48	98.5	98.97	99.11	99.86	99.59	99.32	---	12/1463	64/1463	3/1458	11/1448
21. *S. afghaniensis* NBRC12831^{T}	**99.22**	**99.06**	**99.14**	**99.13**	**99.22**	**99.22**	**98.9**	**98.55**	**96.65**	**97.46**	98.69	98.9	98.41	98.43	98.83	98.9	99.17	98.97	98.9	99.18	---	59/1463	11/1457	9/1448
22. *S. capillispiralis* NBRC 14222^{T}	**95.91**	**95.66**	**95.76**	**95.7**	**95.96**	**95.91**	**95.68**	**95.36**	**93.38**	**94.27**	95.6	95.35	95.24	95.28	94.99	95.39	95.66	95.35	95.49	95.63	95.97	---	64/1457	66/1448
23. *S. levis* NBRC 15423^{T}	**99.57**	**99.42**	**99.5**	**99.49**	**99.57**	**99.57**	**98.83**	**98.47**	**96.65**	**97.39**	98.62	99.66	98.34	98.35	98.9	99.18	99.93	99.86	99.25	99.79	99.25	95.61	---	8/1448
24. *S. stelliscabiei* CFBP 4521^{T}	**99.43**	**99.28**	**99.35**	**99.34**	**99.42**	**99.43**	**98.68**	**98.33**	**96.5**	**97.24**	98.48	99.1	98.48	98.48	99.03	99.1	99.38	99.31	99.52	99.24	99.38	95.44	99.45	---

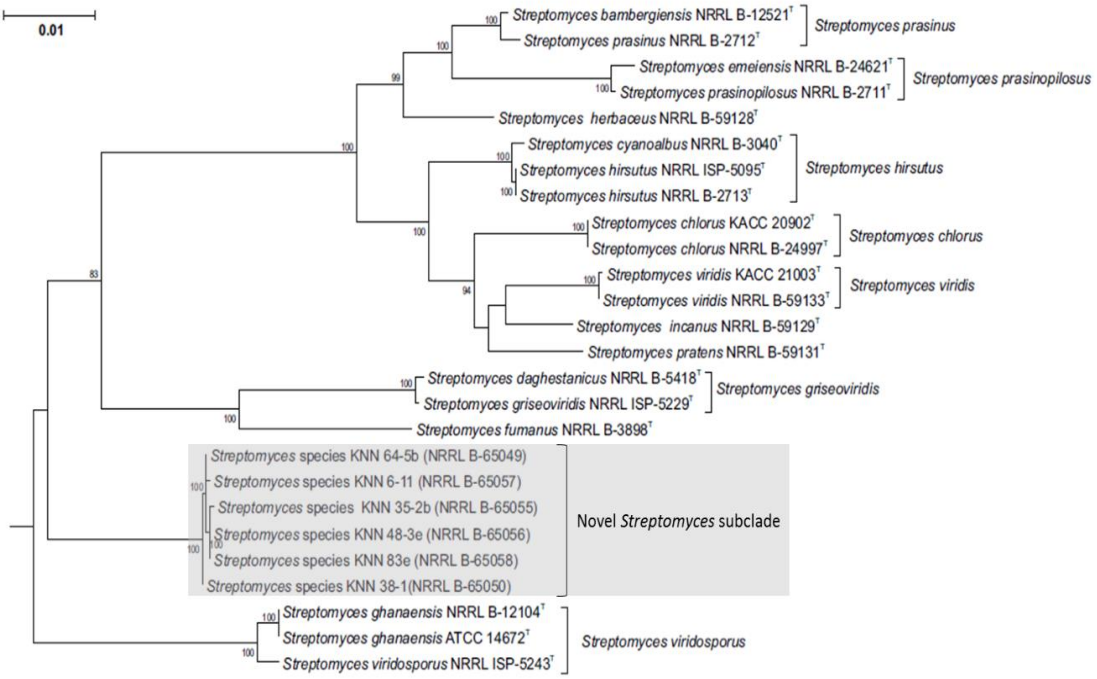

Figure 4.14 Subtree from the *Streptomyces* phylogenetic tree inferred from concatenated partial sequences of the house-keeping genes *atpD, gyrB, recA, rpoB* and *trpB* in MEGA 6 (Tamura et al. 2013) using the maximum-likelihood method based on the General Time Reversible model (Nei and Kumar 2000). There were 2622 positions and 706 strains in the final dataset. Percentages at the nodes represent levels of bootstrap support from 1000 resampled datasets with values less than 60% not shown. Bar marker equals number of substitutions per site.

Table 4.17 MLSA distances for strains phylogenetically near to the novel *Streptomyces* subclade and related isolates. The distances between the isolates and the type strains greater than 0.007 which was defined by Rong and Huang (2012) as equivalent to 70% genomic DNA similarity.

S. prasinus NRRL B-12521TY25A1:Z26																									
S. prasinus NRRL B-2712^{T}	0.003																								
S. emeiensis NRRL B-24621^{T}	0.024	0.026																							
S. prasinopilosus NRRL B-2711^{T}	0.022	0.024	0.003																						
S. herbaceus NRRL B-59128^{T}	0.019	0.021	0.029	0.027																					
S. hirsutus NRRL B-3040^{T}	0.029	0.031	0.043	0.042	0.030																				
S. hirsutus NRRL ISP-5095^{T}	0.028	0.030	0.043	0.041	0.030	0.002																			
S. hirsutus NRRL B-2713^{T}	0.028	0.030	0.043	0.041	0.030	0.002	0.000																		
S. chlorus KACC 20902^{T}	0.035	0.037	0.041	0.040	0.033	0.026	0.025	0.025																	
S. chlorus NRRL B-24997^{T}	0.035	0.037	0.041	0.040	0.033	0.026	0.025	0.025	0.000																
S. viridis KACC 21003^{T}	0.033	0.036	0.046	0.044	0.032	0.016	0.015	0.015	0.021	0.021															
S. viridis NRRL B-59133^{T}	0.034	0.036	0.047	0.044	0.032	0.017	0.016	0.016	0.021	0.021	0.000														
S. incanus NRRL B-59129^{T}	0.033	0.035	0.042	0.041	0.027	0.022	0.021	0.021	0.019	0.019	0.016	0.016													
S. pratens NRRL B-59131^{T}	0.036	0.037	0.043	0.043	0.032	0.024	0.023	0.023	0.022	0.022	0.021	0.021	0.017												
S. daghestanicus NRRL_B-5418^{T}	0.059	0.062	0.065	0.062	0.063	0.065	0.064	0.064	0.068	0.068	0.066	0.067	0.067	0.067											
S. griseoviridis NRRL ISP-5229^{T}	0.059	0.061	0.064	0.061	0.063	0.064	0.063	0.063	0.067	0.067	0.066	0.066	0.066	0.066	0.001										
S. fumanus NRRL B-3898^{T}	0.059	0.059	0.064	0.062	0.062	0.066	0.065	0.065	0.068	0.068	0.065	0.066	0.064	0.069	0.035	0.035									
Streptomyces_species KNN 64.5b NRRL_B-65049	**0.053**	**0.054**	**0.060**	**0.058**	**0.057**	**0.054**	**0.053**	**0.053**	**0.059**	**0.059**	**0.055**	**0.054**	**0.058**	**0.062**	**0.046**	**0.045**	**0.045**								
Streptomyces_species KNN 6.11 NRRL_B-65057	**0.053**	**0.055**	**0.060**	**0.059**	**0.058**	**0.055**	**0.054**	**0.054**	**0.059**	**0.059**	**0.055**	**0.055**	**0.058**	**0.062**	**0.046**	**0.046**	**0.045**	**0.000**							
Streptomyces_species KNN 35.2b NRRL_B-65055	**0.054**	**0.055**	**0.061**	**0.059**	**0.058**	**0.055**	**0.054**	**0.054**	**0.060**	**0.060**	**0.056**	**0.055**	**0.059**	**0.063**	**0.047**	**0.046**	**0.046**	**0.001**	**0.001**						
Streptomyces_species KNN 48.3e NRRL_B-65056	**0.053**	**0.055**	**0.060**	**0.059**	**0.058**	**0.055**	**0.054**	**0.054**	**0.059**	**0.059**	**0.055**	**0.055**	**0.058**	**0.062**	**0.046**	**0.046**	**0.045**	**0.000**	**0.001**	**0.000**					
Streptomyces_species KNN 83.e NRRL_B-65058	**0.053**	**0.055**	**0.060**	**0.059**	**0.058**	**0.055**	**0.054**	**0.054**	**0.059**	**0.059**	**0.055**	**0.055**	**0.058**	**0.062**	**0.046**	**0.046**	**0.045**	**0.000**	**0.001**	**0.000**	**0.000**				
Streptomyces_species KNN 38.1 NRRL_B-65050	**0.053**	**0.054**	**0.060**	**0.058**	**0.057**	**0.054**	**0.053**	**0.053**	**0.059**	**0.059**	**0.054**	**0.054**	**0.057**	**0.062**	**0.045**	**0.045**	**0.044**	**0.000**	**0.001**	**0.001**	**0.001**	**0.001**			
S. ghanaensis NRRL B-12104^{T}	0.055	0.056	0.064	0.062	0.059	0.056	0.055	0.055	0.059	0.059	0.057	0.057	0.061	0.063	0.054	0.054	0.055	0.037	0.038	0.038	0.038	0.038	0.037		
S. ghanaensis ATCC 14672^{T}	0.055	0.056	0.064	0.062	0.059	0.056	0.055	0.055	0.059	0.059	0.057	0.057	0.061	0.063	0.054	0.054	0.055	0.037	0.038	0.038	0.038	0.038	0.037	0.000	
S. viridosporus NRRL ISP-5243^{T}	0.053	0.054	0.062	0.060	0.057	0.053	0.053	0.053	0.058	0.058	0.056	0.056	0.059	0.062	0.054	0.053	0.055	0.039	0.039	0.040	0.039	0.039	0.038	0.004	0.004

Chemotaxonomy

All of the isolates and associated type strains contained *LL*-A_2pm in whole-organism hydrolysates. It can be seen from Table 4.18 that 3 representatives of the novel *Streptomyces* taxon and the type strains of *S. fimbriatus* and *S. ghanaensis* contained complex mixture of saturated and branched chain fatty acids with predominant amounts of *iso*-$C_{16:0}$ (21.3-33.3%). Quantitative differences were found in some components, as exemplified, by *iso*-$C_{14:0}$ (3.3-8.1%), *iso*-$C_{15:0}$ (5.2-11.3%) and *anteiso*-$C_{17:0}$ (6.4-12.3%). A small number of qualitative differences were also found, notably the presence of *iso*-$C_{16:0}$ in isolates KNN 35.1b and KNN 35.2b and C16:0 in isolate KNN 35.2b and the type strain of *S. ghanaensis.*

Two representatives of the novel *Streptomyces* subclade, strains KNN 35.1b and KNN 35.2b, and the type strain of *S. ghanaensis* contained glucose, mannose, ribose and xylose in whole-organism hydrolysates; galactose was also detected in the latter. The polar lipid patterns of these strains consisted of diphosphotidylglycerol, phosphotidylethanolamine, phosphatidylinositol mannosides and a number of unidentified components, a trace of phosphatidylglycerol was detected in isolate KNN 35.1 (Figure 4.14). The predominant menaquinones of strain KNN35.1b consisted of MK9(H_6) (34%), MK9(H_8) (29%) and MK9(H_4) (10%).

Isolates C59, KNN 38.1b, KNN 64.5b and the type strain of *S. fimbriatus* produced whole-organism hydrolysates containing glucose, mannose and ribose, those of isolate C59 and *S. fimbriatus* NRRL B-3175^T also included galactose and a trace of xylose, respectively. These strains shared similar polar lipid patterns, they all contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides (Figure 4.15). Some polar lipids were discontinuously distributed, as exemplified by the presence of hydroxyl-phosphatidylethanolamine in the *S. fimbriatus* strain and several glycolipids in isolate C59. The major menaquinone detected in the type strain of S. fimbriatus and in isolate KNN 64.5b were MK9(H_6) (61%) and MK9(H_8) (15%), the predominant menaquinone in isolate C59 was MK9(H_2) (66%).

Table 4.18 Fatty acid profiles (%) of *Streptomyces* isolates KNN35.1b, KNN 35.2b and KNN 42.f and the type strains of *S. fimbriatus* and *S. ghanaensis.*

Fatty acids	Isolate KNN 35.1b	Isolate KNN 35.2b	Isolate KNN 42.f	*S. ghanaensis* NRRL B-12104^T	*S. fimbriatus* NRRL B-3175^T
Iso-$C_{14:0}$	5.3	7.6	8.1	3.7	3.6
Anteiso- $C_{15:0}$	17.5	15.1	17.8	11.5	8.1
Iso-$C_{15:0}$	11.3	7.2	8.4	5.2	6.4
$C_{16:0}$	4.0	-	6.7	3.0	3.0
Iso-$C_{16:0}$	24.2	21.3	28.6	32.6	33.3
Iso- H $C_{16:0}$	3.0	8.3	-	-	-
$C_{16:0}$	-	8.8	-	3.0	-
Iso-H $C_{16:1}$	-	-	4.4	8.8	6.5
Iso-$C_{17:0}$	6.9	3.4	3.7	2.7	6.1
Anteiso- $C_{17:0}$	9.9	6.4	7.8	12.3	10.0
$C_{17:1}$ CIS 9	-	-	-	1.2	-
$C_{17:1}$ w8c	0.89	-	0.73	1.2	1.1
Anteiso- $C_{17:1}$ w9c	3.4	3.3	3.7	6.6	3.5
Anteiso –$C_{17:1}$	-	-	-	6.6	-
$C_{17:0}$	0.82	-	0.9	0.37	0.53
$C_{17:0}$ 10-methyl	-	-	-	0.20	1.66
$C_{18:0}$	0.29	4.2	1.3	-	0.10
$C_{18:0}$ w9c	-	-	-	-	0.26
Iso- H $C_{18:1}$	0.89	-	-	1.4	1.2
Summed feature 3	2.0	3.3	2.8	4.0	3.8
Summed feature 5		6.1	1.7	-	-
Summed feature 9	6.6	5.1	3.0	3.8	7.9

Trace proportions (< 0.9 %) are only cited for strains where other fatty acids were found beyond this cut-off point.
Summed feature 3, $C_{16:1}$ w7c/$C_{16:1}$ w6c; Summed feature 9, *iso*-$C_{17:1}$ w9c.
KNN 35.2b: Summed feature 5, *iso*-$C_{17:1}$ w9c.
KNN 42.f: Summed feature 5, $C_{18:2}$ w6,9c/*anteiso* B.
S. fimbriatus: Summed feature 4, *Iso*- $C_{17:1}$ I/*anteiso* B.

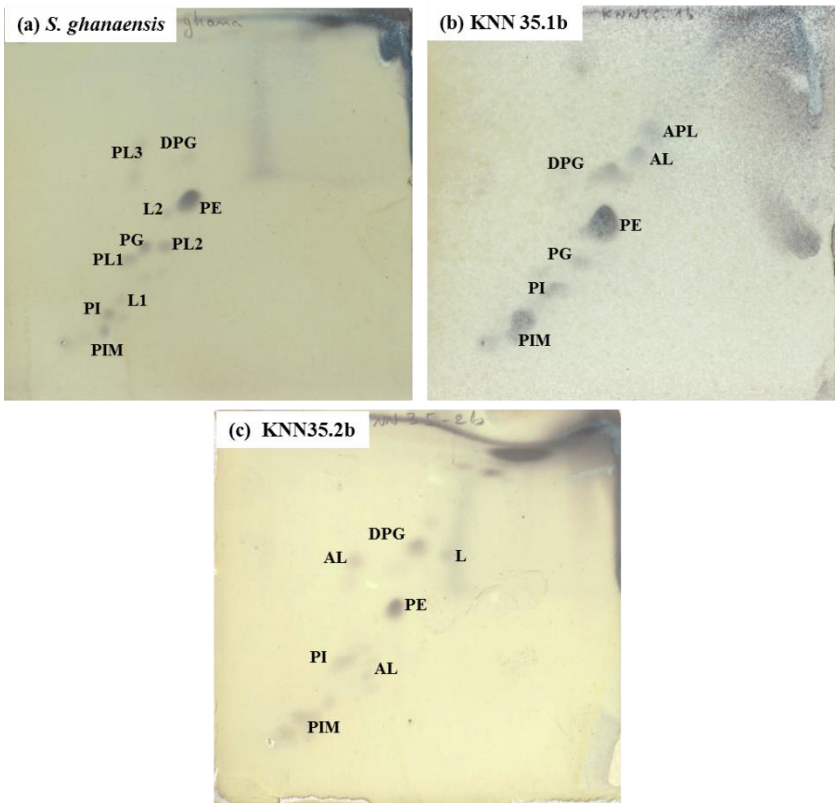

Figure 4.15 Two-dimensional thin-layer chromatography of polar lipids of *Streptomyces* isolates KNN 35.1b, KNN 35.2b and *S. ghanaensis* NRRL B-3175^{T} stained with molybdenum blue spray (Sigma) using the solvent systems cited in the legend to Figure 4.3. Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; methyl-PE, methyl- phosphatidylethanolamine; OH-PE, hydroxyphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; AL, aminolipid; GL, glycolipids, PL, phospholipid and L, unknown lipids.

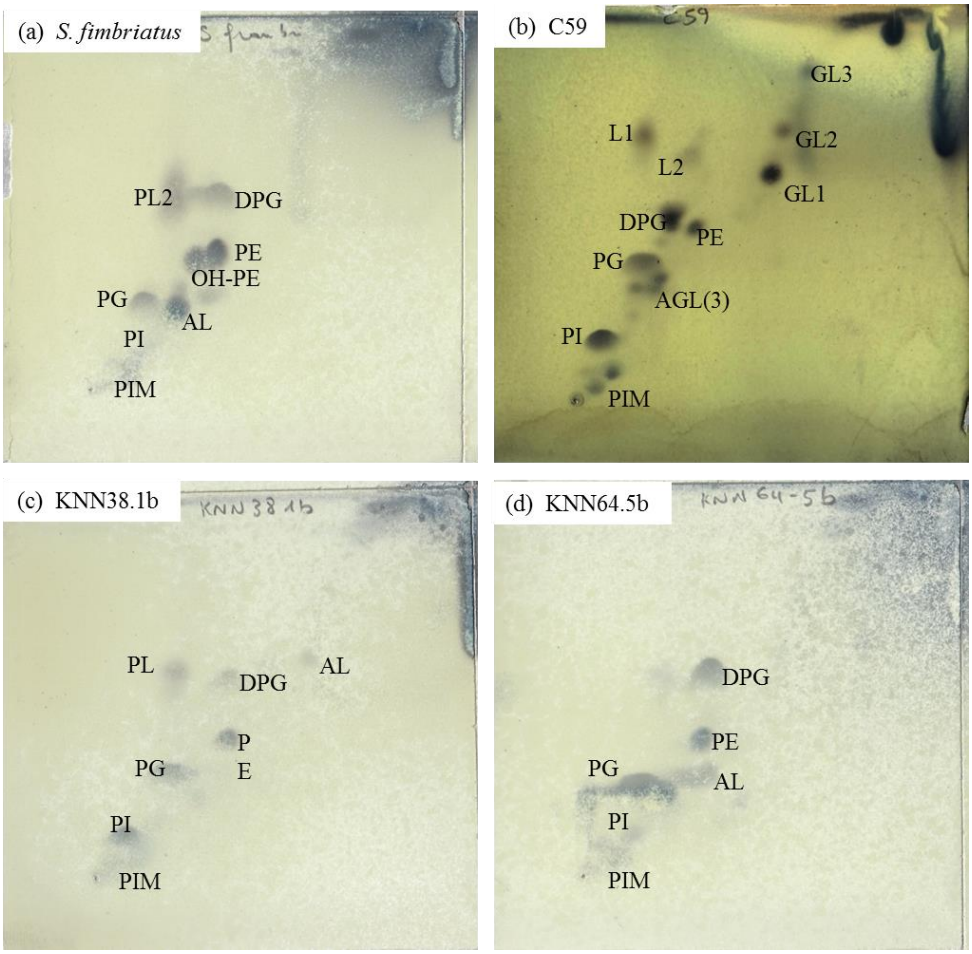

Figure 4.16 Two-dimensional thin-layer chromatography of polar lipids of *Streptomyces fimbriatus* and closely related isolates stained with molybdenum blue spray (Sigma) using the solvent systems cited in the legend to Figure 4.3. Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; methyl-PE, methyl-phosphatidylethanolamine; OH-PE, hydroxyphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; AGL, aminoglycolipid; AL, aminolipid; GL, glycolipids; PL, phospholipid and L, unknown lipids.

Phenotypic properties. The isolates assigned to the novel 16S rRNA subclade had many phenotypic features in common, some of which separate them from the type strain of *S. ghanaensis* (Table 4.19). The isolates, unlike the *Streptomyces ghanaensis* strain, their nearest phylogenetic neighbour, produced N-acetyl-β-glucosaminidase, utilized D-fucose, butyric acid and α-*keto*-butyric acid and grew at 10^{o}C. In contrast, only the *S. ghanaensis* strain formed naphthol-AS-BI-phosphahydrolase and utilized-fucose, D-glucuronic acid and N-acetyl-β-D-mannosamine.

Identical results were obtained for all of the duplicated strains, apart from some of the GEN III BIOLOG microplate tests (Table 4.19 and Table 4.20). All of the isolates and marker strains produced acid phosphatase, cystine arylamidase , β-galactosidase, leucine arylamidase; hydrolysed aesculin and arbutin; degraded adenine, hypoxanthine, Tweens 20 and 40; grew between 20 and 40^{o}C and utilized D-arabitol, γ-amino-butyric acid, β-bydroxy-butyric acid, N-acetyl-D-glucosamine, D-glucose, D-mannose, *myo*-inositol and L-rhamnose, sucrose and D-trehalose and were resistant to aztreonam, . In turn, all of the strains gave negative results for α-fucosidase, α-galactosidase, β-glucosidase and lipase (C14), for allantoin and urea hydrolysis, nitrate reduction, H_2S production, for the degradation of cellulose, chitin, guanine and tributyrin, the utilization of glucuronamide, D-melibiose, 3-*O*-methyl-glucose, mucic acid, quinic acid, D-saccharic acid, D-serine #2, showed resistance to fusidic acid and minocycline and did not grow at 4 and 50^{o}C.

Table 4.19 Phenotypic tests that distinguish isolates KNN 6.11a, KNN 35.1b, KNN 35.2b ,KNN 42.f,KNN 43.e KNN48.3e and KNN 83.e from one another and from *S. ghanaensis* NRRL B-12104^T.

Characteristic	Isolate KNN 6.11a	Isolate KNN 35.1b	Isolate KNN 35.2b	Isolate KNN 42.f	Isolate KNN 43.e	Isolate KNN 83.e	*S. ghanaensis* NRRL B-12104^T
API ZYM tests:							
N-Acetyl-β-glucosaminidase	+	+	+	+	+	+	-
α-Chymotrpsin	-	+	-	-	-	-	-
Esterase (C4)	+	-	-	-	-	+	-
α-Glucuronidase	+	-	+	-	-	+	+
α-Mannosidase	-	+	-	+	-	-	+
Naphthol-AS-BI-phosphohydrolase	-	-	+	-	-	-	+
Trypsin	-	+	-	-	-	-	-
GEN III BIOLOG Microplate tests							
(a) Utilization of sugars:							
N-Acetyl-β-D-mannosamine	-	+	-	-	-	-	+
N-Acetyl-D-galactosamine	+	-	-	-	-	+	+
N-acetyl-neuraminic acid	-	-	-	+	-	-	+
D-Fucose	+	+	+	+	+	+	-
L-Fucose	+	+	+	-	+	+	-
β-Gentiobiose	+	+	+	-	+	+	+

D-Glucose-6-phosphate	-	+	-	+	-	-	+
α-D-Lactose	+	+	+	-	+	-	+
D-Maltose	+	+	+	-	+	+	+
D-Mannitol	+	+	+	-	+	-	+
β-Methyl-D-glucoside	+	+	-	-	-	-	-
D-Salicin	+	-	-	-	-	+	+
D-Sorbitol	+	+	+	-	+	+	-
D-Turanose	+	+	+	-	+	+	+
(b) Utilization of amino acids:							
L-Alanine	+	+	+	+	+	-	+
L-Arginine	+	+	+	+	+	-	-
L-Histidine	+	+	+	+	+	-	+/-
L-Serine	+	+	-	+	-	-	+
(c) Utilization of organic acids:							
Acetoacetic acid	+	+	+	+	+	-	+
Bromo-succinic acid	+	+	-	-	-	-	-
Butyric acid	+	+	+	+	+	+	-
α-*keto*-Butyric acid	+	+	+	+	+	+	-
Citric acid	+	+	+	+	+	+	-
D-Galacturonic acid	+	-	-	-	-	-	-
L-Galactonic acid-γ-lactone	+	-	-	-	-	-	-
α-*keto*-Glutaric acid	+	-	-	+	+	+	+
D-Glucuronic acid	-	-	-	-	-	-	+
α-Hydroxy-butyric acid	+	-	-	+	-	-	-
p-Hydroxy-phenylacetic acid	-	-	-	+	-	-	-
L-Lactic acid	+	-	-	+	-	-	-

D-Malic acid	+	-	-	-	-	-	-
L-Malic acid	+	-	+	+	+	+	-
Methyl pyruvate	-	-	-	+	-	-	-
L-Pyroglutamic acid	+	+	+	+	+	-	-
(d) Utilization of other compounds:							
Inosine	+	-	+	+	+	+	+
Pectin	+	-	-	-	-	-	-
(e) Growth in the presence of:							
Aztreonam	+	+	+	+	+	+	+
Guanidine hydrochloride	+	-	-	-	-	-	-
Lincomycin	+	-	-	-	-	+	-
Niaproof	-	-	-	-	-	+	-
Potassium tellurite	+	+	+	-	+	+	-
Rifamycin SV	+	+	+	-	+	+	-
Sodium chloride (4%, w/v)	+	+	+	-	+	+	-
Sodium formate	-	-	-	+	-	-	-
Sodium lactate (1%)	+	+	-	+	+	+	-
Tetrazolium blue	+	-	-	-	-	+	-
Tetrazolium violet	+	-	-	-	-	+	-
Troleandomycin	+	-	-	-	-	+	-
Vancomycin	+	-	-	-	-	-	-
(f) Growth at:							
pH 5	+	+	-	-	+	+	-
Other phenotypic tests							
(a) Degradation tests:							

Casein	+	+	-	-	-	-	-
(b) Growth at:							
10^{o}C	+	+	+	+	+	+	-
45 ^{o}C	-	-	-	+	-	+	-

+, positive result; -, negative result.

Positive results recorded for all of the isolates and the *Streptomyces ghanaensis* type strain:

- API ZYM tests: acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase lipase (C8), β-galactosidase, leucine arylamidase and valine arylamidase.
- GEN III BIOLOG microplate tests: utilization of acetic acid, *N*-acetyl-D-glucosamine, γ-amino-n-butyric acid, D-arabitol, L-aspartic acid, D-cellobiose, Dextrin, D-fructose, D-galactose, gelatin, D-gluconic acid, D-glucose, 3-*O*-methyl-D-glucose, L-glutamic acid, glycerol, glycl-L-proline, β-hydroxy-butyric acid, *myo*-inositol, D-mannose, D-melibiose, propionic acid, sucrose and D-trehalose, growth at pH6 and in the presence of aztreonam, lincomycin, nalidixic acid, sodium bromate and sodium chloride (1%, w/v).
- Other phenotypic tests: aesculin hydrolysis, arbutin hydrolysis, degradation of adenine, elastin, hypoxanthine, starch, L-tyrosine, Tweens 40, 60 and 80 and growth at 20, 30 and 40^{o}C.

Negative results recorded for all of the isolates and for the *Streptomyces* type strain:

- API ZYM tests: α-fucosidase , α-galactosidase, β-glucosidase, β-glucuronidase and lipase (C14).
- GEN III BIOLOG microplate tests: utilization of D-aspartic acid, D-fructose-6-phosphate, glucuronamide, D-lactic acid methyl ester, mucic acid, quinic acid, D-saccharic acid, D-serine #1, D-serine #2, stachyose and resistance to fusidic acid and minocycline
- Other phenotypic tests: allantoin hydrolysis, nitrate reduction, H_2S and urea hydrolysis, degradation of cellulose, chitin, guanine, tributyrin, uric acid, xanthine, xylan and growth in the presence of sodium chloride (8%, w/v) and at 4 and 50^{o}C.

Table 4.20 Phenotypic profiles of *Streptomyces fimbriatus* NRRL-B3175^{T} and related isolates classified in the *Streptomyces fimbriatus* 16S rRNA gene subclade.

Characteristics	Isolate C59	Isolate KNN 26.b	Isolate KNN 38.1b	Isolate KNN 64.5b	*S.fimbriatus* NRRL B-3175^{T}
API ZYM tests:					
N-Acetyl-β-glucosaminidase	-	+	+	+	+
Esterase (C4), esterase lipase (C8)	+	+	-	+	+
α -Galactosidase	-	-	+	-	-
α –Glucosidase, valine arylamidase	-	-	+	+	-
β-Glucosidase	-	+	-	-	+
Lipase (C14)	+	+	+	-	-
α-Mannosidase	+	+	-	+	+
Naphthol-AS-BI-phosphohydrolase	-	+	-	-	+
GEN III BIOLOG Microplate tests					
(a) Utilization of sugars:					
Acetic acid, D-arabitol, D-cellobiose	+	+	+	+	-
N-Acetyl-β-D-mannosamine, L-fucose, D-glucose-6-phosphate, D-raffinose	-	-	-	-	+
D-Fructose, glycerol, D-trehalose	+	+	+	-	+
D-Fructose-6-phosphate	-	-	-	-	+

D-Fucose	+	+	-	-	+
D-Galactose	-	-	+	+	-
D-Maltose	+	+	-	+	-
D-Mannitol	+	+	+	+	-
β-Methyl-D-glucoside, D-turanose	-	-	-	+	-
Sucrose	-	-	+	-	-
(b) Utilization of amino acids					
L-Alanine	-	-	+	-	-
L-Glutamic acid, L-serine	+	+	+	-	+
(c) Utilization of organic acids:					
Butyric acid	+	+	-	-	+
α-*keto*-Butyric acid	+	+	+	-	-
Citric acid	+	+	-	+	+
D-Glucuronic acid	-	-	+	-	-
L-Glutamic acid	+	-	+	+	+
α-Hydroxy-butyric acid	-	+	+	+	+
(d) Utilization of:					
Gelatin	+	-	+	+	+
Growth in the presence of:					
Lithium chloride	+	+	+	-	+
Nalidixic acid	+	+	+	-	+
Sodium chloride (8%, w/v)	+	+	-	+	-
Sodium lactate (1%)	+	+	-	+	-
Growth at:					
pH 5	+	-	+	-	-

Other phenotypic tests					
(a) Degradation tests:					
Arbutin	-	-	+	-	-
Casein	+	-	+	-	-
Elastin	+	+	+	+	-
Starch	+	+	+	+	-
L-Tyrosine	-	+	+	-	-
Uric acid	+	+	-	-	+
(b) Growth at:					
10^{o}C	-	-	+	+	-
45 ^{o}C	-	-	+	+	-

+, positive result; -, negative result.

Positive results recorded for all of the isolates and for the type strain of *S. fimbriatus:*

- API ZYM tests: acid phosphatase, alkaline phosphatase, cystine arylamidase, β-galactosidase, α-glucuronidase and leucine arylamidase.
- GEN III BIOLOG microplate tests: utilization of acetoacetic acid, *N*-acetyl-D-glucosamine, γ-amino-n-butyric acid, L-aspartic acid, dextrin, β-gentiobiose, D-gluconic acid, D-glucose, gly-pro, L-histidine, β-hydroxy-butyric acid, D-lactic acid methyl ester, α-D-lactose, D-mannose, D-mannitol, *myo*-inositol, propionic acid, L-pyroglutamic acid, L-rhamnose and resistance to aztreonam, growth at pH6 and in the presence of potassium tellurite and sodium chloride (4%, w/v).
- Other phenotypic tests: aesculin and arbutin hydrolysis, degradation of adenine, hypoxanthine, Tweens 40, 60 and 80 and growth at 20, 30 and 40^{o}C.

Negative results recorded for all of the isolates and for the type strain of *S. fimbriatus:*

- API ZYM tests: α-chymotripsin, α-fucosidase, β-glucuronidase, lipase and trypsin.
- GEN III BIOLOG microplate tests: utilization of *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, L-arginine, D-aspartic acid, bromo-succinic acid, D-galacturonic acid, L-galactonic acid-γ-lactone, glucuronamide, α-*keto*-glutaric acid , *p*-hydroxy-phenylacetic acid, inosine, 3-*O*-methyl-D-

glucose, L-lactic acid, D-malic acid, L-malic acid, D-melibiose, methyl pyruvate, mucic acid, pectin, quinic acid, D-salicin, D-saccharic acid, D-serine #1, D-serine #2, stachyose and resistance to fusidic acid, guanidine hydrochloride, lincomycin, minocycline, niaproof, rifamycin SV, tetrazolium blue, tetrazolium violet, troleandomycin and vancomycin and growth in the presence of sodium bromate and sodium formate.

- Other phenotypic tests: Allantoin hydrolysis, nitrate reduction, H_2S and urea hydrolysis, degradation of cellulose, chitin, guanine, xanthine, xylan and tributyrin and growth at 4 and 50^{o}C.

Contrasting BIOLOG results were obtained for:

(a) Isolate C59: utilization of L-glutamic acid and L-serine.
(b) Isolate KNN 26.b: utilization of L-alanine, L-arginine, α-*keto*-butyric acid, citric acid, D-fucose, L-fucose, D-glucose-6-phosphate, D-raffinose, L-serine and growth in the presence of lithium chloride and 1% sodium lactate.
(c) Isolate KNN 38.1b: utilization of L-arginine, D-fucose, L-fucose, α-hydroxy-butyric acid and D-sorbitol.
(d) Isolate KNN 64.5b: utilization of L-alanine, butyric acid, citric acid, L-fucose, gelatin, L-glutamic acid and D-salicin.
(e) *Streptomyces fimbriatus* NRRL-B3175^{T}: utilization of L-arginine, D-fucose, gelatin, D-glucuronic acid, D-salicin, L-serine and D-sorbitol.

4.5 Discussion

Twelve out of 16 Atacama Desert isolates assigned to genera other than *Streptomyces* were found to have 16S rRNA gene sequence similarities with the type strains of their closest phylogenetic neighbours that fell at or below 99.0% threshold which Meier-Kolthoff et al. (2013) recommended as the threshold value below which DNA:DNA relatedness assays were not required. Consequently, these islates were considered to be putatively novel species belonging to the genera *Actinomadura, Amycolatopsis, Cryptosporangium* and *Pseudonocardia.* Most of these isolates were compared with their nearest phylogenetic neighbours using a combination of chemotaxonomic, morphological and phenotypic properties designed to establish whether they merited recognition as novel species.

Two out of the three isolates classified in the genus *Actinomadura*, isolate H59 from the ALMA 2 environmental sample and isolate LB25 from Lomas Bayas soil had 16S rRNA gene sequence similarities against their nearest phylogenetic neighbours that were below 99.0% threshold mentioned above; the exception isolate LB54 from Lomas Bayas soil was very closely related to the type strains of *A. coerulea* and *A. verrucosispora*. Isolate H59 was shown to have chemotaxonomic and morphological properties typical of the genus *Actinomadura* (Trujillo and Goodfellow 2012; Zhao et al. 2015; Abagana et al. 2016). This organism was distinguished readily from *A. napierensis* DSM 44846^{T} and *A. yumaensis* DSM43931^{T}, its closest phylogenetic neighbours, by a broad range of phenotypic properties. In light of these genotypic and phenotypic data isolate H59 is considered to be a new *Actinomadura* species. It seems likely that *Actinomadura* strains are common in Atacama Desert soils as isolates H59, LB25 and LB54 were representatives of multi-membered colour-groups; members of the genus have been isolated previously from hyper-arid Atacama Desert soil (Busarakam 2014). It is also interesting that an actinobacterium from Saharan soil has been validly published as *Actinomadura algeriensis* (Lahoum et al. 2016).

The 4 strains recovered within the evolutionary radiation encompassed by the genus *Amycolatopsis* were isolated from the ALMA 4 environmental sample on glucose-yeast extract agar. Isolate H6 was found to be most closely related to the type strain of *A. pretoriensis* but the remaining strains, isolates H5, H97 and H101, were sharply separated from closely associated marker strains given the 99.0% 16S rRNA threshold recommended by Meier-Kolthoff et al. (2013). Isolate H5 was studied further and found to have chemotaxonomic and morphological properties characteristic of the genus *Amycolatopsis* (Tan and Goodfellow 2012; Klykleung et al. 2015) and was separated

readily from *A. mediterranei* DSM 43304^T and *A. pretoriensis* DSM 44654^T, its closest phylogenetic relatives, by a combination of phenotypic features. These polyphasic taxonomic data indicate that isolate H5 belongs to a novel *Amycolatopsis* species. It seems likely given the present and previous data that *Amycolatopsis* strains are common in Atacama Desert habitats (Okoro et al. 2009; Busarakam 2014). Indeed, members of the rare thermophilic species *A. ruani* and *A. thermalba* have been shown to be major components of extreme hyper-arid soil from the Yungay core region (Busarakam et al. 2016a; Zucchi et al. 2012).

Strain H7 was isolated from an ALMA 4 environmental sample and assigned to colour-group 7 together with two other isolates. The assignment of these isolates to the genus *Cryptosporangium* is interesting as members of this poorly studied taxon have not been isolated previously from Atacama Desert habitats. Isolate H7 was most closely related to two of the six validly published *Cryptosporangium* species; that is, to *C. cibodasense* and *C. minutisporangium.* The organism was shown to have chemotaxonomic and morphological properties consistent with its classification in the genus *Cryptosporangium* (Tamura et al. 1998) and was separated readily from *C. minutisporangium* NBRC 15962^T by a broad range of phenotypic properties. It seems likely that isolate H7 will be found to belong to a new *Cryptosporangium* species, but comparative phenotypic tests are needed to ensure that it can be seperated from *C. cibodasense* NBRC 110976^T, this organism was validly published as a new species after the experimental phase of this project had been completed.

All but one of the isolates recovered in the *Pseudonocardia* 16S rRNA gene tree were representatives of multi-membered taxa. It is particularly interesting that so many pseudonocardiae were isolated from environmental samples collected from Cerro Chajnantor and the Yungay core region as members of this taxon have only been isolated from the Atacama Desert on one previous occasion (Busarakam 2014). Nevertheless, it seems likely that *Pseudonocardia* strains are an integral part of actinobacterial communities in Atacama Desert landscapes, a point undelined in a culture-independent study which revealed that *Pseudonocardia* lineages were common in a high altitude debris field on Llallialaco Volcano in th viccinity of the Chilean-Argentine border (Lynch et al. 2012).

The 9 representative isolates assigned to the four *Pseudonocardia* 16S rRNA subclades along with type strains of associated marker strains were found to have chemotaxonomic and morphological properties in line with their classification in the genus *Pseudonocardia* (Huang and Goodfellow 2015). It seems likely that all but one of

these isolates will be shown belong to novel *Pseudonocardia* species as they show relatively low 16S rRNA gene sequence similarities to their closest phylogenetic neighbours and can be distinguished from them by markedly different phenotypic properties; the exception, isolate H58, is most closely related to the type strain of *P. cypriaca* sharing a 16S rRNA gene sequence with the latter of 99.58%.

A particularly convincing case can be made for cclassifying isolates ATK01, ATK03 and ATK17 as a novel spcecies of *Pseudonocardia*. These strains have key genotypic and phenotypic features in common which separate them from the type strains of *P. bannaensis* and *P. xinjiangensis* their nearest phylogenetic neighbours. It is particularly interesting that unlike the latter they produce spores in vesicles as well as in chains. It is, therefore, proposed that isolates ATK01, ATK03 and ATK17 can be recognized as a novel of *Pseudonocardia* species, *Pseudonocardia yungayensis* sp. nov.. A similar case can be made for recognising isolates H57, H69, H96, H99 and H215 as novel *Pseudonocardia* species. However, isolates H96 and H99, while well separated from the most closely related marker strains, share a 16S rRNA gene sequence similarity above 99.0% hence the need for DNA:DNA pairing studies to establish whether they both merit species status.

The results of this project suggest that high altitude Atacama Desert soils can be expected to be a rich source of novel actinobacteria, not least members of the genera *Actinomadura, Amycolatopsis* and *Pseudonocardia*. Similar deductions have been drawn from surveys of actinobacterial communities in hyper-arid and extreme hyper-arid Atacama Desert soils (Bull and Asenjo 2013; Bull et al. 2016; Busarakam 2014; Okoro et al. 2009) which have led to the valid publication of novel *Lechevalieria* (Okoro et al. 2010), *Modestobacter* (Busarakam et al. 2016b) and *Streptomyces* species (2012b; 2013; Santhanam et al. 2012a).

Busarakam (2014) in her comprehensive survey of filamentous actinobacteria from arid Atacama Desert soils highlighted several putatively novel *Streptomyces* species, the members of which were studied independently in the present study. Six out of the 10 isolates were found to have identical or almost identical 16S rRNA gene sequences and many phenotypic features in common. MLSA analyses of partial sequences of five house-keeping genes showed that these isolates formed a distinct and homogeneous lineage in the universal *Streptomyces* MLSA gene tree that was most closely related to the type strain of *S. ghanaensis*. However, strains assigned to this taxon were distinguished from the *S. ghanaensis* strain based on many of phenotypic properties and quantitative differences in fatty acid components. Consequently, it is proposed that

all six isolates be recognized as a novel *Streptomyces* species, *Streptomyces asenjonii* sp. nov..

The taxonomic status of the four remaining isolates is not so clear-out though they were all assigned to the *S. fimbriatus* 16S rRNA subclade thereby confirming earlier work of Busarakam (2014). Nevertheless, it seems likely that isolate C59, and possibly isolate KNN 26.b are *bona fide* members of *S. fimbriatus* as they have identical or almost identical rRNA gene sequences with the latter; all three of these strains have many phenotypic features in common. However, additional comparative taxonomic studies are needed to confirm these relationships and to clarify the taxonomic status of isolates KNN 38.1b and KNN 64.5b.

Description of *Pseudonocardia yungayensis* sp. nov..

Pseudonocardia yungayensis (yun. gay. en'sis N.L. fem. Adj. *yungayensis*, pertaining to the Yungay region, the source of strains).

Aerobic, Gram-stain-positive actinobacteria which form branched substrate mycelia; aerial hyphae, when formed are white. Strain ATK03 forms aerial hyphae that differentiate into smooth-surfaces spores (1-2µm) that are carried in straight chains (1-2µm) or within sore vesicles. Grows well on oatmeal, tryptone-yeast extract, tyrosine and yeast-extract-malt extract agars. White aerial hyphae and yellow white substrate mycelia are formed on oatmeal agar. Grows from 20ºC to 30ºC, from pH6 to pH7 and n the presence of 4%, v/w sodium chloride. Produces α-mannosidase and naphthol-AS-BI phosphohydrolase and degrades starch. Additional phenotypic features are givel in Table 4.15. the cell wall peptidoglycan contains meso-diaminopimelic acid and the whole-cell sugars are arabinose, galactose, glucose, mannose and ribose. The predominant menaquinone in MK8(H_4) and the major fatty acid iso-hexadecanoic acid (*iso*-$C_{16:0}$). The polar lipid profile includes diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides.

The type stran, ATK03 and strains ATK01 and ATK17 were isolates from an extreme hyper-arid Atacama Desert soil.

Description of *Streptomyces asenjonii* sp. nov..

Streptomyces asenjonii (a. sen. jo'ni. i. N. L. gen. masc., *asenjonii*), named after Juan A. Asenjo in recognition of his promotion of work on Atacama Desert actinobacteria.

Aerobic, Gram-stain-positive actinobacteria which form an extensively branched substrate mycelium that carries aerial hyphae that differentiate into spiral chains of smooth-surfaced spores (1-2µm). Grows from 10ºC to 40ºC , from pH 6 to pH 7 and in the presence of 1%, w/v sodium chloride. Produces acid and alkaline phosphatase, cystine arylamidase, esterase lipase (C8), β-galactosidase, N-acetyl-β-glucosaminidase and leucine and valine arylamidases (API ZYM tests), hydrolyzes aesculin and arbutin, degrades adenine, elastin, hypoxanthine, starch, L-tyrosine and Tween 40 and utilizes butyric acid, α-*keto*-butyric acid, citric acid and is resistant to aztreonam. Additional phenotypic properties are given in Table 4.19. The cell wall peptidoglycan contains *LL*-diaminopimelic acid and the whole-cell sugars are glucose, mannose, ribose and xylose. The major fatty acid is *iso*-hexadecanoic acid (*iso*-$C_{16:0}$) and the rpedominant menaquinones MK9(H_6) and MK9(H_8). The polar lipid profile includes diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides.

The type strain of, KNN 35.2b (=NRRL B-65055^T) and strains KNN 6.11a, KNN 35.1b, KNN 42.f, KNN 48.3e and KNN 83.e were isolated from arid Atacama Desert soil.

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Chapter 5. New specialized metabolites synthesized by novel actinobacteria isolated from soils of the Chajnantor Plateau

5.1 Abstract

Six out of 57 bioactive filamentous actinobacteria, notably isolates H9 and H45, isolated from high altitude Atacama Desert soils were found to produce new specialized metabolites. Large scale fermentation on two production media followed by chromatographic purification and LCMS and NMR analyses led to the isolation of six new and one known diene from isolate H45. The structures of the new compounds, named lentzeosides A-F, were confirmed by HRESIMS and NMR analyses. The purified lentzeosides were found to inhibit HIV-1 integrase activity. Isolate H9 produced a prospective novel peptide and polyunsaturated components based on LCMS and UV spectrophotometric traces. Polyphasic studies designed to establish the taxonomic status of isolates H45 and H9 showed that they had chemotaxonomic and morphological features consistent with their classification in the genera *Lentzea* and *Streptomyces*, respectively. The *Lentzea* strain was found to be most closely related to *Lentzea kentuckyensis* NRRL B-24416^T based on 16S rRNA gene sequence data but was distinguished readily from the latter by a low level of DNA:DNA relatedness and by a broad range of phenotypic properties. Analysis of 16S rRNA gene sequences showed that isolate H9 formed a distinct phyletic line in the *Streptomyces* gene tree. Multilocus sequence analysis based on five housekeeping gene alleles underpinned the separation of the strain from all of its nearest phylogenetic neighbours as did corresponding phenotypic data. In light of these results it is proposed that isolates H45 and H9 be recognized as new species of *Lentzea* and *Streptomyces*, respectively, namely as *Lentzea chajnantorensis* sp. nov. and *Streptomyces aridus* sp. nov., with the type strains H45^T (NCIMB 14966^T =NRRL B-65282^T) and H9^T (NCIMB 14965^T =NRRL B-65268^T), respectively.

5.2 Introduction

Novel filamentous actinobacteria tend to feature in bioprospecting campaigns due to their ability to synthesize new natural products that belong to novel structural classes and have new modes of action (Goodfellow and Fiedler 2010; Barka et al. 2016). The premise that novel actinobacteria from extreme biomes are a rich source of new bioactive compounds

has gained credence with the discovery that isolates from hyper-arid and extreme hyper-arid Atacama Desert soils synthesize new chemical structures with potent biological activity (Bull and Asenjo 2013; Bull et al. 2016), as exemplified by *Streptomyces leeuwenhoekii* strains (Busarakam et al. 2014; Rateb et al. 2011b) which synthesize the atacamycins (Nachtigall et al. 2011), chaxalactins (Rateb et al. 2011a) and chaxapeptin, a new lasso peptide (Elsayed et al. 2015). The type strain of this species has the makings of a key model organism in the search for novel natural products as its whole-genome sequence contains 38 biosynthetic gene clusters that encode for known and undiscovered specialized metabolites (Castro et al. 2015; Gomez-Escribano et al. 2015).

The present study was designed to build upon and extend the pioneering bioprospecting studies on Atacama Desert actinobacteria as outlined above. To this end, extracts of 57 actinobacterial strains isolated from high altitude Atacama Desert soils and 4 from corresponding extreme hyper-arid soils that were known to inhibit the growth of *Bacillus subtilis* and/or *Escherichia coli* strains were the subject of chemical analyses which showed that six of them produced new compounds, notably those synthesized by isolates H9 and H45; these strains were the subject of polyphasic studies which showed that they formed the nuclei of novel species of *Streptomyces* and *Lentzea*, respectively. Detailed chemical screening of extracts from the novel *Lentzea* strain led to the isolation of six novel and one known diene, as well as monoene glycosides. The novel dienes were found to inhibit HIV-1 integrase activity.

5.3 Materials and Methods

5.3.1 *Selection of isolates for bioactivity screening*

Sixty one strains found to inhibit the growth of *Bacillus subtilis* and/or *Escherichia coli* wild type strains in primary plug assays (see Chapter 3) were selected for bioactive compound screening in the Marine Biodiscovery Centre at the University of Aberdeen. The source of the strains, their assignment to colour-groups and their ability to inhibit the growth of a panel of wild type and *Bacillus subtilis* reporter strains are shown in Table 5.1, details of the Chajnantor and Lomas Bayas sampling sites can be found in Table 2.1 in Chapter 2.

Table 5.1 Source, colour-group assignment and bioactivity profiles of Atacama Desert isolates selected for bioactive compound screening.

Soil samples	Isolates	Isolation		Colour groups	Screening	
		Protocol	Medium		Wild type strains	*B. subtilis* reporter strains
A. Chajnantor environmental samples						
1	H90	Pilot 4	Gauze's No. 1	66 ^{S}	a, b, c, e	-
	H91			67 ^{S}	a, e	-
2	H55	Pilot 2	GYEA	51^{S}	a, b, c, e	*ypuA, yvqI, yvgS*
	H59			52 $^{M(3)}$	c, d, e	-
	H60			12 $^{M(31)}$	c, d, e	-
	H61			53 $^{M(2)}$	a, b, c, d, e	-
	H62				a, b, c, e	-
	H68		Oligotrophic	9 $^{M(2)}$	c, d	*yvqI*
	H69			1 $^{M(12)}$	c, d, e	*ypuA*
	H70	Pilot 3	Gauze's No. 1	22 $^{M(3)}$	b, c, e	*ypuA, yvqI, yvgS, yheH*
	H71				a, b, c, d, e	-
	H72			57 ^{S}	a, b, c, e	*ypuA, yvqI, yvgS, yheH, yjaX*
	H73			4 ^{S}	c, d, e	-
	H78			58 ^{S}	c	*yvqI*
	H80			60 ^{S}	c, e	-
	H82			62 ^{S}	c, d, e	*ypuA, yvqI, yvgS, yheH*
	H83			64 ^{S}	a, b, c, e	-
	H84			63 ^{S}	b, c, d, e	*ypuA, yvqI, yvgS, yheH*
	H85			64 ^{S}	b, c, d, e	-
	H87			4 $^{M(3)}$	c	-
	H88			65 ^{S}	b, c, d, e	-
	H93	Pilot 4	Gauze's No. 1	69 ^{S}	a, b, c, d, e	-
	H94			70 ^{S}	c, d, e	*ypuA, yvqI, yvgS, yheH, yjaX*
4	H4	Pilot 1	GYEA	4 $^{M(5)}$	b, c, e	-
	H5			5 ^{S}	c, d, e	-
	H9			49 $^{M(2)}$	b, c, e	-
	H98	Pilot 4	Gauze's No. 1	73 ^{S}	c, d, e	-
6	H10	Pilot 1	Oligotrophic	23 ^{S}	a, b, c, d, e	-
	H11			24 ^{S}	b, c, d, e	*ypuA, yvqI, yvgS, yheH, yjaX*
	H12			25 ^{S}	c, e	-

	H13			12 [M(31)]	c, e	*ypuA, yvqI, yvgS, yheH, yjaX*
	H14			26 [M(2)]	c, e	*ypuA, yvqI, yvgS, yheH, yjaX*
	H15			27 [S]	c, e	*yvqI, yheH*
	H16			28 [M(2)]	c, e	*ypuA*
	H17			29 [S]	c, d, e	*ypuA, yvqI, yvgS, yheH*
	H21			12 [M(31)]	c, d, e	-
	H25		GYEA	36 [M(2)]	b, c, d, e	-
	H26			37 [S]	c, d, e	*yheH*
	H27			38 [S]	c, d, e	*ypuA, yvgS, yheH, yjaX*
	H29			40 [S]	c	*yvgS, yheH, yjaX*
	H34			45 [M(2)]	a, b, c, e	-
	H36			1 [M(11)]	c, d, e	*ypuA, yheH*
	H37			47 [S]	b, c, d, e	-
	H39			49 [M(2)]	a, b, c, d, e	-
	H41		Gauze's No. 1	13 [S]	c, d, e	-
	H43			11 [S]	c	-
	H45			13 [S]	a, b, c, e	-
	H46			14 [S]	a, b, c, d, e	-
	H48			16 [S]	a, b, c, d, e	*yvgS, yheH*
	H49			17 [S]	c, e	*phi105, ypuA, yvqI, yvgS, yheH, yjaX*
	H50			18 [S]	c, e	-
	H51			19 [S]	c, d, e	*ypuA, yvqI, yvgS, yheH*
	H52			20 [S]	b, c, e	-
	H107	Pilot 4		76 [S]	c, d, e	*Phi105, ypuA, yvgS, yheH, yjaX*
	H108			77 [S]	c, e	*Phi105, ypuA, yvqI, yvgS, yheH, yjaX*
	H109			80 [S]	c, d, e	*yvgS, yheH*
	H111			79 [M(2)]	c, e	-
B. Lomas Bayas						
	LB54	Arginine-vitamin agar		19 [S]	c, d, e	-
	LB57			21 [S]	c, d, e	-
	LB62	GYEA		26 [S]	b, c, d, e	-
	LB63			27 [S]	b, c, d	-

-, negative results.

*Colour-group: 1-80, Number of colour-groups; ^{M}, multi-membered colour-group; $^{(1-31)}$, number of isolates in multi-membered colour groups; ^{S}, single-membered colour-groups, see Appendix 1 for details.

*Panel of wild type strains included in standard plug assays: a, *Escherichia coli;* b, *Pseudomonas fluorescens*; c, *Bacillus subtilis;* d, *Staphylococcus aureus;* e, *Saccharomyces cerevisiae.* See Chapter 2 for details.

* *Bacillus subtilis* reporter strains: *Phi105*^{CH} (inhibition of DNA synthesis); *ypuA*^{ER} (inhibition of cell envelope synthesis); *yvqI*^{ER} (inhibition of cell wall synthesis); *yvgS*^{ER} (inhibition of RNA synthesis); *yheH*^{ER} (inhibition of protein synthesis) and *yjaX*^{ER} (inhibition of fatty acid synthesis). (see Chapter 2 for details).

5.3.2 Preliminary detection of bioactive compounds

Fermentation conditions. The 61 strains were grown in shake flasks at 180 revolutions per minute (rpm) for 10 days at 28 ^{o}C in yeast extract-malt extract (Shirling and Gottlieb 1966), media 19 and 410 (Goodfellow and Fiedler 2010) and starch-casein broths (Küster and Williams 1964) which contained a gram of Amberlite beads (Sigma-Aldrich, Gillingham Dorset, UK) per 50 ml of broth for specialized metabolite adsorption.

Extraction of metabolites. The Amberlite beads were separated from the production broths by centrifugation at 4000 rpm for 5 minutes, washed 4-5 times with sterile distilled water, soaked in 50 ml of methanol and sent to Professor Marcel Jaspars at Aberdeen University for preliminary chemical analyses. In Aberdeen, samples were extracted twice with methanol and acetone (2:1, v/v) and the corresponding extracts combined and concentrated *in vacuo*. The resultant preparations were purified by using the Biotage SP1 flash system (Biotage, USA) with a reversed phase 40iM cartridge (KP-C18-HSTM, 40 x 150 mm) with a gradient of methanol in water (5-100% in 12 column volumes), detection of compounds was carried out at 220 and 254 nm.

Chemical analyses of extracts. Each extract was dissolved in methanol to give a final concentration of 0.5mg/mL, filtered and submitted for liquid chromatographic/mass spectrometric analysis (LCMS). High resolution mass spectral data were obtained using a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery, ThermoFiscer, Germany) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela

PDA autosampler and Accela Pump) under 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 and mass range 100–2000 amu (maximum resolution 30000). An Agilent Poroshell 120, EC-C18, 2.1x100 mm, 2.7µm UPLC column was used for the LCMS analyses with a mobile phase of 0-100% methanol over 30 minutes at 0.5 ml/min flow rate. The results were presented as liquid spectrophotometric (LC), mass spectrophotometric (MS) and ultraviolet (UV) traces. Dereplication of the LCMS peaks of the extracts were checked against the Dictionary of Natural Products, V. 23.1 on a DVD, Reaxys online (https://www.reaxys.com/) and the Antibase 2012 (http://www.wiley-vch.de/stmdata/antibase.php) databases to determine whether or not the detected compounds were new.

5.3.3 Screening of novel specialized metabolites produced by isolate H45

Cultivation conditions and preparation of extracts. Two cultivation media were used for large scale fermentation of strain H45, namely medium 410 (calcium carbonate, casamino acid, glucose, glycerol, oatmeal, peptone and yeast extract; Goodfellow & Fiedler, 2010) and modified ISP2 (dextrose, glycerol, malt extract and yeast extract, Rateb *pers. com*.) broths. Following incubation for 7 days, Diaion HP-20 resin was added (Mitsubishi Chemical Co., Italy) to the preparations which were then shaken at 180 rpm for 6 hours prior to centrifugation at 3000 rpm for 15 minutes.

Extraction and isolation. Biomass preparations from each of the production media were extracted with methanol and acetone (2:1, v/v), the extracts combined, concentrated *in vacuo* and purified by using the Biotage SP1 flash system with a gradient of methanol in water (5-100% in 12 column volumes). The eluted fractions were screened using LCMS and ^{1}H NMR and interesting fractions further purified using RP-HPLC. For medium 410, the second fraction was purified using a gradient of MeOH in H_2O/MeOH (95:5, v/v) (25-100% for 25 minutes, flow rate 2.0 mL/minute) to obtain compounds **1** (3.1 mg), **2** (1.0 mg), **3** (2.2 mg), **4** (1.0 mg) and **5** (2.0 mg). In the case of the modified ISP2 broth, the fourth fraction was purified using a gradient of MeOH in H_2O/MeOH (95:5, v/v) (50-100% for 25 minutes, flow rate 2.2 mL/minute) to obtain compounds **1** (3.1 mg), **2** (1.1 mg), **5** (3.4 mg), **6** (3.4 mg), and **7** (2.2 mg).

General experimental procedures. The structures of the purified compounds, which were named lentzeosides, were determined using a range of experimental procedures. Nuclear magnetic resonance (NMR) data were acquired on a Varian VNMRS 600 MHz NMR spectrophotometer and LC-HRESIMS data by using a LTQ/XL Orbitrap coupled to the HPLC system (PDA detector, PDA autosampler, and pump). Optical rotations were recorded using a Bellingham + Stanley ADP410 polarimeter. Medium Pressure Liquid Chromatographic separations were carried out using the Biotage SP1 flash system fitted with a reversed phase 40iM cartridge (KP-C18-HS^{TM}, 40 x 150 mm), the detection was carried out at 220 and 254 nm. For RP-HPLC separations, a SunFire (C_{18}, 250 x 10 mm, 5 µm i.d.) column connected to an Agilent 1200 series binary pump was used and monitored with an Agilent photodiode array detector, detection was carried out at 220, 254, 280, and 320 nm. Acid hydrolyses of the isolated compounds were performed after Nakanishi et al. (1985) HPLC solvents and the authentic sugar samples were obtained from Sigma-Aldrich, the ingredients for fermentation from Oxoid UK and Sigma-Aldrich and the Diaion HP-20 resin from Resindion S.R.L., a subsidiary of Mitsubishi Chemical Co., Binasco, Italy.

5.3.4 Biological activities of novel specialized compounds

Antibacterial activities. The purified lentzeosides were tested against *E. coli* NCIMB 12210 and *S. aureus* NCIMB 12702 using slight modifications of the method described by Domenech et al. (2009). The test strains were grown in nutrient broth to early stationary phase then diluted to give an OD_{650} value of 0.0005. The assays were performed in a 96-well microtiter plate format in triplicate. Both rifampicin and the novel compounds were dissolved in DMSO (Sigma-Aldrich, UK) and the effect of the different dilutions (0.5, 1, 2, 4, 8, 16, 32 µg/ml) on the growth of the *E. coli* and *S. aureus* strains was assessed after 18 hours incubation at 37 °C using a plate reader (Labsystems iEMS MF instrument) at OD_{620}. The MICs were determined as the lowest concentration inhibiting the growth of the *E. coli* and *S. aureus* strains compared to growth in the nutrient broth control. DMSO up to 2.5% was shown to have no antibacterial effect.

Anti-HIV-1 integrase activity. The HIV-1 integrase activity of purified lentzeosides **1-6** were evaluated using an HIV-1 Integrase Assay kit (XpressBio Life Science Products, Frederick, U.S.A), according to manufacturer's protocol. Briefly, biotin-linked HIV-1 LTR U5 donor substrate (DS) DNA was applied to a streptavidin-coated 96-well plate,

the test compounds were added along with target substrate DNA and HIV integrase. The integrase processes the HIV-1 LTR U5 and catalyzes the strand transfer recombination reaction to integrate the DS DNA into the target substrate DNA. The products of these reactions were detected colorimetrically using a HRP-labelled antibody. Sodium azide was used as a positive inhibitory control. Each of the compounds was tested at 4 concentrations (200.0, 50.0, 12.5 and 3.125 µM) in triplicate.

5.3.5 *Isolation and classification of isolates H9 and H45*

Isolates H9 and H45 were found to produce especially interesting novel specialized metabolites hence they were examined in polyphasic studies together with their nearest phylogenetic neighbours (Table 5.2) in order to determine if they formed new centres of taxonomic variation within the genera *Streptomyces* and *Lentzea,* respectively. Strain H9 was isolated from a glucose-yeast extract agar plate that had been inoculated with a suspension of subsurface soil collected at 4000 metres (ALMA 4) on Cerro Chajnantor (Table 5.1), this organism formed a multi-membeed colour-group together with isolate H39. In turn, strain H45 was isolated from a Gauze's No. 1 agar plate inoculated with a subsurface soil sample collected at 5048 metres above sea level on the Chajnantor Plateau (ALMA 6) (Table 5.1) and found to form a single-membered colour-group. All of these strains were grown on ISP 2 agar (Shirling and Gottlieb 1966) for 14 days at 28^{o}C and maintained in 20%, v/v glycerol at -80^{o}C.

Table 5.2 Isolates H9 and H45 and their nearest phylogenetic neighbours.

Strains	Source/ strain histories
A. Genus *Streptomyces*	
Isolate H9 [1,2,3,4]	Subsurface environmental sample (ALMA 4)
S. melanogenes Suguwara and Onuma (1957)	NRRL B- 2072^{T} [2,3]; soil, Japan
S. noboritoensis (Isono et al. 1957)	NRRL B- 12152^{T} [2,3]; soil, Japan
S. polyantibioticus le Roes-Hill and Meyers (2009)	NRRL B- 24448^{T} [2,3]; river bank, South Africa

B. Genus *Lentzea*

Isolate H45[1,2,3,4]	Subsurface environmental sample (ALMA 6)
L. kentuckyensis Labeda et al. (2007)	NRRL B- 24416^{T} [2,3]; NRRL B-24416 ^{T}; J.M. Donahue, LDDC 2876-05; equine placenta, USA

Isolates examined for: [1], cultural properties; [2], chemotaxonomic markers; [3], phenotypic tests; [4], spore chain arrangement and spore surface ornamentation.

Phylogeny. Genomic DNA was extracted from isolates H9 and H45 by using the bead beating method, as described in Chapter 2. 16S rRNA genes were amplified using universal primers, 27f and 1525r (Lane 1991) prior to purification and sequencing. Phylogenetic analyses were carried out using MEGA6 software (Tamura et al. 2013) and phylogenetic trees inferred with the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms. Bootstrap values based on 1000 repeats (Felsenstein 1985) of the neighbour-joining data were generated using the evolutionary distance model of Jukes and Cantor (1969).

Multilocus sequence analyses. Genomic DNA was extracted from isolate H9 and partial sequences of the house-keeping genes *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase beta subunit), *recA* (recombinase A) and *trpB* (tryptophan synthetase, beta subunit) were amplified, sequenced and compared with corresponding sequences held in the MLSA *Streptomyces* database, as described in Chapter 4. The relationships between isolate H9 and its closest phylogenetic neighbours were established using the maximum-likelihood algorithm based on the General Time Reversible model (Nei and Kumar 2000).

Chemotaxonomy. Biomass for the chemotaxonomic studies carried out on the test strains (Table 5.2) was prepared in ISP 2 broth (Shirling and Gottlieb 1966) in shake flasks for 14 days at 28ºC at 180 rpm, washed in distilled water and freeze dried. The strains were examined for the presence of isomers of diaminopimelic acid (A_2pm) using the procedure described by Staneck and Roberts (1974). The fatty acid profiles of isolates H9 and H45, *Streptomyces melanogenes* DSM 40192^{T} and *Streptomyces noboritoensis* NRRL B-12152^{T} were generated at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) using the protocol described by Miller (1982), as

modified by Kuykendall et al. (1988). Standard procedures were used to determine the menaquinone (Collins et al. 1985), polar lipid (Minnikin et al. 1984) and whole cell sugar composition (Lechevalier and Lechevalier 1970) of the test strains using the 'in house' protocols shown in Appendix 4.

DNA-DNA relatedness. Isolate H45 and *Lentzea kentuckyensis* NRRL B- 24416^T were grown in ISP 2 broth (Shirling and Gottlieb 1966) in shake flasks (180 rpm) for 14 days at 28^{o}C and the resultant wet biomass (3g) suspended in *iso*-propanol:water (1:1, v/v). DNA:DNA relatedness experiments were carried out, in duplicate, using the wet biomass preparations, by the identification service at the DSMZ (Braunschweig, Germany). DNA was isolated by chromatography on hydroxyapatite (Cashion et al. 1977) and hybridisation carried out as described by De Ley et al. (1970) with modifications by Huss et al. (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer fitted with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with an *in situ* temperature probe.

Cultural and morphological properties. Isolates H9 and H45 were examined for cultural properties following growth on tryptone-yeast extract , yeast extract-malt-extract, oatmeal, tryptone-yeast extract, glycerol-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1 to 7; Shirling and Gottlieb (1966)) for 2 weeks at 28^{o}C, as described in Chapter 2. Spore chain arrangement and spore surface ornamentation were determined using cultures grown on ISP 3 agar (Shirling and Gottlieb 1966) for 2 weeks at 28^{o}C by scanning electron microscopy, as described by O'Donnell et al. (1993).

Phenotypic tests. The isolates and associated type strains (Table 5.2) were examined for a broad range of phenotypic properties. Inocula were prepared by scraping spores/hyphal fragments from ISP 2 agar plates (Shirling and Gottlieb 1966), which had been incubated at 28^{o}C for 14 days, into 1 ml of ¼ strength Ringer's solution held in bijou bottles. The hyphal material in the suspensions was ground using sterile micropestles in Eppendorf tubes and the resultant preparations resuspended in ¼ strength Ringer's solution to give a turbidity of 5 on the McFarland scale (Murray et al. 1999). Biochemical and degradation tests were carried out, in duplicate, using standard microbiological procedures, as described in Chapter 2. Additional tests were carried out using API ZYM kits (Biomeriuex Co., Hampshire, UK) and GEN III MicroPlates (BIOLOG Inc., Hayward, CA, USA), as described in Chapter 2.

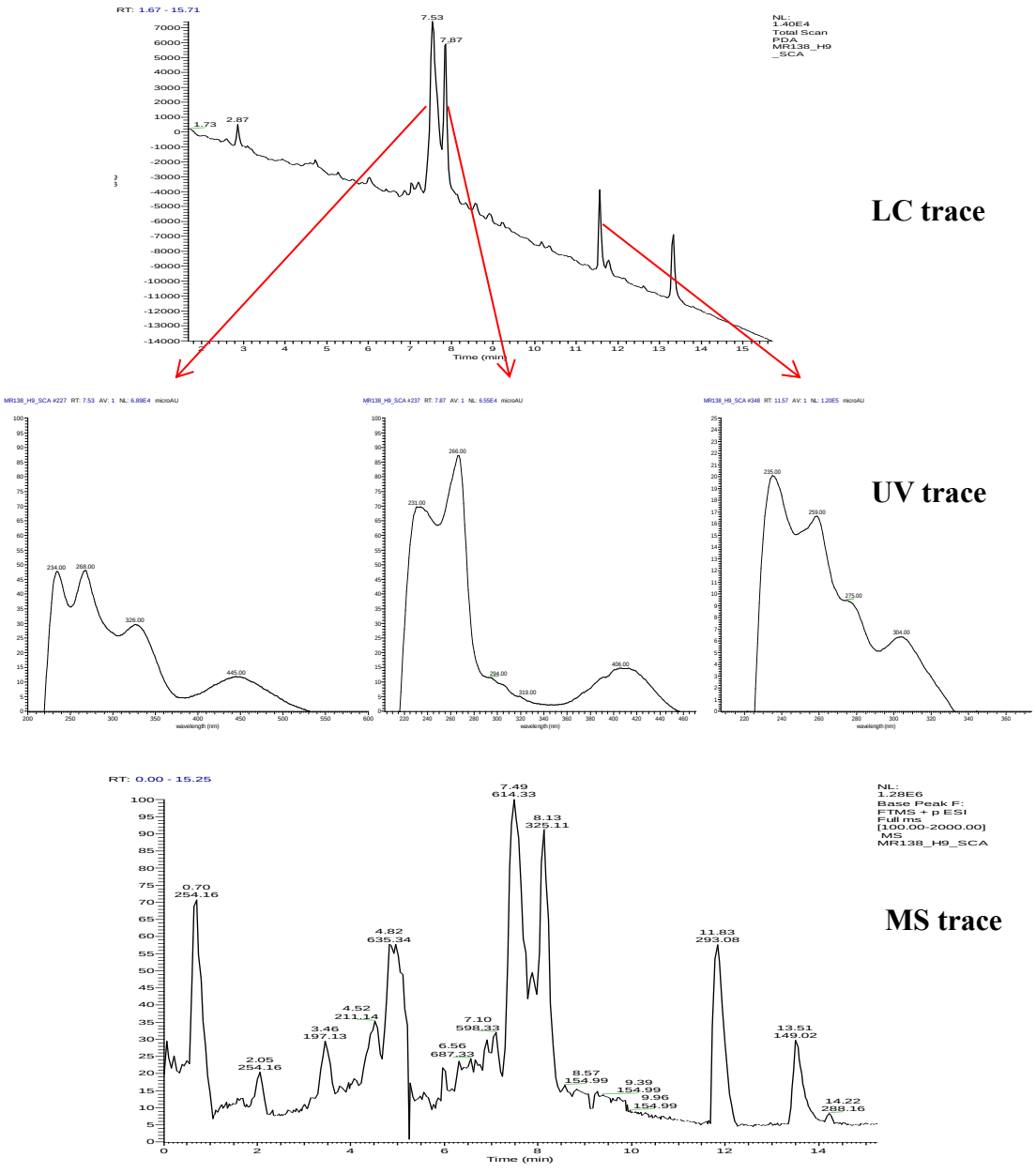
5.4 Results

5.4.1 Screening of initial metabolite extracts

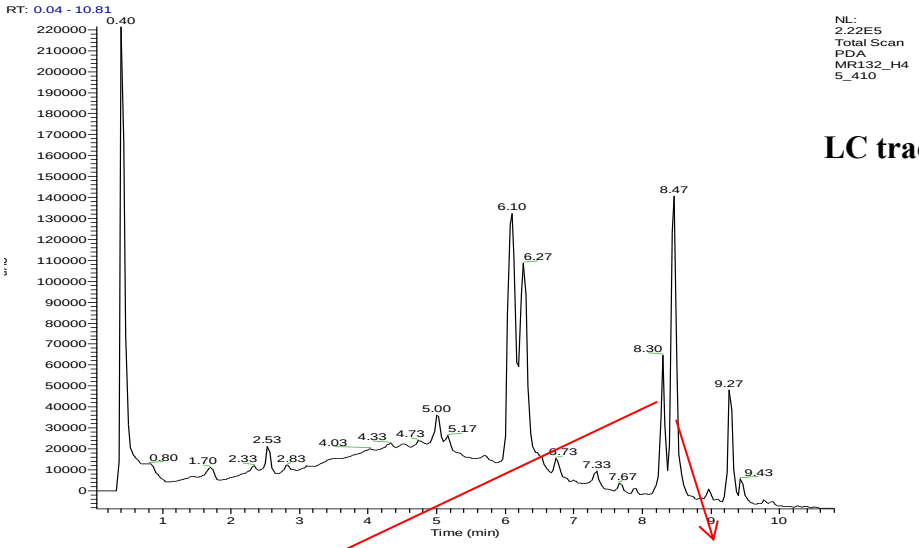
Six out of the 61 strains, namely isolates H9, H15, H27, H45, H46 and H98, were found to produce interesting specialized metabolites following comparison of LCMS peaks of extracts of these strains against the Dictionary of Natural Products, Reaxys online (https://www.reaxys.com/) and the Antibase 2012 (http://www.wiley-vch.de/stmdata/antibase.php) databases. Isolate H9 was found to produce polyunsaturated components, based on LCMS and UV mass spectrophotometric traces, as shown in Figure 5.1a. The compound detected at *m/z* 614.33 was considered to be a novel peptide with a suggested molecular formula $C_{30}H_{43}N_7O_7$ but those at *m/z* 325.11 and 293.08 were similar to prefluorostatin and 2 5-dihydroxy-3,6-diphenyl-1,4-benzoquinone, respectively. Analyses of the extracts of isolate H45 indicated the presence of a conjugated olefinic system based on LCMS and UV spectra (Figure 5.1b), as shown by the presence of novel compounds at *m/z* values 211.14, 652.40, 730.36, 758.4, 889.48, 655.28, 520.34 and 496.34. Extracts of isolates H15, H27 and H98 showed HPLC peaks that indicated the presence of novel specialized metabolites at 6 to 10, 13 to 14 and 8 to 11 minutes, respectively (Figure 5.1c, d and e). Isolate H46 showed a series of polyenes based on the HPLC peaks between 7-13 minutes (*m/z* between 150-620), as shown in Figure 5.1f. It was not practical to follow up all of these interesting leads though it was possible to produce enough material from isolate H45 for chemical identification and biological screening. Further investigations using different media or other approaches, such as microbial co-culture, are needed to increase the titre of specialized metabolites synthesized by the remaining strains in order to enable their isolation and structural characterisation prior to biological screening of the resultant purified compounds.

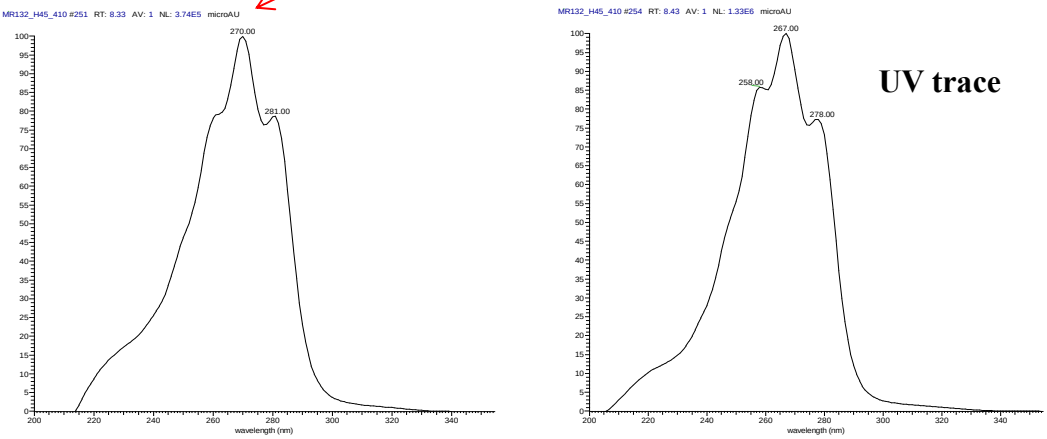
Figure 5.1 Liquid spectrophotometric (LC) and mass spectrophotometric (MS) traces and ultraviolet (UV) spectra of metabolite extracts from isolates after incubation for 10 days at 28ºC.

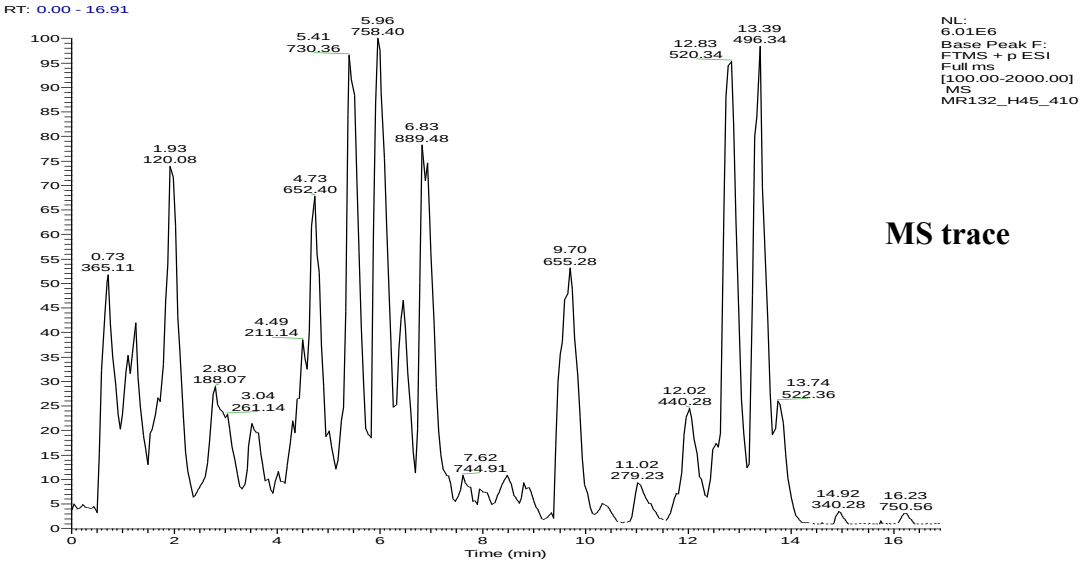
(a) Isolate H9 grown in starch-casein broth



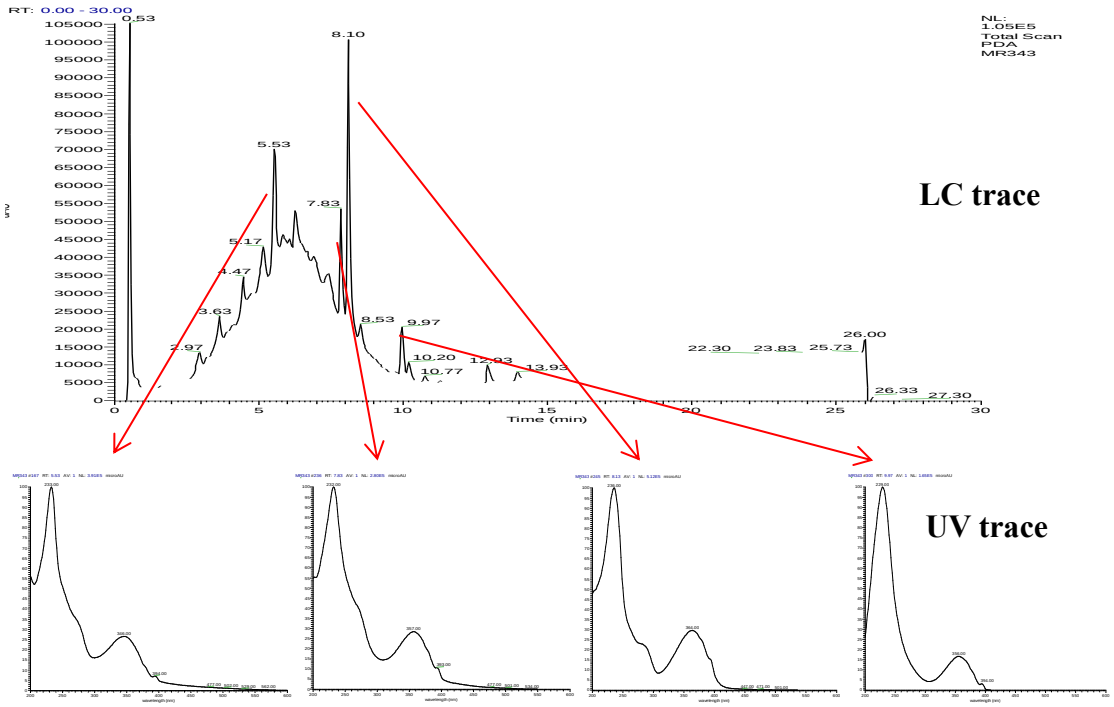
(b) Isolate H45 grown in 410 broth





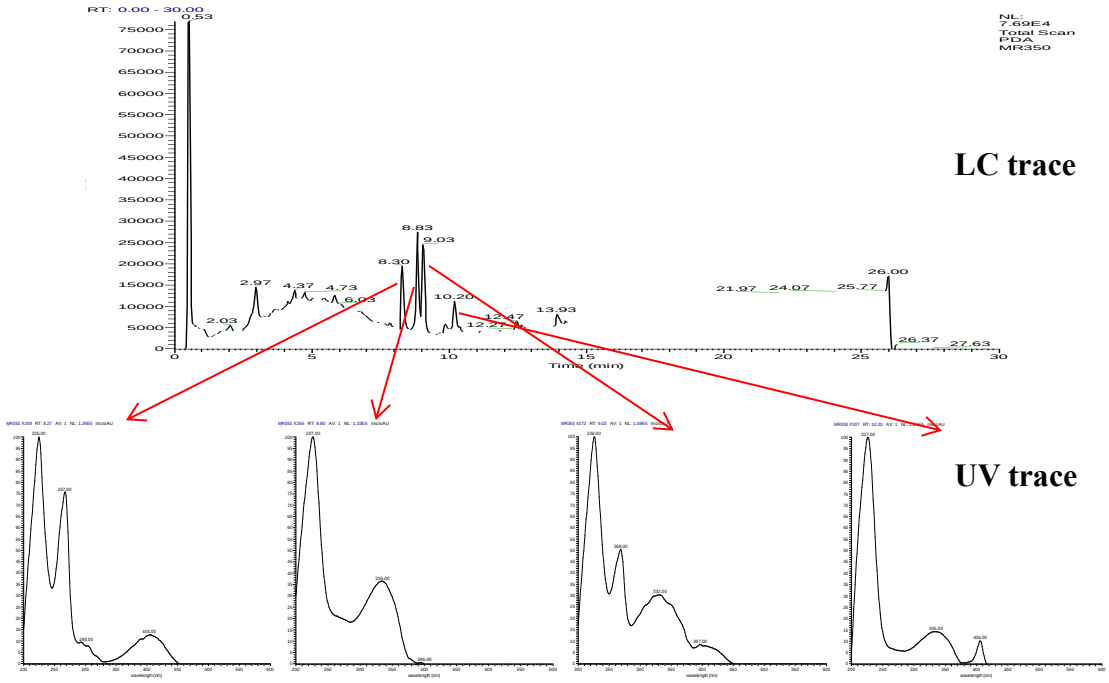


(c) isolate H15 grown in ISP2 broth

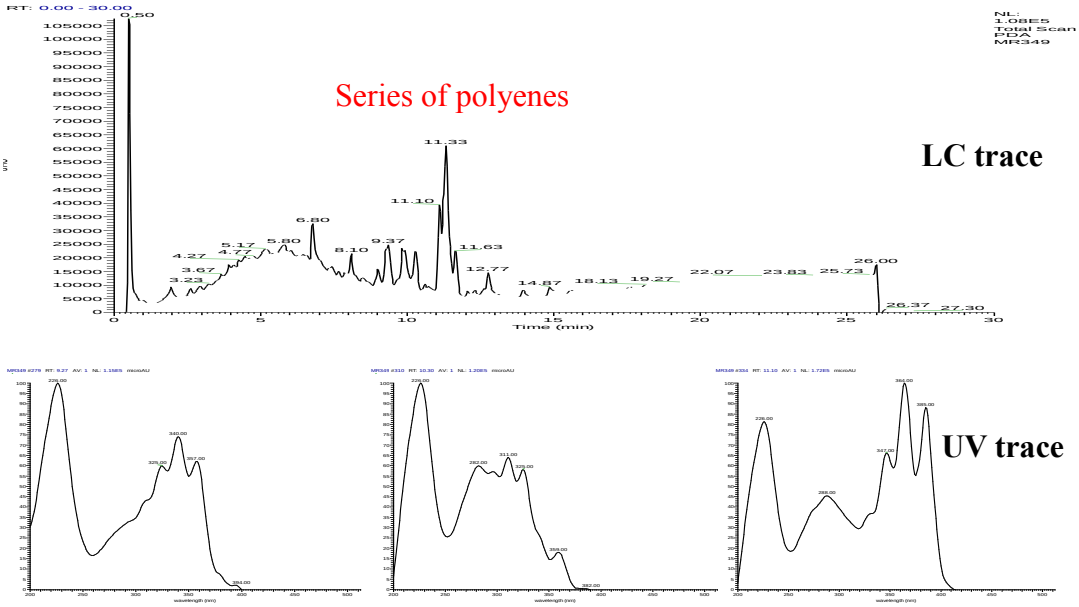


(d) Isolate H27 grown in ISP2 broth

(e) Isolate H98 grown in ISP2 broth



(f) Isolate H46 grown in ISP2 broth



5.4.2 Novel specialised metabolites extracted from isolate H45

The extracts of this isolate were found to produce a specific pattern of 7 specialized metabolites, namely lentzeosides A-G, based on LCMS profiles and associated NMR data (Tables 5.3 and 5.4). Structural elucidation of these compounds was based on HRESIMS, 1D and 2D NMR analyses (Table 5.5; Figures 5.2 and 5.3). Compounds 1 to 6 were found to be novel dienes, compound 7 was a known diene (Tables 5.3 and 5.4), several monoene glycosides were also detected.

Table 5.3 ^{13}C (150 MHz) NMR spectroscopic data for lentzeosides A–F (**1**-**6**) in DMSO-d_6

Position	**1**	**2**	**3**	**4**	**5**	**6**
	δC, mult.	δC, mult.	δC, mult.	δC, mult.	δC, mult.	δC, mult.
1	68.3, CH_2	66.5, CH_2	68.2, CH_2	68.3, CH_2	66.1, CH_2	68.2, CH_2
2	129.6, CH	129.5, CH	129.6, CH	127.1, CH	27.2, CH	27.6, CH
3	127.0, CH	127.2, CH	127.1, CH	132.5, CH	125.4, CH	125.2, CH
4	127.4, CH	127.3, CH	127.4, CH	128.5, CH	133.0, CH	132.9, CH
5	133.7, CH	133.9, CH	133.7, CH	136.5, CH	20.1, CH_2	20.1, CH_2
6	20.5, CH_2	20.5, CH_2	20.5, CH_2	25.0, CH_2	14.2, CH_3	14.2, CH_3
7	14.2, CH_3	14.1, CH_3	14.2, CH_3	13.4, CH_2		
1'	102.1, CH	99.3, CH	102.0, CH	99.1, CH	99.9, CH	102.7, CH
2'	73.7, CH	70.7, CH	73.5, CH	70.7, CH	70.5, CH	73.6, CH
3'	76.4, CH	70.5, CH	76.7, CH	70.5, CH	70.7, CH	76.5, CH
4'	75.3, CH	72.0, CH	68.2, CH	72.0, CH	72.0, CH	75.3, CH
5'	71.5, CH	68.5, CH	76.9, CH	68.5, CH	68.4, CH	71.5, CH
6'	17.9, CH_3	17.9, CH_3	61.1, CH_2	17.9, CH_3	17.9, CH_3	17.9, CH_3

Table 5.4 ^{1}H (600 MHz) NMR spectroscopic data for lentzeosides A–F (**1**-**6**) in DMSO-d_6

Position	**1**	**2**	**3**	**4**	**5**	**6**
	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)
1	4.26 (dd, 13.2, 5.4) 4.08 (dd, 13.1, 5.2)	4.11 (dd, 12.9, 5.3) 3.97 (dd, 12.7, 5.2)	4.32 (dd, 13.7, 5.8) 4.10 (dd, 13.6, 6.5)	4.06 (dd, 12.2, 5.3) 3.91 (dd, 12.4, 5.1)	3.51 (m)	3.66 (m) 3.42 (m)
2	5.71 (dt, 16.1, 6.3)	5.73 (dt, 15.9, 6.1)	5.73 (dt, 15.7, 5.9)	5.63 (dt, 16.1, 5.8)	2.24 (m)	2.26 (m)
3	6.58 (t, 14.5)	6.51 (t, 14.2)	6.59 (dd, 15.2, 11.2)	6.18 (t, 15.3)	5.32 (m)	5.33 (m)
4	5.94 (t, 11.2)	5.96 (t, 11.3)	5.96 (t, 11.1)	6.04 (t, 15.1)	5.42 (m)	5.42 (m)
5	5.42 (dt, 10.1, 6.7)	5.43 (dt, 10.5, 6.2)	5.42 (dt, 10.9, 6.5)	5.75 (dt, 15.5, 6.5)	2.01 (m)	2.01 (m)
6	2.16 (m)	2.16 (m)	2.16 (m)	2.07 (m)	0.92 (t, 7.6)	0.92 (t, 7.6)
7	0.94 (t, 7.5)	0.95 (t, 7.5)	0.94 (t, 7.5)	0.96 (t, 7.4)		
1'	4.15 (d, 7.8)	4.57 (br s)	4.15 (d, 7.9)	4.56 (br s)	4.54 (br s)	4.12 (d, 7.8)
2'	2.98 (t, 7.5)	3.41 (m)	2.97 (m)	3.40 (m)	3.57 (br s)	2.94 (t, 7.9)
3'	3.09 (t, 8.6)	3.60 (br s)	3.12 (t, 7.6)	3.59 (br s)	3.39 (m)	3.08 (t, 8.8)
4'	2.80 (t, 7.6)	3.18 (m)	3.04 (m)	3.17 (m)	3.17 (t, 8.9)	2.79 (t, 8.6)
5'	3.15 (m)	3.38 (m)	3.05 (m)	3.38 (m)	3.37 (m)	3.15 (m)
6'	1.15 (d, 6.1)	1.14 (d, 6.4)	3.66 (dd, 11.5, 5.6) 3.43 (m)	1.13 (d, 6.1)	1.12 (d, 6.3)	1.14 (m)
OH-2'	5.07 (br s)		5.08 (d, 4.4)	4.74 (br s)	4.72 (br s)	4.91 (d, 18.1)
OH-3'	4.96 (br s)		4.91 (d, 3.9)		4.51 (br s)	4.94 (br s)
OH-4'	4.96 (br s)		4.96 (br s)	4.47 (br s)	4.70 (br s)	4.91 (br s)
OH-6'			4.49 (t, 5.7)			

Table 5.5 Analyses and structural elucidation of purified lentzeosides.

Compound	Lentzeoside	Texture	Analyses
1	A	Light yellow gum	$[\alpha]^{20}_{D}$ –25.0 (c 0.15, MeOH); UV (MeOH) λ_{max} (log ε) at 258 (3.2), 268 (3.6) and 280 (2.7) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+Na]^+$ at 281.1351 indicting the molecular formula $C_{13}H_{22}O_5$ (calculated $[M+Na]^+$ ion at *m/z* 281.1359)
2	B	Light yellow gum	$[\alpha]^{20}_{D}$ –31.0 (c 0.18, MeOH); UV (MeOH) λ_{max} (log ε) at 258 (3.1), 270 (3.4) and 280 (2.5) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+Na]^+$ at 281.1351 indicting the molecular formula $C_{13}H_{22}O_5$ (calculated $[M+Na]^+$ ion at *m/z* 281.1359)
3	C	Light yellow gum	$[\alpha]^{20}_{D}$ +38.0 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) at 258 (3.2), 274 (3.8) and 282 (2.9) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+Na]^+$ at 297.1299 indicting the molecular formula $C_{13}H_{22}O_6$ (calculated $[M+Na]^+$ ion at *m/z* 297.1309)
4	D	Light yellow gum	$[\alpha]^{20}_{D}$ –22.0 (c 0.18, MeOH); UV (MeOH) λ_{max} (log ε) at 258 (3.1), 270 (3.4) and 280 (2.5) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+Na]^+$ at 281.1349 indicting the molecular formula $C_{13}H_{22}O_5$ (calculated $[M+Na]^+$ ion at *m/z* 281.1359)
5	E	Colourless gum	$[\alpha]^{20}_{D}$ –29.0 (c 0.15, MeOH); UV (MeOH) λ_{max} (log ε) at 239 (3.6) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+Na]^+$ at 269.1351 indicting the molecular formula $C_{12}H_{22}O_5$ (calculated $[M+Na]^+$ ion at *m/z* 269.1359).

6	F	Colourless gum	$[\alpha]^{20}_D$ –25.0 (c 0.18, MeOH); UV (MeOH) λ_{max} (log ε) at 241 (3.4) nm; ^{1}H and ^{13}C NMR data (Tables 5.3 and 5.4); HRESIMS *m/z* $[M+H]^+$ at 247.1545 indicting the molecular formula $C_{12}H_{22}O_5$ (calculated $[M+H]^+$ ion at *m/z* 247.1540).
7	G	Colourless gum	$[\alpha]^{20}_D$ +9.0 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) at 241 (3.4) nm; HRESIMS *m/z* $[M+H]^+$ at 263.1465 indicting the molecular formula $C_{12}H_{22}O_6$ (calculated $[M+H]^+$ ion at *m/z* 263.1460).

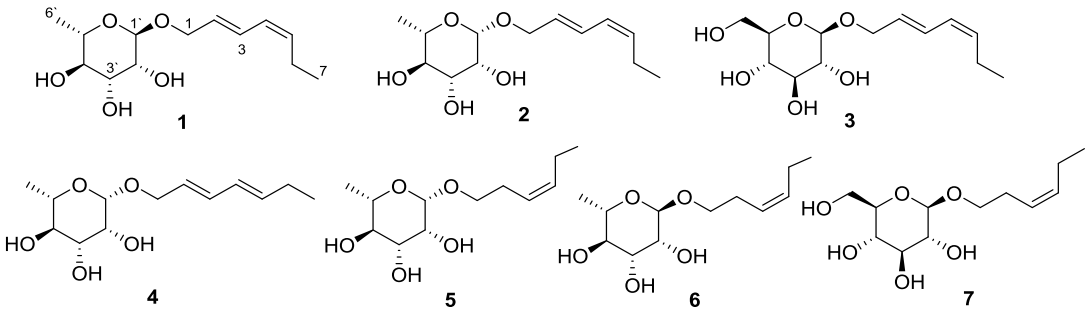


Figure 5.2 Structural elucidation of the seven dienes synthesized by isolate H45.

Compound 1. HRESIMS analysis yielded $[M+Na]^+$ ion at *m/z* 281.1351 predicting a molecular formula of $C_{13}H_{22}O_5$. The analysis of ^{1}H, ^{13}C and multiplicity-edited HSQC spectra indicated the presence of one methylene group (δ_C/δ_H 20.5/2.16) and one oxymethylene group (δ_C/δ_H 68.3/4.26, 4.08), one methyl doublet (δ_C/δ_H 17.9/1.15), one methyl triplet (δ_C/δ_H 14.2/0.94), four olefinic resonances (δ_C/δ_H 133.7/5.42, 129.6/5.71, 127.4/5.94, 127.0/6.58) and five oxymethine groups (δ_C/δ_H 102.1/4.15, 76.4/3.09, 75.3/2.80, 73.7/2.98, 71.5/3.15). Furthermore, the ^{1}H NMR showed hydroxy groups at δ_H 4.8-5.1. The COSY correlations of H_2-1 through H_3-7 indicated two conjugated olefins with a terminal ethyl group and established the first substructure, (2*E*,4*Z*)-heptadien-1-ol (Figure 5.3). This substructure was confirmed by the HMBC correlations of H_2-1/C-3, H-2/C-4, and H_3-7 to both C-5 and C-6 (Figure 5.3). The COSY correlations of H-1` through H-5` and H-5`/$H_3$-6` and the HMBC correlation of H-1`/C-5` suggested a hexose sugar as a second substructure. Furthermore, the COSY data revealed the connectivity of three hydroxy groups by the correlations of OH-2`/H-2`, OH-3`/H-3` and OH-4`/H-4`. Finally, the connectivity of the two substructures was confirmed by the HMBC correlation of

H-1`/C-1. The sugar moiety of **1** was assigned as *β*-L-rhamnopyranoside based on the optical rotation data, the coupling constant analysis of the anomeric proton H-1` (*J* = 7.82 Hz), as well as the agreement of the ^{13}C NMR data and the ROESY correlations with the reported data (Fujimoto and Isomura 1988) The ROESY correlations of H-2/H-4, H-3/H_2-1, H-4/H-5 and H-3/H_2-6 indicated an *E*-configuration of the olefinic group C-2/C-3 and a *Z*-configuration of the olefinic group C-4/C-5 (Figure 5.3). Following a search of the natural product databases it was concluded that **1** is a new metabolite for which the name lentzeoside A is proposed.

Compound 2. Shares the chemical formula with **1** as $C_{13}H_{22}O_5$ based on the HRESIMS analysis that gave $[M+Na]^+$ ion at *m/z* 281.1351. The ^{1}H and ^{13}C NMR together with the multiplicity-edited HSQC spectra indicated a close similarity to that of **1** with the presence of two methyl groups, one methylene group, one oxygenated methylene group, four olefinic resonances, and five oxymethine groups. The COSY correlations of H_2-1 through H_3-7 indicated the same moiety as in **1**. The COSY correlations of H-1` through $H_3$-6` and the HMBC correlations of H-5`/C-3` and H-1`/C-5` suggested a hexose sugar as a second substructure. The connectivity of the two substructures was confirmed by the HMBC correlation of H-1`/C-1. The sugar moiety of **2** was assigned as *α*-L-rhamnopyranoside based on the optical rotation data, the very small coupling of the anomeric proton and the agreement of the ^{13}C NMR data and ROESY correlations with reported data (Cao et al. 2015). Based on these data compound **2** is identical to **1** except in the relative configuration at the sugar moiety which indicates a new metabolite for which the name lentzeoside B is proposed.

Compound 3. The molecular formula $C_{13}H_{22}O_6$ for this compound was established on the basis of HRESIMS analysis that afforded $[M+Na]^+$ ion at *m/z* 297.1299 indicating one more oxygen atom than **1**. The ^{1}H, ^{13}C NMR and multiplicity-edited HSQC spectra showed a close similarity to that of **1** indicating an identical diene moiety but a different hexose sugar. The COSY correlations of OH-2`/H-2`, OH-3`/H-3`, OH-4`/H-4` and OH-6`/$H_2$-6` revealed the connectivity of four hydroxy groups. The connectivity of the two substructures was confirmed by the HMBC correlation of H-1` to C-1. The sugar moiety in **3** was assigned as *β*-D-glucopyranoside based on the optical rotation data and the coupling constant analysis of the anomeric proton (*J*=7.9 Hz) as well as the agreement of the ^{13}C NMR data and ROESY correlations with reported data (Fujimoto and Isomura

1988). Following a search of the natural product databases it was concluded that **3** is a new metabolite for which the name lentzeoside C is proposed.

Compound 4. The HRESIMS analysis provided $[M+Na]^+$ ion at *m/z* 281.1349 indicating a molecular formula of $C_{13}H_{22}O_5$ which is identical to that of **2**. The analysis of 1D, COSY, multiplicity-edited HSQC spectra and optical rotation data indicated *α*-L-rhamnopyranoside sugar moiety as in **2** but a different diene moiety. The COSY correlations of H_2-1/H-2, H-2/H-3, H-3/H-4, H-4/H-5, H-5/H_2-6 and H_2-6/H_3-7 indicated a conjugated diene with a terminal ethyl group (Figure 5.3). The connectivity of the two substructures was confirmed by the HMBC correlation of H-1`/C-1. The ROESY correlations of H_2-1/H-3, H-3/H-5, H-2/H-4 and H-4/H_2-6 indicated *E*-configurations for the olefinic groups C-2/C-3 and C-4/C-5 (Figure 5.3). Following a search of the natural product databases it was concluded that **4** is a new metabolite for which the name lentzeoside D is proposed.

Compound 5. The molecular formula was deduced as $C_{12}H_{22}O_5$ as HRESIMS analysis of $[M+Na]^+$ at *m/z* 269.1351 indicated one carbon atom less than **2**. Analysis of 1D and multiplicity-edited HSQC spectra indicated the presence of two methyl groups (δ_C/δ_H 17.9/1.12, 14.2/0.92,), two methylene groups (δ_C/δ_H 27.2/2.24, 20.1/2.01), one oxymethylene group (δ_C/δ_H 66.1/3.51), one double bond (δ_C/δ_H 133.0/5.42, 125.4/5.32) and five oxymethine groups (δ_C/δ_H 99.9/4.54, 72.0/3.17, 70.7/3.39, 70.5/3.57, 68.4/3.37). The COSY correlations of H_2-1 through H_3-6 indicated an olefin with a terminal ethyl group on one side and a bridging oxyethyl on the other side. The COSY correlations of H-1` through H-4` and H-5`/$H_3$-6` together with the HMBC correlations of H-5`/C-1` and H-4`/C-6` suggested a hexose sugar as a second substructure. The connectivity of the two substructures was confirmed by the HMBC correlation of H-1`/C-1 (Figure 5.3). The optical rotation data with chemical shift comparison indicated the sugar moiety of **5** to be *α*-L-rhamnopyranoside identical to that of **2**. The ROESY correlations of H_2-1/H-3, H-4/H_3-6 and H_2-2/H_2-5 indicated an *E*-configuration of the olefinic group C-3/C-4 (Figure 5.4). Following the search of the natural product databases it was concluded that **5** is a new metabolite for which the name lentzeoside E is proposed.

Compound 6. The HRESIMS analysis provided $[M+H]^+$ ion at *m/z* 247.1545 indicating a molecular formula of $C_{12}H_{22}O_5$ identical to that of **5**. The analysis of 1D and 2D NMR spectra indicated an identical aglycone moiety to **5**. The COSY correlations of H-1`/H-2`, H-2`/H-3`, H-3`/H-4`, H-4`/H-5` and H-5`/$H_3$-6` and the HMBC correlation of H-5`/C-1` suggested a hexose sugar moiety. The connectivity of the two substructures was confirmed by the HMBC correlation of H_2-1/C-1`. The optical rotation data with chemical shift comparison indicated the sugar moiety of **6** to be *β*-L-rhamnopyranoside identical to that of **1**. Following a search of the natural product databases it was concluded that **6** is a new metabolite for which the name lentzeoside F is proposed.

Compound 7. The molecular formula $C_{12}H_{22}O_6$ was established on the basis of HRESIMS analysis that afforded $[M+H]^+$ ion at *m/z* 263.1465. The analysis of 1D and 2D NMR spectral data and database searches for substructures indicated **7** was (*Z*)-3-hexenyl glucoside which was previously isolated from different natural sources of plant origin, such as *Epimedium grandiflorum* (Francis et al. 2004).

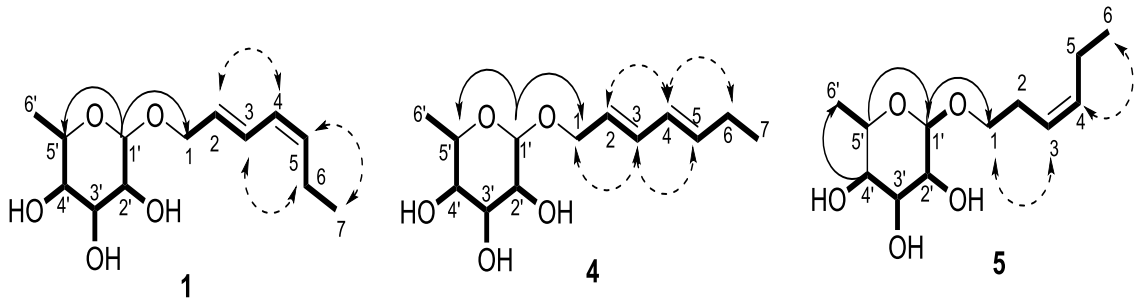


Figure 5.3 Key COSY (——), HMBC (⌒) and ROESY (⇢) correlations of compounds **1**, **4**, and **5**.

5.4.3 Biological testing of novel specialized metabolites

Antimicrobial tests. None of the lentzeosides showed activity against the *E. coli* and *S. aureus* strains up to the final dilution of 32μg/ml. In contrast, the positive control, rifampicin, showed an MIC of 1.1 μg/mL against the *E. coli* strain and an MIC of 0.25-0.5 μg/mL against the *S. aureus* strain.

HIV assay. Lentzeosides C, D and E inhibited HIV integrase activity with an IC_{50} 21 μM, 16 μM and 21 μM, respectively. Lentzeosides A, B and F were not found to give 50 % inhibition of enzyme activity up to 100 μM concentration even though mild inhibition of enzyme activity was detected using these compounds.

5.4.4 Classification of isolate H45

Cultural and morphological properties. In general, the isolate grew well on all of the ISP media producing white/ pinkish or white aerial hyphae and slightly yellow/ light brown substrate mycelia (Table 5.6). The colonies were entire, raised, round and smooth with entire margins. Aerial hyphae fragmented into rod-shaped elements with smooth surfaces after growth on oatmeal agar (Figure 5.4).

Table 5.6 Growth and cultural characteristics of isolate H45 on ISP media after incubation for 14 days at 28°C.

Characteristic	ISP						
	1	2	3	4	5	6	7
Growth	+++	+++	+++	+++	+++	++	+++
Aerial hyphae	White	White	Pinkish white	Pinkish white	Yellowish white	White	White
Substrate mycelium	Slightly yellow	Slightly yellow	Pale yellow	Light brown	Slightly yellow	Slightly yellow	Slightly yellow

+++ abundant growth; ++ very good growth. Diffusible pigments were not produced on any of the media.

Figure 5.4 Scanning electron micrograph of isolate H45 showing fragmentation of aerial hyphae into rod-shaped elements following growth on oatmeal agar for 10 days at 28^{o}C. Bar 1µm.

Phylogenetic analyses. The isolate formed a distinct branch at the periphery of the *Lentzea* 16S rRNA gene tree, a result that was supported by all the tree-making algorithms and by a 57% bootstrap value (Figure 5.5). The strain was most closely related to *L. kentuckyensis* NRRL B-24416^{T}, these strains shared a 16S rRNA gene similarity of 99.0%, a value that corresponds to 14 nucleotide (nt) differences at 1360 locations (Table 5.12). The isolate showed 16S rRNA gene sequence similarities with the remaining *Lentzea* type strains within the range 96.7% to 98.5%, values that are equivalent to between 14 to 46 nt differences. The corresponding relationships between the isolate and the type strains of *Lechevalieria* species fell within the range 97.8% to 98.7% 16S rRNA gene similarity, values corresponding to 18 to 32 nt differences (Table 5.7).

Chemotaxonomy. Whole-organism hydrolysates of isolate H45 and *L. kentuckyensis* NRRL B-24416^{T} contained *meso*-A_2pm, galactose and ribose; mannose was also detected in isolate H45. The predominant isoprenologues was MK9(H_4) (52%) and MK9(H_4) (12%) was also detected. The fatty acid profile of isolate H45 consisted of major proportions of (>10%) *iso*-$C_{15:0}$ (22.48%), *iso*-$C_{16:0}$ (19.21%) and $C_{17:0}$ (13.69%), minor proportions (>2.8%) of *iso*-$C_{14:0}$ (6.97%), *iso*-$C_{15:0}$ (6.15%), $C_{15:0}$ (8.99%), $C_{16:0}$ (7.96%) and *anteiso*-$C_{17:0}$ (6.54%) and trace amounts (<0.5%) of $C_{13:0}$ (0.27%), $C_{14:0}$ (0.34%), $C_{16:1}$ w7c/15 *iso* 2OH (1.84%), *iso*-$C_{15:0}$ (6.15%), $C_{16:0}$ methyl (1.22%), *iso*-$C_{17:0}$ (0.71%), $C_{17:1}$ w8c (1.90%), $C_{17:1}$ (1.12%) and $C_{17:0}$ methyl (0.60%). The polar lipid pattern included diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and several unidentified components, as shown in Figure 5.6.

DNA: DNA relatedness. The DNA:DNA relatedness value between isolate H45 and *L. kentuckyensis* was 37.6% ± 4.0.

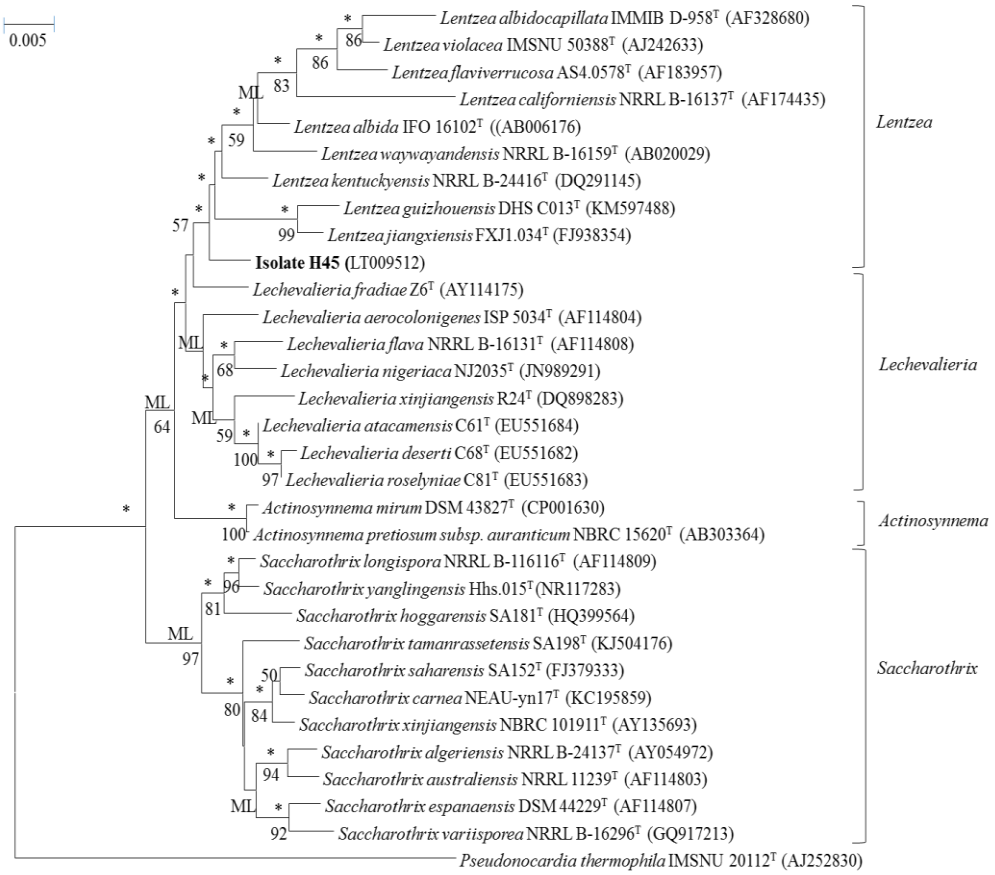


Figure 5.5 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolate H45 and the type strains of *Lentzea* and *Lechevalieria* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates branches of the tree that were recovered using the maximum-likelihood 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.

Table 5.7 Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences showing relationships between isolate H45 and the type strains of closely related *Actinosynnema*, *Lechevalieria*, *Lentzea* and *Saccharothrix* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1. Isolate H45	**---**	**22/1421**	**24/1437**	**23/1420**	**24/1420**	**18/1389**	**20/1444**	**32/1421**	**22/1435**	**29/1385**	**22/1419**	**46/1406**	**42/1397**	**32/1389**	**30/1421**	**14/1360**	**35/1419**	**27/1420**	**23/1421**	**23/1421**	**30/1403**	**30/1420**	**37/1421**	**40/1420**	**39/1420**	**39/1420**	**38/1396**	**42/1398**	**39/1360**	**43/1420**	**43/1405**	**105/1418**
2. *Lechevalieria aerocolonigenes* ISP 5034^T	**98.5**	---	25/1452	29/1455	24/1460	20/1407	36/1461	34/1459	30/1461	20/1402	31/1453	49/1438	46/1437	39/1406	38/1461	18/1400	38/1459	26/1460	24/1461	24/1455	32/1443	31/1460	41/1461	46/1460	43/1453	42/1460	38/1426	47/1429	42/1400	40/1460	43/1445	104/1458
3. *Lechevalieria atacamensis* C61^T	**98.3**	98.3	---	6/1452	22/1451	23/1406	35/1470	30/1452	6/1466	14/1401	24/1450	45/1437	44/1430	39/1406	34/1452	22/1393	32/1450	32/1452	28/1452	26/1452	38/1435	38/1451	43/1452	44/1451	43/1450	45/1451	41/1425	43/1428	42/1393	46/1451	50/1437	100/1449
4. *Lechevalieria deserti* C68^T	**98.4**	98.0	99.6	---	26/1454	28/1406	33/1455	34/1455	2/1455	19/1401	28/1452	49/1437	48/1432	44/1406	38/1455	26/1395	36/1453	37/1455	32/1455	30/1454	42/1437	42/1454	47/1455	48/1454	47/1452	49/1454	45/1425	47/1428	46/1395	50/1454	54/1439	104/1452
5. *Lechevalieria flava* NRRL B-16131^T	**98.3**	98.4	98.5	98.2	---	28/1406	37/1470	27/1458	27/1460	17/1401	40/1452	59/1437	55/1449	50/1405	52/1473	31/1413	53/1471	36/1472	33/1473	32/1454	41/1455	41/1472	43/1470	44/1469	43/1452	46/1472	43/1425	49/1428	45/1413	44/1472	40/1457	107/1470
6. *Lechevalieria fradiae* Z6^T	**98.7**	98.6	98.4	98.0	98.0	---	31/1407	26/1407	27/1407	33/1402	24/1405	47/1406	48/1405	36/1405	29/1407	19/1378	39/1405	30/1406	23/1407	22/1407	32/1406	31/1406	36/1407	41/1406	38/1405	39/1406	37/1404	40/1406	37/1378	42/1406	44/1405	105/1404
7. *Lentzea guizhouensis* DHS C013^T	**98.6**	97.5	97.6	97.7	97.5	97.8	---	44/1459	34/1475	40/1402	33/1453	45/1438	51/1447	27/1406	19/1471	30/1410	31/1469	37/1470	40/1471	38/1455	47/1453	47/1470	54/1471	49/1470	48/1453	48/1470	47/1426	45/1429	43/1410	49/1470	50/1455	111/1468
8. *Lechevalieria nigeriaca* NJ2035^T	**97.8**	97.7	97.9	97.7	98.2	98.2	97.0	---	33/1459	30/1402	44/1453	66/1438	61/1435	51/1406	51/1459	29/1398	59/1457	49/1458	41/1459	39/1455	45/1441	45/1458	54/1459	57/1458	58/1453	55/1458	44/1426	51/1429	41/1398	50/1458	48/1443	103/1456
9. *Lechevalieria roselyniae* C81^T	**98.5**	98.0	99.6	99.9	98.2	98.1	97.7	97.7	---	18/1402	27/1453	48/1438	48/1437	42/1406	39/1461	26/1400	37/1459	37/1460	33/1461	29/1455	43/1443	43/1460	48/1461	49/1460	46/1453	50/1460	44/1426	46/1429	46/1400	51/1460	55/1445	105/1458
10. *Lechevalieria xinjiangensis* R24^T	**97.9**	98.6	99.0	98.6	98.8	97.7	97.2	97.9	98.7	---	37/1400	47/1401	50/1400	47/1400	47/1402	27/1377	39/1400	33/1401	28/1402	27/1402	39/1401	39/1401	43/1402	44/1401	45/1400	48/1401	45/1399	50/1401	47/1377	47/1401	50/1400	105/1399
11. *Lentzea albida* IFO 16102^T	**98.5**	97.9	98.3	98.1	97.3	98.3	97.7	97.0	98.1	97.4	---	39/1436	34/1429	28/1404	25/1453	12/1392	25/1451	23/1452	32/1453	30/1453	39/1435	40/1452	45/1453	52/1453	47/1451	51/1453	46/1424	45/1427	43/1392	45/1452	49/1437	97/1451
12. *Lentzea albidocapillata* IMMIB D-958^T	**96.7**	96.6	96.9	96.6	95.9	96.7	96.9	95.4	96.7	96.7	97.3	---	35/1428	25/1405	46/1438	42/1391	18/1436	41/1437	47/1438	47/1438	63/1434	62/1437	63/1438	62/1437	59/1436	65/1437	64/1425	62/1428	57/1391	65/1437	67/1436	108/1435
13. *Lentzea californiensis* NRRL B-16137^T	**97.0**	96.8	96.9	96.7	96.2	96.6	96.5	95.8	96.7	96.4	97.6	97.6	---	36/1405	58/1450	45/1412	34/1448	40/1450	44/1450	44/1431	58/1449	57/1449	58/1447	63/1446	61/1429	66/1449	67/1424	66/1427	66/1412	66/1449	68/1448	121/1447
14. *Lentzea flaviverrucosa* AS4.0578^T	**97.7**	97.2	97.2	96.9	96.4	97.4	98.1	96.4	97.0	96.6	98.0	98.2	97.4	---	32/1406	30/1377	11/1404	24/1406	42/1406	42/1406	52/1405	51/1405	54/1406	53/1405	50/1404	56/1405	54/1403	56/1405	50/1377	56/1405	58/1404	107/1403
15. *Lentzea jiangxiensis* FXJ1.034^T	**97.9**	97.4	97.7	97.4	96.5	97.9	98.7	96.5	97.3	96.7	98.3	96.8	96.0	97.7	---	21/1413	36/1472	46/1473	46/1474	40/1455	51/1456	52/1473	54/1471	51/1470	48/1453	53/1473	46/1426	47/1429	46/1413	56/1473	58/1458	111/1471
16. *Lentzea kentuckyensis* NRRL B-24416^T	**99.0**	98.7	98.4	98.1	97.8	98.6	97.9	97.9	98.1	98.0	99.1	97.0	96.8	97.8	98.5	---	36/1412	23/1413	27/1413	23/1394	30/1413	31/1412	36/1410	40/1409	38/1392	42/1412	36/1389	43/1390	45/1411	47/1412	48/1412	99/1410
17. *Lentzea violacea* IMSNU 50388^T	**97.5**	97.4	97.8	97.5	96.4	97.2	97.9	96.0	97.5	97.2	98.3	98.8	97.7	99.2	97.6	97.5	---	27/1471	42/1473	39/1453	55/1455	54/1472	57/1469	56/1468	52/1451	59/1472	56/1425	53/1427	50/1412	56/1472	60/1456	106/1470
18. *Lentzea waywayandensis* NRRL B-16159^T	**98.1**	98.2	97.8	97.5	97.6	97.9	97.5	96.6	97.5	97.6	98.4	97.2	97.2	98.3	96.9	98.4	98.2	---	35/1473	33/1454	41/1455	41/1472	49/1470	50/1469	45/1452	49/1472	46/1425	46/1428	41/1413	49/1472	49/1457	107/1470
19. *Actinosynnema mirum* DSM 43827^T	**98.4**	98.4	98.1	97.8	97.8	98.4	97.3	97.2	97.7	98.0	97.8	96.7	97.0	97.0	96.9	98.1	97.2	97.6	---	2/1455	27/1456	26/1474	29/1471	36/1470	35/1453	36/1474	37/1426	44/1429	40/1413	40/1474	41/1458	106/1472
20. *Actinosynnema pretiosum subsp. auranticum* NBRC 15620^T	**98.4**	98.4	98.2	97.9	97.8	98.4	97.4	97.3	98.0	98.1	97.9	96.7	96.9	97.0	97.3	98.4	97.3	97.7	99.9	---	27/1437	26/1454	29/1455	36/1454	35/1453	38/1454	37/1426	42/1429	38/1394	39/1454	40/1439	103/1452
21. *Saccharothrix longispora* NRRL B-116116^T	**97.9**	97.8	97.4	97.1	97.2	97.7	96.8	96.9	97.0	97.2	97.3	95.6	96.0	96.3	96.5	97.9	96.2	97.2	98.2	98.1	---	7/1455	15/1453	24/1452	25/1435	21/1455	22/1426	34/1428	28/1413	32/1455	36/1454	104/1453
22. *Saccharothrix yanglingensis* Hhs.015^T	**97.9**	97.9	97.4	97.1	97.2	97.8	96.8	96.9	97.1	97.2	97.3	95.7	96.1	96.4	96.5	97.8	96.3	97.2	98.2	98.2	99.5	---	16/1470	23/1469	24/1452	20/1473	19/1425	28/1428	23/1412	27/1473	35/1457	101/1471
23. *Saccharothrix hoggarensis* SA181^T	**97.4**	97.2	97.0	96.8	97.1	97.4	96.3	96.3	96.7	96.9	96.9	95.6	96.0	96.2	96.3	97.5	96.1	96.7	98.0	98.0	99.0	98.9	---	19/1470	22/1453	24/1470	25/1426	37/1429	32/1410	35/1470	40/1455	109/1468
24. *Saccharothrix saharensis* SA152^T	**97.2**	96.9	97.0	96.7	97.0	97.1	96.7	96.1	96.6	96.9	96.4	95.7	95.6	96.2	96.5	97.2	96.2	96.6	97.6	97.5	98.4	98.4	98.7	---	9/1453	7/1472	22/1426	27/1429	19/1410	26/1470	29/1455	111/1469
25. *Saccharothrix xinjiangensis* NBRC 101911^T	**97.3**	97.0	97.0	96.8	97.0	97.3	96.7	96.0	96.8	96.8	96.8	95.9	95.7	96.4	96.7	97.3	96.4	96.9	97.6	97.6	98.3	98.4	98.5	99.4	---	14/1453	17/1425	20/1428	15/1393	19/1453	24/1438	107/1450
26. *Saccharothrix carnea* NEAU-yn17^T	**97.3**	97.1	96.9	96.6	96.9	97.2	96.7	96.2	96.6	96.6	96.5	95.5	95.5	96.0	96.4	97.0	96.0	96.7	97.6	97.4	98.6	98.6	98.4	99.5	99.0	---	19/1426	26/1429	17/1413	23/1474	26/1458	112/1473
27. *Saccharothrix tamanrassetensis* SA198^T	**97.3**	97.3	97.1	96.8	97.0	97.4	96.7	96.9	96.9	96.8	96.8	95.5	95.3	96.2	96.8	97.4	96.1	96.8	97.4	97.4	98.5	98.7	98.3	98.5	98.8	98.7	---	18/1426	15/1390	20/1426	26/1425	101/1423
28. *Saccharothrix algeriensis* NRRL B-24137^T	**97.0**	96.7	97.0	96.7	96.6	97.2	96.9	96.4	96.8	96.4	96.9	95.7	95.4	96.0	96.7	96.9	96.3	96.8	96.9	97.1	97.6	98.0	97.4	98.1	98.6	98.2	98.7	---	11/1391	19/1429	28/1428	108/1426
29. *Saccharothrix australiensis* NRRL 11239^T	**97.1**	97.0	97.0	96.7	96.8	97.3	97.0	97.1	96.7	96.6	96.9	95.9	95.3	96.4	96.7	96.8	96.5	97.1	97.2	97.3	98.0	98.4	97.7	98.7	98.9	98.8	98.9	99.2	---	15/1413	21/1413	96/1410
30. *Saccharothrix espanaensis* DSM 44229^T	**97.0**	97.3	96.8	96.6	97.0	97.0	96.7	96.6	96.5	96.7	96.9	95.5	95.5	96.0	96.2	96.7	96.2	96.7	97.3	97.3	97.8	98.2	97.6	98.2	98.7	98.4	98.6	98.7	98.9	---	13/1458	107/1471
31. *Saccharothrix variisporea* NRRL B-16296^T	**96.9**	97.0	96.5	96.3	97.3	96.9	96.6	96.7	96.2	96.4	96.6	95.3	95.3	95.9	96.0	96.6	95.9	96.6	97.2	97.2	97.5	97.6	97.3	98.0	98.3	98.2	98.2	98.0	98.5	99.1	---	109/1455
32. *Pseudonocardia thermophila* IMSNU 20112^T	**92.6**	92.9	93.1	92.8	92.7	92.5	92.4	92.9	92.8	92.5	93.3	92.5	91.6	92.4	92.5	93.0	92.8	92.7	92.8	92.9	92.8	93.1	92.6	92.4	92.6	92.4	92.9	92.4	93.2	92.7	92.5	---

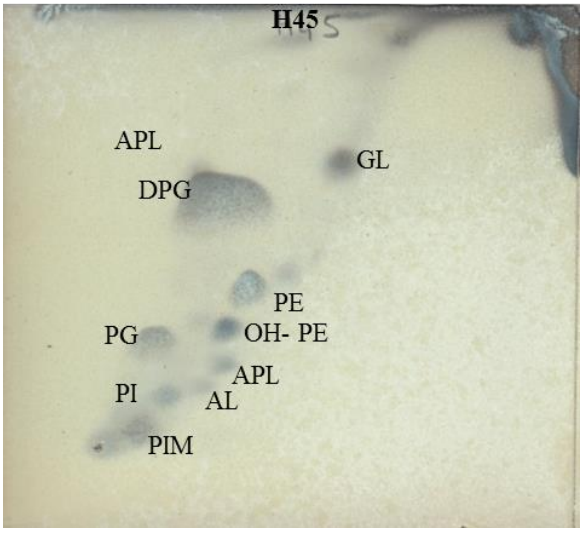


Figure 5.6 Two-dimensional thin-layer chromatography of polar lipids of isolate H45 stained with molybdenum blue spray (Sigma). Chloroform : methanol : water (32.5:12.5:2.0, v/v) was used in the first direction and chloroform : acetic acid : methanol : water (40.0:7.5:6.0:2.0, v/v) in the second direction. Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; OH-PE, hydroxyphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; AL, aminolipid; APL, aminophospholipids and GL, glycolipid.

Phenotypic properties. Identical results were obtained for the duplicated phenotypic analyses carried out on isolate H45 and *L. kentuckyensis* NRRL B-24416^T, apart from some of the BIOLOG tests. It is evident from Table 5.8 that these strains can be distinguished using a broad range of phenotypic properties.

Table 5.8 Phenotypic characteristics that differentiate isolate H45 from *Lentzea kentuckyensis* NRRL B-24416^T.

Characteristics	Isolate H45	*L. kentuckyensis* NRRL B-24416^T
API ZYM test:		
α-Fucosidase	-	+
GEN III BIOLOG microplates:		
Utilization of:		
Acetoacetic acid,	+	-
γ-amino-n-Butyric acid, citric acid, α-*keto*-glutaric acid,	-	+
D-Fucose, D-sorbitol	+	-
α-D-Lactose, L-rhamnose	-	+

Inhibition test:		
Potassium tellurite	-	+
Phenotypic tests:		
Degradation test (%, v/v):		
Tween 80	+	-
Growth in the presence of:		
Sodium chloride (4%, w/v)	+	+*
Growth at:		
40ºC	+	-

+, positive result; -, negative result

* Feature taken from Labeda et al. (2007)

Positive results recorded for isolate H45 and *L. kentuckyensis* NRRL B-24416^T:

- API ZYM tests: acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, β-glucosidase, α-glucuronidase, lipase (C14), leucine arylamidase, α-mannosidase, N-acetyl-β-glucosaminidase, trypsin and valine arylamidase.
- GEN III BIOLOG microplate tests: utilization of β-gentiobiose, acetic acid, bromo-succinic acid, dextrin, D-arabitol, L-alanine, D-cellobiose, D-fructose, L-fucose, D-galactose, D-gluconic acid, D-glucose, gelatin, glycerol, L-histidine, L-malic acid, D-maltose, D-mannose, D-mannitol, D-melibiose, *myo*-inositol, *N*-acetyl-D-glucosamine, propionic acid, D-salicin, D-serine #2, sucrose, D-trehalose and D-turanose; sensitive to aztreonam and lithium chloride; growth at pH6 and in the presence of sodium chloride (1%, w/v).
- Other phenotypic tests: aesculin hydrolysis, degradation of casein, elastin, hypoxanthine, starch, L-tyrosine, Tweens 40 and 60 and growth at 10ºC, 20ºC and 30ºC.

Negative results recorded for isolate H45 and *L. kentuckyensis* NRRL B-24416^T:

- API ZYM tests: β-glucuronidase and naphthol-AS-BI-phosphohydrolase.
- GEN III BIOLOG microplate tests: utilization of *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, L-arginine, D-aspartic acid, butyric acid, D-fructose-6-phosphate, L-galactonic acid-γ-lactone, D-glucose-6-phosphate, glucuronamide, α-hydroxy-butyric acid, *p*-hydroxy-phenylacetic acid, D-lactic acid methyl ester, L-lactic acid, L-lactic acid, D-malic acid, 3-*O*-methyl-D-glucose, β-methyl-D-glucoside, mucic acid, D-saccharic acid, D-serine #1 and stachyose and resistance to guanidine hydrochloride, minocycline, niaproof, tetrazolium violet, tetrazolium blue

and troleandomycin, but does not grow in the presence of sodium formate or 1% sodium lactate.

- Other phenotypic tests: allantoin hydrolysis, nitrate reduction, H_2S production, urea hydrolysis, degradation of adenine, cellulose, chitin, guanine, uric acid, xanthine, xylan and tributyrin and growth at 4°C, 45°C and 50°C.

Contrasting BIOLOG results obtained for:

(d) isolate H45: utilization of β-hydroxybutyric acid, D-raffinose, *N*-acetyl-β-D-mannosamine and L-pyroglutamic acid and growth in the presence of sodium bromate.

(e) *L. kentuckyensis* NRRL B-24416^T: utilization of α-*keto*-butyric acid, D-galacturonic acid, D-glucuronic acid, glycyl-L-proline, inosine, L-aspartic acid, L-glutamic acid, L-serine, methyl pyruvate, pectin, quinic acid and resistance to lincomycin and nalidixic acid.

5.4.5 Classification of isolate H9

Cultural and morphological properties. The isolate grew well on ISP media 1 to 7 producing a range of aerial spore mass, substrate mycelial and diffusible pigment colours (Table 5.9). Colonies were entire, raised, round and smooth with filamentous margins. The isolate formed spiral chains of spores with smooth surfaces, as shown in Figure 5.7.

Table 5.9 Growth and cultural characteristics of *Streptomyces* isolate H9 on ISP media after 14 days at 28°C.

Characteristic	ISP						
	1	2	3	4	5	6	7
Growth	++	+++	++	++	+++	++	++
Aerial spore mass	None	None	White	White (edge) and pale yellow pink (middle)	Light brown gray	None	None
Substrate mycelium	Brown black	Brown black	Dark gray brown	Brown gray	Brown black	Dark olive brown	Black

Diffusible pigments	None	None	None	None	Mild brown	Mild brown	Brown black

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

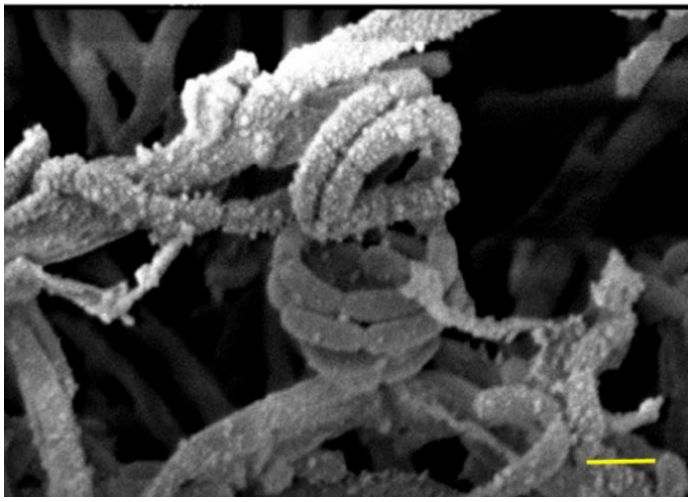


Figure 5.7 Scanning electron micrograph of isolate H9 showing spiral chains of smooth surfaced spores following growth on oatmeal agar at 28ºC for 10 days. Bar 1µm.

Phylogenetic analyses. The isolate formed a distinct branch at the periphery of a well-delineated subclade in the *Streptomyces* 16S rRNA gene tree together with *Streptomyces crystallinus* NBRC 15401^T, *Streptomyces melanogenes* NBRC 12890^T and *Streptomyces noboritoensis* NRRL B-12152^T, relationships that were supported by all of the tree-making algorithms, but not by a high bootstrap value (Figure 5.8). The isolate was most closely related to the type strains of *S. melanogenes* and *S. noboritoensis*, which had identical 16S rRNA gene sequences, it shared 16S rRNA gene similarities with these organisms of 98.6%, a value that corresponds to 20 nt dfferences at 1424 and 1423 locations, respectively (Table 5.10). The isolate shared 16S rRNA gene similarities with the remaining phylogenetically close strains shown in Table 5.4 within the range 97.7 to 98.5%, values that correspond to between 21 and 31 nt differences (Table 5.10).

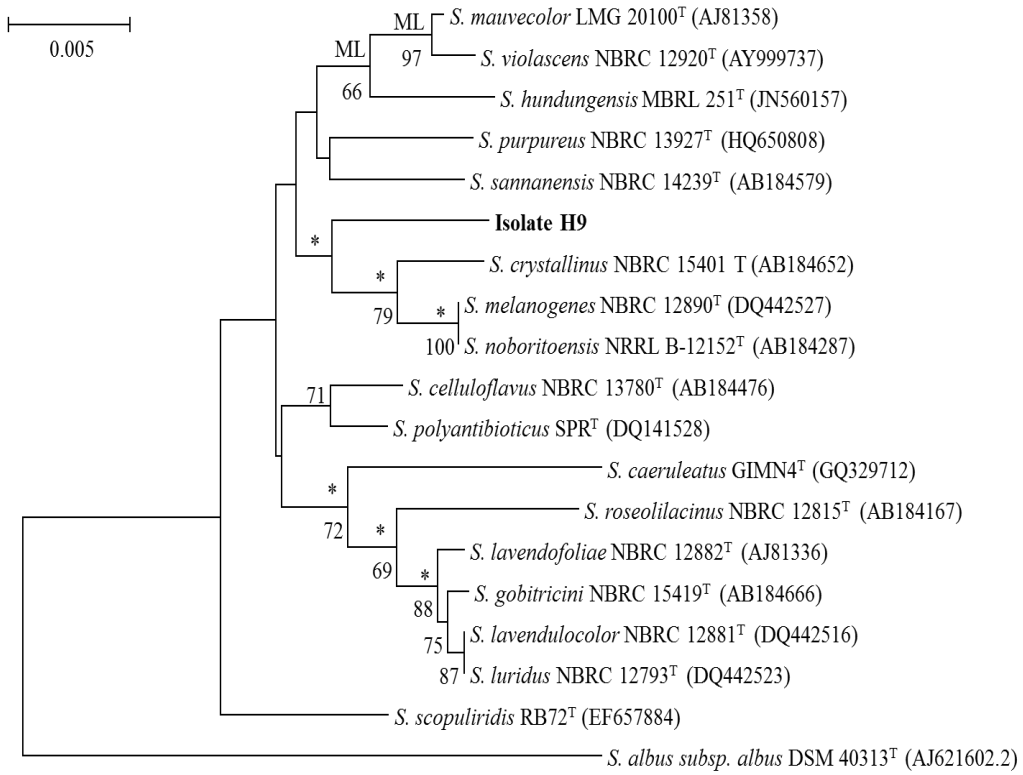


Figure 5.8 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolate H9 and closely related type strains of *Streptomyces* species. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making methods; ML indicates branches of the tree that were 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.

Table 5.10Nucleotide similarities (%) and differences based on almost complete 16S rRNA gene sequences between isolate H9 and closely related *Streptomyces* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Isolate H9	**---**	**66/1425**	**29/1392**	**29/1424**	**22/1424**	**22/1425**	**27/1424**	**22/1425**	**22/1425**	**21/1421**	**24/1423**	**20/1424**	**25/1401**	**26/1425**	**28/1424**	**30/1420**	**31/1329**	**24/1419**	**20/1423**
2. *S. albus subsp. albus* DSM 40313^{T}	**95.37**	---	72/1397	56/1453	53/1454	61/1462	69/1460	62/1461	60/1443	60/1439	65/1459	59/1460	69/1429	60/1462	66/1452	65/1435	57/1355	59/1455	59/1473
3. *S. caeruleatus* GIMN4^{T}	**97.92**	94.85	---	30/1396	35/1397	25/1397	31/1397	23/1397	25/1397	25/1397	29/1396	31/1397	27/1395	35/1397	25/1397	30/1397	32/1333	33/1392	31/1396
4. *S. celluloflavus* NBRC 13780 ^{T}	**97.96**	96.15	97.85	---	21/1451	27/1453	21/1451	26/1452	27/1442	27/1438	20/1450	22/1451	18/1419	26/1453	31/1446	24/1434	20/1346	16/1446	22/1450
5. *S. crystallinus* NBRC 15401 ^{T}	**98.46**	96.35	97.49	98.55	---	24/1454	29/1454	26/1453	25/1441	25/1437	28/1453	14/1454	31/1422	21/1454	32/1449	32/1435	38/1349	21/1449	14/1453
6. *S. gobitricini* NBRC 15419 ^{T}	**98.46**	95.83	98.21	98.14	98.35	---	21/1463	3/1464	2/1443	2/1439	23/1462	18/1463	24/1431	28/1465	16/1452	27/1435	27/1358	27/1457	18/1462
7. *S. hundungensis* MBRL 251 ^{T}	**98.1**	95.27	97.78	98.55	98.01	98.56	---	22/1462	21/1441	21/1437	10/1463	16/1463	23/1452	25/1463	32/1452	21/1435	23/1376	13/1457	16/1462
8. *S. lavendofoliae* NBRC 12882 ^{T}	**98.46**	95.76	98.35	98.21	98.21	99.8	98.5	---	3/1443	3/1438	21/1461	20/1462	23/1430	27/1464	16/1451	26/1435	26/1357	25/1456	20/1461
9. *S. lavendulocolor* NBRC 12881 ^{T}	**98.46**	95.84	98.21	98.13	98.27	99.86	98.54	99.79	---	0/1438	23/1440	19/1441	24/1413	28/1443	13/1440	27/1435	25/1340	27/1436	19/1440
10. *S. luridus* NBRC 12793 ^{T}	**98.52**	95.83	98.21	98.12	98.26	99.86	98.54	99.79	100	---	23/1436	19/1437	24/1414	28/1439	13/1437	27/1435	25/1341	27/1432	19/1436
11. *S. mauvecolor* LMG 20100 ^{T}	**98.31**	95.54	97.92	98.62	98.07	98.43	99.32	98.56	98.4	98.4	---	17/1462	22/1431	21/1462	31/1451	14/1434	18/1358	3/1456	17/1461
12. *S. melanogenes* NBRC 12890 ^{T}	**98.6**	95.96	97.78	98.48	99.04	98.77	98.91	98.63	98.68	98.68	98.84	---	25/1431	27/1463	29/1452	21/1435	27/1358	21/1457	0/1462
13. *S. polyantibioticus* SPR ^{T}	**98.22**	95.17	98.06	98.73	97.82	98.32	98.42	98.39	98.3	98.3	98.46	98.25	---	29/1431	32/1425	26/1413	16/1375	25/1425	25/1430
14. *S. purpureus* NBRC 13927 ^{T}	**98.18**	95.9	97.49	98.21	98.56	98.09	98.29	98.16	98.06	98.05	98.56	98.15	97.97	---	33/1452	23/1435	29/1358	16/1457	27/1462
15. *S. roseolilacinus* NBRC 12815 ^{T}	**98.03**	95.45	98.21	97.86	97.79	98.9	97.8	98.9	99.1	99.1	97.86	98	97.75	97.73	---	32/1435	34/1352	35/1447	29/1451
16. *S. sannanensis* NBRC 14239 ^{T}	**97.89**	95.47	97.85	98.33	97.77	98.12	98.54	98.19	98.12	98.12	99.02	98.54	98.16	98.4	97.77	---	18/1340	18/1430	21/1434
17. *S. scopuliridis* RB72 ^{T}	**97.67**	95.79	97.6	98.51	97.18	98.01	98.33	98.08	98.13	98.14	98.67	98.01	98.84	97.86	97.49	98.66	---	21/1352	27/1357
18. *S. violascens* NBRC 12920^{T}	**98.31**	95.95	97.63	98.89	98.55	98.15	99.11	98.28	98.12	98.11	99.79	98.56	98.25	98.9	97.58	98.74	98.45	---	21/1456
19. *S. noboritoensis* NRRL B-12152^{T}	**98.59**	95.99	97.78	98.48	99.04	98.77	98.91	98.63	98.68	98.68	98.84	100	98.25	98.15	98	98.54	98.01	98.56	---

Multilocus sequence analysis. It can be seen from Figure 5.9 that *Streptomyces* strain H9 is most closely related to the type strains of *S. melanogenes*, *S. noboritoensis* and *S. polyantibioticus*, relationships that are supported by a 100% bootstrap value; the type strain of *S. crystallinus* is loosely associated with this lineage. *Streptomyces* strain H9 shared MLSA distances much greater than 0.007 with all of these strains (Table 5.11) indicating that it merits recognition as a distinct species. In contrast, the type strains of *S. melanogenes* and *S. noboritoensis* shared a MLSA evolutionary distance of only 0.004 showing that they should be classified in the same species.

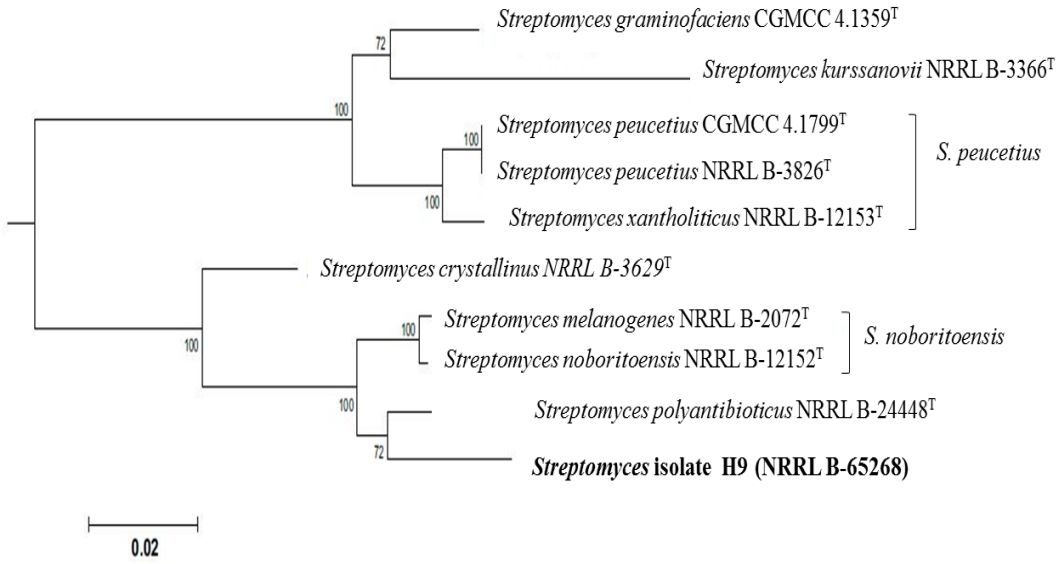


Figure 5.9 *Streptomyces* sub-tree derived from the phylogenetic tree inferred from concatenated partial sequences of the house-keeping genes *atpD, gyrB, recA, rpoB* and *trpB* in MEGA 6 (Tamura et al. 2013) using the maximum-likelihood method based on the General Time Reversible model (Nei and Kumar 2000). The final dataset consisted of 2622 positions and 706 strains. Percentages at the nodes represent levels of bootstrap support from 1000 resampled datasets, values less than 60% are not shown. The proposed new species is indicated in bold. Bar, equals number of substitutions per site.

Table 5.11 MLSA distances for strains phylogenetically near to isolate H9 and related isolates.

Strain	MLSA (Kimura 2-parameter) Distance								
Streptomyces halstedii CGMCC 4.1359^T	-								
Streptomyces kurssanovii NRRL B-3366^T	0.063								
Streptomyces peucetius CGMCC 4.1799^T	0.040	0.073							
Streptomyces peucetius subspecies peucetius NRRL_B-3826^T	0.040	0.073	0.000						
Streptomyces xantholiticus NRRL B-12153^T	0.038	0.076	0.014	0.014					
Streptomyces crystallinus NRRL B-3629^T	0.094	0.123	0.093	0.093	0.097				
Streptomyces melanogenes NRRL B-2072^T	0.112	0.142	0.109	0.109	0.114	0.049			
Streptomyces noboritoensis NRRL B-12152^T	0.113	0.142	0.106	0.106	0.111	0.048	0.004		
Streptomyces polyantibioticus NRRL B-24448^T	0.110	0.139	0.107	0.107	0.110	0.060	0.035	0.035	
***Streptomyces species* H9 (NRRL B-65268)**	**0.111**	**0.138**	**0.107**	**0.107**	**0.110**	**0.054**	**0.034**	**0.034**	**0.039**

Chemotaxonomy. The wall peptidoglycan of *Streptomyces* isolate H9 and the type strains of *S. melanogenes*, S. *noboritoensis* and S. *polyantibioticus* contained *LL*-A_2pm. Whole organism hydrolysates of these strains contained glucose, mannose and ribose; traces of xylose were detected in *S. noboritoensis* NRRL B-12152^T. It can be seen from Table 5.12 that all three strains exhibited major proportions of *anteiso*-$C_{15:0}$, *iso*-$C_{16:0,}$ $C_{16:0}$ and *anteiso*-$C_{17:0}$ fatty acids though quantitative differences were apparent. Such differences were also found between most of the remaining components while a few trace components were discontinously distributed. *Streptomyces* isolate H9 and the type strains of *S. melanogenes* and *S. noboritoensis* gave similar polar lipid patterns as they contain diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, hydroxyphosphatidylethanolamine was detected in isolate H9 and the type strain of *S. melanogenes* and phosphatidylethanolamine in isolate H9 and the type strain of *S. noboritoensis*; while unidentified components were discontinuously distributed (Figure 5.10). Isolate H9 produced major proportions of MK9(H_8) (30%), MK9(H_6) (23%) and MK9(H_4) (16%).

Table 5.12 Fatty acid profiles (%) of *Streptomyces* isolate H9 and the type strains of *Streptomyces melanogenes* and *Streptomyces noboritoensis.*

Fatty acid	Isolate H9	*S. melanogenes* NRRL B- 2072^T	*S. noboritoensis* NRRL B-12152^T
$C_{12:0}$	-	0.07	-
Iso-$C_{12:0}$	-	0.04	-
$C_{13:0}$	-	0.06	-
Anteiso-$C_{13:0}$	-	0.15	-
Iso-$C_{13:0}$	0.1	0.32	0.11
$C_{14:0}$	0.12	0.4	0.26
Iso-$C_{14:0}$	5.68	2.33	4.20
$C_{15:0}$	0. 32	2.28	0.55
Anteiso- $C_{15:0}$	34.55	24.14	21.63
Iso-$C_{15:0}$	2.09	12.30	15.40
$C_{15:0}$ w6c	-	0.05	-
Iso- $C_{16:0}$	19.37	12.74	19.42
Iso- H $C_{16:0}$	-	0.15	4.50
Summed feature 3	0.56	1.30	0.85
$C_{16:0}$	10.91	11.66	8.62
$C_{16:1}$ w9c	-	1.04	-
Iso-$C_{17:0}$ w9c	0.70	2.11	2.61
Anteiso-$C_{17:0}$ w9c	0.30	1.65	1.73
Iso-$C_{17:0}$	5.73	9.59	8.62
Anteiso-$C_{17:0}$	17.89	15.01	9.93
$C_{17:1}$ w8c	0.16	0.70	-
$C_{17:0}$	0.5	1.42	0.40
$C_{17:0}$ 2OH	-	0.08	-
Iso-$C_{18:0}$	0.81	0.26	0.43
$C_{18:0}$	0.21	0.10	-
$C_{18:0}$ w9c	-	0.06	-

-, fatty acid not detected; Summed feature 3: 16:1 w7c/15 iso 2 OH.

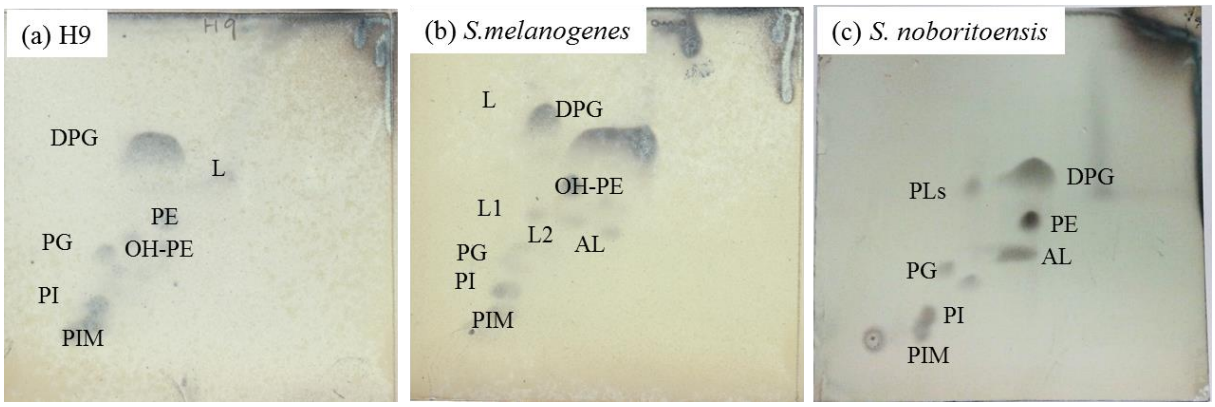


Figure 5.10 Two-dimensional thin-layer chromatography of polar lipids of (a) isolate H9, (b) *Sterptomyces melanogenes* NRRL B-2072^T and (c) *Streptomyces noboritoensis* NRRL B- NRRL B-12152^T stained with molybdenum blue spray (Sigma) using the solvent systems cited in the legend to Figure 5.6. Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; OH-PE, hydroxy phosphatidylethanolamine; PGL, phosphoglycolipid; PG, phosphatodylglycerol, PI, phosphatidylinositol; PIM, phosphatidyl mannosides; PL, phospholipids; AL, aminolipid; and L, unknown lipids.

Phenotypic properties. Identical results were obtained for many of the tests carried out in duplicate on *Streptomyces* isolate H9, *S. melanogenes* NRRL B- 2072^T, *S. noboritoensis* NRRL B-12152^T and *S. polyantibioticus* NRRL B- 24448^T though conflicting results were obtained for some of the BIOLOG tests (Table 5.13). It can also be seen from this Table that isolate H9 can be distinguished from the *S. melanogenes, S. noboritoensis* and *S. polyantibioticus* strains using a broad range of phenotypic properties though it is also apparent that all of the strains have many properties in common.

Table 5.13 Phenotypic properties that differentiate *Streptomyces* isolate H9 from *Streptomyces melanogenes* NRRL B- 2072^T, *S. noboritoensis* NRRL B-12152^T and *Streptomyces polyantibioticus* NRRL B-24448^T.

Phenotypic tests	Isolate H9	*S. melanogenes* NRRL B- 2072^T	*S. noboritoensis* NRRL B-12152^T	*S. polyantibioticus* NRRL B-24448^T
Morphology:				
Spore chains	Spiral	Straight to flexuous[a]	Straight to flexuous[b]	Spores held within sporangia[c]
API ZYM tests:				
α-Chymotrypsin	-	-	-	+
Esterase (C4)	+	-	+	+
β-Galactosidase	+	+	-	+
α-Glucuronidase	+	+	-	+
GEN III BIOLOG microplates:				
Utilization of:				
N-acetyl-D-Galactosamine, D.fructose, inosine, D-mannose	+	+	+	-
L-Arginine	-	-	+	+
D-Aspartic acid, *N*-acetyl-β-D-mannosamine	-	+	-	+
Citric acid	-	-	+	+
D-Fructose-6-phosphate	-	+	-	+
D-Fucose, D-raffinose	+	-	-	-
Guanidine	-	-	+	-
β-hydroxy-Butyric acid	+	-	+	+
β-methyl-D-Glucoside	-	+	-	-
Pectin	+	-	-	-
L-Pyroglutamic acid	+	-	-	+
D-Salicin	-	-	-	+
D-Turanose	+	+	-	-
Inhibition tests:				
Sodium bromate	+	+	+	-
Sodium lactate (1%)	-	-	-	+
Tetrazolium blue	-	-	+	-
Growth in the presence of:				
Sodium chloride (4%, w/v)	+	+	+	-
Growth at:				
pH 5	-	-	-	+
Phenotypic tests:				
Biochemical test:				

Allantoin hydrolysis	-	-	+	+
Degradation tests:				
Arbutin	-	+	-	+
Casein	+	-	-	+
Elastin	-	-	+	+
Hypoxanthine, L-tyrosine	-	+	+	+
Starch	+	-	+	+
Uric acid	+	+	+	-
Tween 40	+	-	+	+
Tween 80	+	-	-	+
Growth at:				
40^{o}C	+	-	-	-

+, positive result; -, negative result.

[a], [b] and [c], data taken from Kämpfer (2012), Isono et al. (1957) and le Roes-Hill and Meyers (2009), respectively.

Positive results recorded for *Streptomyces* isolate H9, *S. melanogenes* NRRL B-2072^{T}, *S. noboritoensis* NRRL B-12152^{T} and *S. polyantibioticus* NRRL B-24448^{T}:

- API ZYM tests: acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase lipase (C8), β-glucosidase, leucine arylamidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase and valine arylamidase.
- GEN III BIOLOG microplates: utilization of L-alanine, γ-amino-n-butyric acid, L-aspartic acid, α-*keto*-butyric acid, L-fucose, α-*keto*-glutaric acid, β-gentiobiose, acetic acid acetoacetic acid, dextrin, D-cellobiose, D-galactose, D-gluconic acid, D-glucose, L-glutamic acid, glycerol, gly-pro, L-histidine, L-malic acid, D-melibiose, 3-O-methyl-D-galactose, propionic acid, L-serine, stachyose, D-trehalose and growth in the presence of potassium tellurite, rifamycin SV and sodium chloride (1%, w/v) .
- Other phenotypic tests: aesculin hydrolysis, adenine degradation and growth at 10^{o}C, 20^{o}C and 30^{o}C

Negative results recorded for *Streptomyces* isolate H9, *S. melanogenes* NRRL B-2072^{T}, *S. noboritoensis* NRRL B-12152^{T} and *S. polyantibioticus* NRRL B-24448^{T}:

- API ZYM tests: α-fucosidase, α-galactosidase, β-glucuronidase and trypsin.
- GEN III BIOLOG microplates: utilization of *N*-acetyl-neuraminic acid, D-arabitol, butyric acid, D-galacturonic acid, L-galactonic acid-γ-lactone, glucuronamide, α-D-lactose, D-malic acid, D-mannitol, mucic acid, quinic acid,

L-rhamnose, D-saccharic acid, D-salicin, D-serine #1, D-serine #2, D-sorbitol and stachyose; resistance to fusidic acid, guanidine hydrochloride, lincomycin, minocycline, niaproof, sodium formate, tetrazolium violet, tetrazolium blue, troleandomycin and vancomycin and growth in the presence of sodium chloride (8%, w/v) and at pH 6.

- Other phenotypic tests: H_2S production, nitrate reduction, urea hydrolysis, degradation of celllulose, chitin, guanine, xanthine, xylan and tributyrin and growth at 4°C, 45°C and 50°C.

Contrasting BIOLOG results obtained for:

(a) Isolate H9: utilization of 3-O-methyl-D-glucose, α-hydroxy-butyric acid and butyric acid and resistance to lithium chloride, nalidixic acid and sodium formate.

(b) *S. melanogenes* NRRL B- 2072^T: utilization of D-maltose and resistance to aztreonam and nalidixic acid.

(c) *S. noboritoensis* NRRL B-12152^T: utilization of bromo-succinic acid, D-glucose-6-phosphate, gelatin, D-maltose and methyl pyruvate and resistance to minocycline.

(d) *S. polyantibioticus* NRRL B-24448^T: utilization of D-fructose, 3-O-methyl-D-glucose, p-hydroxy-phenylacetic acid, D-glucuronic acid, D-lactic acid methyl ester, L-lactic acid and L-rhamnose and resistance to aztreonam, potassium tellurite and rifamycin SV.

5.5 Discussion

The present study provides further evidence that de-replicated filamentous actinobacteria, notably streptomycetes, isolated from Atacama Desert soils are a rich source of novel bioactive compounds (Bull and Asenjo 2013; Bull et al. 2016). Indeed, over 10% of the 57 de-replicated actinobacteria isolated from previously unexplored high altitude soils from Cerro Chajnantor and known to inhibit the growth of wild type *Bacillus subtilis* and/or *Escherichia coli* strains produced new specialized metabolites, notably ones synthesized by isolates H9 and H45, strains shown to represent novel species of *Streptomyces* and *Lentzea,* respectively.

It is especially interesting that the *Lentzea* strain was found to synthesize six novel dienes, lentzeosides A-F, as previously discovered specialized metabolites from Atacama Desert actinobacteria were restricted to the genus *Streptomyces*, notably from *S. leeuwenhoekii* strains which form a deep-seated clade within the evolutionary radiation occupied by the genus *Streptomyces* (Busarakam et al. 2014). Although the genus *Lentzea* was validly published over 20 years ago (Yassin et al. 1995) little is known about its biological properties, apart from the discovery that *Lentzea sp.* strain 7887 is able to biotransform FR901459, a novel derivative of cyclosporine (Sasamura et al. 2015).

Chemical screening of *Lentzea* strain H45 on two production media led to the isolation of six new and one known diene and the known compound (Z)-3-hexenyl glucoside. The structural motif of lentzeosides A-D have not been reported in nature though those of lentzeosides E and F have been previously detected, albeit from plant sources (Francis et al. 2004). It was particularly interesting that some of the novel lentzeosides inhibited HIV integrase activity *in vitro*. Treatment of this immune-deficient disease usually involves combination therapy based on different drugs that target different stages of the viral replication cycle in order to overcome resistance development due to the high mutation rate of the virus (Maes et al. 2012). HIV integrase is one of the key enzymes in the virus replication cycle as it is responsible for the integration of the reverse transcribed viral cDNA into the host cell genome (Sherman and Greene 2002). Raltegravir® is the first Federal Drug Agency clinically approved HIV integrase inhibitor used for the treatment of HIV infection in treatment-experimented adult patients who showed evidence of viral replication and HIV-1 strain resistance to multiple antiretroviral agents (Hicks and Gulick 2009). It is especially interesting that lentzeosides C, D and E showed moderate inhibitory activity compared with lentzeosides A, B and F despite the close structural similarities of these compounds.

In addition to the phylogenetic data, isolate H45 was shown to have chemotaxonomic and morphological properties in line with its assignment to the genus *Lentzea* (Yassin et al. 1995; Cao et al. 2015; Li et al. 2012) as it formed a branched substrate mycelium, aerial hyphae that fragmented into rod-shaped elements, produced whole-cell hydrolysates rich in A_2pm, galactose, mannose and ribose, complex mixtures of *iso* and *anteiso*-fatty acids, predominant amounts of tetrahydrogenated menaquinones with nine isoprene units and a polar lipid pattern that

included diphosphatidylglycerol, phosphatidylethanolamine, hydroxyl-phosphatodylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The isolate was most closely related to *L. kentuckyensis* NRRL B-24416^T given 16S rRNA gene sequence data, but was distinguished from the latter based on low levels of DNA:DNA relatedness, differences in fatty acid profiles and by a range of phenotypic characteristics. On the basis of these data it is proposed that isolate H45 represents a novel species of *Lentzea* for which the name *Lentzea chajnantorensis* sp. nov. is proposed.

Similarly, isolate H9 was found to have chemotaxonomic and morphological properties consistent with its classification in the genus *Streptomyces* (Kämpfer 2012; Busarakam et al. 2014) , as exemplified by its ability to form extensively branched substrate hyphae and masses of spore bearing aerial hyphae on ISP media, to produce whole-cell hydrolysates rich in LL-A_2pm, complex mixtures of saturated and unsaturated fatty acids and predominant proportions of tetra-, hexa- and octahydrogenated menaquinones. The organism exhibited a broad range of phenotypic properties, including cultural and morphological features found to be especially useful in distinguishing between *Streptomyces* species by Labeda and his colleagues (2012) in their phylogenetic analyses of the type strains *Streptomyces* species and related taxa. Isolate H9 is only loosely associated with its closest phylogenetic neighbours based on comparative 16S rRNA gene sequence data, a distinctness that was underlined by corresponding MLSA data derived from concatenated sequences of the five housekeeping alleles, and by a range of phenotypic features, notably cultural and morphological characteristics. The isolate can be separated readily from *S. melanogenes* NRRL B-2072^T, *S. noboritoensis* NRRL B-12152^T and *S. polyantibioticus* NRRL B-24448^T by its ability to form spiral chains of spores, utilization of N-acetyl-D-galactosamine, D-fructose, D-mannose and pectin and growth at 40^{o}C. In turn, the type strains of these species can be distinguished from the isolate by their ability to form straight to flexious chains of spores and by their ability to degrade hypoxanthine and L-tyrosine. . In light of these genotypic and phenotypic features it is proposed that isolate H9 be recognised as a new *Streptomyces* species, namely *Streptomyces aridus* sp. nov..

Description of *Lentzea chajnantorensis* sp. nov.

Lentzea chajnantorensis (chaj.nan.tor.en'sis. N. L. fem. adj. *chajnantorensis,* named after the Chajnantor Plateau, the source of the isolate).

Aerobic, Gram-positive actinobacterium which tends to form branched, pale yellow substrate mycelia and white to pinkish white aerial hyphae on ISP media; soluble pigments are not formed on these media. Aerial hyphae fragment into rod-shaped elements. Grows from 10-40ºC, optimally at 28ºC, from pH 4-10, optimally ~pH 7.0 and in the presence of up to 5%, w/v sodium chloride. Additional phenotypic properties are cited in the text and in Table 5.6 and Table 5.8. Chemotaxonomic features are typical of the genus *Lentzea.* Produces novel dienes, lentzeosides A-F. Lentzeosides C, D and E inhibit HIV integrase activity.

The type strain, H45^T (=NCIMB 14966^T = NRRL B-65282^T) was isolated from a subsurface soil sample collected at 5048 metres above sea level on the Chajnantor Plateau, near San Pedro de Atacama in north eastern Chile. The GenBank accession number of the 16S rRNA gene sequence of isolate H45^T is LT009512.

Description of *Streptomyces aridus* sp. nov.

Streptomyces aridus (a'ri. dus. L. masc. adj *aridum,* dry, referring to the isolation of the strain from arid soil.

Aerobic, Gram-stain-positive, catalase-positive actinobacterium which forms an extensively branched substrate mycelium that bears aerial hyphae that differentiate into spiral chains of smooth spores (1-1.5µm x 0.5 µm) on oatmeal agar. A brown black diffusible pigment is produced on yeast-extract- malt extract agar. Grows from 10-40ºC, optimally ~28ºC, from pH 5 – 10, optimally ~pH 7.0 and in the presence of up to 2.5%, w/v NaCl. Additional phenotypic features are cited in the text and in Table 5.9 and Table 5.13. Chemotaxonomic characteristics are typical of the genus *Streptomyces.* Produces polyunsaturated compounds and a novel metabolite with the provisional molecular formula $CH_{30}H_{43}N_7O_7$.

The type strain, H9^T (=NCIMB 14965^T =NRRL B-65268^T) was isolated from a subsurface soil sample collected at 4000 metres above sea level on Cerro Chajnantor,

near San Pedro de Atacama in north eastern Chile. The GenBank accession number for the 16S rRNA gene sequence of isolate H9 is LT594571.

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Chapter 6. Actinobacterial Rare Biospheres and Dark Matter Revealed in Habitats of the Chilean Atacama Desert

6.1 Abstract

The Atacama Desert is one of the most extreme biomes on Earth with the aridity of its core region considered to represent the dry limit for life. Metagenomic analysis of the distribution of actinobacteria at hyper-arid and extreme hyper-arid locations in the Desert has exposed remarkable taxonomic diversity. The extent of actinobacterial 'dark matter' in this biome is evidenced by a detected increase of 16% inthese bacteria at the Family rank than is currently validated. Rank-abundance analyses indicated that hyper- and extreme hyper-arid soils were high-diversity habitats and that the great majority of designated 'rare' genera were always rare. A core actinobacterial microbiome common to both habitats was composed of members of the sub-order *Geodermatophilales* (namely *Blastococcus, Geodermatophilus, Modestobacter*) the families *Micrococcaceae, Micromonosporaceae, Propionibacteriaceae, Streptomycetaceae*), the genus *Sporichtya* and three unclassified genera. The great majority of detected taxa have not been recovered by culture dependent methods, neither, with very few exceptions, has their functional ecology started to be explored. However, a microbial seed bank of this magnitude may have significance in Atacama soil ecosystem resilience while in addition offering propitious opportunities for biotechnology discovery programmes.

6.2 Introduction

Interest in extremophilic and extremotrophic microorganisms has grown over the past forty years as microbiologists recognized that extreme environments were capable of sustaining life. It is now known that the extremobiosphere encompasses all of the physico-chemical variables on Earth and that many microorganisms, including actinobacteria, have evolved tolerances to extreme conditions (Bull 2011). Members of the phylum *Actinobacteria* (Goodfellow et al., 2012a) are found throughout the extremobiosphere, not least in desert soils which account for nearly a quarter of terrestrial ecosystems of which a third are classified as either hyper-arid (ratio of mean

annual rainfall to mean annual evaporation (MAR) less than 0.05) or extreme hyper-arid (ratio < 0.002) (Houston 2006).

The Atacama Desert is the oldest and continuously driest non-polar environment on Earth and apart from its aridity it is unique in its range of habitats, its geology and geochemistry, its elevation and topography, and its radiation intensities (Bull et al. 2016). Small numbers of cultivable, but taxonomically diverse actinobacteria have been recovered from a range of Atacama soils (Okoro et al. 2009; Busarakam 2014), regoliths and rock surfaces, including ones considered to represent "the dry limit of microbial life" (Navarro-González et al. 2003), and from which have been isolated several new species and natural products with encouraging bioactivities (Nachtigall et al. 2011; Elsayed et al. 2015) and whole genome sequences determined (Gomez-Escribano et al. 2015; Busarakam et al. 2016b).

High throughput molecular techniques have recently been applied to microbial community analyses in the Atacama Desert, notable examples being those of Neilson et al. (2012) who found that actinobacteria were dominant in hyper-arid margin soils, and Crits-Christoph et al. (2013) who studied bacterial colonization patterns along a longitudinal moisture gradient; both of these groups employed the Roche GS-FLX sequencing system. The present study was developed to be a first step in such an approach specifically to actinobacterial diversity in the Atacama Desert based on 454 pyrosequencing. This particular platform was chosen due to its high classification efficiency (Claesson et al. 2010), a decision that is supported by the findings of Bowen et al. (2012) who found that deep pyrosequencing of 16S tags was well suited for distinguishing site specific similarities and differences among rare taxa.

The primary aim of this study was to apply Whittaker's classic diversity indices (Whittaker 1960) to actinobacterial diversity of a hyper-arid and extreme hyper-arid landscape in the Atacama Desert at five distinct habitats covering an altitude range of approximately 900-2500 mabsl. The analyses were restricted to genus and family taxonomic ranks but no attempt was made to correlate local diversity (*sensu* α-diversity, Whittaker *et. al.*, 2001) with environmental factors. This desert landscape was considered to be ideal for exploring the rare biosphere concept, and the notion that actinobacterial signatures can be used to define habitats. The results obtained reinforce the view that rather than being devoid of life the Atacama Desert is a microbial treasure trove waiting to be explored.

6.3 Materials and Methods

6.3.1 Sample collection and research landscape

Soil samples Table 6.1 were collected from selected locations in the Atacama Desert landscape Figure 6.1 between 2010 and 2014, as described in Chapter 2. Samples were collected aseptically with implements sterilized in the field with ethanol and contained in sterile polycarbonate bottles. Sampling was made between 11:00 and 16:00 hours when temperature and relative humidity at the lower elevation sites ranged between 34°-38°C and 5-20% respectively, and 30°-33ºC and 3-18%, respectively at the higher altitudes. Following transport to the UK all samples were stored at 4ºC.

The Yungay area is the extreme hyper-arid core of the Atacama Desert and is considered to be the closest analogue of Martian soils on Earth (Opfell and Zebal 1967). A large proportion of the area is encrusted with halite and super-rich in nitrates, while certain slopes on the Cerros Aguas Blancas present evidence of water erosion in geological time. Two Yungay sites were sampled: (1) nitrate rich soil from a tamarisk oasis (Y2); and (2) transect samples from the Cerros Aguas Blancas (CAB series, Y6) WSW of the Cerro Caballo Muerto. (3) The Lomas Bayas region another extreme hyper-arid environment and a centre of copper mining located north east of Antofagasta. Samples (LB) were collected at non-mining sites. (4) Sampling was made on the eastern slope of Cerro Paranal adjacent to Route B-710 linking Antofagasta and the coastal village of Paposo. (5) The Salar de Atacama is the largest salt flat in Chile within which the Laguna Chaxa is an area of open water and highly crystalline salt encrusted soils. Samples (CHX) were collected from the halite soils. (6) The Valle de la Luna is an extreme hyper-arid area in the Cordillera de la Sal; the sample (VDL) was collected at a sand formation site. Small diversities and low numbers ($10^2 – 10^3$ cfu g^{-1} soil) of culturable actinobacteria have been recovered from each of these sampling sites (Okoro et al. 2009; Busarakam 2014).

Table 6.1 Sampling sites

Research location	Sampling site and code	Collection date	Sample description
Yungay	(1) Tamarisk oasis		
	Y2	11.11.2010	Degraded tamarisk leaves and surface nitrate soil
	(2) Cerros Aguas Blancas		
	Y6_1	13.11.2010	Extreme hyper-arid site, fine white soil (Surface 2cm)
	Y6_2	13.11.2010	Sub-surface (30 cm)
	Y6_3	13.11.2010	Sub-surface (100 cm)
	CAB2	30.10.2011	Surface (2cm)
	CAB3	30.10.2011	Surface (2cm)
Lomas Bayas	LB1	26.10.2012	Extreme hyper-arid, surface soil
	LB3	27.02.2014	
Cerro Paranal	POP2	30.10.2011	Coarse sandy hyper-arid surface soil
Salar de Atacama	Laguna Chaxa		
	CHX1	26.10.2012	Hyper-arid halite (Surface 2 cm)
	CHX2	26.10.2012	Sub-surface (30 cm)
	CHX3	26.10.2012	Soil colonized by cyanobacteria
Cordillera de la Sal	Valle de la Luna		
	VDL	05.10.2004	Extreme hyper-arid, sand

The precise locations of these sites can be found in Table 2.1, Chapter2.

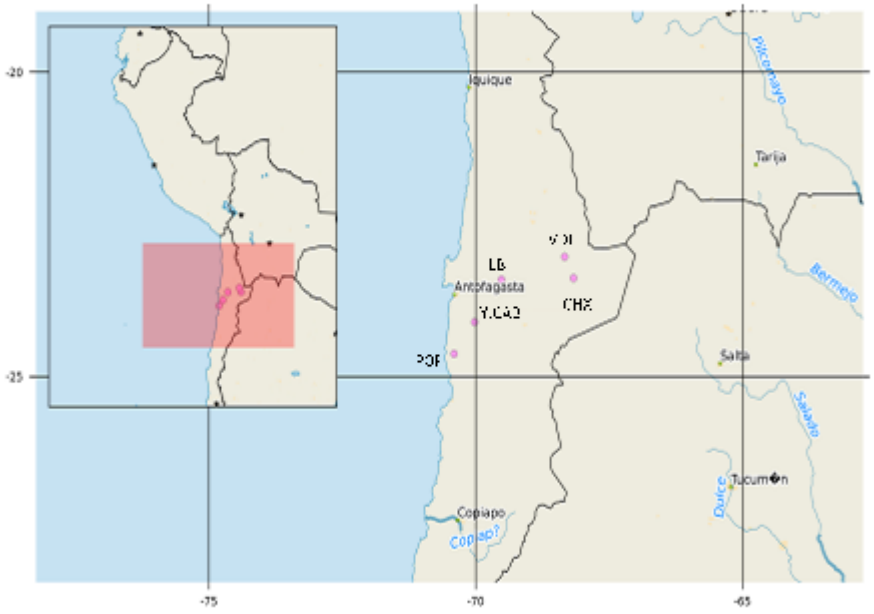


Figure 6.1 Location of Atacama Desert Research in northern Chile; site codes: Yungay (Y); Cerros Aguas Blancas (CAB); Lomas Bayas (LB); Cerro Paranal (POP); Salar de Atacama (CHX); Cordillera de la Sal (VDL).

6.3.2 DNA extraction, PCR amplification and pyrosequencing

Total community DNA was extracted from all of the environmental samples using the proprietary UltraClean Soil DNA extraction kit (MOBIO Laboratories, Inc., USA) following the manufacturer's protocol, and stored at -20°C. Soil extractions were made in a laminar flow chamber using sterilized equipment in order to avoid contamination. The quality of community DNA preparations was checked by agarose gel electrophoresis, as described in Chapter 2. Actinobacterial specific regions in community DNA preparations were amplified using the primers Com2xf (5'-AAA CTC AAA GGA ATT GAC GG-3') and Ac1186r (5'- CTT CCT CCG AGT TGA CCC-3') Schäfer et al. (2010). Unique, sample-specific barcodes (10 bp) were attached to the 5' end of the forward primer. Four replicates were constructed from each of the environmental samples; the latter were assigned individual barcodes.

Polymerase chain reactions and purification of the amplified products were achieved as described in Chapter 2 and the purified products kept at -20°C prior to use. Sample concentrations were quantified using a Qubit® Fluorometer (Invitrogen, CA)

and adjusted to give a final concentration of 100µg/mL; sample quality was checked using an Agilent Bioanalyzer by NewGene Ltd. (Newcastle upon Tyne, UK). Pyrosequencing was made using the Roche GS-FLX+ system (454 Life Sciences, Branford, CT) at the WM Keck Center for Comparative and Functional Genomics, University of Illinois, USA, as described in the manufacturer's protocol.

6.3.3 Bioinformatic analysis

High quality reads were processed using CLCommunity v3.30 software (www.chunlab.com) at ChunLab Inc., Seoul National University, Seoul, Korea), as described in Chapter 2. Rarefaction and rank-abundance analyses, Shannon diversities and Chao1 richness estimations were calculated using the CLCommunity software. Venn diagrams were constructed using the Mothur version 1.37.4 program (http://www.mothur.org/).

6.4 Results and Discussion

Over 90k sequence reads were obtained from the 12 sites representing 67 actinobacterial families, 34% of which could not be assigned to validly published taxa and hence are regarded as putatively novel candidate families (Appendix 6, Table A). The large majority of identified families belonged to the class *Actinobacteria* (Goodfellow, 2012b) but in addition a few representatives of deep lineage actinobacteria belonging to the classes *Acidimicrobia* (Clark and Norris 2015) and *Nitriliruptoria* (Ludwig et al. 2012) were detected. The total diversity at the generic rank numbered 297 of which only 61% of which were assigned to validly published taxa (Appendix 6, Table B).

Pyrosequencing data for microbiota diversities in other world deserts show significant variations in the occurrence of actinobacteria. Thus, sandy Asian deserts such as the Gobi, Gurbantünggütt and Taklamaken are reported to have small (2-18%) actinobacterial communities (An et al. 2013; Li et al. 2015). In contrast, soils of certain western deserts such as the Atacama and Sonoran are heavily dominated by actinobacterial phylospecies (75-88%, Neilson *et al.,* 2012; Crits-Christoph *et al.,* 2013). Culture-dependent values obtained for the sites examined in the present study are 50-70% (hyper-arid) and up to 100% in certain extreme hyper-arid samples (Valle de la Luna). Some caution should be exercised in reviewing such measures of

dominance, or otherwise, as actinobacterial counts are influenced significantly by the selective media used for their isolation (Okoro et al. 2009). The taxonomic megadiversity of actinobacteria in the Atacama Desert landscape as revealed for the first time in this study argues strongly for their successful adaptation to extreme resource-depleted conditions.

6.4.1 Actinobacterial taxon richness and diversity coverage

Rarefaction analysis. Construction of rarefaction curves enabled comparison of genus richness and gauge the extent to which total diversity had been recovered at each of the Atacama Desert sites (Figure 6.2). In most cases the major extent of the actinobacterial community diversities had been captured by the sequencing campaign at the 1500-sequence level but in one case (CAB3) the sequence level obtained fell well below this value and likely compromised subsequent analyses of this site. Whereas some of the curves closely approached asymptotes, others remained curvilinear indicating that considerable diversity remained to be sequenced. Thus it is important to recognize that taxon counts are strictly comparable only when richness is asymptotic. The efficiencies of sequence capture in a few of the sites examined (Yungay Y6.1, Y6.2, Y6.3; Chaxa Laguna CHX1, CHX2) were not influenced by sampling depth.

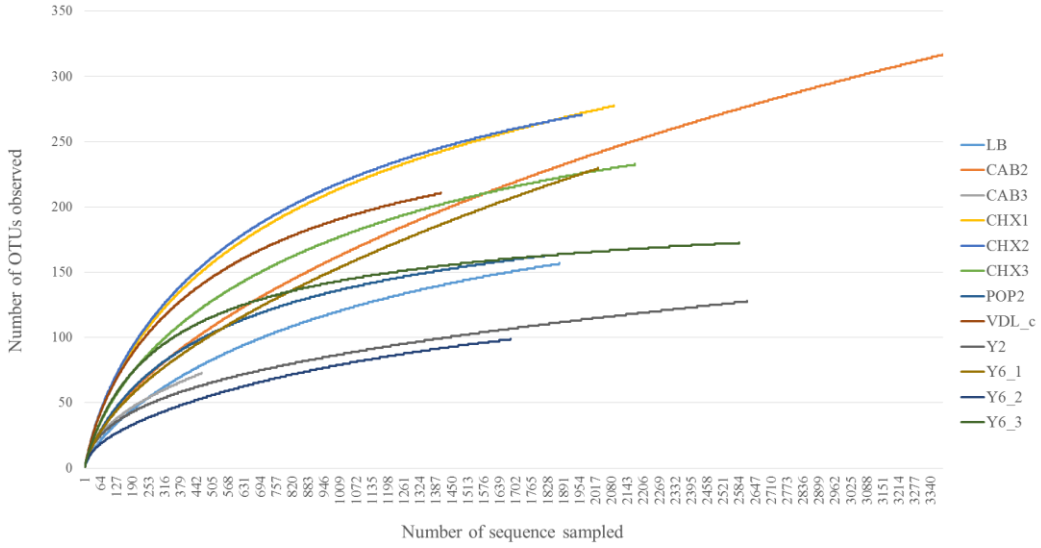


Figure 6.2 Rarefaction analysis of the extreme hyper-arid and hyper- arid OTUs at 94.5-97% phylogenetic similarity.

Application of Good's library coverage index indicated that 90% to 97% of genera were recovered at all sites. Table 6.2 presents total OTUs (total observed validly published genera, plus unidentified genera) and α-diversity indices.

Table 6.2 Observed OTUs and α-diversity indices

Sampling site and depth (cm)	Observed OTUs (Genera)	Chao1 Richness	Shannon Index *(H)*	Simpson Diversity (1-*D)*
1. Extreme hyper-arid locations[1]				
Yungay				
Y6_1 (2)	186	316	4.01	0.948
Y6_2 (30)	87	134	2.78	0.873
Y6_3 (100)	159	222	3.87	0.949
CAB2 (2)	175	331	3.73	0.941
CAB3 (2)	63	110	3.40	0.952
Lomas Bayas				
LB (2)	162	264	3.54	0.901
Cordillera de la Sal				
VDL (2)	185	242	4.29	0.964
2. Hyper-arid locations[2]				
Laguna Chaxa				
CHX1 (2)	222	306	4.48	0.977
CHX2 (30)	208	295	4.38	0.966
CHX3[3] (2)	171	247	3.73	0.932
Cerro Paranal				
POP2 (2)	157	228	3.40	0.890
Yungay Oasis				
Y2[4] (2)	89	137	3.21	0.935

[1] MAR 0.002, [2]MAR 0.009, [3] Partly colonized by cyanobacteria, [4]Tamarisk grove

Only a small decrease (*ca.* 7% average) was found in the observed OTUs recovered from surface soil samples as a function of increased aridity, a somewhat unexpected result given the generally held view that the more extreme an environment the lower the number of taxa. Comparable data for other desert environments are largely lacking but increased salinity was not observed to be a determinant of species richness in saline lakes of the Monegros Desert in NE Spain (Casamayor et al. 2013). Neither was a major change in overall actinobacterial ecological diversity (*H*, 1-*D*) observed between extreme hyper-arid and hyper-arid locations, a result indicating that they are dominated by relatively few abundant taxa. Chao 1 predicted richness was high at both types of location exceeding the observed OUT numbers by 63% (extreme hyper-arid) and 41% (hyper-arid) and providing further evidence of considerable diversity yet to be sequenced. It should be noted that non-parametric richness estimators such as Chao 1 predict counts of the number of taxa (in this case genera and families) present at a site but cannot be used to compare the genetic diversity between sites (Stach and Bull 2005).

Rank-abundance distributions. For the purpose of defining rank-abundances in Atacama Desert soils, a relative cut-off of 0.1% was used (Pedrós-Alió 2012; Lynch and Neufeld 2015) below which threshold a rare actinobacterial biosphere can be defined; and an arbitrary threshold of 10% to define abundant taxa (Ugland and Gray 1982; Shade et al. 2012; Lynch and Neufeld 2015). Rank-abundance curves for all of the sampling sites are shown in Figure 6.3a while typical curves for extreme hyper-arid and hyper-arid sites are shown in Figure 6.3b.

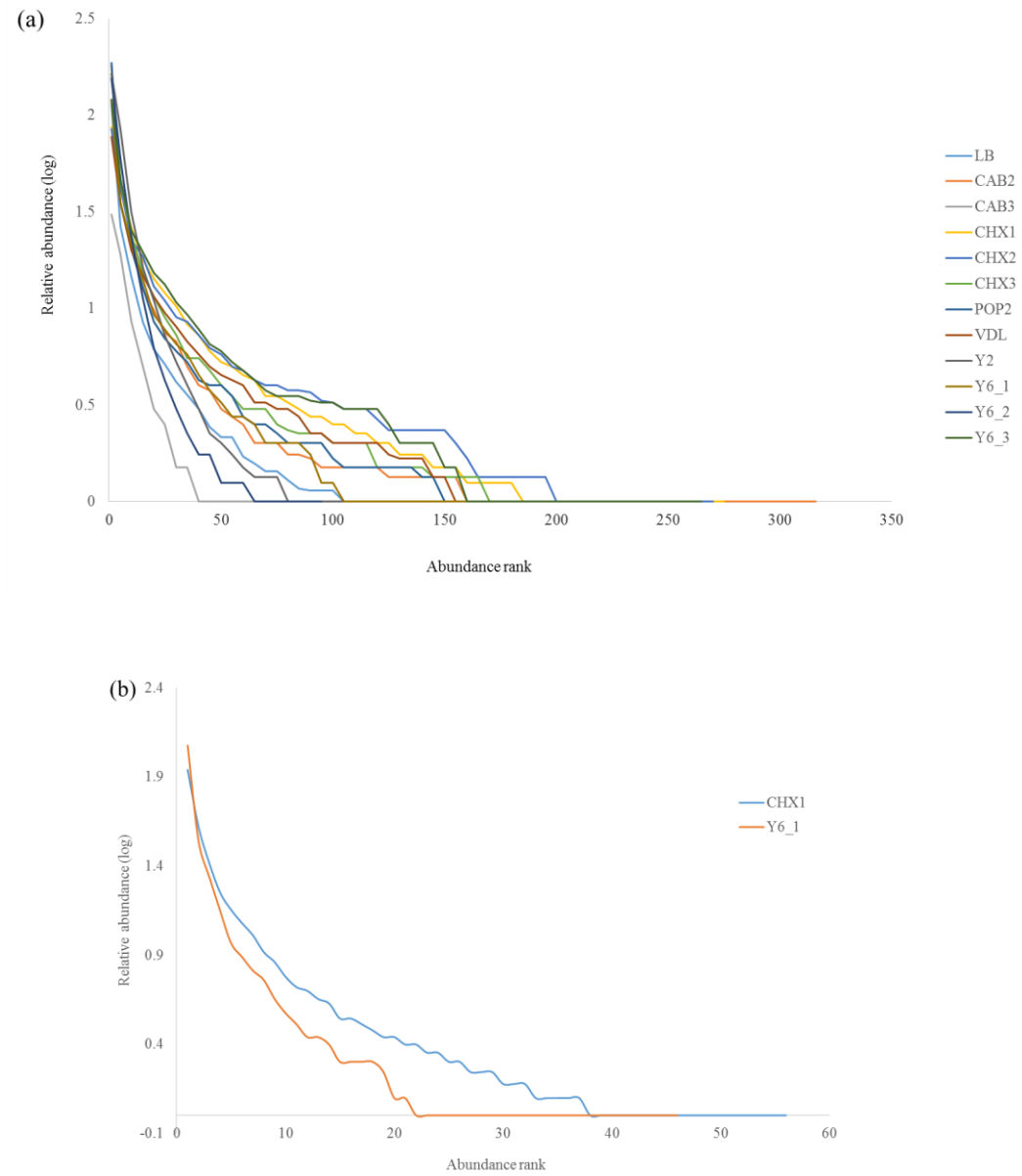


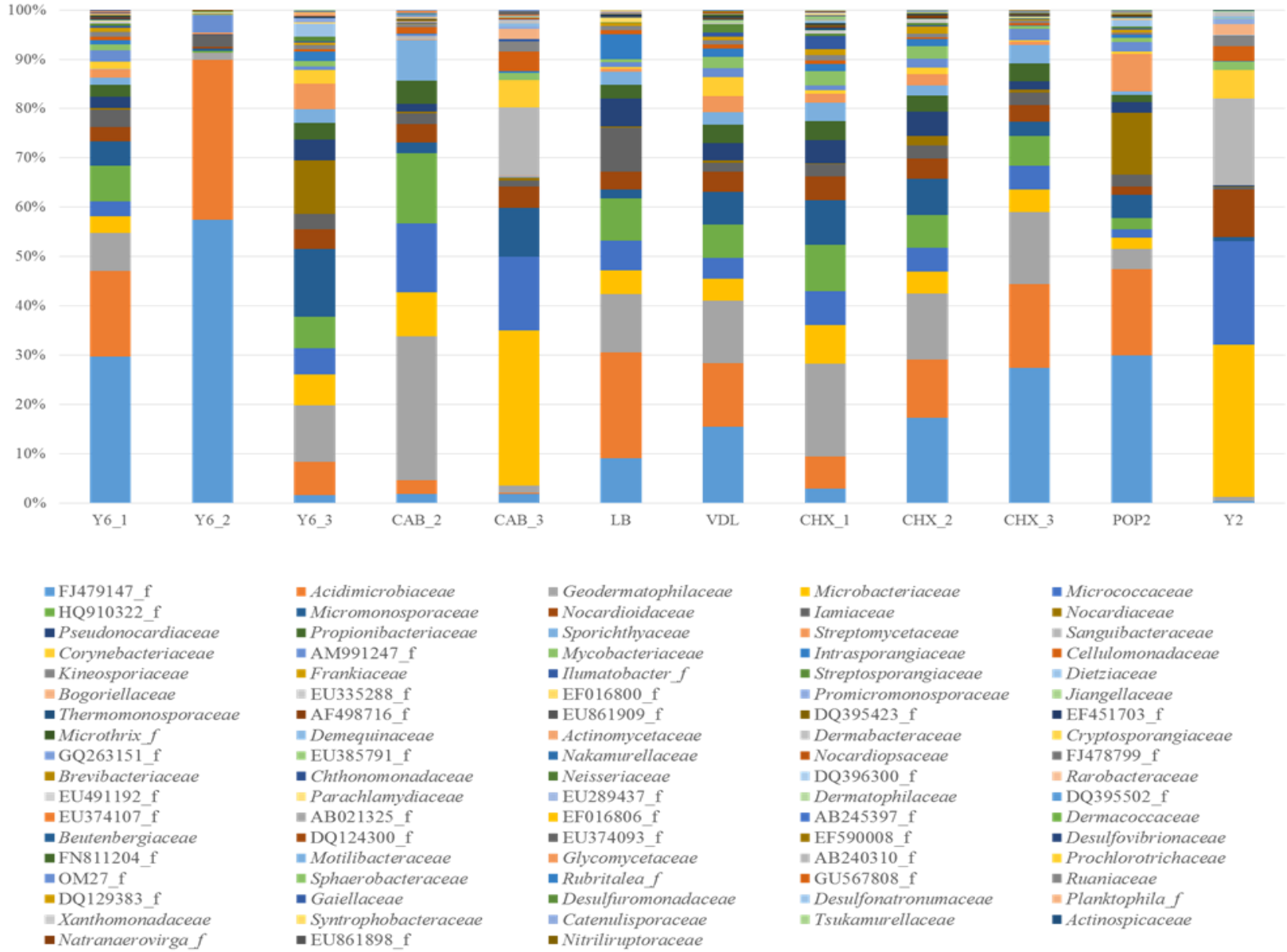
Figure 6.3 Rank-abundance curves. (a) All sites, (b) extreme hyper-arid (Y6.1) vs. hyper-arid (CHX1) surface community rank-abundance profiles.

Salient points arising from this analysis: (1) the majority of curves indicate high-diversity environments, i.e. shallow curves and long tails representing a rare biosphere; (2) the great majority of rare generic OTUs in the Atacama Desert habitats were always rare (> 95%) and were not found as abundant at any of the environments examined; (3) the proportions of abundant and rare actinobacterial taxa were similar at extreme hyper-arid and hyper-arid sites (9-17% and 9-15% abundant vs. 46-62% and 48-61% rare genera respectively, illustrative curves are shown in Figure 6.3b; (4) on the basis of a small number of comparisons, the composition of the abundant

communities in surface, sub-surface and deep samples was similar (e.g., Cerros Aguas Blancas sites at 2, 30 and 100 cm were dominated by *Blastococcus, Verrucosispora* and unidentified OTUs FJ479147, HQ674860 and HQ910322; while Laguna Chaxa sites at 2 and 30 cm were dominated by *Arthrobacter*, *Blastococcus* and unidentified OTU HQ910322); (5) the taxonomic constancy of abundant OTUs observed as a function of soil depth argues for long term habitat stability and minimal atmospheric aerosol dispersal of Atacama Desert microbiota, a conclusion supported by aerosol optical depth measurements (Cordero et al. 2016); and (6) at generic and family ranks a very much higher number of unassigned OTUs were evident in the rare biosphere confirming that the Atacama Desert landscape represents a vast reservoir of microbial dark matter.

6.4.2 Taxonomic diversity

The profiles of actinobacterial family lineages throughout the landscape locations were similar and dominated by members of the families *Acidimicrobiaceae, Geodermatophilaceae, Iamiaceae, Microbacteriaceae, Micrococcaceae, Micromonosporaceae, Nocardiaceae* and *Nocardioidaceae* and two unidentified taxa, FJ479147_f and HQ910322_f (Figure 6.4a). The relative abundance of the top ten families (Figure 6.4b) shows clearly how community structure was affected by soil depth (Y6 and CHX series) and how at two sites (Y2, CAB3) the communities were dominated by OTUs belonging to the family *Microbacteriaceae*. At this stage there are insufficient data to interpret the shifts in the structures of the latter communities except to note that site Y2 was partially vegetated, and that variations in community structure observed at sites Y6.1, CAB2 and CAB3 reinforce the importance of recognising spatial and temporal factors in habitat sampling (Aguilera et al. 2016). The majority of the most abundant identified families belong to the orders *Acidimicrobiales, Geodermatophiales, Micrococcinales* and *Micromonosporiales* (Goodfellow et al. 2012a; Sen et al. 2014) actinobacterial taxa notable for their extremophilic and extremotrophic members (Bull 2011). Currently 58 validly published families are recognised in the phylum *Actinobacteria* (www.bacterio.net at 19/05/2016); consequently the additional 30 taxa recorded as 'families_f' constitute a massive element of actinobacterial dark matter in the Atacama landscape.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Y6_1
Y6_2
Y6_3
CAB_2
CAB_3
LB
VDL
CHX_1
CHX_2
CHX_3
POP2
Y2
FJ479147_f
Acidimicrobiaceae
Geodermatophilaceae
Microbacteriaceae
Micrococcaceae
HQ910322_f
Micromonosporaceae
Nocardioidaceae
Iamiaceae
Nocardiaceae
Pseudonocardiaceae
Propionibacteriaceae
Sporichthyaceae
Streptomycetaceae
Sanguibacteraceae
Corynebacteriaceae
AM991247_f
Mycobacteriaceae
Intrasporangiaceae
Cellulomonadaceae
Kineosporiaceae
Frankiaceae
Ilumatobacter_f
Streptosporangiaceae
Dietziaceae
Bogoriellaceae
EU335288_f
EF016800_f
Promicromonosporaceae
Jiangellaceae
Thermomonosporaceae
AF498716_f
EU861909_f
DQ395423_f
EF451703_f
Microthrix_f
Demequinaceae
Actinomycetaceae
Dermabacteraceae
Cryptosporangiaceae
GQ263151_f
EU385791_f
Nakamurellaceae
Nocardiopsaceae
FJ478799_f
Brevibacteriaceae
Chthonomonadaceae
Neisseriaceae
DQ396300_f
Rarobacteraceae
EU491192_f
Parachlamydiaceae
EU289437_f
Dermatophilaceae
DQ395502_f
EU374107_f
AB021325_f
EF016806_f
AB245397_f
Dermacoccaceae
Beutenbergiaceae
DQ124300_f
EU374093_f
EF590008_f
Desulfovibrionaceae
FN811204_f
Motilibacteraceae
Glycomycetaceae
AB240310_f
Prochlorotrichaceae
OM27_f
Sphaerobacteraceae
Rubritalea_f
GU567808_f
Ruaniaceae
DQ129383_f
Gaiellaceae
Desulfuromonadaceae
Desulfonatronumaceae
Planktophila_f
Xanthomonadaceae
Syntrophobacteraceae
Catenulisporaceae
Tsukamurellaceae
Actinospicaceae
Natranaerovirga_f
EU861898_f
Nitriliruptoraceae

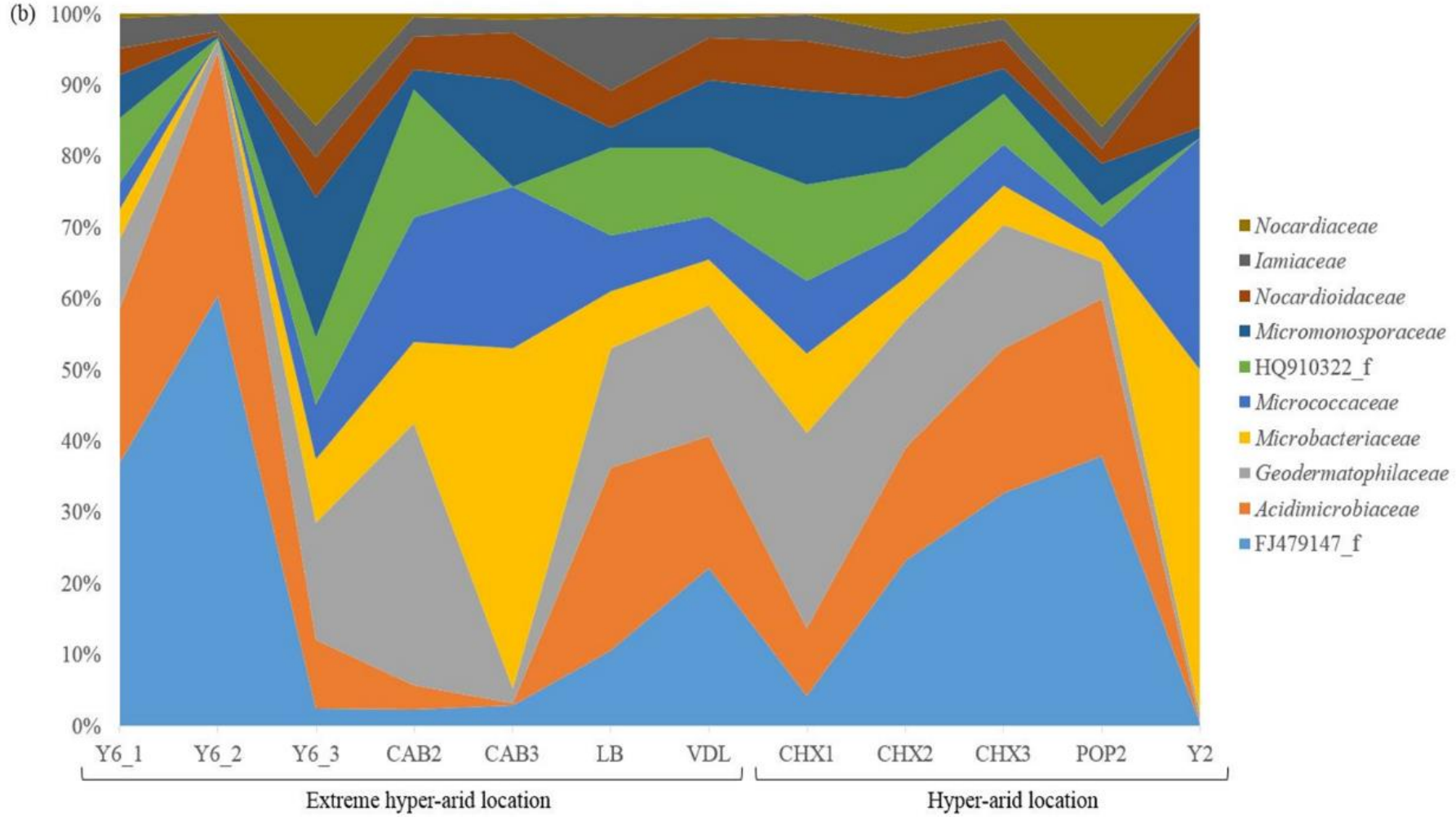
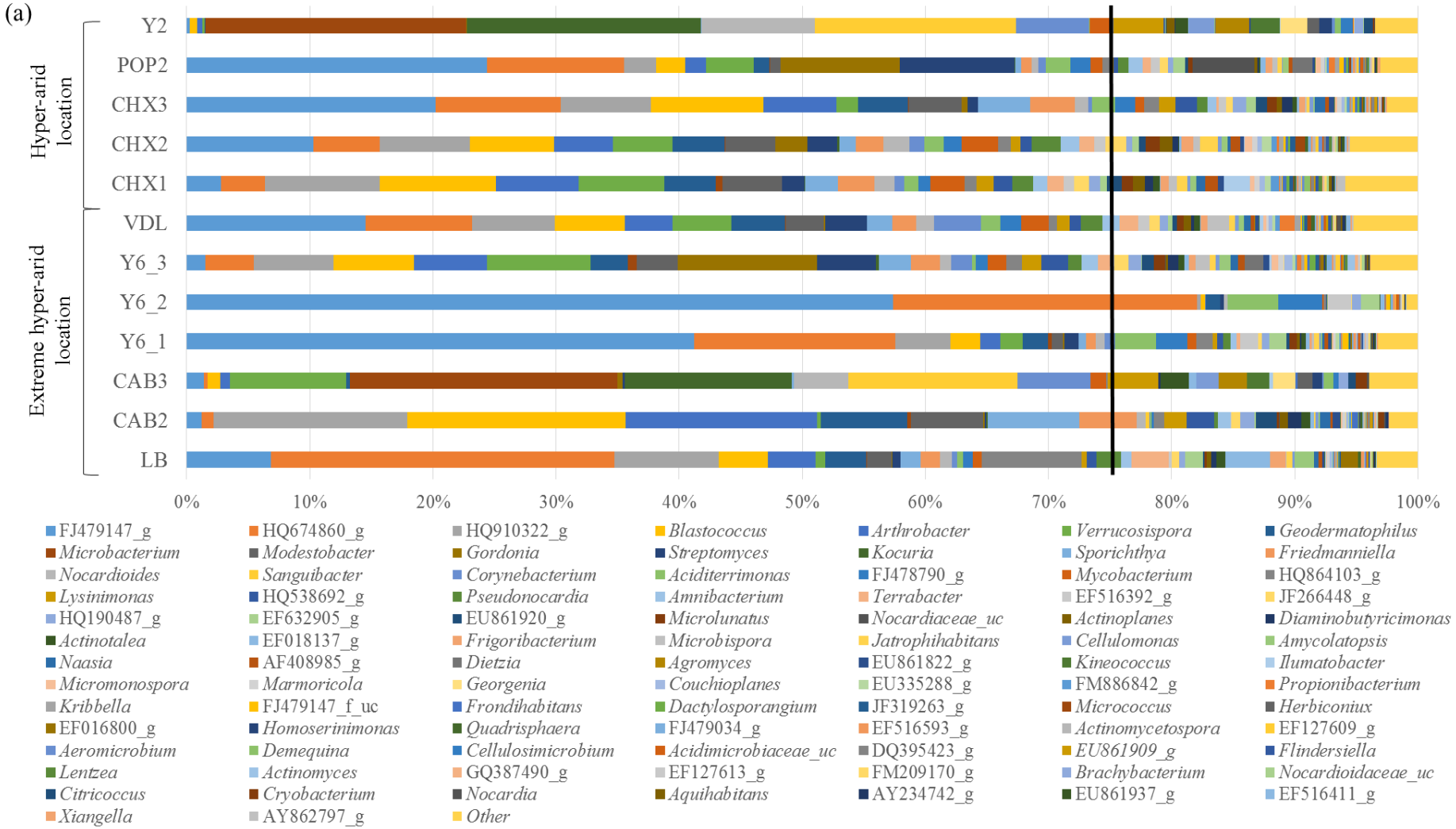


Figure 6.4 Relative abundance of actinobacterial families detected in Atacama Desert locations. (a) Relative abundance of all 88 detected families, (b) relative abundance of the top 10 most frequently detected families.

The total number of generic OTUs in each sample is too large to render relative abundance diagrams readily decipherable at the 0.1% cut-off level, consequently data presentation is shown for 1% and 2.5% cut-off levels in Figures 6.5; the full list of the 297 genera is contained in Appendix 6. The genera dominating surface soils were *Arthrobacter, Blastococcus, Freidmanniellla, Geodermatophilus, Modestobacter, Sporichthya and Verrucosipsora,* and three unidentified putatively novel taxa: FJ479147_g, HQ674860_g and HQ910322_g. Although the proportions of these genera varied from site to site, generally they comprised 75% of the community (Figure 6.5a) and, predictably, showed high correspondence with family dominance at the same sites. Figure 6.5b defines the distinctive nature of sites CAB3 and Y2 within which the genera *Kocuria, Microbacterium* and *Sanguibacter* dominated and comprised 48% and 58% of these communities, respectively. The few genera recorded as deep lineage actinobacteria (*Aciditerrimonas, Iamia, Ilumatobacter, and Nitriliruptoraceae*) were detected at very low read levels at certain sites and can be regarded as constituents of the rare actinobacterial biosphere (see below) in the Atacama Desert landscape. Although the family *Acidimicrobiaceae* was found to be among the dominant families, *Ilumatobacter* was the only member genus detected and in most cases as a constituent of the rare biosphere.



*OTUs that were detected below 1% are grouped as 'Other'.

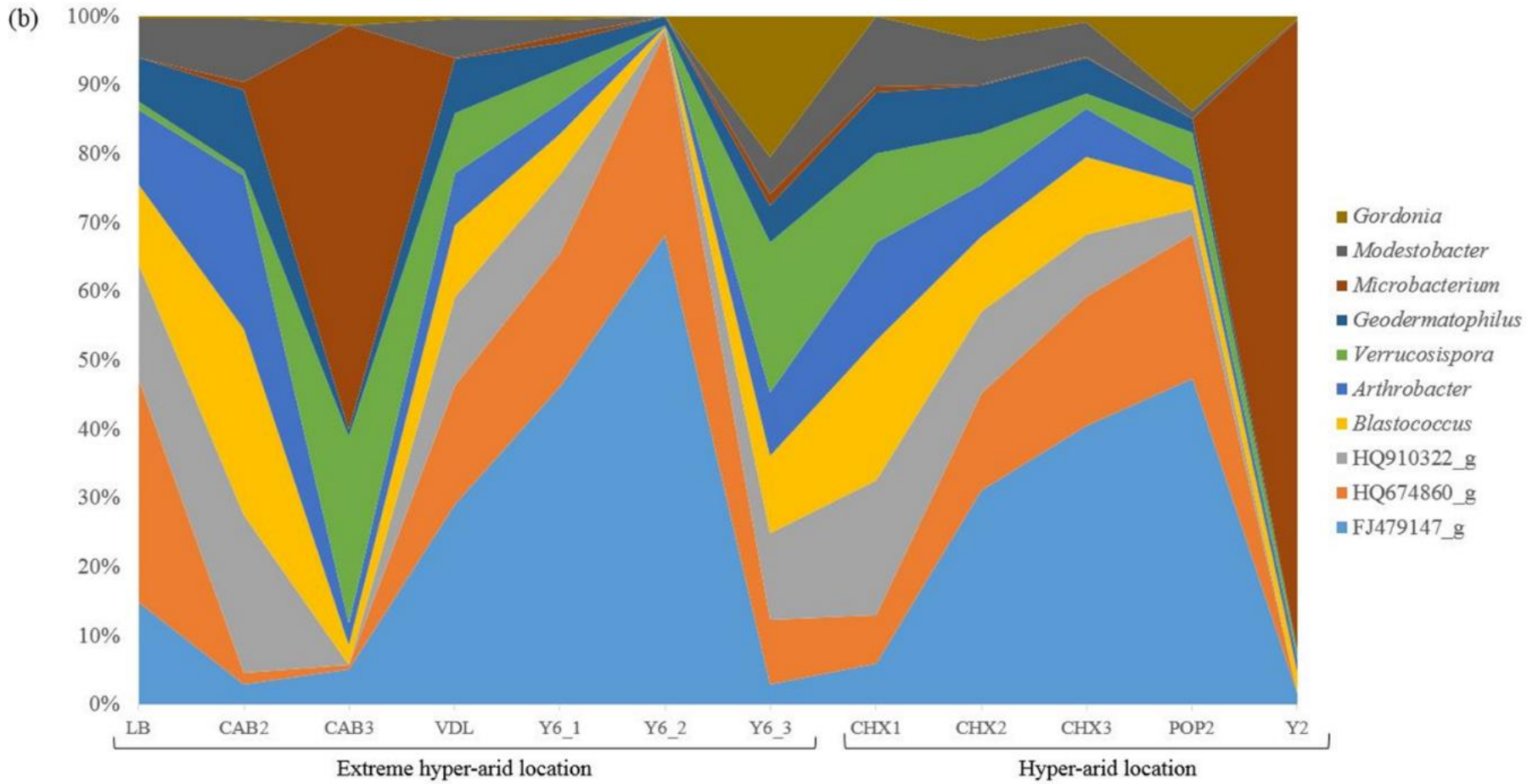


Figure 6.5 Relative abundance of actinobacterial genera detected in Atacama Desert locations. (a) Relative abundance shown at 1% cut off; (b) Relative abundance shown at 2.5% cut off. Genera below these thresholds were summed as 'Other'.

6.4.3 Habitat specificity and co-occurrence

Venn diagram analyses showed that the proportion of surface community genera shared between sites within extreme hyper-arid (32%) and within hyper-arid (37%) locations was comparable (Figure 6.6). However, similar analyses highlighted the effect of vegetation on hyper-arid community composition and of soil depth on extreme hyper-arid shared community composition reducing them to 14% and 22% respectively.

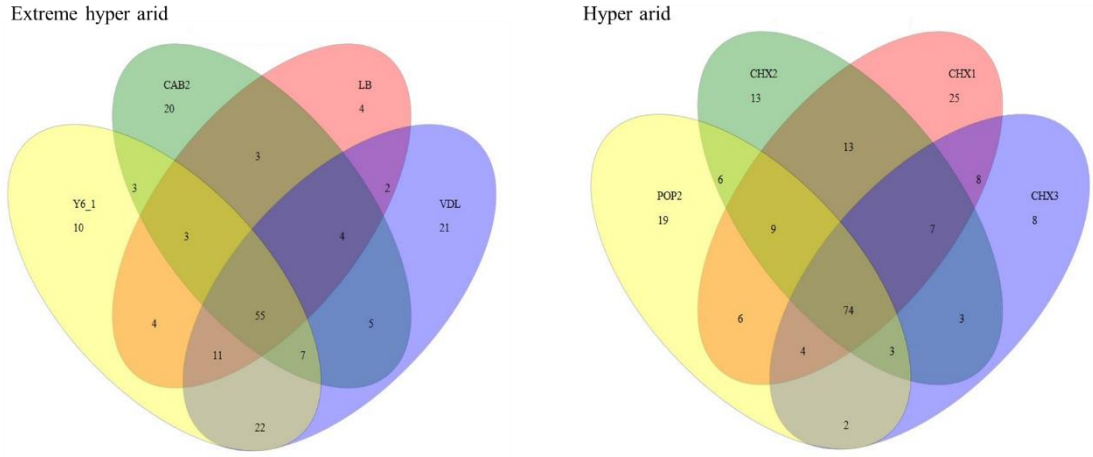


Figure 6.6 Venn diagrams showing proportions of shared and unique actinobacterial genera at extreme hyper-arid and hyper-arid sites.

Shade and Hendelsman (2012) consider the Venn diagram as a 'reasonable first exploration' of defining the 'core microbiome' of a habitat and their definition of the term is followed here to describe organisms shared across the Atacama Desert landscape. Although the focus of this study is solely on the phylum *Actinobacteria* it is still credible to construct a core microbiome based upon this dominant component of the Atacama Desert microbiota. Figure 6.6 indicates that 55 and 74 respectively of the OTUs were shared in extreme hyper-arid and hyper-arid surface sites. Considering only the 10% most abundant OTUs, the core actinobacterial biomes share 70% of their generic composition (Appendix 6).

The top clusters of most abundant OTUs are almost identical in the two biomes (Table 6.3), suggesting an actinobacterial signature for and postulating that they play a key role in ecosystem function within these Atacama Desert soils.

Table 6.3 Most abundant genera of the extreme hyper-arid and hyper-arid core microbiomes*

Extreme hyper arid	Hyper arid
FJ479147_g	FJ479147_g
HQ674860_g	HQ674860_g
Blastococcus	*Microbacterium*
HQ910322_g	*Kocuria*
Arthrobacter	*Sanguibacter*
Verrucosispora	*Verrucosispora*
Geodermatophilus	HQ910322_g
Modestobacter	*Nocardioides*
Microbacterium	*Gordonia*
Sporichthya	*Blastococcus*
Friedmanniella	*Corynebacterium*
Streptomyces	*Arthrobacter*
Sanguibacter	*Streptomyces*
Kocuria	*Aciditerrimonas*
Nocardioides	FJ478790_g

*The order of genera indicates their comparative dominance within each microbiome.

6.5 Conclusions

The results reported here confirm that actinobacteria constitute a major and frequently dominant component of desert soils. Remarkable, however, is the megadiversity of this phylum throughout hyper-arid and extreme hyper-arid habitats in the world's driest desert such that the metagenomics data set revealed a 16% greater coverage of actinobacteria at the family rank than that currently recognized. Similar actinobacterial dark matter was evident at the generic level where 40% of the captured diversity wasnot ascribable to validly published genera. Equally remarkable are the high proportions of unidentified (*No-Rank*) and very low abundance OTUs, features that are also found in other extreme and poorly researched ecosystems (Yang et al. 2012; Riquelme et al. 2015). Although caution is required in interpreting such metagenomic

data (Hedlund et al. 2014) this study provides a sound base from which to explore an astonishing and unpredicted desert microbiota.

Taxonomic composition of actinobacterial communities did not differentiate between the hyper-arid and extreme hyper-arid habitats but an actinobacterial core microbiome dominated by FJ479147_g, HQ674860_g, HQ910322_g *Arthrobacter, Blastococcus, Friedmanniella, Geodermatophilus, Modestobacter, Streptomyces, Sporichthya,* and *Verrucosispora* clearly defined this Atacama Desert landscape.

Exploration of the microbial world has recently been revolutionized by the advent of rapid and cheap DNA sequencing technology. In the past assessments of species 'rarity' were largely subjective and relied on culture-dependent experiments but now the ability to construct and analyse very large rank-abundance data sets has enabled rare biospheres to be defined with confidence. However, it is important to recognise that, as Bowen et al. (2012) remarked, truly rare sequences in environmental samples may escape detection due to incomplete sequencing. The actinobacterial rare biosphere determined within the Atacama Desert landscape is the first to be reported for highly arid environments and accords with the few broader microbial surveys of other deserts such as Sonoran Desert (Andrew et al. 2012). A notable feature of the Atacama rare biosphere is the high frequency of taxonomically unassigned OTUs confirming that this landscape contains a vast reservoir of actinobacterial dark matter. Although the functional ecology of the Atacama's actinobacteria remains to be studied it provides a considerable microbial seed bank whose role in soil ecosystem resilience warrants investigation. This hypothesis is given credence by, for example, the recent report that members of rare bacterial taxa became dominant in spring sediment microcosm experiments following exposure to environmental stressors (Coveley et al. 2015).

Acknowledgements

The pyrosequencing was carried out with help from Dr. Ian Singleton (School of Biology, Newcastle University), the sequence processing and bioinformatics analysis by Professor Jongsik Chun, Dr. Byung-yang Kim and colleagues (ChunLab) and the map and some of the analyses were under the guidance of Dr. Roy Sanderson (School of Biology, Newcastle University) while technical assistance was provided by Mrs. Ros Brown (School of Biology, Newcastle University).

Chapter 7. Characterization of culture-independent actinobacterial communities in high altitude Atacama Desert soils

7.1 Abstract

Little is known about the composition of actinobacterial communities in high altitude Atacama Desert soils. Consequently, a pilot 454 pyrosequencing survey was undertaken to determine the composition of actinobacterial communities in surface and subsurface soils collected at three altitudes on Cerro Chajnantor. The results revealed an astonishing level of taxonomic diversity that was composed of abundant and low abundant phylotypes. Some abundant phylotypes were found in soils from all of the sampling sites but others were discontinuously distributed. Actinobacterial community composition was found to be influenced by certain environmental variables notably altitude and redox potential. The results of this study have implications for ecological studies and bioprospecting campaigns.

7.2 Introduction

In general, culture-independent methods are now favoured over culture-dependent procedures as they reveal more complete and much higher levels of prokaryotic diversity in natural habitats. Metagenomic analyses of Atacama Desert habitats have been skewed towards examination of soil samples taken from in and around the extreme hyper-arid core of the Yungay region (Connon et al. 2007; Neilson et al. 2012; Crits-Christoph et al. 2013); in all of these surveys actinobacteria were found to be the predominant element in prokaryotic communities. In the two latter studies it was particularly interesting that actinobacterial taxa that belonged to the most deep-seated lineages in the actinobacterial 16S rRNA gene tree were to the fore, as exemplified by genera classified in the orders *Acidimicrobiales, Coriobacteriales, Rubrobacteriales* and *Solirubrobacteriales.*

Few culture-independent studies have been design to reveal prokaryotic diversity in high altitude Atacama Desert habitats (Costello *et al*., 2009; Lynch *et al.*, 2012). In the first of these studies Costello and her colleagues found sizeable actinobacterial populations in hyper-arid, non-fumerolic soil from the Socompa Volcano adjacent to the Chilean-Argentine border. In turn, Lynch et al. (2012) found that a metagenome generated from community 16S rRNA from a high altitude debris

field on Llullaillaco Volcano, also sited near the Chilean-Argentine border, was almost conpletely composed of actinobacetrial lineages, notably one belonging to the genus *Pseudonocardia.*

The present pyrosequencing study was undertaken to determine the extent of actinobacterial diversity in surface and subsurface soil collected from three altitudes on Cerro Chajnantor. As in the earlier study (see Chapter 6) analyses were restricted to generic and family ranks though in this case the effect of environmental variables on actinobacterial community composition was an integral part of the study.

7.3 Materials and Methods

7.3.1 Sampling sites

Six soil samples, designated ALMA (Atacama Large Millimeter Array) 1-6 were collected from surface and subsurface soil at three altitudes on the Cerro Chajnantor, as shown in Table 7.1. All of the samples were stored at 4^{o}C when received in the UK.

Table 7.1 The Cerro Chajnantor sampling sites.

Location	Environmental sample		Altitude (msl)	Biome
Cerro Chajnantor	ALMA	1	3018	Surface soil (2cm)
		2		Subsurface soil (30cm)
		3	4000	Surface soil (2cm)
		4		Subsurface soil (30cm)
		5	5046	Surface soil (2cm)
		6		Subsurface soil (30cm)

7.3.2 Physico-chemical properties

The conductivity, pH, redox potential , moisture and organic matter content of each of the ALMA soil samples was determined by Professor Barbara Andrews (University of Chile, Santiago) using standard ‘in house’ protocols.

7.3.3 DNA extraction, PCR amplification and pyrosequencing

Each of the ALMA soil samples was ground in a pestle and mortar prior to the extraction of total community DNA. The extraction, PCR amplification and pyrosequencing procedures were carried out, as described in Chapter 2.

7.3.4 Analysis of pyrosequence data

The high quality sequence reads were processed at Chunlab Inc., Seoul National University, Seoul, Korea, as described in Chapter 2. Taxonomic nomenclature was based on validly published names (http://www.bacterio.net/). The OTU table was generated at genus level using <Taxonomic Composition> option in CLCommunity v3.30 software (www.chunlab.com).

7.3.5 Actinobacterial community structure

Alpha diversity analyses, including rarefaction, rank abundance and taxonomic composition, were carried out using the CLCommunity software, as described in Chapter 2. The identified genera were checked manually from the OTU table and analyzed to establish their composition in each of the ALMA soil samples. A heatmap was generated using the 'heatplus' package from Bioconductor (Ploner, 2015) and hierarchical clustering achieved using the Bray-Curtis dissimilarity matrix from the 'vegan' package using R software. Venn diagrams were generated using the sharedotus parameter from Mothur v1.37.4 software (http://www.mothur.org/).

7.3.6 Multivariate statistical analyses

The OTU table was standardised to zero mean and unit standard deviation prior to analyses using the *R* package vegan followed by multivariate analysis, as described in Chapter 2. To observe the actinobacterial community composition in each of the ALMA soil samples and to find similarities between them, unconstrained ordination, principal component analysis (PCA) was used. Redundancy analysis (RDA) was carried out to determine the effect of environmental factors (altitude, conductivity, organic matter, pH and redox potential) on actinobacterial communities in the ALMA soil samples. In this analysis, the best fit of the response variables (community

structure) to the explanatory variables (environmental parameters) was followed by a permutation test to observe the significance of the relationships.

7.4 Results and Discussion

7.4.1 Physico-chemical properties of ALMA soil samples

The results of the physico-chemical analyses are shown in Table 7.2. Only the ALMA 6 soil gave a reading for moisture content. The pH of the soil samples collected at the lowest altitude (ALMA 1 and 2) were higher than those recorded from the soil samples taken from the two higher altitudes (ALMA 3 – 6). The pH of the subsurface soil samples at the two lower altitudes (ALMA 2 and 4) was higher than the corresponding surface samples (ALMA 1 and 3). Redox potential, a measure of electron availability in environmental samples (Fiedler et al. 2007), was higher at the surface (ALMA 1, 3 and 5) compared to the corresponding subsurface samples (ALMA 2, 4 and 6). Conductivity, a measure of soil salinity (Friedman 2005) was higher in ALMA 2 compared to ALMA 1 soil but contrasting readings were found at the two higher altitudes where conductivity in the surface samples (ALMA 3 and 5) was higher than that in the subsurface samples (ALMA 4 and 6). The percentage of organic matter in the subsurface ALMA 2 and 4 samples was higher than that in the surface samples (ALMA 1 and 3) but the percentage was lower in the subsurface ALMA 6 sample compared with surface ALMA 5 sample.

Table 7.2 Physico-chemical properties of the ALMA soil samples

ALMA site	Physico-chemical properties				
	pH	Redox potential (Eh[mv])	Conductivity (µS/cm)	Moisture content (%)	Organic matter content (%)
1	7.08	360	215	0	1.67
2	7.63	336	326	0	3.67
3	6.55	360	239	0	3.33
4	6.70	335	108	0	3.67
5	6.73	369	54	0	2.00
6	6.30	365	44	4.67	0.67

7.4.2 Actinobacterial community composition

Rarefaction analyses. Rarefaction curves enable comparisons to be made of taxonomic richness and estimates of the extent to which total diversity has been recovered. Curves constructed for the ALMA soils (Figure 7.1) show a greater actinobacterial richness in the ALMA 2 and 4 subsurface soil samples compared with the corresponding surface soil samples (ALMA 1 and 3). At 5048 m, the ALMA 6 subsurface soil sample showed a slightly greater actinobacterial richness when compared to the ALMA 5 surface soil sample. In general, the curves are asymptotic indicating that the majority of the generic diversity was captured by the sequencing campaign.

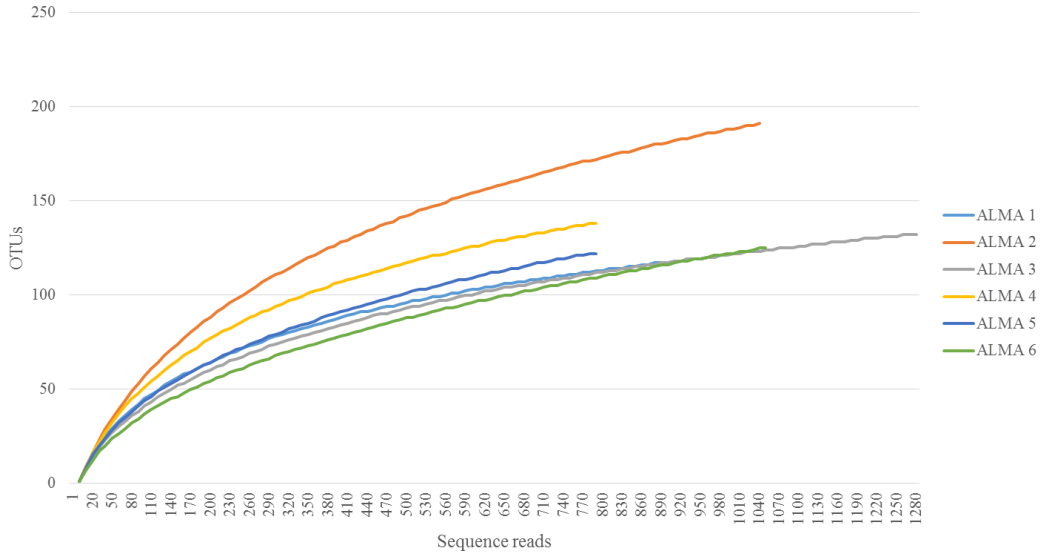


Figure 7.1 Rarefaction analyses of ALMA OTUs showing the relation between the increase in bacterial OTUs and the number of randomly sampled sequences in the ALMA soil samples.

Rank abundance. Rank abundancy curves were determined for all of the Cerro Chajnantor soils, as shown in Figure 7.2. As in the analyses of the actinobacterial taxa in the hyper- and extreme hyper-arid Atacama Desert soils the curves are indicative of high diversity habitats (shallow curves and long tails representing a rare actinobacterial biosphere). The proportions of abundant and rare actinobacterial taxa were similar in all of the ALMA soil samples.

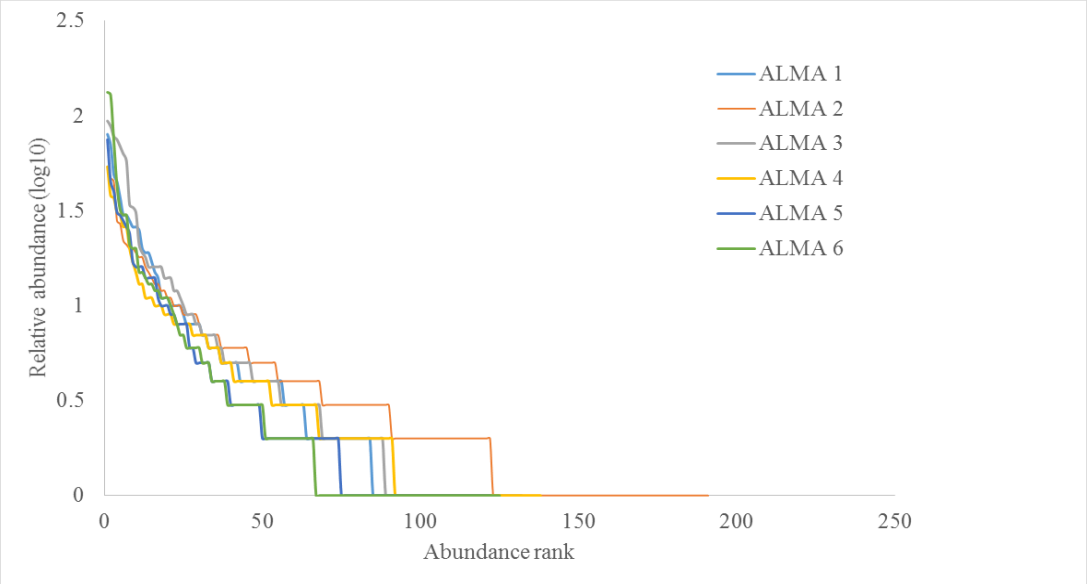


Figure 7.2 Rank-abundance curves of actinobacterial communities detected in ALMA soil samples.

Taxonomic composition. The OTUs recovered from the Cerro Chajnantor sampling sites were affiliated with 245 actinobacterial genera of which 138 (56%) were identified to known taxa, as shown in Figure 7.3 and Appendix 7; the balance of 107 (44%) are unidentified genera. The 10 most common genera detected in the ALMA soil samples were *Arthrobacter*, *Blastococcus*, *Friedmanniella*, *Geodermatophilus*, *Jatrophibitans*, *Modestobacter*, *Mycobacterium*, *Nocardioides*, *Pseudonocardia* and *Terrabacter*.

The effect of altitude and sampling depth on OTU abundance is exemplified in Figure 7.4. Members of the genera *Blastococcus*, *Geodermatophilus* and *Modestobacter* of the family *Geodermatophilaceae* together with *Friedmanniella* of the family *Propionibacteriaceae* were found to be abundant in the surface soil samples (ALMA 1, 3 and 5) compared with the corresponding subsurface samples (ALMA 2,4 and 6). In contrast, genus *Pseudonocardia* was more abundant in the subsurface soils (ALMA 2, 4 and 6) than in the corresponding surface soil samples (ALMA 1, 3 and 5), the remaining genera were discontinuously distributed; members of all of these taxa are present in the Cerro Chajnantor soils. The genus *Friedmanniella* has not been isolated or previously detected in Atacama Desert soils though it is interesting that the type and only strain of *Friedmanniella antarctica* was isolated from a sandstone sample at 1600m above ocean level in the Asgard Range of the Transantartic Mountains (Schumann et al. 1997).

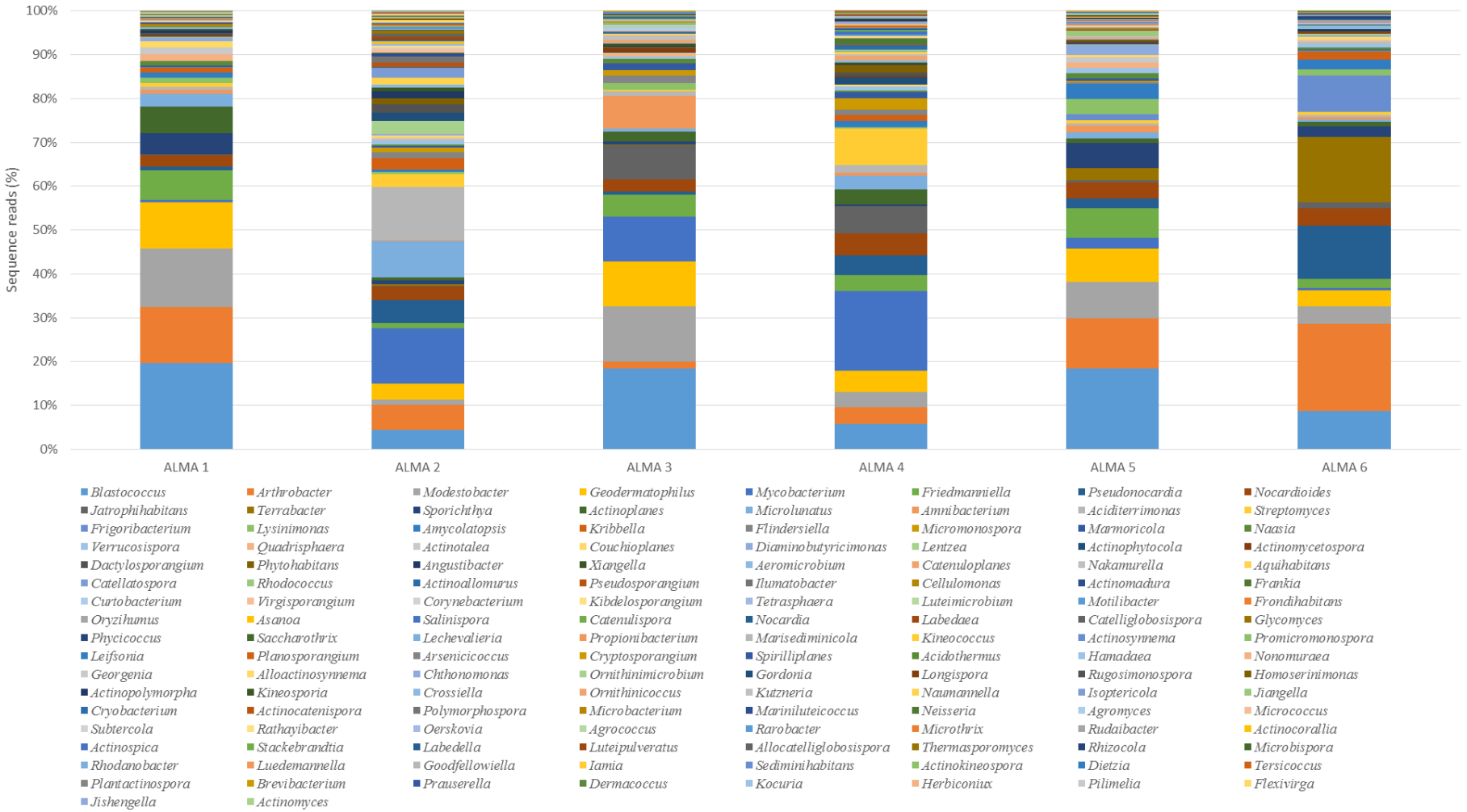


Figure 7.3 Relative abundance of known actinobacterial genera detected in the ALMA soil samples.

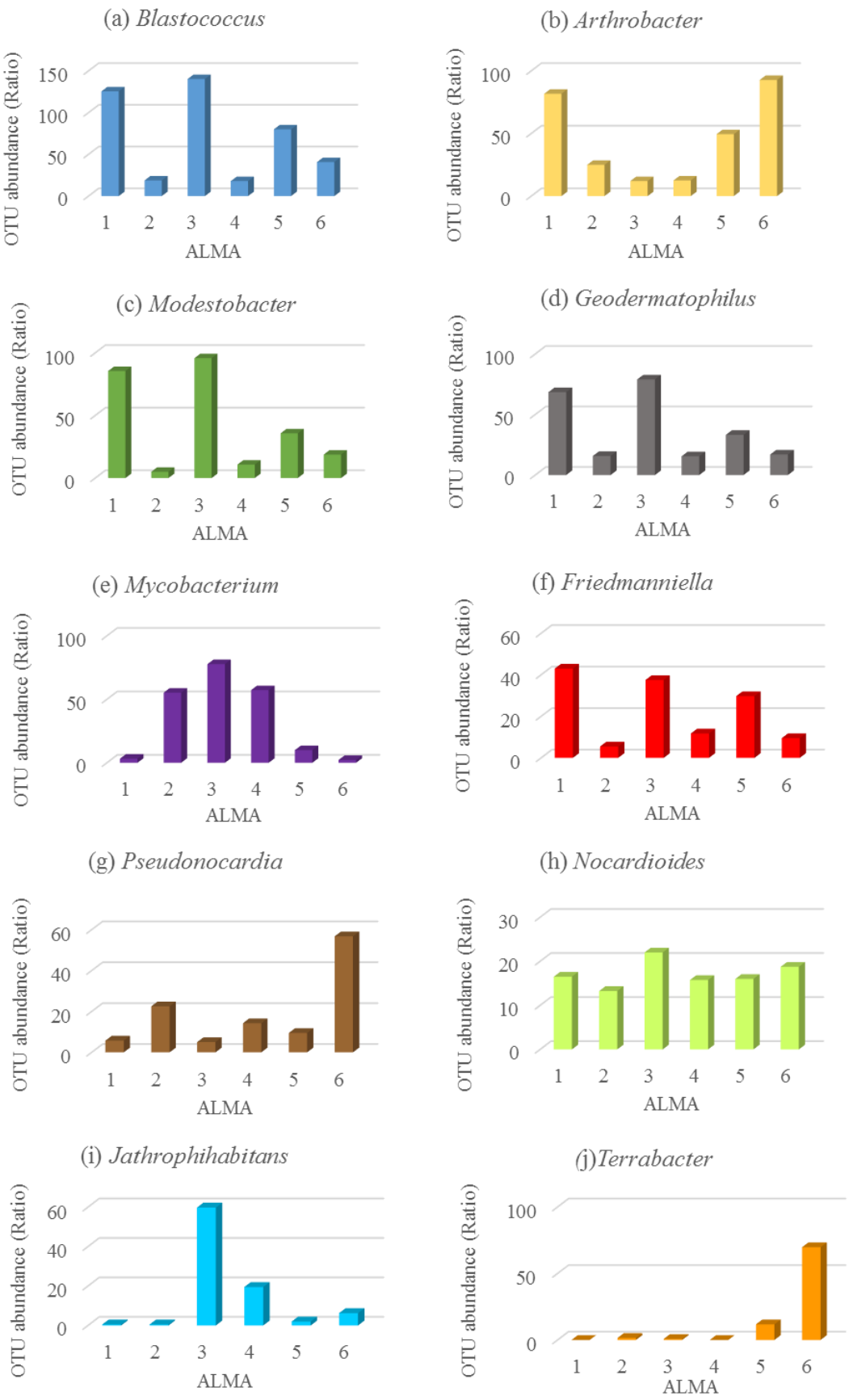


Figure 7.4 OTU abundance (%) of the top 10 representative actinobacterial genera detected in the ALMA soil samples.

Venn diagrams. The shared communities at the different altitudes and soil depths are shown in the Venn diagrams (Figure 7.5). The total of shared genera in the surface (ALMA 1, 3 and 5) and subsurface (ALMA 2, 4 and 6) soil samples were 44 and 48, respectively; this represents 33 and 36% of the total number of the identified actinobacterial genera. These results provide further evidence that actinobacterial genera are distributed across all of the ALMA soils. It is also interesting that quite large numbers of previously unknown genera were detected in each of the soil samples (12-24%), the highest numbers of these taxa were detected in the ALMA 2 surface and ALMA 3 subsurface soil samples, namely 34 and 32 genera, respectively.

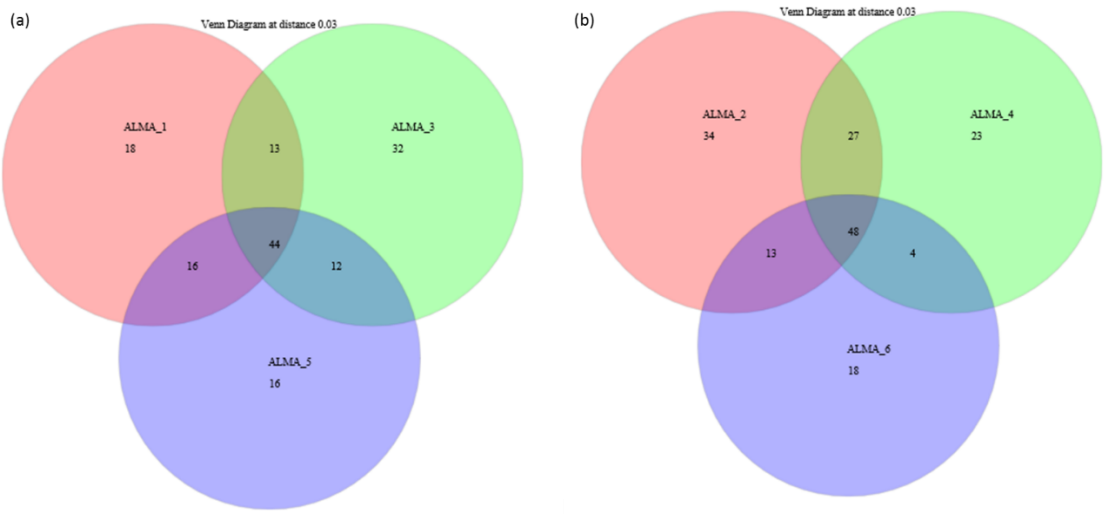


Figure 7.5 Venn diagram showing unique and shared OTUs found between (a) surface and (b) subsurface soil samples collected from Cerro Chajnantor locatons.

7.4.3 Actinobacterial community variation between ALMA soil samples

Heatmap. It can be seen from Figure 7.6 that the 138 validly published genera shown to be most abundant in the Cerro Chajnantor soils can be assigned to two groups, one composed of genera abundant in two surface soil samples (ALMA 1 and 5) and in the high altitude ALMA 6 subsurface soil sample. The second group encompasses genera seen to be abundant in two subsurface soil samples (ALMA 2 and 4) and in the ALMA 3 surface soil. Those genera found to be abundant in the first group are *Actinotalea* (family *Cellulomonadaceae*, order *Micrococcales*), *Cellulomonas* (as for *Actinotalea*), *Frondihabitans* (*Micrococcaceae, Micrococcales*), *Ornithinicoccus* (*Intrasporangiaceae, Micrococcales*) and *Sporichtya* (*Sporichtyaceae, Frankiales*). Similarly, the genera *Agrococcus* (*Microbacteriaceae, Micrococcales*), *Brevibacterium* (*Brevibacteriaceae, Mcrococcales*), *Gordonia* (*Nocardiaceae, Corynebacteriales*),

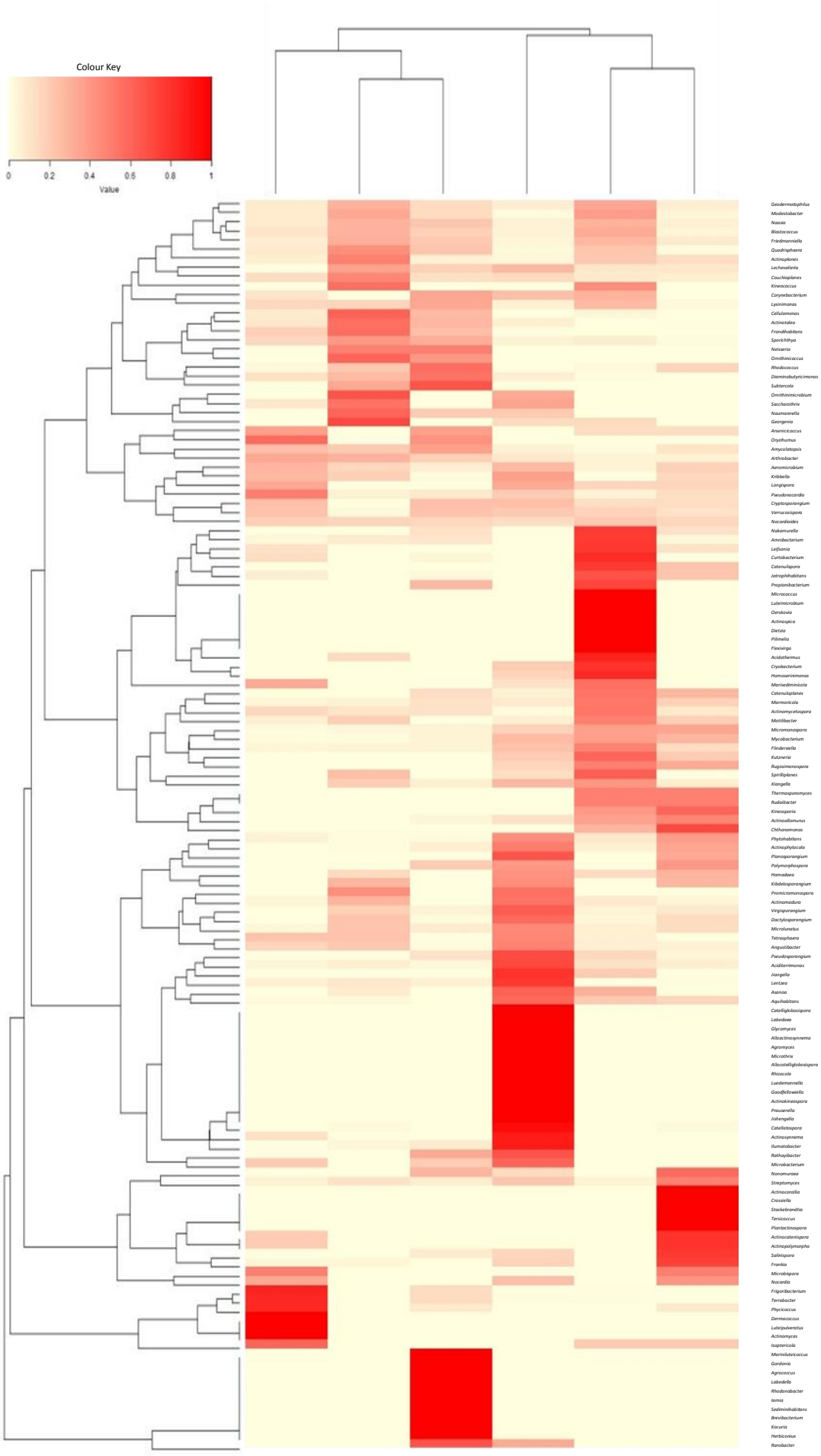
Herbiconiux (*Microbacteriaceae, Micrococcales*), *Mariniluteococcus* (*Propionibacteriaceae, Propionibacteriales*), *Rarobacter* (*Rarobacteriaceae, Micrococcales*) and *Sediminihabitans* (*Sanguinibacteriaceae, Micrococcales*) were more abundant in the ALMA 5 soil sample and *Actinomyces* (*Actinomycetaceae, Actinomycetales*), *Dermacoccus* (*Dermatococcaceae, Micrococcales*), *Frigoribacterium* (*Microbacteriaceae, Micrococcales*), *Isoptericola* (*Promicromonosporaceae, Micrococcales*), *Luteipulveratus* (*Dematococcaceae, Micrococcales*), *Phycicoccus* (*Intrasporangiaceae, Micrococcales*) and *Terrabacter* (as for *Physiococcus*). It is interesting that 16 out of these 22 genera belong to the order *Micrococcales*, the members of which are amycelial. The less abundant genera in group 1 were similar in all of the soil samples.

The abundant genera found in group 2 were *Actinokineospora* (*Pseudonocardiaceae, Pseudonocardiales*), *Actinosynnema* (*Pseudonocardiaceae, Pseudonocardiales*), *Alloactinosynnema* (*Micromonosporaceae, Micromonosporaciales*), *Catellatospora* (*Micromonosporaceae, Micromonosporaciales*), *Catelliglobosispora* (*Micromonosporaceae, Micromonosporales*), *Goodfellowia* (*Pseudonocardiaceae, Pseudonocardiales*), *Glycomyces* (*Glycomycetaceae, Glycomycetales*), *Illumatobacter* (*Illumatobacter*_f, *Acidomicrobiales*), *Jishengella* (*Micromonosporaceae, Micromonosporaciales*), *Labadaea* (*Micromonosporaceae, Micromonosporaciales*), *Luedemanniella* (*Micromonosporaceae, Micromonosporaciales*), *Microbacterium* (*Microbacteriaceae, Micrococcales*), *Microthrix* (*Microthrix*_f, *Acidimicrobiales*), *Prauserella* (*Pseudonocardiaceae, Pseudonocardiales*), *Rhizocola* (*Micromonosporaceae, Micromonosporaciales*) and *Rathayibacter* (*Microbacteriaceae, Micrococcales*). The composition of this group is markedly different to that of group 1 as 11 out of the 17 abundant genera belong to either the order *Micromonosporales* or the order *Pseudonocardiales.*

Finally, the genera *Acidothermus* (*Frankiaceae, Frankiales*), *Actinospica* (*Actinospicaceae, Catenulisporales*), *Amnibacterium* (*Microbacteriaceae, Micrococcales*), *Catenulispora* (*Micromonosporaceae, Micromonosporaciales*), *Cryobacterium* (*Microbacteriaceae, Micrococcales*), *Dietzia* (*Dietziaceae, Corynebacteriales*), *Flexivirgo* (*Dermatococcaceae, Micrococcales*), *Homoserinimonas* (*Microbacteriaceae, Micrococcales*), *Jatrophihabitans* (*Frankiaceae, Frankiales*), *Leifsonia* (*Microbacteriaceae, Micrococcales*), *Luteimicrobium* (*Promicromonosporaceae, Micrococcales*), *Micrococcus* (*Micrococcaceae,*

Micrococcales), *Nakamurella* (*Nakamurellaceae, Frankiales*), *Oerskovi*a (*Sanguibacteriaceae, Micrococcales*), *Pilimelia* (*Micromonosporaceae, Micromonosporales*) and *Propionibacterium* (*Propionibacteriaceae, Propionibacteriales*) were abundant in the ALMA 3 soil sample whereas in the corresponding subsurface soil sample (ALMA 4) the genera *Actinocorralia* (*Thermomonosporaceae, Streptosporangiales*), *Crossiella* (*Pseudonocardiaceae, Pseudonocardiales*), *Stackebrandtia* (*Glycomycetaceae, Glycomycetales*), *Tersicoccus* (*Micrococcaceae, Micrococcales*) and *Plantactinospora* (*Micromonosporaceae, Micromonosporaciales*) were abundant. Finally the taxa found to be abundant in the ALMA 4 soil sample were *Actinocatenispora* (*Micromonosporaceae, Micromonosporaciales*), *Actinopolymorpha* (*Propionibacteriaceae, Propionibacteriales*), *Salinispora* (*Micromonosporaceae, Micromonosporaciales*) and *Frankia* (*Frankiaceae, Frankiales*).

The generic phylotypes shown to be abundant in the ALMA soil samples represent an astonishing taxonomic diversity even without taking into account the putatively novel genera and the extent of the actinobacterial dark matter. Indeed, the abundant phylotypes represent 21 out of the 42 families and 10 out of the 14 orders classified in the class *Actinobacteria* in the current edition of *Bergey's Manual of Systematic Bacteriology* (Goodfellow *et al.,* 2012). These results are in good agreement with those of other culture-independent surveys in showing that microbial communities are extremely diverse (Yang *et al.,* 2012), including ones in arid Atacama Desert soils (Neilsen *et al.,* 2012; Crits-Christoph *et al.,* 2013). Such studies have also noted that certain phylotypes are discontinuously distributed across sampling sites; in the present study it was particularly interesting that actinobacteria with the capacity to exhibit complex morphologies were associated with particular soil samples while other with simpler morphologies prevailed at other sites on Cerro Chajnantor. It has been speculated that the extensive prokaryotic diversity in arid desert soils is a function of competition for scarce trophic resources and the ability of microorganisms to adapt to extreme environmental conditions (Kutovaya *et al.,* (2015) There is also evidence that in highly diverse biomes elements of the rare biosphere can act as a back up system that respond to changed environmental circumstances (Coveley et al. 2015)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Haasia
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Friedmanniella
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Actinoplanes
Lechevalieria
Couchioplanes
Kineococcus
Corynebacterium
Lysinimonas
Cellulomonas
Actinotalea
Frondihabitans
Sporichthya
Neisseria
Ornithinicoccus
Rhodococcus
Diaminobutyricimonas
Subtercola
Ornithinimicrobium
Saccharothrix
Naumannella
Georgenia
Arsenicicoccus
Oryzihumus
Amycolatopsis
Arthrobacter
Aeromicrobium
Kribbella
Longispora
Pseudonocardia
Cryptosporangium
Verrucosispora
Nocardioides
Nakamurella
Amnibacterium
Leifsonia
Curtobacterium
Catenuloispora
Jatrophihabitans
Propionibacterium
Micrococcus
Luteimicrobium
Dietzia
Pilimelia
Flexivirga
Acidothermus
Cryobacterium
Homoserinimonas
Marisediminicola
Catenulisporales
Marmoricola
Actinomycetospora
Motilibacter
Micromonospora
Mycobacterium
Flindersiella
Kutzneria
Rugosimonospora
Spirillospora
Xiangella
Thermosporomyces
Rudaibacter
Kineosporia
Actinoallomurus
Chthonomonas
Phytohabitans
Actinophytocola
Planosporangium
Polymorphospora
Hamadaea
Kibdelosporangium
Promicromonospora
Actinomadura
Virgisporangium
Dactylosporangium
Microlunatus
Tetrasphaera
Angustibacter
Pseudosporangium
Aciditerrimonas
Jiangella
Lentzea
Asanoa
Aquihabitans
Catelliglobosispora
Labedaea
Glycomyces
Allocatenosynnema
Agromyces
Microthrix
Allocatelliglobosispora
Rhizocola
Luedemannella
Goodfellowiella
Actinokineospora
Prauserella
Jishengella
Catellatospora
Actinosynnema
Iamia
Rathayibacter
Microbacterium
Nonomuraea
Streptomyces
Actinocorallia
Crossiella
Stackebrandtia
Tersicoccus
Plantactinospora
Actinocatenispora
Actinopolymorpha
Salinispora
Frankia
Microbispora
Nocardia
Frigoribacterium
Terrabacter
Phycicoccus
Dermacoccus
Luteipulveratus
Actinomyces
Isoptericola
Marinilutеicoccus
Gordonia
Agrococcus
Labedella
Rhodonobacter
Iamia
Sediminihabitans
Brevibacterium
Kocuria
Herbiconiux
Rarobacter

Figure 7.6 Hierarchical heatmap showing actinobacterial distribution of validly published genera among the six ALMA soil samples. The double hierarchical dendrogram shows the actinobacterial distribution and the heatmap represents the relative percentage of each actinobacterial genus within each sample. The relative abundance values ~~for~~ are indicated by the colour intensity as shown in the legend on the top left corner.

Principal component analysis. The actinobacterial communities in the soil samples collected from the three locations on Cerro Chajnantor were the subject of principalcomponent analysis (PCA). Twenty six percent of the total between sample variance (Figure 7.7, PC1 versus PC2) showed that the samples fell into two groups that were broadly in accord with the results shown in the heatmap; one of the groups was composed of the actinobacterial communities in ALMA 1, 5 and 6 soils samples, as shown at the top of the PCA plot while those found in the ALMA 2, 3 and 4 soil samples are located at the foot of the plot. The distribution of representatives genera found in abundance in the ALMA soil samples is shown in the second PCA plot (Figure 7.8). In general, good correlation was found between the results of this analysis and those shown in the heat map. It can, for instance, be seen that the genera *Cellulomonas, Dermacoccus, Frondihabitans, Phylococcus* and *Sporichtya*, representatives of heat map group 1 (ALMA 1, 5 and 6), are grouped together at the top left corner of the PCA plot. In contrast, representatives of group 2, namely the genera *Actinospica, Catenulispora, Cryobacterium, Curtobacterium, Flexivirga, Leifsonia, Luteimicrobium, Micrococcus* and *Propionibacterium* are located at the bottom left corner of the PCA plot. Less variation was found between the composition of the actinobacterial communities in the surface and subsurface soils at the higher altitudes on Cerro Chajnantor, as shown in Figure 7.9. It can also be seen from the box plot that there is less variation in actinobacterial community composition in the surface soil samples (ALMA 1, 3 and 5) at the different altitudes than between the corresponding subsurface soil samples (ALMA 2, 4 and 6).

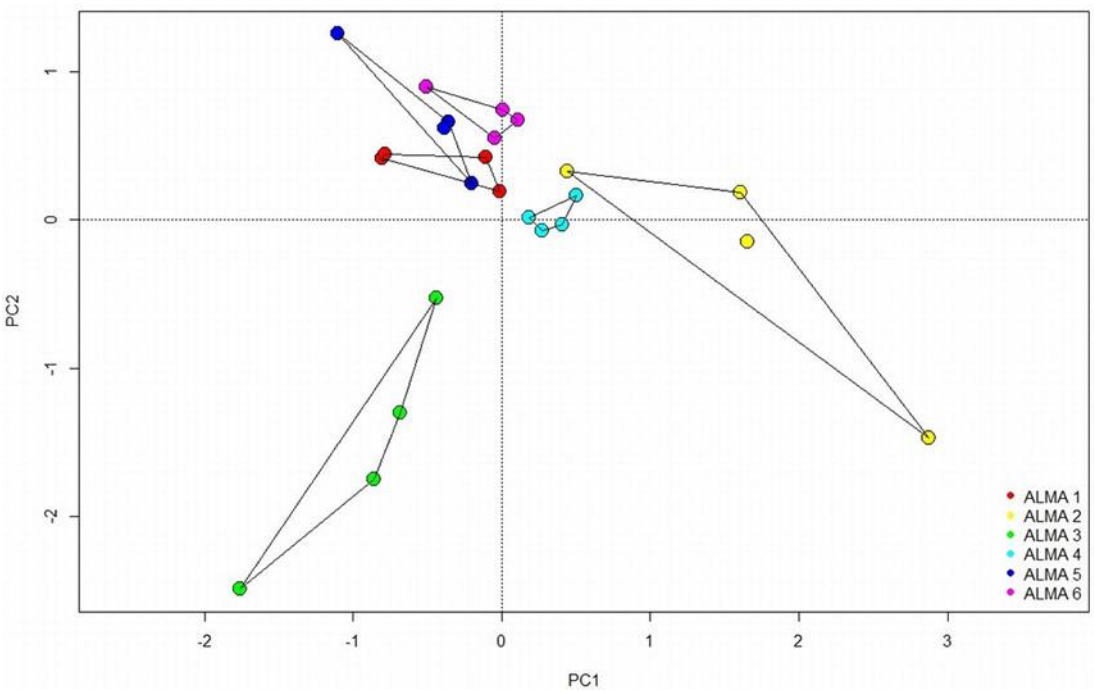

Figure 7.7 Principal component analysis of actinobacteria detected in surface and subsurface soils collected from three sampling sites on Cerro Chajnantor.

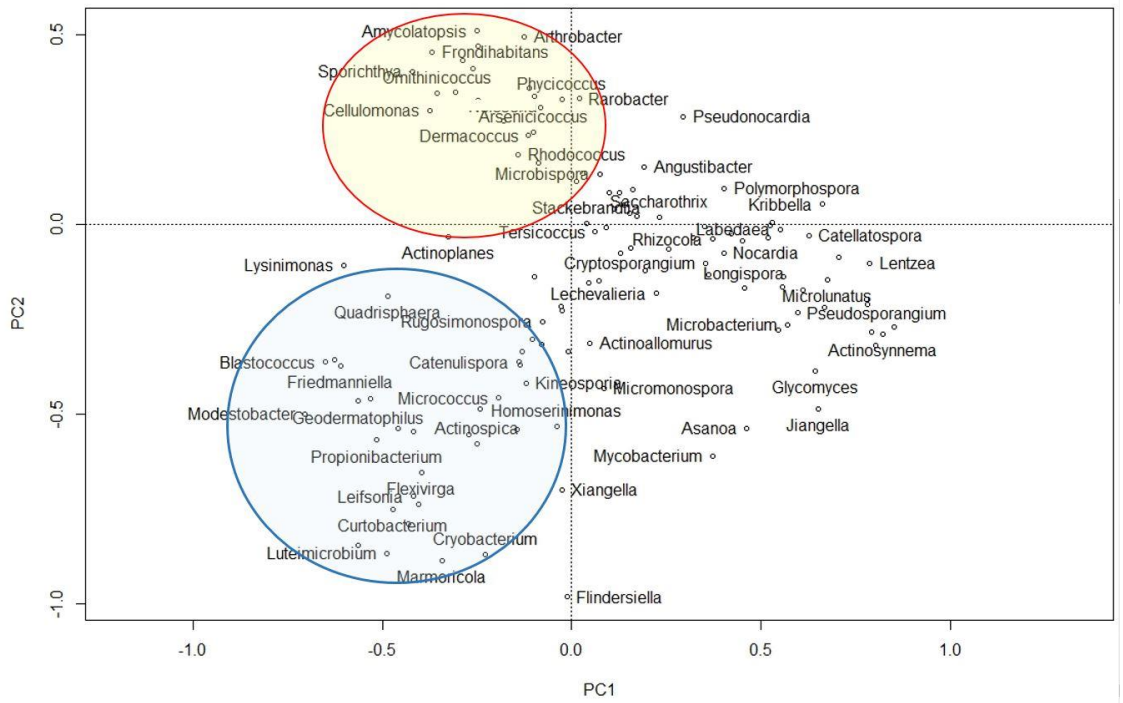

Figure 7.8 Principal component analysis of actinobacterial genera detected in surface and subsurface soils collected from three sampling sites on Cerro Chajnantor. Red circle, group 1; blue circle, group 2.

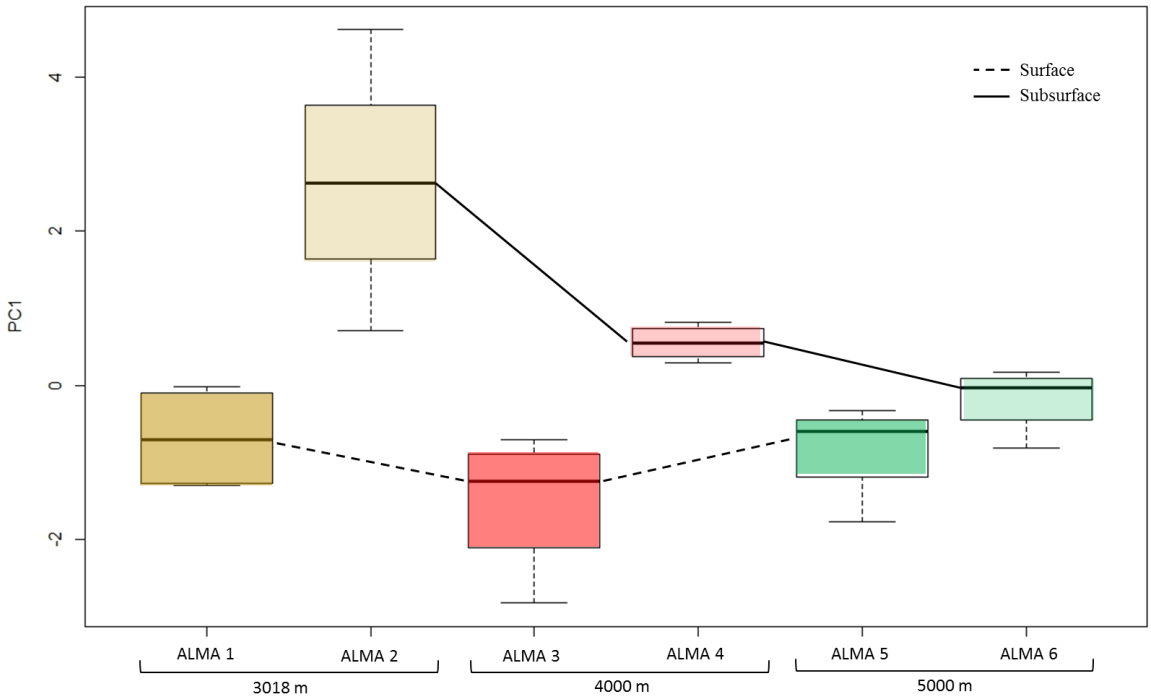

Figure 7.9 Box plot summarizing pyrosequencing data of actinobacteria detected in the ALMA environmental samples. The interquartile range is represented by the outer bounds of the boxes, the median is represented by the midline (black), and the whiskers represent the minimum and maximum values.

The relationship between actinobacterial community composition and the environmental variables (altitude, conductivity, organic matter, pH and redox potential) was determined using the first two axes in the RDA biplot which accounted for 26% of the total variance (Figure 7.10). Correlation was found between altitude and redox potential, but these variables were negatively correlated with conductivity and organic matter content as shown by the arrows, no correlation was found between pH and the other environmental variables. It is also evident that the actinobacterial communities in the ALMA 1, 5 and 6 soil samples, as was the case with the heat map and PCA plot, are grouped together as seen on the lower left side of the RDA plot, these communities are influenced by altitude and redox potential. Currently, the actinobacterial communities in the remaining soil samples (ALMA 2, 3 and 4), as in the earlier analyses, are grouped together as shown on the right side of the RDA plot; the actinobacterial communities in those soils are influenced by conductivity, organic matter content and pH. The RDA ANOVA permutation test revealed statistically significant effects ($p<0.05$) of the

environmental variables on actinobacterial community composition in the ALMA soil samples, the F ratios and p values are given in Table 7.2.

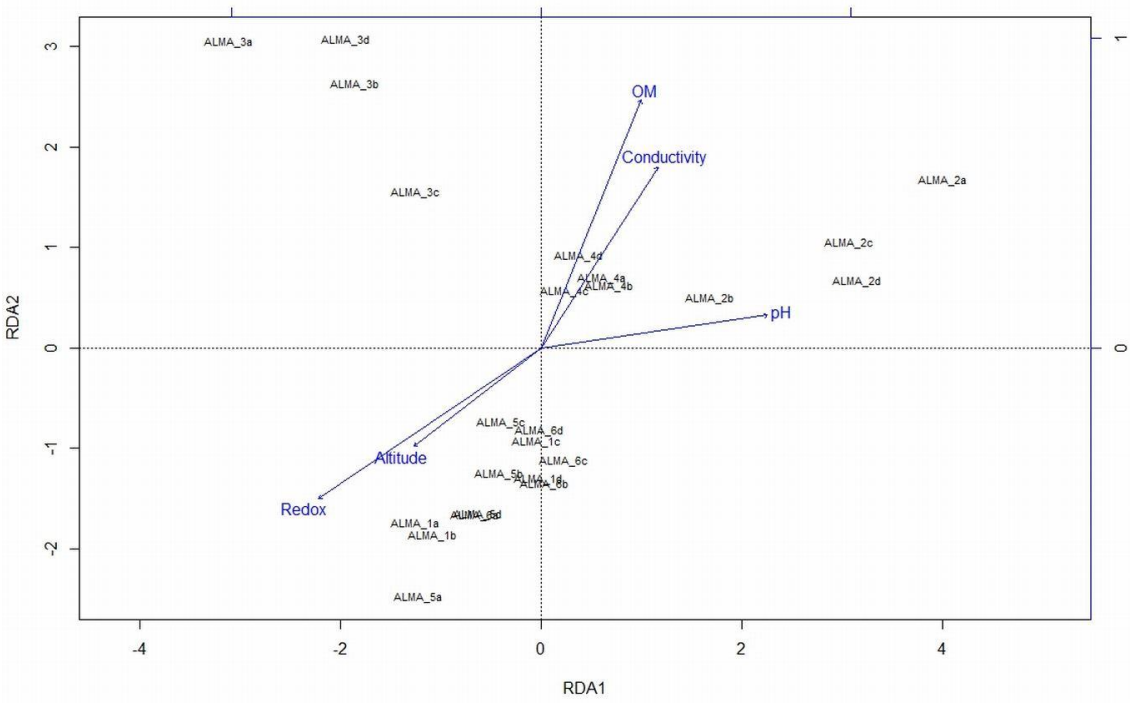

Figure 7.10 Redundancy analysis (RDA) biplot of ALMA sites showing the relationship between actinobacterial community composition and environmental variables. Arrows represent environmental variables. a, b, c and d are replicates of each of the ALMA soil samples.

Table 7.2 The RDA permutation test of ALMA environmental variables.

Environmental variable	F ratio	P value
Altitude	1.6716	0.008
pH	2.0878	0.002
Redox	2.2539	0.001
Conductivity	1.6550	0.004
Organic matter	1.6143	0.018

A number of key deductions can be drawn from the results of this pioneering survey of actinobacterial taxonomic diversity in high altitude Atacama Desert soils. These soils, like those from hyper- and extreme hyper-arid regions of the Atacama Desert, contain complex, taxonomically diverse actinobacterial communities composed of small numbers of abundant generic phylotypes and low numbers of innumerable phylotypes that represent an important component of the bacterial dark matter. The discovery that

actinobacterial communities on Cerro Chajnantor are influenced by certain environmental variables has wide reaching implications for biogeographical, ecological and bioprospecting studies. To this end, effective isolation and characterization procedures are needed to recover representative high quality strains for bioactivity screens. This is a tall order as effective procedures have still to be found to isolate taxa known to be prevalent in high altitude Atacama Desert soils, not least genera classified in families belonging to the order *Micrococcales.*

Acknowledgement

Many thanks are due to Dr. Roy Sanderson (School of Biology, Newcastle University) for his invaluable assistance with the multivariate statistical analyses.

Chapter 8. Major outcomes and perspectives for future work

8.1 Major outcomes

The major objectives of this project were realised and in some respects exceeded as (a) taxonomically diverse filamentous actinobacteria with an ability to synthesize a broad range of bioactive compounds were isolated from high altitude and extreme hyper-arid Atacama Desert soils; (b) strains isolated from these habitats and from a previously examined hyper-arid soil were shown to belong to novel species based on 16S rRNA gene sequence data; (c) selected presumptively novel isolates were proposed as new species following extensive polyphasic taxonomic studies; (d) representatives of the novel species were shown to synthesize novel natural products; (e) metagenomic analyses of community DNA highlighted an amazing actinobacterial diversity in a selection of Atacama Desert soils and (f) key environmental variables were found to influence actinobacterial community composition in the high altitude Atacama Desert soils.

The present study shows that the high altitude Cerro Chajnantor and the extreme hyper-arid Lomas Bayas biomes contain small numbers of taxonomically diverse filamentous actinobacteria, results in excellent agreement with those of corresponding culture-dependent studies on the actinobacterial microbiota of hyper-arid and extreme hyper-arid soils (Okoro *et al.*, 2009; Busarakam, 2014). Further, as in these earlier studies, representatives of dereplicated taxa were found to belong to new species, notably ones assigned to the genus *Streptomyces*. It was particularly interesting that presumptively novel species of *Cryptosporangium, Kineococcus, Microbispora* and *Nocardiopsis* species were isolated, as members of these taxa have not been previously cultivated from Atacama Desert soils. However, in accord with the aforementioned studies presumptively novel *Actinomadura, Amycolatopsis, Pseudonocardia* and *Streptomyces* species were detected indicating that members of these taxa are common in the Atacama Desert landscape.

Polyphasic taxonomic studies have revolutionized actinobacterial systematics (Goodfellow *et al*., 2012) and when applied to presumptive novel strains isolated from arid Atacama Desert soils have led to the valid publication of *Lechevalieria, Modestobacter* and *Streptomyces* species (Bull & Asenjo, 2013; Bull *et al*., 2016; Busarakam *et al.*, 2016a). Corresponding studies on selected strains isolated from the Cerro Chajnantor and from hyper- and extreme hyper-arid soils provide strong grounds for recognizing several actinobacterial novel taxa, namely *Lentzea chajnantorensis* sp.

nov., *Pseudonocardia yungayensis* sp. nov., *Streptomyces aridus* sp. nov. and *Streptomyces asenjonii* sp. nov.. The designation of isolate H45 as the presumptive type strain of *L. chajnantorensis* is particularly significant as this organism was found to synthesize several novel dienes, the lentzeosides, some of which inhibit HIV-1 integrase activity. This discovery provides further evidence for the hypothesis that extreme environmental conditions give rise to unique actinobacterial diversity which is the basis of novel chemistry (Bull & Stach, 2007; Bull, 2003; Goodfellow *et al.*, 2013).

This and previous biosystematic surveys of Atacama Desert actinobacteria underline the fact that polyphasic taxonomic studies are laborious and time-consuming and cannot be applied to resolve the taxonomic provenance of large numbers of putatively novel isolates. This point is self-evident given the fact that only eight of the many presumptively Atacama Desert isolates have been validly published following polyphasic studies. Serious taxonomic bottlenecks such as these are now being addressed by the application of whole-genome sequencing and associated bioinformatic tools, not least the use of whole genome sequence data for establishing generic and species boundaries, as exemplified by recent studies on the genera *Amycolatopsis* (Tang *et al.,* 2015), *Rhodococcus* (Sangal et al. 2016) and *Streptomyces* (Girard *et al.,* 2014, 2015).

The results of the antimicrobial plug assays are encouraging as they show that a clear majority of strains isolated from the Cerro Chajnantor and Lomas Bayas soil samples inhibited the growth of one or more of the panel of wild type strains, notably that of the *E. coli* and *P. fluorescens* strains. Similar results were reported by Busarakam (2014) in corresponding screens of strains isolated from arid Atacama Desert soils. Taken together these data show that dereplication of taxonomically diverse strains isolated from Atacama Desert soils provide a practical and powerful way of identifying organisms with the capacity to produce bioactive compounds. In addition, more refined analyses based on the use of *B. subtilis* reporter strains were used to detect isolates that inhibited cell envelope, cell wall, DNA and fatty acid synthesis. It can be concluded that the screening campaigns carried out in this and the earlier study by Busarakam (2014) provide an effective way of generating high quality strains for chemical dereplication procedures designed to identify novel lead compounds worthy of further study in the search for new specialized metabolites.

Not surprisingly, the twin track approach used in this study to reveal the extent of actinobacterial diversity in several Atacama Desert soils provides strong backing for the widely held view that culture-dependent procedures seriously underestimate the extent of prokaryotic diversity in natural habitats (Bull et al., 2000; Bull & Stach, 2003). Indeed,

an overwhelming majority of the actinobacterial taxa detected in the metagenomic analyses were not recovered using the culture-dependent procedures. Nevertheless, a comparison of metagenomic data with the results of culture-dependent studies conducted by Busarakam (2014) showed that both approaches revealed the presence of substantial populations of *Blastococcus*, *Geodermatophilus* and *Modestobacter* strains in extreme hyper-arid Atacama Desert soils. In contrast, the failure of the culture-dependent methods applied in this and previous surveys (Okoro *et al.,* 2009; Busarakam, 2014) to detect genera classified in validly published genera assigned to the families *Micrococcaceae, Micromonosporaceae* and *Propionibacteriaceae,* in the metagenomic analyses underpin the power of the culture-independent procedure used in this study.

Another example of the power of pyrosequencing was the unexpected discovery that actinobacterial communities in Atacama Desert habitats consist of abundant phylotypes which form a major proportion of the total 16S rRNA gene pool and an incredible high number of low abundance phylotypes, the actinobacterial rare biosphere that make up the balance of the gene pool. The extent and taxonomic significance of this rare biosphere in the Atacama Desert landscape is underlined by the observation that it is composed mainly by seldomly isolated and previously unknown actinobacteria taxa. The significance of these tiny populations of taxonomically diverse actinobacteria has still to be ascertained though it is very interesting that in the present study the type strain of *L. chajnantorensis*, a member of both the rare biosphere and the Atacama's actinobacterial dark matter, was found to synthesize the novel lentzeosides. It can be concluded that the Atacama Desert biome is particularly well suited for biosystematic studies as it should be relatively easy to devise innovative isolation procedures to recover taxa from hyper-arid and extreme hyper-arid soils as isolation plates should be free of fast-growing bacteria. Even so, it is clear from this and the earlier studies on the actinobacterial microflora of Atacama Desert habitats that new approaches are needed to isolate elements of vast numbers of previously uncultivated taxa known to be present in these soils. The use of genome and metabolic pathway analyses to determine microbial nutrient requirements offers a possible way forward (Carini et al. 2013; Oberhardt et al. 2015).

The metagenomic analyses of actinobacterial communities in the high altitude Cerro Chajnantor soils provides a fascinating insight into the environmental factors that influence the distribution and abundance of the constituent taxa. It is particularly interesting that actinobacteria with the ability to form mycelia and spores were abundant at certain sampling sites whereas phylotypes belonging to genera encompassing coccoid,

rod and pleomorphic micromorphologies were abundant at the other sites. Such results can be expected to inform ecological and bioprospecting studies.

8.2 Perspectives for future work

- Valid publication of putatively novel strains belonging to poorly studies taxa, such as the genera *Actinomadura*, *Cryptosporangium* and *Kineococcus*, and of representatives of key taxa, such as the genera *Pseudonocardia* and *Streptomyces*, using polyphasic taxonomic procedures.
- Biosystematic studies of taxa, such as those assigned to the order *Micrococcales*, that have not been associated previously with Atacama Desert habitats.
- Development and application of innovative isolation/cultivation methods to study whole organism physiology of selected Atacama Desert actinobacteria.
- Application of functional genomics to explore ecological traits in representatives of novel actinobacterial species, to caste light on the roles they play in the desert ecosystem.
- Harness the biotechnological potential of Atacama Desert "actinobacterial dark matter".
- Generation of 16S rRNA oligonucleotide probes to chart the distribution of rare taxa, such as the genus *Rubrobacter*, across the Atacama Desert landscape.
- Further work on drug leads designed to raise the titre of putatively novel specialized metabolites to enable structural characterization of purified compounds.
- Genome mining of representatives of poorly studied genera isolated from high altitude Atacama Desert soils to identify new metabolite pathways.

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Appendix 1

Assignment of isolates to colour-group

Assignment of strains isolated from ALMA and Lomas Bayas 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 2 weeks and 3 days, respectively.

A. ALMA

Colour group	Cultural characteristic			Codes and source of isolates						Number of isolates
				ALMA						
	Aerial spore mass colour	Substrate mycelia colour	Colour of diffusible pigment	1	2	3	4	5	6	
1.	White (263)	White (263)	-		H1*, H54, H56*, H57*, H58*, H67*, H69*, H74,				H36*	12

					H75, H76, H77					
2.	White (263)	Dark yellow brown (78)	-		H2*					1
3.	Olive gray (113)	Mild olive brown (95)	-		H3					1
4.	Yellow white (92)	Mild yellow brown (72)	-	H153	H4, H73, H86, H87					5
5.	White (263)	Light orange yellow (70)	-				H5*			1
6.	White (263)	Slight yellow (84)	-				H6*, H106			2
7.	-	Mild yellow (80)	-				H7*, H104, H105			3
8.	Medium gray (265)	Black (267)	-				H8			1
9.	Yellow white (92)	Mild yellow brown (77)	-	H126	H68					2
10.	Light yellow brown (76)	Dark brown (59)	Slight yellow brown (74)						H41¥ *, H42¥	2
11.	Olive gray (113)	Pale yellow (89)	--						H43*	1
12.	White (263)	Yellow white (92)	-	H119, H123, H125, H135,	H60, H89	H171, H172, H173,		H193, H199, H205*, H213,	H44*, H13*, H21	32

				H136, H142, H143, H145, H147, H148, H150, H159, H161, H163, H168		H174 H176		H214, H215*		
13.	Yellow white (92)	Pale orange yellow (73)	-						H45*	1
14.	Light gray yellow brown (79)	Brown gray (64)	-						H46*	1
15.	Olive gray (113)	Gray greenish yellow (105)	-						H47*	1
16.	Light brown gray (63)	Dark yellow brown (78)	Dark gray yellow (91)						H48	1
17.	Light gray (264)	Mild yellow (87)	-						H49	1
18.	Light greenish yellow olive (109)	Deep olive yellow (69)	Brilliant orange yellow (67)						H50	1
19.	Light yellow brown (76)	Dark gray yellow brown (81)	-						H51*	1
20.	Yellow white (92)	Light orange yellow (70)	-						H52	1

21.	Pale orange yellow (73)	Dark yellow brown (78)	Deep brown (56)						H53*	1
22.	Medium gray (265)	Gray yellow brown (80)	-		H70, H71, H85					3
23.	Dark gray (266)	Dark gray yellow (91)	-						H10*	1
24.	Medium gray (265)	Dark gray yellow (91)	Very yellow (82)						H11	1
25.	Light brown gray (63)	Gray yellow (9)	-						H12*	1
26.	Yellow gray (93)	Gray yellow (90)	-					H181	H14*	2
27.	Medium gray (265)	Light olive brown (94)	-						H15	1
28.	Dark gray (266) & white (263)	Gray yellow (90)	-	H132					H16	2
29.	Light gray brown (60)	Brown black (65)	-						H17*	1
30.	Light gray yellow brown (79)	Dark olive brown (96)	-						H18	1
31.	Light gray (264)	Yellow white (92)	-						H19	1
32.	Light gray yellow brown (79)	Dark brown (59)	-						H20	1
33.	Light gray olive (109)	Deep orange yellow (69)	Very orange yellow (66)						H22	1

34.	Gray olive (110)	Dark olive (108)	-						H23	1
35.	Light olive greenish yellow (112)	Greenish yellow gray yellow (115)	-						H24	1
36.	Yellow white (92)	Yellow white (92)	-		H64				H25	2
37.	Dark gray (66)	Yellow white (92)	-						H26*	1
38.	Light olive greenish yellow (112)	Slight yellow (84)	Very yellow (82)						H27	1
39.	Light brown greenish yellow (63)	Light gray olive (109)	-						H28*	1
40.	Gray yellow brown (80)	Brown black (65)	Brown gray (64)						H29	1
41.	Light gray brown (60)	Dark yellow brown (78)	Mild yellow brown (77)						H30	1
42.	Medium gray (265)	Yellow white (92)	-						H31*	1
43.	Dark gray 9266) & white (263)	Brilliant yellow (67)	Light orange yellow (70)						H32	1
44.	Medium gray (265)	Deep yellow pink (27)	-						H33*	1
45.	Light gray yellow brown (79)	Mild yellow brown (77)	-	H146					H34*	2

46.	Light brown gray (63)	Dark gray brown (81)	Mild yellow brown (77)						H35	1
47.	Brown gray (64) & white (263)	Dark orange yellow (72)	Dark yellow (88)						H37*	1
48.	Yellow white (92)	Brown black (65)	Dark yellow brown (78)						H38	1
49.	Yellow white (92)	Dark yellow brown (78)	-				H9*		H39	2
50.	Black (267) & white (263)	Mild yellow (87)	-						H40*	1
51.	Medium gray (265)	Mild yellow brown (77)	Gray yellow brown (80)		H55					1
52.	-	Yellow white (92)	-	H166	H59*		H99*			3
53.	Yellow gray (93) edge & Olive gray (113) centre	Mild yellow brown (77)	-		H61*, H62*					2
54.	Very yellow pink (25)	Dark orange yellow (72)	-		H63*					1
55.	Olive gray (113)	Mild yellow brown (77)	Light olive brown (94)		H65*					1
56.	Dark olive brown (96)	White (263)	-		H66*					1
57.	Dark gray (266) centre &yellow white (92) edge	Dark yellow brown (78)	Gray yellow brown (80)		H72*					1

58.	Yellow white (92)	Dark orange yellow (72)	Dark orange yellow (72)		H78					1
59.	Yellow gray (93)	Mild yellow brown (77)	-		H79					1
60.	Dark yellow (88) centre & yellow white (92) edge	Gray yellow (90)	-		H80					1
61.	-	Slight gray yellow (99)	Pale gray yellow (104)		H81					1
62.	Pale gray (233)	Dark dray yellow (91)	-		H82					1
63.	Medium gray (265)	Mild olive brown (95)	Very green yellow (97)		H84					1
64.	White (263)	Mild yellow brown (77)	Light gray yellow brown (70)		H83					1
65.	White (263)	Gray purple (228)	i. p Gray (232)		H88					1
66.	Gray yellow brown (80)	Gray yellow brown (80)	Light yellow brown (79)	H90*						1
67.	Medium gray (265)	Yellow gray (93)	-	H91*						1
68.	White (263)	Pale yellow (89)	-	H92						1
69.	Medium gray (265) & white (263)	Light olive gray (112)	-		H93*					1
70.	Light brown gray (63)	Light olive gray (112)	-		H94					1

71.	Yellow white (92)	White (263)	-			H95*, H96*				2
72.	Light orange (52)	Mild orange (53)	-				H97*			1
73.	-	Pale gray yellow (104)	Pale gray yellow (104)				H98			1
74.	White (263)	Dark orange yellow (72)	-				H100			1
75.	Yellow white (92)	Brilliant orange yellow (67)	-				H101*, H102, H103			3
76.	Red gray (22)	Dark gray yellow (91)	Light yellow (86)						H107	1
77.	Dark gray (266)	Mild yellow brown (77)	-						H108	1
78.	Light olive gray (112) & white (263)	Slight yellow brown (74)	Slight yellow (84)						H109	1
79.	Medium gray (265)	Mild yellow brown (77)	-						H111, H112	2
80.	Light gray (264)	Light gray olive (109)	-						H110*	1
81.	Medium gray (265)	Gray yellow (90)	-						H113	2
82.	Yellow gray (93)	Mild yellow brown (77)	Gray yellow (90)	H114						1
83.	Olive gray (113) middle & white (263) edge	Dark gray yellow (91)	Gray yellow (90)	H115						1

84.	Very dark bluish green (166)	Mild yellow green (136)	-	H116, H124, H127, H128, H152, H154						6
85.	Pale yellow (89) & Light gray (264)	Slight yellow (84)	Mild yellow (87)	H117						1
86.	Pale yellow (89) & Dark blue gray (192)	Mild olive brown (95)	-	H118						1
87.	Pale greenish yellow (104)	Light yellow (86)	-	H120						1
88.	Pale yellow pink (31)	Mild red brown (43)	-	H121						1
89.	Yellow gray (93)	Dark orange yellow (72)	Grey yellow (90)	H122						1
90.	White (263)	Mild red brown (43)	Brown pink (33)	H129						1
91.	White (263)	Pale yellow (89)	-	H130						1
92.	Medium gray (265)	Deep yellow brown (75)	Gray yellow (90)	H131						1
93.	Dark yellow brown (78)	Yellow white (92)	-	H133						1
94.	White (263)	Light grey yellow brown (79)	-	H134, H137						2

95.	Light gray (264)	Dark yellow brown (78)	Dark gray yellow (91)	H139, H140, H141, H155, H156						5
96.	Light gray yellow brown (79)	Gray yellow (90)	-	H138						1
97.	White (263)	Yellow gray (93)	Gray yellow (90)	H144						1
98.	Light Gray (264)	Yellow white (92)	Pale yellow (89)	H151						1
99.	Dark purple gray (234)	Yellow white (92)	-	H157						1
100.	Light gray (264)	Dark gray yellow brown (82)	Gray yellow (90)	H158						1
101.	Dark blue gray (187)	Blue gray (919)	-	H160						1
102.	Light gray (264)	Dark gray yellow brown (81)	Gray yellow brown (80)	H162						1
103.	Light gray (264)	Gray yellow (90)	-	H164						1
104.	Pale yellowish pink (31)	Slight orange yellow (68)	-	H165						1
105.	-	Deep red brown (41)	-	H167						1
106.	Medium gray (265)	Light olive gray (112)	-			H175				1

107.	Gray yellow brown (80)	Yellow gray (93)	-			H177				1
108.	Light gray (264)	Gray yellow brown (80)	-	H178, H179						2
109.	Yellow gray (93)	Slight yellow (84)	-	H180						1
110.	Light gray (264) middle & white (263) edge	Light olive gray (112)	-	H182						1
111.	Light gray (264)	Deep yellow brown (75)	Mild yellow brown (77)	H183, H184						2
112.	Light gray (264)	Gray yellow brown (80)	-	H185, H203, H217, H218						4
113.	Light gray (264) middle & white (263) edge	Yellow gray (93)	-	H186, H188, H202, H204						4
114.	Light yellow brown (77) & white (263)	Dark gray yellow (91)	-	H220, H223						2
115.	Medium gray (265)	Light olive gray (112)	-	H189, H190, H191, H192, H194, H195,						8

				H196 H198						
116.	Medium gray (265) & white (263)	Light gray olive (109)	-	H200, H201, H209, H210, H211, H219, H222						7
117.	Light gray (264) middle & white (263) edge	Slight yellow (84)	Very yellow (82)	H206						1
118.	Black (267)	Black (267)	-	H207						1
119.	Light gray yellow brown (79) middle & white (263) edge	Mild yellow brown (77)	Gray yellow (90)	H212						1
120.	Light gray yellow brown (79)	Brown black (65)	Dark yellow brown (78)	H216						1
121.	Brown gray (64)	Light gray olive (109)	Gray yellow (90)	H221						1

¥, produced melanin on ISP 6 medium; *, isolates subject to 16S rRNA gene sequencing

B. Lomas Bayas

Colour group	Cultural characteristic			Isolate	Number of isolates
	Aerial spore mass colour	Substrate mycelia colour	Colour of diffusible pigment		
1.	Pale yellow green (121)	Gray yellow (90)	-	LB13, LB36, LB37, LB40, LB43, LB44, LB46	7
2.	White (263)	Dark gray yellow (91)	-	LB45	1
3.	White (263)	Yellow white (92)	-	LB16, LB64, LB65	3
4.	Light gray (264)	Mild yellow (87)	Light gray yellow brown (79)	LB2*, LB6*, LB24	3
5.	Light gray (264)	Mild reddish orange (37)	-	LB9, LB14, LB15, LB17, LB18, LB27, LB28, LB30	8
6.	Light gray (264)	Deep yellow brown (75)	-	LB21, LB22, LB26	3
7.	Light yellow pink (28)	Mild yellow pink (29)	Pale yellow pink (31)	LB1*, LB29	2
8.	Slight orange (50)	Mild orange (53)	-	LB39	1
9.	Deep orange (51)	Mild orange (53)	-	LB32	1
10.	Light orange yellow (70)	Pale orange yellow (73)	-	LB19, LB4*	2

11.	Dark orange yellow (72)	Mild yellow brown (77)	-	LB42	1
12.	Deep yellow brown (75)	Mild yellow brown (77)	-	LB41	1
13.	Mild yellow (87) & white (263)	Dark yellow (88)	-	LB33*, LB34	2
14.	Yellow white (92)	Yellow white (92)	-	LB7*, LB20*, LB25*	3
15.	Yellow gray (93)	Light olive gray (112)	-	LB8*	1
16.	Yellow gray (93)	Mild yellow brown (77)	-	LB35	1
17.	Mild orange yellow (71)	Mild orange yellow (71)	-	LB49, LB51, LB52, LB53	4
18.	Pale yellow pink (31)	Light orange yellow (70)	-	LB50	1
19.	Pale yellow pink (31) & white (263)	Pale orange yellow (73)	-	LB54*	1
20.	Dark gray (266)	Gray red orange (39)	Pale green yellow (104)	LB55	1
21.	Olive gray (113)	Mild brown (58)	Mild yellow brown (77)	LB56*	1
22.	Pale yellow pink (31)	Yellow white (92)	-	LB57	1
23.	White (263)	Slight yellow (84)	-	LB58	1
24.	White (263)	White (263)	-	LB59, LB60	2

25.	Dark gray (266)	Gray red orange (39)	Pale green yellow (104)	LB61	1
26.	Slight yellow brown (74)	Slight yellow brown (74)	Slight yellow (84)	LB62	1
27.	Dark purple gray (234) middle & black (267) edge	Blackish purple (230)	Gray red brown (46)	LB63	1
28.	Yellow white (92) edge & Dark gray purple (288) middle	Yellow white (92)	-	LB66	1
29.	Dark olive brown (96)	Yellow gray (92)	-	LB38	1
30.	Pale purplish blue (203)	Slightly reddish brown (40)	-	LB31	1
31.	Yellow gray (93)	Gray yellow (90)	-	LB23	1
32.	-	Light orange (52)	-	LB10	1
33.	-	Brown orange (54)	-	LB11	1
34.	Light gray (264)	Mild red brown (43)	Pale yellow (89)	LB12	1
35.	-	Mild orange (53)	-	LB3	1

*Isolates subject to 16S rRNA gene sequencing

Appendix 2

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.

A) Culture media

1. 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

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

3. 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.

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

5. 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.

6. 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

7. Tyrosine agar (ISP medium 7; Shirling & Gottlieb, 1966)
Glycerol……... 15.0 g
L-Tyrosine (Difco)……..... 0.5 g
L- sparagine (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 …..…….……..... 1.0 ml
Agar……..... 20.0 g
pH…….…..……7 .2 - 7.4

8. Modified Bennett's agar (Jones, 1949)

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

9. Pushpa agar (Agrawal, unpublished)

Lab Lemco……..1 g
Tryptose…… 2 g
Yeast extract…….2 g
Starch……. .0.1 g
Glucose …….. 10 g
$CaCO_3$ ……. 0.1 g
*Trace salt solution …….. 0.1 mL
Agar ……... 15 g
pH …….. 7.2

B) Isolation media

1. Arginine-vitamin agar (El Tarabily *et al*., 1996)

L-arginine…….0.3 g
Glucose……1.0 g
Glycerol……...1.0 g
K_2HPO_4……..0.3 g
$MgSO_4.7H_2O$…….0.2 g
NaCl……0.3 g
*Trace salt solution……..1.0 ml
Agar…….18 g
pH……6.4

2. Gauze'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 ……7.2+0.2

3. 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
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
icotinamide …….……... 10 mg
Pantothenic acid (B5) …….....… 10 mg
Pyridoxine (B6) ……. 10 mg
Riboflavin a choline (B2) …….……..……10 mg
Thiamin (B1) …….…… 10 mg
Cyanocobalamin (B12) ……..... 0.5 mg
Folic acid ……...…..... 0.5 mg

4. 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

5. Peptone-yeast extract agar (Goodfellow & Dawson, 1977)

Peptone…….5 g
Yeast extract……3 g
Agar……..15 g
dH_2O……1.0 L
pH……7.2+- 0,2

6. Raffinose-histidine agar (Vickers *et al*., 1984)

Raffinose……10 g
Histidine…….1 g
K_2HPO_4…….…….1 g
$MgSO_4.7H_2O$……..0.5 g
$FeSO_4.7H_2O$……...0.01 g
Agar……12 g
dH_2O……..1.0 L
pH …….7.0 – 7.2

7. 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

8. Tapwater agar

Tapwater……..1.0 L
Agar…….20 g

C) Phenotypic test media

1. 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 were tyndallised in distilled water and arbutin were prepared and autoclave prior adding to the melted basal medium to give a final concentration of 0.1% (w/v).

2. 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
Allantoin was prepared in distilled water, autoclaved at 121°C for 20 minutes 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.

3. 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

4. Nitrate reduction medium (Gordon & Mihm, 1962)

KNO_3…….. 1.0 g
Lab LEMCO…….…….. 2.4 g
dH_2O…….……...1.0 L
pH…….…….. .0
Three ml amounts of the media were dispensed into bijoux bottles.
Reagent A: 0.8g sulfanilic acid in 100ml of 5 M acetic acid
Reagent B: 0.6g α-naphthylamine in 100 ml of 5M acetic acid

5. Tributyrin agar

Tributyrin agar base (Sigma T3688)……..... 23.0 g
Glycerol tributyrate (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.

6. 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.

Appendix 3

Buffers

1. Buffer media for pH tolerance tests (Xue et al., 2005)

Glucose-yeast extract-malt extract (ISP medium 2; Shirling & Gottlieb, 1966) and Modified Bennett's agar (Agrawal) were used as basal medium. The pH of the media were tested using pH meter and adjusted with pH Buffers prepared in distilled water as listed below:

pH	Acidic	Alkali
4-5	0.1M citric acid	0.1M sodium citrate
6-8	1 N HCl	0.1M NaOH
		0.1M KH_2PO_4
9-10	1 N HCl	0.1M Na_2CO_3
		0.1M $NaHCO_3$
11	1 N HCl	0.05M Na_2HPO_4
		0.1M NaOH

2. Congo red (0.1%) for cellulose degradation

Stock solution:
Congo red …… 5 g
Saturated NaCl* …….. 500 ml

*Saturated NaCl
NaCl ……. 30 g
dH_2O ……. 200 ml
mix well, and add ethanol ……. 800 ml

Working solution:
Congo red stock solution …… 10 ml
1% NaOH ……. 90 ml

3. Nitrate reduction reagents

Reagent A:
Sulphanilic acid …….. 0.8 g
Acetic acid ……. 100 ml
Reagent B:
α-Napthylamine ……. 0.8 g
Acetic acid ……. 100 ml

Appendix 4

Chemotaxonomic methods

A) Diaminopimelic acid analysis by TLC (Staneck & Roberts, 1974)

Hydrolysis (Use nitrile gloves and glasses)

1. Weight out 5-10 mg freeze-dried cell material into a screw capped Duran GL 14 tube.
2. Add 200 µl 6N HCl.
3. Incubate at 100 °C for 12-16 hours.
4. Remove insoluble material by centrifugation (2999 rpm for 5 minutes) and transfer supernatant to a Fiolax Schott tube.
5. Dry sample in an incubator at 60-70 ^{O}C.
6. Dissolve the final residue in 100µl water.

Thin-layer chromatography

1. Chromatography tank must be prepared 1 day in advance to create a uniform solvent-*saturated atmosphere.* Place 2 strips of filter paper (Camag 022.5243) on both sides of the tank and add methanol: distilled water: 6 N HCl: pyridine (80: 26: 4: 10 v/v).
2. Draw a pencil line 1 cm above the bottom of an aluminium-backed sheet (20 x 20 cm diameter, No. 5716 Merck, Darmstadt, Germany).
3. Apply each sample and a mixture of the controls, namely 0.01M LL- and *meso*-A_2pm (Sigma) onto the sheets using a capillary pipette, 1 cm from the base of the sheet.
4. Develop the TLC plate until solvent front is 2 cm from the top of the plate (takes about 3 hours).
5. Air-dry chromatogram in a fume hood, spray with ninhydrin reagent (0.2%, w/v ninhydrin in acetone) and heat at 100^{o}C for 5 minutes.
6. The isomers of diaminopimelic acid (A_2pm) appear as dark purple to brown spots that have a lower Rf (retention factor) than other amino acids, the latter give blue to purple coloured spots. The Rf values of the A_2pm spots run in the

order: 3-hydroxy-A2pm, *meso*-A_2pm and LL-A_2pm (from lowest to highest). The A_2pm isomers of test strains are identified by comparison with the A_2pm standards.

B) Isoprenoid quinones extraction (Collins 1985)

1. Weight 200-500 mg of freeze-fried biomass and add to Schlott 618 tube.
2. Add 25 ml chloroform: methanol (2:1,v/v).
3. Leave overnight on a rotary shaker.

   NEXT DAY
4. Wait until the cells are deposited at the bottom of the tube and filter extract using a small funnel lined with filter paper (Sartorious 388) (fold filter paper 4 times).
5. Dry the extract in the evaporator at 40 °C.
6. Add chloroform: methanol (2:1, v/v) to the remaining cells and leave for an hour on a rotary shaker, then filter.
7. Combine and dry the two extracts
8. Dissolve the extract in 400 µl acetone.
9. Load extract onto a TLC plate (TLC silica gel 60 F 254.1.05735.0004, Merck, UK) using a Pasteur pipette. Prepare 2 samples per plate (4 cm apart). Close the tubes with paraffin.
10. Run samples in a chromatography tank containing petroleum ether: diethyleter (85: 15 v/v). Always use fresh solvent.
11. Air-dry chromatogram and examine under ultraviolet light.
12. Scrape isoprenoid quinone spot from the gel plate and add to a glass syringe containing a filter.
13. Place the glass syringe in an evaporator tube and add diethylether twice to elute the menaquinones.
14. Dry extract using the evaporator at 40 °C.
15. Dissolve the residue in isopropanol.
16. Transfer the extract to a small vial.
17. Isoprenoid quinones identified by 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).

18. 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 traces integrated using a C-R6A Chromatopac Integrator (Shimadzu Co.).

C) Extraction and analysis of whole cell sugars (Lechevalier & Lechevalier, 1970)

Hydrolysis (Use nitrile gloves and glasses)

1. Weight out 25 mg freeze-dried cell material into a Teflon-lined screw-capped tube.
2. Add 1 ml 1N H_2SO_4
3. Heat at 95 °C for 2 hours.
4. After cooling at room temperature, add a Pasteur pipette volume of saturate $Ba(OH)_2$ and transfer to a 10 ml beaker.
5. Adjust pH to 5.0-5.5using saturated $Ba(OH)_2$ or 1N H_2SO_4. As the pH is initially very low and the volume is small, use pieces of pH indicator paper to detect pH values.
6. Transfer sample to a hydrolysis tube and centrifuge at 1800 rpm for 5 minutes.
7. Add the liquid phase to a Falcon tube.
8. Freeze sample at an angle of 45 ° for 2-3 hours at -20 °C.
9. Freeze-dry in vacuum overnight.
10. Dissolve the residue in 0.5 ml distilled water and transfer to an Eppendorf tube.

Thin-layer chromatography

1. Chromatography tank must be prepared 1 day in advance to create a uniform solvent-saturated atmosphere. Place two strips of filter paper (Camag 022.5243) on both sides of the tank. Add ethylacetate: pyridine: water (100: 35: 25, v/v).
2. A pencil line was drawn 1 cm above the bottom of the aluminium-backed sheets (20 x 20 cm diameter; No. 5716, Merck, Darmstadt, Germany).

3. Apply 5 µl of each sample (2 cm) and sugar standard (1%, w/v [arabinose, galactose, D-(+) glucose, D-mannose, rhamnose, ribose, xylosein pyridine]) onto the plate using a gas-chromatography syringe 1.5 cm from the baseline and dry with a hair-dryer.
4. Develop the TLC plate for 2 hours (until solvent front is 1 cm from the top).
5. Air-dry under a fume hood for 30 minutes prior to repeating the whole procedure.
6. Air-dry and spray with aniline phthalate reagent and heat at 105 ^{o}C for 5 minutes.
7. Hexose and pentose sugars appear as brown-red and grey-green spots, respectively. Sugar pattern are identified by comparison against the standard sugars.

D) Extraction and analysis of polar lipids (Minnikin *et al.* (1984) as modified by Kroppenstedt & Goodfellow (2006))

Hydrolysis (Use nitrile gloves and glasses)

1. Weigh out about 100 mg freeze-dried cells into a 10 ml Teflon-lined screw capped tube.
2. Add 2 ml aqueous methanol (10 ml of 0.3 % aqueous NaCl to 90 ml methanol)
3. + 2 ml petroleum ether (bp. 60-80 °C).
4. Mix for 15 minutes on a rotary shaker.
5. Centrifuge at 1800 rpm for 5 minutes at room temperature and save the upper layer for isoprenoid quinone analyses and keep at – 20 ^{o}C in the dark. Quinones are degraded by light.
6. Add 1 ml petroleum ether (bp. 60-80 °C) to the aqueous phase, mix for 15 minutes and centrifuge for 5 minutes. The upper layer may be kept at -20^{o}C in the dark prior to analyses for isoprenoid quinone.
7. Mix the lower phase using a vortex and heat for 5 minutes in a boiling water bath (99 °C).
8. Cool at 37 °C for 5 minutes.
9. Add 2.3 ml of chloroform: methanol: 0.3%, v/v aqueous NaCl (90: 100:30, v/v/v) and mix for 1 hour on a rotary shaker.

10. Centrifugeat 1800 rpm for 5 minutes. Transfer the supernatant to a clean 10 ml screw-capped tube.
11. Add 0.75 ml chloroform: methanol: 0.3%, v/v aqueous NaCl (50: 100: 40, v/v/v) to the pellet (cells) and mix for 30 minutes, centrifuge and combine the solvents (discard cells).
12. Add 1.3 ml chloroform and 1.3 ml 0.3 % NaCl to the combined extracts and mix carefully (20 seconds at 2700 rpm by vortexing)
13. Centrifuge at 1800 rpm for 5 minutes and transfer the lower layers directly to an evaporator tube (Fiolax Schott tube).
14. Concentrate to dryness in the evaporator (water bath at 40 °C) for 20-30 minutes.
15. Dissolve the lipid extracts in 0.4 ml chloroform: methanol (2:1, v/v). Keep at -20 °C.

2D-Thin-layer chromatography

*Check humidity, do not run TLC if H_R is above 60 %.

1. Place two chromatography tanks each containing 2 strips of filter paper on the sides in a tray connected to water recirculator. Adjust temperature to 25 ^{o}C.
2. Load 20 µl of polar lipid extract to the corners of 4 aluminium TLC plates (Macherey-Nagel size 10 x 10 Ref. 818135) using a chromatography syringe and dry with a hair-dryer.

   <u>First run</u>
3. Add the following solvent to the first chromatography tank: chloroform: methanol: water (65:25:4, v/v/v) using glass pipettes**.**
4. Place the 4 TLC plates into the same chromatography tank and develop until solvent front reaches the top of the plates.
5. Leave to air-dry for 30 minutes under fume hood.

   <u>Second run</u>
6. Add the following solvent to the second chromatography tank: chloroform: glacial acetic acid: methanol: distilled water (80:12:15:4, v/v/v/v) using glass pipettes.
7. Place the 4 TLC plates in the same chromatography tank and develop until solvent front reaches the top of the plates.

8. Leave to air-dry for 30 minutes under a fume hood.
9. The chromatograms were developed using the differential stains shown below.

Detection of lipids

- Plate 1: Detection of all lipids
    - Spray with 5% (v/v) molibdatophosphoric acid in ethanol (Sigma P1518, Merck 100480).
    - Char at 150 ^{o}C for 20 minutes then at 170 ^{o}C for 20 minutes, to detect all of the lipids.
    - Cool at room temperature. Lipids appear as black spots.
    - Scan plate and label spots using a pencil.
- Plate 2: Detection of amino-groups
    - Spray with 0.2 % (w/v) ninhydrin in acetone or methanol.
    - Char at 100 ^{o}C for 10 minutes, to detect lipids with free amino groups.
    - Cool at room temperature. Phosphatidylethanolamine (PE) and phosphatidyl-N-methylethanolamine (PME) appear as dark pink spots on a white background.
    - Scan plate and label spots using a pencil.
    - Spray the same sheet with 1.3% molybdenum blue (Sigma 119 KG123) diluted with 4.5M sulphuric acid (1:1, v/v).
    - Dry at room temperature. Phosphorus-containing lipids appear as blue spots on a white background.

  The lipids on each of the TLC plates were identified by comparing their mobilities with those of standard lipids (Sigma, PH-9).
- Plate 3: Detection of glyco-groups
        - Spray with α-naphtho-sulphuric acid (40.5 ml ethanol, 4 ml distilled water, 6.5 ml concentrate sulphuric acid and 10.5 ml α-naphthol (15 g/l in ethanol))
    - Char at 80 ^{o}C for 10 minutes to detect lipids with glycolyl groups.
    - Cool at room temperature. Phosphatidylinositol mannosides (PIDM) and glycolipids appear as brown spots on a white background.
    - Scan plate.
- Plate 4: Detection of choline-groups (Tindall 1990)

- Gently spray with Dragendorff reagent (0.11 M potassium iodide and 0.6 mM bismuth subnitrate in 3.5 M acetic acid).
- Dry at room temperature. Phosphatidylcholine (PC) appears as an orange spot on a yellow background.
- Scan plate.

Appendix 5

Antimicrobial screening of actinobacterial strains isolated from the Cerro Chajnantor and Lomas Bayas sampling sites

Table 1 Inhibition zones (mm) produced by actinobacteria isolated from the ALMA and Lomas Bayas environmental samples in the plug assays after incubation on Luria Bertani agar overnight at 28ºC.

Colour-groups		Isolates	Panel of wild type strains									
			E. coli		*P. fluorescens*		*B. subtilis*		*S. aureus*		*S. cerevisiae*	
			ISP2	ISP3	ISP2	ISP3	ISP2	ISP3	ISP2	ISP3	ISP2	ISP3
		ALMA 1										
Multi-membered	4	H153	-	-	-	-	14	-	14	11	15	-
	12	H136	-	-	-	23	-	-	-	-	-	-
		H142	13	13	21	16	19	18	27	25	21	20
		H150	-	-	22	21	20	17	16	12	17	16
		H151	-	-	14	-	12	-	15	-	15	-
		H161	18	-	25	19	24	21	22	-	25	-
	45	H146	-	-	18	21	18	8	13	19	15	16
	84	H116	-	-	-	-	-	12	13	12	-	15
		H124	-	-	-	-	-	15	15	15	12	23
		H127	-	-	-	-	-	12	-	12	-	18
		H128	-	-	-	-	-	12	-	13	-	17
		H152	-	-	-	-	12	14	-	16	-	20
		H154	-	-	-	-	11	16	13	16	-	-
	94	H134	-	-	19	21	22	20	20	14	20	17
		H137	-	-	17	29	23	20	19	13	21	17
	95	H139	-	-	-	12	16	13	-	16	-	-
		H140	-	-	-	-	14	13	-	22	-	-
		H141	-	-	-	-	15	13	15	17		-
		H155	-	-	-	-	15	-	-	-	-	-
		H156	-	-	16	-	16	14	23	16	13	-
Single-membered		H90	-	-	-	15	-	10	-	-	23	23
		H91	-	-	-	-	11	-	-	-	15	13
		H114	-	-	-	-	-	10	-	13	-	-
		H115	-	-	-	15	22	18	22	15	27	19
		H117	-	-	-	-	-	17	-	12	-	
		H118	-	-	-	-	-	12	-	13	-	20
		H120	-	-	-	12	12	20	12	-	-	16
		H121	-	-	16	20	16	16	16	16	-	20
		H122	12	13	19	20	17	19	13	14	23	24
		H129	-	-	-	-	15	16	-	-	-	-
		H131	-	-	-	-	-	11	-	14	-	-
		H144	-	-	17	21	17	18	15	13	16	13
		H151	-	11	-	19	-	14	-	13	-	

		H157	-	-	-	-	-	11	-		-	-
		H158	-	-	-	-	17	12	-	16	12	-
		H160	-	-	-	-	12	12	14	12	-	-
		H162	-	-	-	-	17	15	13	17	13	-
		H164	-	-	-	-	-	12	-	-	-	-
		H165	20	-	26	19	23	20	23	13	24	15
		H167	-	-	-	-	14	14	12	13	-	-
		ALMA 2										
Multi-membered	1	H56	-	-	-	-	-	-	-	-	14	-
		H69	-	-	-	-	12	-	12	-	12	-
		H74	-	-	-	-	15	-	-	-	-	-
	4	H73	-	-	-	-	17	-	16	-	19	-
		H87	-	-	-	-	15	-	-	-	-	-
	9	H68	-	-	-	-	11	-	12	-	-	-
	12	H60	-	-	-	-	25	-	20	-	25	-
		H89	-	-	-	15	-	-	-	-	-	-
	22	H70	-	-	16	13	12	14	-	-	14	18
		H71	-	11	19	17	14	16	-	12	32	30
		H85	-	-	16	-	18	20	12	13	20	-
	36	H64	-	-	-	-	-	-	-	-	12	-
	52	H59	-	-	-	-	25	-	20	-	40	13
	53	H61	12	13	17	15	16	15	-	11	19	22
		H62	12	12	17	13	16	14	-	-	23	16
Single-membered		H55	15	-	14	-	19	12	11	-	21	22
		H63	-	-	-	-	-	-	-	-	15	11
		H65	-	-	-	-	-	-	-	-	23	12
		H72	-	15	15	16	13	15	-	-	19	21
		H78	-	-	-	-	14	-	-	-	-	-
		H79	-	-	-	-	-	-	-	-	-	-
		H80	-	-	-	-	11	-	-	-	11	11
		H81	-	-	-	-	-	-	-	-	22	-
		H82	-	-	-	-	19	14	14	-	24	21
		H83	-	12	13	14	11	16	-	-	18	23
		H84	-	-	-	13	26	24	22	22	30	26
		H88	-	-	20	-	21	-	20	-	24	-
		H93	12	11	17	17	15	14	-	11	25	19
		H94	-	-	-	-	18	13	12	-	22	20
		ALMA 3										
Single-membered		H175	-	-	-	-	14	-	14	-	-	-
		ALMA 4										
Multi-membered	4	H4	-	-	-	13	-	10			14	16
	49	H9	-	-	18	-	16	12	-	-	18	15
	52	H99	-	-	14	-	-	-	-	-	-	-
	75	H101	-	-	-	-	-	-	-	-	11	-
		H103	-	-	-	-	-	-	-	-	-	14
Sin		H5	-	-			12	12	12	10	18	16

		H8	-	-	-	-	-	-	-	-	17	-
		H97	-	-	-	-	-	-	27	-	28	-
		H98	-	-	-	-	17	-	14	-	20	-
ALMA 5												
multi-membered	12	H193	-	-	-	-	18	17	16	-	16	18
		H199	-	-	24	24	36	32	30	34	-	27
	108	H178	-	-	-	-	-	17	-	-	-	-
		H179	-	-	-	-	-	11	24	-	20	-
	111	H183	-	-	-	-	-	15	-	18	-	-
		H184	-	-	-	-	-	14	-	16	-	-
	112	H185	-	15	-	-	19	18	-	15	-	16
		H203	-	-	-	-	-	16	-	-	16	-
		H217	-	-	-	-	18	20	-	-	-	12
	113	H186	-	-	-	-	-	14	-	-	-	18
		H188	-	-	-	15	20	20	-	-	20	-
		H202	-	-	-	-	-	14	-	-	16	-
		H204	-	-	-	-	17	13	-	-	-	-
	114	H220	-	-	-	-	-	20	12	13	-	14
		H223	-	-	-	-	-	15	-	12	-	14
	115	H189	-	-	-	-	-	17	-	-	-	-
		H190	-	-	-	-	-	13	-	-	-	-
		H192	-	-	-	-	-	15	18	-	12	15
		H194	-	-	-	-	-	17	-	-	-	20
		H195	-	-	-	-	20	17	16	-	-	20
	116	H200	-	-	-	-	16	16	-	-	-	-
		H209	-	-	18	-	17	20	15	-	-	20
		H219	-	-	-	-	-	18	-	-	-	16
		H222	-	-	-	-	-	-	14	-	15	17
Single-membered		H180	-	-	-	12	12	14	15	-	-	-
		H206	-	-	25	25	25	32	26	34	-	30
		H212	-	-	16	-	20	-	-	-	22	-
		H216	-	-	-	13	17	-	17	-	-	-
		H221	-	-	20	20	23	23	16	18	-	-
		ALMA 6										
Multi-membered	1	H36	-	-	-	-	14	-	11	-	15	-
	10	H41	-	-	-	-	12	-	14	-	18	-
		H42	-	-	-	-	-	-	-	-	11	14
	12	H13	-	-	-	-	24	21	-	-	27	25
		H21	-	-	-	-	11	-	11	-	14	-
	26	H14	-	-	-	-	21	20	-	-	29	23
	28	H16	-	-	-	-	15	-	-	-	19	14
	29	H17	-	-	-	-	-	11	-	11	-	15
	36	H25	-	-	13	-	11	-	14	-	20	17
	45	H34	-	12	16	17	12	18	-	-	15	26
	49	H39	-	11	19	12	17	14	15	-	21	20
	79	H111	-	-	-	-	-	20	-	-	-	18
		H112	-	-	-	-	-	-	-	-	19	-
		H10	-	12	18	15	26	14	28	-	30	19

Single-membered		H11	-	-	-	20	30	24	30	22	30	25
		H12	-	-	-	-	15	-	-	-	20	-
		H15	-	-	-	-	18	-	-	-	22	-
		H19	-	-	-	-	-	-	-	-	11	-
		H20	-	-	-	-	-	-	12	-	-	-
		H23	-	-	-	-	-	12	23	16	15	-
		H26	-	-	-	-	20	-	19	-	27	-
		H27	-	-	-	-	30	28	27	24	31	30
		H29	-	-	-	-	-	10	-	-	-	-
		H31	-	-	-	-	-	-	-	-	19	16
		H33	-	-	-	-	-	-	-	-	10	20
		H37	-	-	13	13	20	17	18	17	20	20
		H38	-	-	-	-	-	-	16	-	-	18
		H43	-	-	-	-	23	-	-	-	-	-
		H45	14	12	15	11	12	12	-	-	22	15
		H46	-	-	-	-	11	-	-	-	13	11
		H48	-	-	-	-	-	11	-	-	-	17
		H49	-	-	-	-	20	-	-	-	25	-
		H50	-	-	-	-	11	-	-	-	12	-
		H51	-	-	-	-	-	11	-	10	14	13
		H52	-	-	12	-	14	13	-	-	16	16
		H107	-	-	-	-	16	-	-	13	22	12
		H108	-	-	-	-	-	17	-	-	18	18
		H109	-	-	-	-	-	16	17	13	17	16
Lomas Bayas												
Multi-membered	1	LB36	-	-	15	-	15	-	-	-	-	-
		LB13	-	-	-	13	-	14	-	-	-	-
	3	LB16	-	-	-	-	-	12	-	14	-	-
		LB65	-	-	-	-	-	-	16	-	-	-
	4	LB2	-	-	-	-	14	19	-	-	-	-
		LB24	-	-	-	-	18	20	-	-	-	-
	5	LB14	-	-	-	-	-	-	-	-	-	15
		LB15	-	-	-	-	-	-	-	14	-	-
		LB27	-	-	-	-	16	16	-	15	13	16
		LB28	-	-	-	-	-	-	-	19	-	13
		LB30	-	-	-	-	-	-	-	14	-	-
	6	LB21	-	-	-	-	17	18	18	15	20	-
		LB22	-	-	-	-	17	17	17	-	15	-
		LB26	-	-	-	-	-	-	15	16	-	-
	7	LB1	-	-	-	-	-	15	-	-	-	-
		LB29	-	-	-	-	-	-	-	-	-	16
	13	LB34	-	-	-	-	12	-	-	12	-	-
	14	LB20	-	-	-	-	-	-	16	-	-	-
		LB25	-	-	-	20	-	-	-	-	-	12
	24	LB59	-	-	-	-	-	25	-	-	-	-
Single-membered		LB12	-	-	-	-	-	-	-	20	-	-
		LB23	-	-	-	-	19	-	-	-	18	-
		LB31	-	-	-	-	-	-	-	21	-	13
		LB32	-	-	-	-	-	-	-	-	-	16
		LB35	-	-	-	-	-	-	15	16	-	-

	LB45	-	-	-	-	-	-	10	10	-	-
	LB54	-	-	-	-	-	11	-	14	-	11
	LB57	-	-	-	-	-	13	-	10	-	12
	LB62	-	-	-	20	-	11	-	10	-	19
	LB63	-	-	-	20	-	13	-	15	-	-

-, negative results.

Strains negative against all of the wild type strains:

ALMA 1:

Multi-membered colour-groups: H119[12], H123[12], H125[81], H126[9], H132[28], H135[12], H143[12], H145[12], H147[12], H148[12], H163[12], H166[52], H168[12].

Single-membered colour-groups: H92, H130, H133, H138, H149.

ALMA 2:

Multi-membered colour-groups: H1[1], H57[1], H58[1], H67[1], H75[1], H76[1], H77[1], H86[4].

Single membered colour-groups: H2, H3, H66, H79.

ALMA 3:

Multi-membered colour-groups: H95[71], H96[71], H171[12], H172[12], H173[12], H174[12] and H176[12].

Single-membered colur-groups: H177.

ALMA 4:

Multi-membered colour-groups: H6[6], H7[7], H102[75], H104[7], H105[7] and H106[6].

Single-membered colour-groups: H100.

ALMA 5:

Multi-membered colour-groups: H181[26], H191[115], H196[115], H198[115], H201[116], H205[12], H210[116],

H211[116], H213[12], H214[12], H215[12] and H218[112].

Single-membered colour-groups: H182, H197 and H207.

ALMA 6:

Multi-membered colour-groups: H44[12] and H54[1].

Single-membered colour-groups: H18, H22, H24, H28, H30, H32, H35, H40, H47, H53, H110 and H113.

Lomas Bayas:

Multi-membered colour-groups: LB4[10], LB6[4], LB7[14], LB9[5], LB17[5], LB18[5], LB19[10], LB33[13], LB37[1], LB40[1], LB43[1], LB44[1], LB46[1], LB49[17], LB51[17], LB52[17], LB53[17], LB60[24] and LB64[3].

Single-membered colour-groups: LB3, LB8, LB10, LB11, LB38, LB39, LB41, LB42, LB50, LB55, LB56, LB58, LB61 and LB66.

Table 2 Diameter of zones of inhibition (mm) produced by strains isolated from the Cerro Chajnantor and Lomas Bayas sampling sites against *B. subtilis* reporter strains following incubation on Luria Bertani agar overnight at 28ºC. Strains giving blue halos are shown in blue.

Colour-groups		Isolates	Inhibition zones (mm)					
			Reporter genes					
			phi105	*ypuA*	*yvqI*	*yvgS*	*yheH*	yjaX
ALMA 1								
Multi-membered	4	H153*	-	15	16	15	-	15
	12	H142*	-	23	18	27	15	25
		H150*	-	25	25	25	24	26
		H159*	-	18	18	19	16	13
		H161*	-	19	20	19	20	20
	45	H146*	-	21	23	23	22	21
	52	H166	-	-	15	-	-	-
	84	H116*	-	13	16	16	16	17
		H124*	-	17	10	18	14	15
		H127*	-	15	-	13	14	-
		H128*	-	-	-	13	15	-
		H152*	-	16	16	17	-	18
		H154*	-	18	18	17	16	19
	94	H134*	-	25	26	25	24	20
		H137*	-	24	22	25	-	24
	95	H139*	-	16	16	15	25	18
		H140*	-	17	-	16	18	17
		H141*	-	17	13	16	15	18
Single-membered		H114*	-	10	-	-	-	-
		H115*	-	18	15	20	24	22
		H117*	-	14	18	19	15	13
		H118*	-	14	-	15	-	-
		H120*	-	14	18	14	12	12
		H121*	-	20	18	15	22	21
		H122*	-	22	22	20	32	19
		H151*	-	-	-	-	14	-
		H157*	-	-	-	28	20	-
		H158*	-	18	14	16	-	-
		H160*	-	12	-	14	-	-
		H162*	-	14	-	15	17	-
		H164*	-	-	-	12	-	-
		H167*	14	14	12	13	11	14

ALMA 2								
Multi-membered	1	H69*	22	13	14	15	13	11
	9	H68*	14	-	13	-	10	10
	22	H70*	-	18	24	25	30	25
		H85*	19	22	22	24	26	22
	52	H59*	32	26	25	24	25	24
Single-membered		H3	-	-	11	10	12	-
		H55*	-	19	24	26	28	26
		H72*	-	15	15	20	14	18
		H78*	16	13	16	13	14	14
		H82*	24	16	15	15	17	16
		H84*	25	27	27	30	24	24
		H94*	-	20	21	22	23	22
ALMA 3								
Multi-membered 12		H172	-	-	-	-	-	17
		H176	-	20	-	-	-	-
Single-membered		H175*	14	12	-	15	-	15
		H177	-	11	-	-	-	-
ALMA 4								
Multi-membered	4	H4*	-	20	21	18	22	25
	49	H9*	-	17	21	15	21	23
	52	H99	-	20	23	22	26	-
	75	H103	20	14	16	15	15	14
Single-membered		H5*	-	19	21	15	19	20
		H97	-	25	21	20	21	22
ALMA 5								
Multi-membered	1	H184*	-	13	-	-	-	-
	2	H203*	-	-	-	-	15	11
		H217*	-	-	-	13	-	-
	3	H188*	-	17	-	14	-	15
		H204*	12	12	12	14	15	12
	5	H190*	-	-	18	-	15	-
		H194*	-	-	-	-	12	11
		H195*	13	10	-	13	-	-
	6	H222	13	12	-	-	-	-
	10	H179*	-	11	-	-	-	-
	11	H183*	-	12	-	-	-	-
		H185*	14	20	-	21	14	13
		H186*	-	15	-	-	-	-
		H220	15	11	-	12	-	-
	12	H199*	-	22	-	22	24	20
	26	H181	12	-	-	-	-	-

	116	H211	20	19	-	-	-	15
Single-membered		H180*	-	14	-	-	-	-
		H182	-	-	-	-	-	15
		H206*	-	18	-	15	16	20
		H212*	-	10	-	-	-	-
		H216*	-	12	-	14	-	-
		H221*	-	19	-	-	-	-
ALMA 6								
Multi-membered	1	H36*	-	14	-	14	14	-
	10	H41*	-	-	-	-	-	15
		H42	-	-	10	-	-	-
	12	H13*	-	22	18	20	25	20
	26	H14*	-	28	29	24	27	23
	28	H16*	-	11	-	-	-	-
	29	H17*	-	10	12	12	12	-
	36	H25*	12	-	-	-	-	-
	45	H34*	-	21	23	21	24	25
	49	H39*	-	21	28	23	-	25
	79	H112	12	14	14	13	14	11
Single-membered		H11	-	33	11	13	15	25
		H15*	-	-	11	-	12	11
		H18	-	-	11	10	-	-
		H23*	-	15	15	-	-	-
		H26*	15	18	16	14	17	16
		H27*	-	25	-	32	14	13
		H29*	-	11	-	12	14	12
		H32	-	10	-	-	10	-
		H37*	-	21	-	22	-	25
		H43*	-	-	-	-	-	12
		H45*	-	14	-	13	-	16
		H46*	-	11	14	12	-	-
		H48*	-	17	15	15	15	16
		H49*	-	13	13	16	19	20
		H51*	-	10	10	10	12	-
		H52*	-	20	24	22	25	25
		H107*	21	16	15	15	16	15
		H108*	16	13	14	11	12	13
		H109*	23	20	18	17	16	16
Lomas Bayas								
Multi-membe	1	LB13*	15	-	11	-	-	-
		LB43	16	-	-	-	-	-
		LB44	14	-	-	-	-	-

		LB46	16	-	-	-	-	-
	3	LB64	-	15	-	18	-	-
		LB65	-	10	-	-	-	-
	4	LB2*	-	14	-	14	-	-
		LB6	-	11	-	21	20	-
		LB24*	-	10	-	13	-	14
	5	LB9	-	-	23	-	-	-
	6	LB21*	-	20	-	24	-	19
		LB22*	-	19	-	18	17	19
		LB26	-	20	-	19	15	20
	7	LB29	-	-	-	-	12	-
	13	LB34*	-	16	-	-	-	-
	14	LB7	11	-	23	-	-	-
		LB20	13	16	-	18	-	-
		LB25	-	10	-	-	-	-
	24	LB12	-	-	11	-	-	-
Single-membered		LB8	-	-	22	-	-	-
		LB11	-	10	12	-	-	-
		LB23	-	12	-	11	17	-
		LB35	-	19	-	19	16	20
		LB45	16	-	-	-	-	-
		LB50	-	-	-	-	16	-
		LB62*	-	12	-	-	-	-

-, negative result.

*, isolate grown on ISP2 and/or ISP3 showed activity against wild type *Bacillus subtilis* in primary screening.

Strains negative against all of the *Bacillus subtilis* reporter strain:
([1-81], number of multi-membered colour-groups)

ALMA 1:
Multi-membered colour group: H119[12], H123[12], H125[81], H126[9], H132[28], H135[12], H136[12], H143[12], H145[12], H147[12], H148[12], H155*[95], H156*[95], H163[12], H168[12]
Single-membered colour-group: H90*, H91*, H92, H130, H133, H138, H149, H129*, H131*, H144*, H165*

ALMA 2
Multi-membered colour-group: H1[1], H56[1], H57[1], H58[1], H60*[12], H61*[53], H62*[53], H64[36], H67[1] H71*[22], H73*[4], H74*[1], H75[1], H76[1], H77[1], H86[4], H87*[4], H89[12]
Single-membered colour-group: H2, H63, H65, H66, H79, H80*, H81, H83*, H88*, H93*

ALMA 3:
Multi-membered: H95[71], H96[71], H171[12], H173[12], H174[12]

ALMA 4:
Multi-membered: H6[6], H7[7], H102[75], H104[7], H105[7], H106[6]
Single-membered: H8, H98*, H100

ALMA 5:

Multi-membered: H178*[12], H189*[11], H191[115], H192*[5], H193*[12], H196[115], H198[115], H200*[11], H201[116], H202*[3], H205[12], H209*[6], H210[116], H213[12], H214[12], H215[12], H218[112], H219*[6], H223*[4]
Single-membered: H197, H207

ALMA 6:

Multi-membered: H21*[12], H44[12], H54[1], H111*[79]
Single-membered: H10, H12*, H19, H20, H22, H24, H28, H30, H31, H33, H35, H38, H40, H47, H50*,
H53, H110, H113

Lomas Bayas:

Multi-membered: LB1*[7], LB4[10], LB14[5], LB15[5], LB16*[3], LB17[5], LB18[5], LB19[10], LB27*[5], LB28[5], LB30[5], LB33[13], LB36*[1], LB37[1], LB40[1], LB49[17], LB51[17], LB52[17], LB53[17], LB59*[24], LB60[24],
Single-membered: LB3, LB10, LB31, LB32, LB38, LB39, LB41, LB42, LB54*, LB55, LB56, LB57*, LB58, LB61, LB63*, LB66, LB67

Appendix 6

A) Operational taxonomic units (OTUs) detected from hyper- and extreme hyper-arid environmental samples at family level

No.	Taxonomy	Family
1.	FJ478799_c;;FJ478799_o;;	FJ479147_f
2.	*Acidimicrobiia;;Acidimicrobiales*	*Acidimicrobiaceae*
3.	*Actinobacteria_c;;Frankiales*	*Geodermatophilaceae*
4.	*Actinobacteria_c;;Micrococcales*	*Microbacteriaceae*
5.	*Actinobacteria_c;;Micrococcales*	*Micrococcaceae*
6.	*Actinobacteria_c;;Frankiales*	HQ910322_f
7.	*Actinobacteria_c;;Micromonosporales*	*Micromonosporaceae*
8.	*Actinobacteria_c;;Propionibacteriales*	*Nocardioidaceae*
9.	*Acidimicrobiia;;Acidimicrobiales*	*Iamiaceae*
10.	*Actinobacteria_c;;Corynebacteriales*	*Nocardiaceae*
11.	*Actinobacteria_c;;Pseudonocardiales*	*Pseudonocardiaceae*
12.	*Actinobacteria_c;;Propionibacteriales*	*Propionibacteriaceae*
13.	*Actinobacteria_c;;Frankiales*	*Sporichthyaceae*
14.	*Actinobacteria_c;;Streptomycetales*	*Streptomycetaceae*
15.	*Actinobacteria_c;;Micrococcales*	*Sanguibacteraceae*
16.	*Actinobacteria_c;;Corynebacteriales*	*Corynebacteriaceae*
17.	*Acidimicrobiia;;Acidimicrobiales*	AM991247_f
18.	*Actinobacteria_c;;Corynebacteriales*	*Mycobacteriaceae*
19.	*Actinobacteria_c;;Micrococcales*	*Intrasporangiaceae*
20.	*Actinobacteria_c;;Micrococcales*	*Cellulomonadaceae*
21.	*Actinobacteria_c;;Kineosporiales*	*Kineosporiaceae*

22.	*Actinobacteria_c;;Frankiales*	*Frankiaceae*
23.	*Acidimicrobiia;;Acidimicrobiales*	*Ilumatobacter_f*
24.	*Actinobacteria_c;;Streptosporangiales*	*Streptosporangiaceae*
25.	*Actinobacteria_c;;Corynebacteriales*	*Dietziaceae*
26.	*Actinobacteria_c;;Micrococcales*	*Bogoriellaceae*
27.	*Actinobacteria_c;;Frankiales*	EU335288_f
28.	*Actinobacteria_c;;EF016800_o*	EF016800_f
29.	*Actinobacteria_c;;Micrococcales*	*Promicromonosporaceae*
30.	*Actinobacteria_c;;Jiangellales*	*Jiangellaceae*
31.	*Actinobacteria_c;;Streptosporangiales*	*Thermomonosporaceae*
32.	*Actinobacteria_c;;Streptosporangiales*	AF498716_f
33.	*Actinobacteria_c;;Frankiales*	EU861909_f
34.	*Acidimicrobiia;;Acidimicrobiales*	DQ395423_f
35.	*Actinobacteria_c;;Corynebacteriales*	EF451703_f
36.	*Acidimicrobiia;;Acidimicrobiales*	*Microthrix_f*
37.	*Actinobacteria_c;;Micrococcales*	*Demequinaceae*
38.	*Actinobacteria_c;;Actinomycetales*	*Actinomycetaceae*
39.	*Actinobacteria_c;;Micrococcales*	*Dermabacteraceae*
40.	*Actinobacteria_c;;Frankiales*	*Cryptosporangiaceae*
41.	*Actinobacteria_c;;Frankiales*	*Nakamurellaceae*
42.	*Actinobacteria_c;;Streptosporangiales*	*Nocardiopsaceae*
43.	FJ478799_c;;FJ478799_o	FJ478799_f
44.	*Actinobacteria_c;;Micrococcales*	*Brevibacteriaceae*
45.	*Acidimicrobiia;;Acidimicrobiales*	DQ396300_f
46.	*Actinobacteria_c;;Micrococcales*	*Rarobacteraceae*
47.	*Acidimicrobiia;;Acidimicrobiales*	EU491192_f
48.	*Actinobacteria_c;;Micrococcales*	*Dermatophilaceae*

49.	*Acidimicrobiia;;Acidimicrobiales*	DQ395502_f
50.	EU374107_c;;EU374107_o	EU374107_f
51.	*Actinobacteria_c;;Frankiales*	AB021325_f
52.	*Actinobacteria_c;;EF016806_o*	EF016806_f
53.	*Actinobacteria_c;;Frankiales*	AB245397_f
54.	*Actinobacteria_c;;Micrococcales*	*Dermacoccaceae*
55.	*Actinobacteria_c;;Micrococcales*	*Beutenbergiaceae*
56.	EU374107_c;;EU374107_o	EU374093_f
57.	*Acidimicrobiia;;Acidimicrobiales*	FN811204_f
58.	*Actinobacteria_c;;Motilibacter_o*	*Motilibacteraceae*
59.	*Actinobacteria_c;;Glycomycetales*	*Glycomycetaceae*
60.	*Actinobacteria_c;;Micrococcales;;Ruaniaceae*	*Ruaniaceae*
61.	*Acidimicrobiia;;Acidimicrobiales;;*DQ129383_f	DQ129383_f
62.	*Rubrobacteria;;Gaiellales;;Gaiellaceae*	*Gaiellaceae*
63.	*Actinobacteria_c;;Planktophila_o;;Planktophila_f*	*Planktophila_f*
64.	*Actinobacteria_c;;Catenulisporales;;Catenulisporaceae*	*Catenulisporaceae*
65.	*Actinobacteria_c;;Corynebacteriales;;Tsukamurellaceae*	*Tsukamurellaceae*
66.	*Actinobacteria_c;;Catenulisporales;;Actinospicaceae*	*Actinospicaceae*
67.	*Nitriliruptoria;;Nitriliruptorales;;Nitriliruptoraceae*	*Nitriliruptoraceae*

B) Operational taxonomic units (OTUs) detected from hyper- and extreme hyper-arid environmental samples at genus level

No.	Taxonomy	Genus
1	FJ478799_c;;FJ478799_o;;FJ479147_f	FJ479147_g
2	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	HQ674860_g
3	*Actinobacteria_c;;Frankiales;;*HQ910322_f	HQ910322_g
4	*Actinobacteria_c;;Frankiales;;Geodermatophilaceae*	*Blastococcus*
5	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Arthrobacter*
6	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Verrucosispora*
7	*Actinobacteria_c;;Frankiales;;Geodermatophilaceae*	*Geodermatophilus*
8	*Actinobacteria_c;;Frankiales;;Geodermatophilaceae*	*Modestobacter*
9	*Actinobacteria_c;;Corynebacteriales;;Nocardiaceae*	*Gordonia*
10	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Microbacterium*
11	*Actinobacteria_c;;Streptomycetales;;Streptomycetaceae*	*Streptomyces*
12	*Actinobacteria_c;;Frankiales;;Sporichthyaceae*	*Sporichthya*
13	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Friedmanniella*
14	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Kocuria*
15	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Nocardioides*
16	*Actinobacteria_c;;Micrococcales;;Sanguibacteraceae*	*Sanguibacter*
17	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	HQ864103_g
18	*Actinobacteria_c;;Corynebacteriales;;Corynebacteriaceae*	*Corynebacterium*
19	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	*Aciditerrimonas*
20	*Acidimicrobiia;;Acidimicrobiales;;*AM991247_f	FJ478790_g
21	*Actinobacteria_c;;Corynebacteriales;;Mycobacteriaceae*	*Mycobacterium*
22	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Lysinimonas*
23	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Pseudonocardia*
24	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	HQ538692_g

25	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Amnibacterium*
26	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Terrabacter*
27	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	JF266448_g
28	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	EF632905_g
29	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	EF516392_g
30	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	HQ190487_g
31	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	EF018137_g
32	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	EU861920_g
33	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Microlunatus*
34	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Frigoribacterium*
35	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Actinoplanes*
36	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Diaminobutyricimonas*
37	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Amycolatopsis*
38	*Actinobacteria_c;;Corynebacteriales;;Nocardiaceae*	*Nocardiaceae*_uc
39	*Actinobacteria_c;;Micrococcales;;Cellulomonadaceae*	*Actinotalea*
40	*Actinobacteria_c;;Frankiales;;Frankiaceae*	*Jatrophihabitans*
41	*Actinobacteria_c;;Streptosporangiales;;Streptosporangiaceae*	*Microbispora*
42	*Actinobacteria_c;;Micrococcales;;Cellulomonadaceae*	*Cellulomonas*
43	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Naasia*
44	*Actinobacteria_c;;Frankiales;;Sporichthyaceae*	AF408985_g
45	*Actinobacteria_c;;Corynebacteriales;;Dietziaceae*	*Dietzia*
46	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	EU861822_g
47	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Agromyces*
48	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Kineococcus*
49	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	*Ilumatobacter*
50	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Marmoricola*
51	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Micromonospora*

52	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Couchioplanes*
53	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	FM886842_g
54	*Actinobacteria_c;;Micrococcales;;Bogoriellaceae*	*Georgenia*
55	*Actinobacteria_c;;Frankiales;;*EU335288_f	EU335288_g
56	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Propionibacterium*
57	*Actinobacteria_c;;*EF016800_o;;EF016800_f	EF016800_g
58	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Kribbella*
59	FJ478799_c;;FJ478799_o;;FJ479147_f	FJ479147_f_uc
60	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Frondihabitans*
61	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Dactylosporangium*
62	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	JF319263_g
63	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Quadrisphaera*
64	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Micrococcus*
65	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	EF516593_g
66	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	FJ479034_g
67	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Homoserinimonas*
68	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Actinomycetospora*
69	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Aeromicrobium*
70	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	EF127609_g
71	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Herbiconiux*
72	*Actinobacteria_c;;Frankiales;;*EU861909_f	EU861909_g
73	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	*Acidimicrobiaceae*_uc
74	*Acidimicrobiia;;Acidimicrobiales;;*DQ395423_f	DQ395423_g
75	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Flindersiella*
76	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	*Cellulosimicrobium*
77	*Actinobacteria_c;;Micrococcales;;Demequinaceae*	*Demequina*
78	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Lentzea*

79	*Actinobacteria_c;;Actinomycetales;;Actinomycetaceae*	*Actinomyces*
80	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	EF127613_g
81	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	FM209170_g
82	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	GQ387490_g
83	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Nocardioidaceae*_uc
84	*Actinobacteria_c;;Corynebacteriales;;*EF451703_f	EF451703_g
85	*Actinobacteria_c;;Frankiales;;Frankiaceae*	AY234742_g
86	*Actinobacteria_c;;Streptosporangiales;;*AF498716_f	EU861937_g
87	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	*Aquihabitans*
88	*Actinobacteria_c;;Jiangellales;;Jiangellaceae*	EU735662_g
89	*Actinobacteria_c;;Corynebacteriales;;Nocardiaceae*	*Nocardia*
90	*Acidimicrobiia;;Acidimicrobiales;;Microthrix_f*	EF516411_g
91	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Xiangella*
92	*Actinobacteria_c;;Micrococcales;;Dermabacteraceae*	*Brachybacterium*
93	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	AY862797_g
94	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Pseudosporangium*
95	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Micromonosporaceae*_uc
96	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Pseudonocardiaceae*_uc
97	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Citricoccus*
98	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Leifsonia*
99	*Actinobacteria_c;;Frankiales;;Geodermatophilaceae*	*Geodermatophilaceae*_uc
100	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Angustibacter*
101	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	EU753662_g
102	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Phytohabitans*
103	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Agrococcus*
104	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Cryobacterium*
105	*Actinobacteria_c;;Streptosporangiales;;Thermomonosporaceae*	*Actinomadura*

106	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Catellatospora*
107	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Tetrasphaera*
108	*Actinobacteria_c;;Streptosporangiales;;Thermomonosporaceae*	*Actinoallomurus*
109	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	*Promicromonospora*
110	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Phycicoccus*
111	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Pontimonas*
112	*Actinobacteria_c;;Frankiales;;Cryptosporangiaceae*	GQ088405_g
113	*Actinobacteria_c;;Frankiales;;Sporichthyaceae*	*Sporichthyaceae*_uc
114	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Oryzihumus*
115	*Actinobacteria_c;;Jiangellales;;Jiangellaceae*	*Jiangella*
116	*Actinobacteria_c;;Frankiales;;Frankiaceae*	*Frankiaceae*_uc
117	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	GQ487899_g
118	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Lechevalieria*
119	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Actinophytocola*
120	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Virgisporangium*
121	*Actinobacteria_c;;Frankiales;;Sporichthyaceae*	DQ413131_g
122	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Catenuloplanes*
123	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Curtobacterium*
124	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Arsenicicoccus*
125	*Actinobacteria_c;;Frankiales;;*HQ910322_f	AY250885_g
126	*Actinobacteria_c;;Micrococcales;;Brevibacteriaceae*	*Brevibacterium*
127	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	GQ402597_g
128	*Actinobacteria_c;;Streptosporangiales;;Nocardiopsaceae*	*Nocardiopsis*
129	*Actinobacteria_c;;Corynebacteriales;;Nocardiaceae*	*Rhodococcus*
130	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Planosporangium*
131	FJ478799_c;;FJ478799_o;;FJ478799_f	EU132929_g
132	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	EU289436_g

133	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	AY093455_g
134	*Acidimicrobiia;;Acidimicrobiales;;*EU491192_f	EU491192_g
135	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Janibacter*
136	*Actinobacteria_c;;Frankiales;;*HQ910322_f	HQ910322_f_uc
137	*Actinobacteria_c;;Frankiales;;Frankiaceae*	*Frankia*
138	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Kineosporiaceae*_uc
139	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Thermasporomyces*
140	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	EF016795_g
141	*Actinobacteria_c;;Micrococcales;;Dermatophilaceae;*	*Piscicoccus*
142	*Actinobacteria_c;;Corynebacteriales;;*EF451703_f;	EF451703_f_uc
143	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	GQ487905_g
144	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Marisediminicola*
145	*Actinobacteria_c;;Frankiales;;Nakamurellaceae*	*Nakamurella*
146	*Actinobacteria_c;;Micrococcales;;Rarobacteraceae*	*Rarobacter*
147	*Acidimicrobiia;;Acidimicrobiales;;*AM991247_f	AM991247_g
148	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Micrococcaceae_uc*
149	*Actinobacteria_c;;Micrococcales;;Sanguibacteraceae*	*Sediminihabitans*
150	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Hamadaea*
151	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	AJ863196_g
152	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Ornithinicoccus*
153	*Actinobacteria_c;;Streptosporangiales;;*AF498716_f	AF498716_g
154	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Ornithinimicrobium*
155	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Polymorphospora*
156	*Acidimicrobiia;;Acidimicrobiales;;*AM991247_f	AM991247_f_uc
157	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Rugosimonospora*
158	*Acidimicrobiia;;Acidimicrobiales;;Microthrix_f*	FN554394_g
159	*Actinobacteria_c;;Jiangellales;;Jiangellaceae*	*Jiangellaceae*_uc

160	*Actinobacteria_c;;Streptomycetales;;Streptomycetaceae*	*Streptomycetaceae*_uc
161	*Bacteria;;;Actinobacteria;;Acidimicrobiia;;Acidimicrobiales;;*DQ396300_f	EF076187_g
162	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	*Iamiaceae*_uc
163	*Actinobacteria_c;;Frankiales;;Nakamurellaceae*	FM874283_g
164	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Plantibacter*
165	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Auritidibacter*
166	*Acidimicrobiia;;Acidimicrobiales;;*DQ395502_f	DQ395502_g
167	EU374107_c;;EU374107_o;;EU374107_f	FJ712481_g
168	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Saccharothrix*
169	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Labedaea*
170	*Actinobacteria_c;;Frankiales;;Cryptosporangiaceae*	*Cryptosporangium*
171	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Catelliglobosispora*
172	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Rathayibacter*
173	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Actinosynnema*
174	*Acidimicrobiia;;Acidimicrobiales;;Acidimicrobiaceae*	GU168000_g
175	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	FM209069_g
176	*Actinobacteria_c;;*EF016806_o;;EF016806_f	EF016806_g
177	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Spirilliplanes*
178	*Actinobacteria_c;;Streptosporangiales;;*AF498716_f	AF498716_f_uc
179	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Rothia*
180	*Actinobacteria_c;;Frankiales;;*AB245397_f	AB245397_g
181	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	*Luteimicrobium*
182	*Actinobacteria_c;;Streptosporangiales;;Streptosporangiaceae*	*Nonomuraea*
183	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	FJ379331_g
184	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Microbacteriaceae*_uc
185	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Jishengella*
186	*Actinobacteria_c;;Frankiales;;*AB021325_f	FN687458_g

187	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Naumannella*
188	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Luedemannella*
189	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Subtercola*
190	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Crossiella*
191	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	EF423344_g
192	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Propionibacteriaceae*_uc
193	EU374107_c;;EU374107_o;;EU374093_f	EU374093_g
194	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Thermotunica*
195	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Nesterenkonia*
196	*Acidimicrobiia;;Acidimicrobiales;;*DQ396300_f	DQ396300_g
197	*Actinobacteria_c;;Corynebacteriales;;Corynebacteriaceae*	*Corynebacteriaceae*_uc
198	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Kibdelosporangium*
199	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Pseudoclavibacter*
200	*Actinobacteria_c;;Propionibacteriales;;Nocardioidaceae*	*Actinopolymorpha*
201	*Acidimicrobiia;;Acidimicrobiales;;*FN811204_f	FN811204_g
202	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Knoellia*
203	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Alloactinosynnema*
204	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Rudaibacter*
205	*Actinobacteria_c;;Motilibacter_o;;Motilibacteraceae*	*Motilibacter*
206	*Actinobacteria_c;;Jiangellales;;Jiangellaceae*	JF727732_g
207	*Actinobacteria_c;;Frankiales;;*AB021325_f	AB021325_g
208	*Actinobacteria_c;;Micrococcales;;Dermacoccaceae*	*Luteipulveratus*
209	*Acidimicrobiia;;Acidimicrobiales;;*DQ395502_f	FJ229917_g
210	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Aquiluna*
211	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Kineosporia*
212	*Actinobacteria_c;;Frankiales;;Cryptosporangiaceae*	*Cryptosporangiaceae*_uc
213	*Acidimicrobiia;;Acidimicrobiales;;*DQ395423_f	DQ269060_g

214	*Actinobacteria_c;;Micrococcales;;Sanguibacteraceae*	*Oerskovia*
215	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Longispora*
216	*Actinobacteria_c;;Micrococcales;;Rarobacteraceae*	*Rarobacteraceae*_uc
217	*Actinobacteria_c;;Micrococcales;;Beutenbergiaceae*	*Serinibacter*
218	EU374107_c;;EU374107_o;;EU374107_f	EU374107_g
219	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Okibacterium*
220	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Salinibacterium*
221	*Actinobacteria_c;;Glycomycetales;;Glycomycetaceae*	*Glycomyces*
222	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Chryseoglobus*
223	*Actinobacteria_c;;Frankiales;;Frankiaceae*	*Acidothermus*
224	*Actinobacteria_c;;Streptomycetales;;Streptomycetaceae*	*Streptacidiphilus*
225	*Acidimicrobiia;;Acidimicrobiales;;Iamiaceae*	*Iamia*
226	*Actinobacteria_c;;Micrococcales;;Beutenbergiaceae*	*Miniimonas*
227	*Actinobacteria_c;;Frankiales;;Sporichthyaceae*	EU644212_g
228	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	DQ532175_g
229	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Saccharopolyspora*
230	AB240310_c;;AB240310_o;;AB240310_f	AB240310_f_uc
231	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Phytomonospora*
232	FJ478799_c;;FJ478799_o;;FJ478799_f	FJ478799_f_uc
233	*Acidimicrobiia;;Acidimicrobiales;;Microthrix_f*	*Microthrix*
234	*Actinobacteria_c;;Corynebacteriales;;Mycobacteriaceae*	*Mycobacteriaceae*_uc
235	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Microcella*
236	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	*Promicromonosporaceae*_uc
237	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Labedella*
238	*Acidimicrobiia;;Acidimicrobiales;;*DQ129383_f	DQ129383_g
239	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Actinocatenispora*
240	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Rhizocola*

241	*Actinobacteria_c;;Frankiales;;*EU861909_f	EU861909_f_uc
242	*Acidimicrobiia;;Acidimicrobiales;;Ilumatobacter_f*	AJ863237_g
243	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Allocatelliglobosispora*
244	*Actinobacteria_c;;Micrococcales;;Dermacoccaceae*	*Dermacoccus*
245	*Acidimicrobiia;;Acidimicrobiales;;Microthrix_f*	*Microthrix*_f_uc
246	*Actinobacteria_c;;Streptosporangiales;;Thermomonosporaceae*	*Spirillospora*
247	*Actinobacteria_c;;Frankiales;;Cryptosporangiaceae*	*Fodinicola*
248	*Actinobacteria_c;;Streptosporangiales;;Thermomonosporaceae*	*Actinocorallia*
249	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	*Pseudokineococcus*
250	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Kutzneria*
251	*Actinobacteria_c;;Streptosporangiales;;Nocardiopsaceae*	*Thermobifida*
252	*Actinobacteria_c;;Micrococcales;;Ruaniaceae*	*Ruania*
253	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Tessaracoccus*
254	*Actinobacteria_c;;Micrococcales;;Cellulomonadaceae*	*Cellulomonadaceae*_uc
255	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Intrasporangiaceae*_uc
256	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Saccharomonospora*
257	*Nitriliruptoria;;Nitriliruptorales;;Nitriliruptoraceae*	*Nitriliruptoraceae*_uc
258	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Intrasporangium*
259	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Brooklawnia*
260	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	*Humibacillus*
261	*Actinobacteria_c;;Kineosporiales;;Kineosporiaceae*	DQ532344_g
262	*Actinobacteria_c;;Micrococcales;;Sanguibacteraceae*	*Sanguibacteraceae*_uc
263	*Actinobacteria_c;;Jiangellales;;Jiangellaceae*	*Haloactinopolyspora*
264	*Acidimicrobiia;;Acidimicrobiales;;*DQ396300_f	DQ396300_f_uc
265	*Actinobacteria_c;;Propionibacteriales;;Propionibacteriaceae*	*Mariniluteicoccus*
266	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	EF157137_g
267	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Umezawaea*

269	*Rubrobacteria;;Gaiellales;;Gaiellaceae*	*Gaiellaceae*_uc
270	*Actinobacteria_c;;Corynebacteriales;;Dietziaceae*	*Dietziaceae_uc*
271	*Actinobacteria_c;;Corynebacteriales;;Tsukamurellaceae*	*Tsukamurella*
272	*Actinobacteria_c;;Catenulisporales;;Catenulisporaceae*	*Catenulispora*
273	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Mycetocola*
274	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Humibacter*
275	*Actinobacteria_c;;Frankiales;;*AB021325_f	AB021325_f_uc
276	*Actinobacteria_c;;Micrococcales;;Microbacteriaceae*	*Glaciibacter*
277	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Plantactinospora*
278	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Salinispora*
279	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Actinokineospora*
280	*Actinobacteria_c;;Actinomycetales;;Actinomycetaceae*	*Actinomycetaceae_uc*
281	*Actinobacteria_c;;Pseudonocardiales;;Pseudonocardiaceae*	*Allokutzneria*
282	*Actinobacteria_c;;Micrococcales;;Intrasporangiaceae*	JN588609_g
283	*Actinobacteria_c;;Frankiales;;Frankiaceae*	EU132518_g
284	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Zhihengliuella*
285	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	FN667447_g
286	*Actinobacteria_c;;Micrococcales;;Dermacoccaceae*	*Rudaeicoccus*
287	*Actinobacteria_c;;Planktophila_o;;Planktophila_f*	*Planktophila*
288	*Actinobacteria_c;;Streptomycetales;;Streptomycetaceae*	*Kitasatospora*
289	*Actinobacteria_c;;Micrococcales;;Micrococcaceae*	*Tersicoccus*
290	*Actinobacteria_c;;Corynebacteriales;;Mycobacteriaceae*	*Hoyosella*
291	*Actinobacteria_c;;Streptosporangiales;;Nocardiopsaceae*	*Nocardiopsaceae*_uc
292	*Actinobacteria_c;;Micromonosporales;;Micromonosporaceae*	*Asanoa*
293	*Actinobacteria_c;;Glycomycetales;;Glycomycetaceae*	*Glycomycetaceae*_uc
294	*Actinobacteria_c;;Corynebacteriales;;Corynebacteriaceae*	*Turicella*
295	*Actinobacteria_c;;Micrococcales;;Beutenbergiaceae*	*Salana*

296	*Actinobacteria_c;;Catenulisporales;;Actinospicaceae*	*Actinospica*
297	*Actinobacteria_c;;Micrococcales;;Promicromonosporaceae*	*Myceligenerans*

Appendix 7

Operational taxonomic units (OTUs) detected from Cerro Chajnantor soil samples at genus level

No.	Taxonomy	Genus
1.	*Frankiales;;Geodermatophilaceae*	*Blastococcus*
2.	*Micrococcales;;Micrococcaceae*	*Arthrobacter*
3.	*Frankiales;;Geodermatophilaceae*	*Modestobacter*
4.	*Frankiales;;Geodermatophilaceae*	*Geodermatophilus*
5.	*Corynebacteriales;;Mycobacteriaceae*	*Mycobacterium*
6.	*Propionibacteriales;;Propionibacteriaceae*	*Friedmanniella*
7.	*Pseudonocardiales;;Pseudonocardiaceae*	*Pseudonocardia*
8.	*Propionibacteriales;;Nocardioidaceae*	*Nocardioides*
9.	*Frankiales;;Frankiaceae*	*Jatrophihabitans*
10.	*Micrococcales;;Intrasporangiaceae*	*Terrabacter*
11.	*Frankiales;;Sporichthyaceae*	*Sporichthya*
12.	*Micromonosporales;;Micromonosporaceae*	*Actinoplanes*
13.	*Propionibacteriales;;Propionibacteriaceae*	*Microlunatus*
14.	*Micrococcales;;Microbacteriaceae*	*Amnibacterium*
15.	*Acidimicrobiales;;Acidimicrobiaceae*	*Aciditerrimonas*
16.	*Streptomycetales;;Streptomycetaceae*	*Streptomyces*
17.	*Micrococcales;;Microbacteriaceae*	*Frigoribacterium*

18.	*Micrococcales;;Microbacteriaceae*	*Lysinimonas*
19.	*Pseudonocardiales;;Pseudonocardiaceae*	*Amycolatopsis*
20.	*Propionibacteriales;;Nocardioidaceae*	*Kribbella*
21.	*Propionibacteriales;;Nocardioidaceae*	*Flindersiella*
22.	*Micromonosporales;;Micromonosporaceae*	*Micromonospora*
23.	*Propionibacteriales;;Nocardioidaceae*	*Marmoricola*
24.	*Micrococcales;;Microbacteriaceae*	*Naasia*
25.	*Micromonosporales;;Micromonosporaceae*	*Verrucosispora*
26.	*Kineosporiales;;Kineosporiaceae*	*Quadrisphaera*
27.	*Micrococcales;;Cellulomonadaceae*	*Actinotalea*
28.	*Micromonosporales;;Micromonosporaceae*	*Couchioplanes*
29.	*Micrococcales;;Microbacteriaceae*	*Diaminobutyricimonas*
30.	*Pseudonocardiales;;Pseudonocardiaceae*	*Lentzea*
31.	*Pseudonocardiales;;Pseudonocardiaceae*	*Actinophytocola*
32.	*Pseudonocardiales;;Pseudonocardiaceae*	*Actinomycetospora*
33.	*Micromonosporales;;Micromonosporaceae*	*Dactylosporangium*
34.	*Micromonosporales;;Micromonosporaceae*	*Phytohabitans*
35.	*Kineosporiales;;Kineosporiaceae*	*Angustibacter*
36.	*Micromonosporales;;Micromonosporaceae*	*Xiangella*
37.	*Propionibacteriales;;Nocardioidaceae*	*Aeromicrobium*
38.	*Micromonosporales;;Micromonosporaceae*	*Catenuloplanes*
39.	*Frankiales;;Nakamurellaceae*	*Nakamurella*

40.	*Acidimicrobiales;;Iamiaceae*	*Aquihabitans*
41.	*Micromonosporales;;Micromonosporaceae*	*Catellatospora*
42.	*Corynebacteriales;;Nocardiaceae*	*Rhodococcus*
43.	*Streptosporangiales;;Thermomonosporaceae*	*Actinoallomurus*
44.	*Micromonosporales;;Micromonosporaceae*	*Pseudosporangium*
45.	*Acidimicrobiales;;Ilumatobacter_f*	*Ilumatobacter*
46.	*Micrococcales;;Cellulomonadaceae*	*Cellulomonas*
47.	*Streptosporangiales;;Thermomonosporaceae*	*Actinomadura*
48.	*Frankiales;;Frankiaceae*	*Frankia*
49.	*Micrococcales;;Microbacteriaceae*	*Curtobacterium*
50.	*Micromonosporales;;Micromonosporaceae*	*Virgisporangium*
51.	*Corynebacteriales;;Corynebacteriaceae*	*Corynebacterium*
52.	*Pseudonocardiales;;Pseudonocardiaceae*	*Kibdelosporangium*
53.	*Micrococcales;;Intrasporangiaceae*	*Tetrasphaera*
54.	*Micrococcales;;Promicromonosporaceae*	*Luteimicrobium*
55.	*Motilibacter_o;;Motilibacteraceae*	*Motilibacter*
56.	*Micrococcales;;Microbacteriaceae*	*Frondihabitans*
57.	*Micrococcales;;Intrasporangiaceae*	*Oryzihumus*
58.	*Micromonosporales;;Micromonosporaceae*	*Asanoa*
59.	*Micromonosporales;;Micromonosporaceae*	*Salinispora*
60.	*Catenulisporales;;Catenulisporaceae*	*Catenulispora*
61.	*Corynebacteriales;;Nocardiaceae*	*Nocardia*

62.	*Pseudonocardiales;;Pseudonocardiaceae*	*Labedaea*
63.	*Micromonosporales;;Micromonosporaceae*	*Catelliglobosispora*
64.	*Glycomycetales;;Glycomycetaceae*	*Glycomyces*
65.	*Micrococcales;;Intrasporangiaceae*	*Phycicoccus*
66.	*Pseudonocardiales;;Pseudonocardiaceae*	*Saccharothrix*
67.	*Pseudonocardiales;;Pseudonocardiaceae*	*Lechevalieria*
68.	*Propionibacteriales;;Propionibacteriaceae*	*Propionibacterium*
69.	*Micrococcales;;Microbacteriaceae*	*Marisediminicola*
70.	*Kineosporiales;;Kineosporiaceae*	*Kineococcus*
71.	*Pseudonocardiales;;Pseudonocardiaceae*	*Actinosynnema*
72.	*Micrococcales;;Promicromonosporaceae*	*Promicromonospora*
73.	*Micrococcales;;Microbacteriaceae*	*Leifsonia*
74.	*Micromonosporales;;Micromonosporaceae*	*Planosporangium*
75.	*Micrococcales;;Intrasporangiaceae*	*Arsenicicoccus*
76.	*Frankiales;;Cryptosporangiaceae*	*Cryptosporangium*
77.	*Micromonosporales;;Micromonosporaceae*	*Spirilliplanes*
78.	*Frankiales;;Frankiaceae*	*Acidothermus*
79.	*Micromonosporales;;Micromonosporaceae*	*Hamadaea*
80.	*Streptosporangiales;;Streptosporangiaceae*	*Nonomuraea*
81.	*Micrococcales;;Bogoriellaceae*	*Georgenia*
82.	*Pseudonocardiales;;Pseudonocardiaceae*	*Alloactinosynnema*
83.	*Micrococcales;;Intrasporangiaceae*	*Ornithinimicrobium*

84.	*Corynebacteriales;;Nocardiaceae*	*Gordonia*
85.	*Micromonosporales;;Micromonosporaceae*	*Longispora*
86.	*Micromonosporales;;Micromonosporaceae*	*Rugosimonospora*
87.	*Micrococcales;;Microbacteriaceae*	*Homoserinimonas*
88.	*Propionibacteriales;;Nocardioidaceae*	*Actinopolymorpha*
89.	*Kineosporiales;;Kineosporiaceae*	*Kineosporia*
90.	*Pseudonocardiales;;Pseudonocardiaceae*	*Crossiella*
91.	*Micrococcales;;Intrasporangiaceae*	*Ornithinicoccus*
92.	*Pseudonocardiales;;Pseudonocardiaceae*	*Kutzneria*
93.	*Propionibacteriales;;Propionibacteriaceae*	*Naumannella*
94.	*Micrococcales;;Promicromonosporaceae*	*Isoptericola*
95.	*Jiangellales;;Jiangellaceae*	*Jiangella*
96.	*Micrococcales;;Microbacteriaceae*	*Cryobacterium*
97.	*Micromonosporales;;Micromonosporaceae*	*Actinocatenispora*
98.	*Micromonosporales;;Micromonosporaceae*	*Polymorphospora*
99.	*Micrococcales;;Microbacteriaceae*	*Microbacterium*
100.	*Propionibacteriales;;Propionibacteriaceae*	*Mariniluteicoccus*
101.	*Micrococcales;;Microbacteriaceae*	*Agromyces*
102.	*Micrococcales;;Micrococcaceae*	*Micrococcus*
103.	*Micrococcales;;Microbacteriaceae*	*Subtercola*
104.	*Micrococcales;;Microbacteriaceae*	*Rathayibacter*
105.	*Micrococcales;;Sanguibacteraceae*	*Oerskovia*

106. *Micrococcales;;Microbacteriaceae* *Agrococcus*
107. *Micrococcales;;Rarobacteraceae* *Rarobacter*
108. *Acidimicrobiales;;Microthrix_f* *Microthrix*
109. *Micrococcales;;Microbacteriaceae* *Rudaibacter*
110. *Streptosporangiales;;Thermomonosporaceae* *Actinocorallia*
111. *Catenulisporales;;Actinospicaceae* *Actinospica*
112. *Glycomycetales;;Glycomycetaceae* *Stackebrandtia*
113. *Micrococcales;;Microbacteriaceae* *Labedella*
114. *Micrococcales;;Dermacoccaceae* *Luteipulveratus*
115. *Micromonosporales;;Micromonosporaceae* *Allocatelliglobosispora*
116. *Propionibacteriales;;Nocardioidaceae* *Thermasporomyces*
117. *Micromonosporales;;Micromonosporaceae* *Rhizocola*
118. *Streptosporangiales;;Streptosporangiaceae* *Microbispora*
119. *Micromonosporales;;Micromonosporaceae* *Luedemannella*
120. *Pseudonocardiales;;Pseudonocardiaceae* *Goodfellowiella*
121. *Acidimicrobiales;;Iamiaceae* *Iamia*
122. *Micrococcales;;Sanguibacteraceae* *Sediminihabitans*
123. *Pseudonocardiales;;Pseudonocardiaceae* *Actinokineospora*
124. *Corynebacteriales;;Dietziaceae* *Dietzia*
125. *Micrococcales;;Micrococcaceae* *Tersicoccus*
126. *Micromonosporales;;Micromonosporaceae* *Plantactinospora*
127. *Micrococcales;;Brevibacteriaceae* *Brevibacterium*

128.	*Pseudonocardiales;;Pseudonocardiaceae*	*Prauserella*
129.	*Micrococcales;;Dermacoccaceae*	*Dermacoccus*
130.	*Micrococcales;;Micrococcaceae*	*Kocuria*
131.	*Micrococcales;;Microbacteriaceae*	*Herbiconiux*
132.	*Micromonosporales;;Micromonosporaceae*	*Pilimelia*
133.	*Micrococcales;;Dermacoccaceae*	*Flexivirga*
134.	*Micromonosporales;;Micromonosporaceae*	*Jishengella*
135.	*Actinomycetales;;Actinomycetaceae*	*Actinomyces*