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NOVEL ACTINOBACTERIAL DIVERSITY IN KAZAKHSTAN DESERTS SOILS AS A SOURCE OF NEW DRUG LEADS

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ABSTRACT

Discovering new metabolites, notably antibiotics, by isolation and screening novel actinomycetes from extreme habitats gave extraordinary results that can be adapted in the future for healthcare. However, it was little attention payed to desert soils in Central Asia, such as from Kazakhstan.
Taxonomic approach was to isolate selectively, dereplicate and classify actinomycetes from two Kazakhstan Deserts (Betpakdala and Usturt Plateu). The most representative isolates from colour-groups were describe via 16S rRNA gene sequence analysis.
Relatively large number, of strains from environmental soil samples were classified into *Streptomyces* genera. Moreover, three strains from two different soil samples were identified as relatively close to *Pseudonocarida* genera. All representative isolates were screened for bioactive compound against wild type microorganisms, as a result, of it can be interpreted that approximately half of screened strains are likely to produce metabolites which inhibits cell growth.
The results of this project demonstrate for the first time that arid regions of Kazakhstan soils are rich reservoirs of cultivable novel actinobacteria with the capacity to produce bioactive compounds that can be developed as drug leads for medicine.

Keywords: Actinomycetes, Kazakhstan, 16sRNA gene sequence, *Streptomyces* genera

INTRODUCTION

According to World Health Organization (WHO) antibiotic resistance has become a huge problem that needs immediate attention (**WHO 2014**). New antibiotics are urgently needed to kill multi-drug resistant microbial pathogens and to treat life threatening diseases, such as cancer (**Genilloud 2014**). For example, *Klebsiella pneumonia* clones are carbapenemase-resistant, which appears as a major problem in the USA (**Kumarasamy *et al.* 2010**). An estimated two million patients in the USA are infected every year by drug-resistant pathogens and around 23000 of them die **(PharmTech 2013).**
Microbial natural products are still considered to be the most promising source of new drugs (**Cragg and Newman 2013**). **Shoichet (2013)** states that natural products contain a range of chemical structures optimised for biological interactions. However, compounds should follow "the rule of five" (**Lipinski *et al.* 2012**). Natural products are evolutionarily selected by their ability to enter and inhibit or kill bacterial cells and are therefore better than synthetically designed drugs which are basically not "tailored for microbial biology" (**Wright 2014**).
Amongst prokaryotes, members of the phylum *Actinobacteria*, notably *Streptomyces* strains, remain a unique source of novel bioactive compounds of therapeutic interest (**Lucas *et al.* 2012**). It is now known that the genomes of actinobacteria contain many biosynthetic gene clusters that code for known or predicted specialist metabolites (**Goodfellow and Fiedler 2010**). Moreover, natural habitats contain a wealth of undiscovered actinobacteria (**Stach *et al.* 2003**).
Despite these discoveries it has become increasingly difficult to find new bioactive compounds from actinobacteria isolated from well-studied habitats as screening such organisms leads to a costly rediscovery of known metabolites (**Busti *et al.* 2006**). One way of addressing these problems is to selectively isolate, dereplicate (assign in to groups), and screen representatives of novel actinomycetes from neglected and unexplored habitats. This taxonomic approach to bioprospecting has been used to isolate actinobacteria from extreme biomes on the premise that, harsh environmental conditions will give rise to novel actinobacteria with the capacity to synthesise novel metabolites **(Goodfellow and Fiedler 2010; Goodfellow 2013**). This hypothesis is tested by selecting, dereplicating and screening actinomycetes from marine habitats, notably deeper sediments. To date, novel actinomycetes from desert and marine ecosystems have been found to be a rich source of new drug leads (**Manivasagan et al. 2013**).
It is known that actinomycetes are common in extreme habitats, such as acidic soils (**Kim *et al.* 2003**) or deep sediments (**Maldonado *et al.* 2005**). However, little attention has been paid to actinomycetes diversity from desert soils, yet deserts cover almost one-quarter of the Earth's land surface.
The deserts are the arid and hyper-arid areas of lands; generally ecoregions contain organisms that adapted to survive in arid environmental regions and geographically with large areas of soil or rock with low organic matter.
Actinobacteria have been isolated from desert soils, including the soils from the Amargosa desert in Nevada (**Luedemann 1968**), the Mojave desert in southwest United States **(Garrity *et al.* 1996**), the Mongolian desert (**Kurapova *et al.* 2012**), the Namibian desert (**Wink *et al.* 2003**), the Sahara desert (**Zitouni *et al.* 2005; Meklat *et al.* 2011**), the Taklamakan desert in Xinjiang province, China **(Luo *et al.* 2012**), the Thar desert in Rajastan, India (**Harwani 2013**) and the desert ecosystem in the northeast of the Qinghai-Tibet plateau **(Ding *et al.* 2013**). However, Kazakhstan desert soil has not been explored for actinomycetes diversity.
The Kazakhstan Republic, located in Central Asia neighbouring the Russian Federation and China is a landlocked country, with high mountain ranges along the eastern and south-eastern borders. Deserts and semi-deserts make up around 58% of the country.
Deserts are divided into two geomorphological regions: Turan lowland, Plato Usturt and Betpak-Dala (**Turuzbekova 2014**). Within these, many geological landscapes exist: sand (Karakum), rocky (Betpak-Dala), gravelly (Ustyurt), clay (Hungry Steppe), saline (Kelkor) deserts (**www.zoogeo365.ru 2011**).
According to **Gvozdetskii and Mihailov (1978)** the average temperature in the Betpakdala desert and in the Usturt Plateau is 26-28°C. In some years, the temperature reaches 40-60°C in the Usturt Plateau. Average annual rainfall is less than 120 mm and 150 mm. Usturt Plateau is located between the Caspian and Aral seas. It is semi-enclosed by steep cliffs. The Usturt Plateau is 300 m plus above sea level and generally flat with a number of shallow shafts (Muzbel, Karabaur) and hollows. Sarmatian limestone is the parent rock, which weathers into gray-brown solonetzic soils, interbedded with gypsum.
The Betpakdala desert is located between the lower reaches of the rivers Chu and Sarysu on the western shore of Lake Balkhash. The western part of Betpakdala –

is an elevated plain of flat-lying folded Mesozoic and Paleogene loose rocks. Above lie predominantly marine and continental Paleogene sediments (sand, sandstone, clay, gravel). Flat sections alternate with undrained depressions in the form of logs and closed cavities with salt marshes.

Very little attention has been past to the microbial flora of Kazakhstan Deserts. **Kutovaya *et al.* (2015)** states that two dominant groups of bacteria are represented in the Aridic Calcisols extreme hyper arid desert region: *Proteobacteria* (43.9%) and *Actinobacteria* (10%). Therefore, it is likely that the same amount of *Actinobacteria* can be isolated from other arid regions, such as Ustyurt Plateau.

MATERIALS AND METHODS

Soil samples

Two soil samples from the Kazakhstan Desert were studied (Table 2.1.)**.** The soil samples from Kazakhstan was provided by the Director of Usturt National Preserve (Mangistauskaya region) and head of city administration Sholakkorgan, Sozak district, Southern Kazakhstan region (Betpakdala desert). The soil samples were stored in plastic bags at room temperature.

Table 2.1 Sources of soil samples

Sample code	Source	Latitude	Longitude	Collection date
UKZ	Usturt Desert	43°50'00.0"N	55°16'00.0"E	05.01.2015
BKZ	Betpakdala Desert	46°02'00.0"N	70°12'00.0"E	05.01.2015

The bulk pH values of the soil samples were determined following the procedure described by **Reed and Cummings (1945)**. Two grams of each soil sample were transferred to 50 ml beakers and mixed thoroughly with demineralized water. The soil samples were left for 2 hours, then the pH of each soil was determined, in triplicate, using a glass electrode pH meter (Model 320 Mettler-Toledo AG, CH.8603, Schwerzenbach, Switzerland).

The soil samples were added to pre-weighed silica crucibles and the weight of the crucibles plus soil samples accurately taken. The samples were then dried to constant weight in a hot oven of 105°C and re-weighed. This procedure was carried out in triplicate and the moisture content of the samples recorded as the percentage loss of weight in the three measurements. The crucibles plus dry soil were then placed in a muffle furnace and the temperature slowly raised to 700°C and kept constant for 30 minutes in order to burn off the organic matter prior to overnight cooling and reweighing. The average percentage loss as weighted for three measurements was record as the organic matter content.

Selective isolation and enumeration of putative actinomycetes

A gram of each soil sample was added to 4.5 ml of 1/4 strength Ringer's solution (9 g NaCl, 0.42 g KCl, 0.48 g $CuCl_2$, 2 g $NaHCO_3$, 4 l H_2O) to give a $10^{-1/2}$. The $10^{-1/2}$ samples were shaken on a tumble-shaker (IKA®KS 260 basic, Germany) at 300 motion/min for 30 minutes then the soil samples pre-heated in a water bath at 55°C for 20 minutes. 10^{-1} and 10^{-2} dilution was then prepared in 1/4 strength Ringer's solution. Aliquots (100µl) of each dilution were spread over the surface of three sets of isolation plates that had been dried for 15 minutes, as recommended by **Vicker *et al.* (1984)**. In all cases three plates per dilution were inoculated and incubated at 28°C for 21 days. Then the number of presumptive actinomycetes and bacteria was counted and the results expressed as number of the colony forming units (cfu) per gram dry weight soil.

Table 2.2 Selective media used for the isolation of bacteria and actinomycetes

Media	Antibiotic	Target organisms	Reference
Peptone-yeast agar	Cyclohexamide (25 µg/ml) and nystatin (25 µg/ml)	Bacteria	**Goodfellow (1966)**
Humic acid agar	Cyclohexamide (25 µg/ml) and nystatin (25 µg/ml)	Rare actinomycetes	**Hayakawa and Nonomura (1987)**
Humic acid agar modified	Cyclohexamide (25 µg/ml), nystatin (25 µg/ml) and naladixic acid (25 µg/ml)	Rare actinomycetes	**Hayakawa and Nonomura (1987)**
Starch - casein agar	Cyclohexamide (25 µg/ml) and nystatin (25 µg/ml)	Common *Streptomyces*	**Küster and Williams (1964)**
Modified starch-casein agar	Cyclohexamide(25 µg/ml), nystatin (25 µg/ml), naladixic acid (25 µg/ml) and oxyteracycline (25 µg/ml)	Rare *Streptomyces*	**Kim *et al.* (2011)**
SM1 agar	Cyclohexamide (25 µg/ml), nystatin (25 µg/ml), neomycin (4 µg/ml) and sorbitol (1%, w/v)	*Amycolatopsis strains*	**Tan *et al.* (2006)**

Selection, purification and maintenance of isolates and marker cultures

The selective isolation plates were examined both by eye and by using a binocular microscope (Olympus Optical co., Ltd, Tokyo, Japan). One hundred forty five colonies representing different colony types of the presumptive actinomycetes were taken from the isolation plates using sterile toothpicks and inoculated onto yeast extract-malt extract (ISP2 agar, **Shirling and Gottlieb (1966)**) plates which were incubated at 28°C for 14 days. The incubated colonies were checked for purity and if pure were used to prepare stock cultures for each strain by transferring mycelial growth and spores from each purified isolate into vacuum tubes containing 0.75 ml of 20% (w/v) sterile glycerol solution (**Williams and Wellington 1982**). The frozen glycerol cultures were stored at -20°C to provide a convenient source of inoculum for preservation in 3 copies and future use.

Preliminary characterization of presumptive actinomycetes

Colour grouping

One hundred fifteen of the representative isolates were inoculated onto oatmeal agar (ISP3 medium, Shirling and Gottlieb (1966)) and peptone-yeast extract-iron agar (ISP6 medium, **Shirling and Gottlieb (1966)** and incubated at 28°C for 14 days. After incubation, the isolates were examined by eye and aerial spore mass colour, reverse substrate mycelial and diffusible pigment using a National Bureau of Standards (NBS) Colour Name Chart (**Kelly 1958**). The peptone-yeast extract-iron agar plates were examined to determine whether the isolates produced characteristic dark coloured melanin pigments. The isolates were assigned to 19 multi-membered and 61 single-membered colour-groups based on the recorded properties.

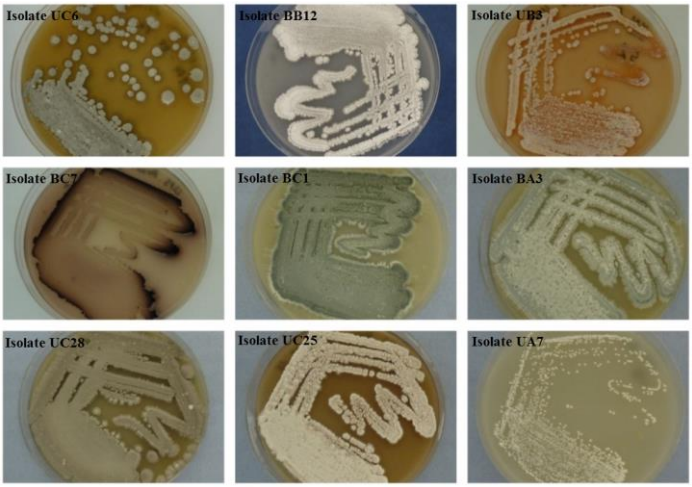

Figure 2.1 Selected actinomycetes growing on oatmeal agar after incubation at 28°C for 14 days, showing aerial spore mass and diffusible pigment colour

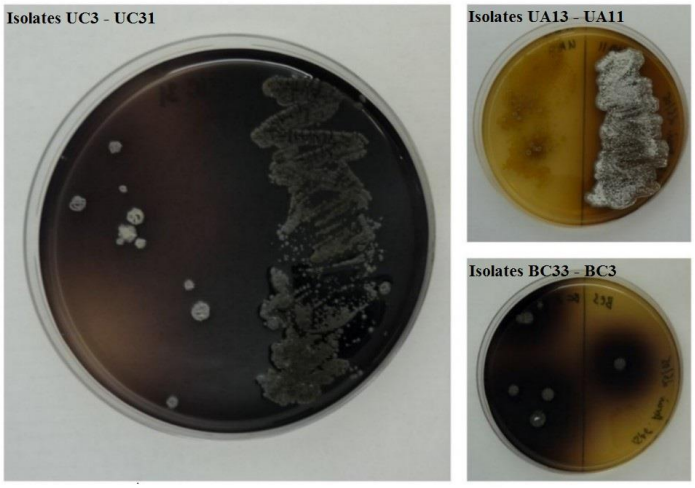

Figure 2.2 Melanin production of selected actinomycetes growing on peptone-yeast extract iron agar after incubation at 28°C for four days

Extraction of genomic DNA, PCR amplification and analysis of 16S rRNA genes

DNA extraction

Genomic DNA was extracted from twenty seven representatives of different colour groups by using a bead beating method. One or two loop fulls of each isolate biomass was transferred into a tube containing 3g of acid washed glass beads (0.1 mm diameter) and 500 µl of sterile water. The resultant preparations were treated for 30 seconds at 5.5 m/s, placed in ice for 2 minutes, and the procedure repeated. Next the tubes were centrifuge, for 3 minutes at 13000 rpm and the supernatant containing the DNA extracts transferred into fresh tubes and kept at -20°C until required.

Quality of extracted DNA

The quality of the DNA products were checked by agarose gel electrophoresis (1%, w/v agarose in 0.5x TBE, 40 minutes). The gels were stained with ethidium bromide (0.5 µg/ml) and loaded with four µl of DNA extracts mixed with two µl of 6x loading dye (0.5 µg/ml, Sigma). The sizes of the DNA fragments were compared with a 1000bp molecular size marker (Gene Ruler^{TM} MBI Fermentas, Vilnius, Lithuania). After electrophoresis, the gels were visualised using the Gel Doc EZ system (BioRad).

PCR

One or two µl of each DNA product was used as a DNA template in a 25 µl polymerase chain reaction (PCR) which contained 1x buffer (10x buffer: 160 mM $(NH_4)_2SO_4$, 670 mMTris-HCL, 0.1% Tween-20), a mixture of dNTP's containing 0.125 mM of each of the four dNTP's, and 200 µM of each forward and reverse primer (27F and 1525R, respectively), 1.5 µM of $MgCl_2$ and 1.25 Taq polymerase; positive and negative controls were run together; the negative control was sterile water and positive control was a known DNA sample. The PCR reaction were carried out as follows: initial denaturation at 95°C for a minute, 35 cycles of 95°C for a minute, 55°C for a minute and 72°C for a minute, and finally at 72°C for five minutes. The PCR products were checked for purity, as mentioned above, and the preparations kept at -20°C until as required.

Purification of PCR products

The PCR products were purified for sequencing by using ExoSAP-IT enzyme kits (USB, Corporation, Ohio, USA), according to the manufactur's protocol. Each PCR product (5 µl) was vortexed with 2 µl of ExoSAP-IT and then incubated at 37°C for 15 minutes to degrade any remaining primers and nucleotides. The preparations were then incubated at 80°C for 15 minutes to inactivate any remaining ExoSAP-IT and purified products stored at -20°C until required. GENEIUS Company of the Newcastle University was carried determination sequences of almost complete 16S rRNA genes.

Phylogenetic analyses

ABI format files of each 16S rRNA gene sequence were obtained from each primer. The files were edited *via* Finch TV(C) version 1.4.0, which presents sequence results as a long side electropherogram reading of each nucleotide. Poor quality regions were deleted from the beginning and the end of each of the 16S rRNA gene sequences. The 16S rRNA sequences were assembled using the CAP3 Sequence Assembly Program **(Huang and Madan 1999)** and aligned against corresponding sequences of the most phylogenetically related type strain taken from the EzTaxon software (http://www.ezbiocloud.net/eztaxon **, Chun *et al.* (2007)**). The aligned sequences were used to generate phylogenetic trees using the neighbour-joining algorithm (**Saitou and Nei 1987**) from the MEGA5 software programme (**Tamura *et al.* 2011**). The topologies of the resultant trees were evaluated in bootstrap values analyses **(Felsenstein 1985**) based on 1000 resampling of the neighbour-joining dataset. 16s rRNA gene sequence data was also analysed with maximum-likelihood and maximum-parsimony methods. Maximum-likelihood methods are based on evaluation of the likelihood for the give evolutionary model of the observed trees, where maximum-parsimony methods tree is used to find the most parsimonious tree among all possible tree topologies with minimal overall number of changes (**Felsenstein 1985**). The *Actinomadura* 16S rRNA gene trees were rooted using *Thermomonospora curvata* DSM 43183^{T} (CP001738), the *Pseudonocardia* 16S gene tree using *Pseudonocarida cypriaca* KT2142^{T} (HQ157191) and the Streptomyces 16S rRNA gene trees using *Streptomyces albus* NRRL B-2365^{T} (DQ026669).

Screening for bioactivity. Plug assays

The 115 representatives strains, representing 19 multi-membered and 61 single-membered colour groups were screened for antimicrobial activity against a panel of wild type microorganisms using a standard plug assay **(Fiedler 2004)**.
All of the isolates were grown on oatmeal agar (ISP3 medium, **Shirling and Gottlieb (1966)**) for 14 days at 28°C to using the following procedure:

- Five individual plugs from each representative isolate were transferred using of 1 ml sterile tips and placed into large Petri dishes labelled as *Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus,* and *Saccharomyces cerevisiae*.
- Lysogeny broth (50 ml) mixed with 50 ml of nutrient agar in 5 separate sterile bottles and 100 µl of the *B. subtilis, E. coli, P. fluorescens, S. aureus,* and *S. cerevisiae* wild type strains was added to the appropriate bottle.
- The mix of nutrient agar and lysogeny broth was poured carefully into corresponding Petri dishes until the bottom of agar plugs were covered. Plates were incybated overnight for 28°C then checked for the presence of inhibition zones around the agar plugs.

Reporter strains

Sixty three isolates which showed activity against the panel of strains in the plug assays were tested against *B. subtilis* reporter strains designed to check specific modes of action against targeted cells.

- Six plugs were taken from each isolate plate, as explained before, and transferred to Petri dishes labelled with the codes of the six *B. subtilis* reporter strains (Tab. 2.3).
- 50 ml of Luria broth (LB), 50 µl of erythromycin, 50 µl of X-gal and 50 µl of each reporter strain were added to 50 ml of nutrient agar; for the *phi*105^{CH} reporter strain 50 µl of chloramphenicol was used instead of erythromycin.

The resultant media were carefully poured into Petri dishes until all of the plugs were covered; additional positive controls are shown in Table 2.3. Positive results were recorded when a blue halo was formed round zones of inhibition. The reporter genes are induced by bioactive substances that cause the cleavage X-gal to galactose and 5-bromo-4-chloro-3-hydroxyindole. The presence of a blue halo around inhibition zones which is due to this latter compound of protein lacZ produced by β-galactosidase activity indicated the mode of action of the bioactive compounds.

Table 2.3 *Bacillus subtilis* reporter strains and positive controls

Reporter genes	Positive control	Target
YvgS	Rifampicin	RNA synthesis
YupA	Cefoxitin	Cell envelope synthesis
YheH	Naladixic acid	Sporulation inhibitor
YvqI	Bacitracin	Cell wall synthesis
YiaX	Triclosan	Fatty acid synthesis
Phi105	Naladixic acid	DNA synthesis

RESULTS

Physico-chemical properties of the environmental samples

The pH, moisture and organic matter values of the Betpakdala and Usturt environmental soil samples are shown in Table 3.1.

Table 3.1 Physico-chemical properties of environmental samples

Soil samples	pH	Moisture content (%)	Organic matter content (%)
UKZ	7.0±0.5	0.094	0.43
BKZ	7.8±0.3	0.066	0.31

Enumuration, detection of isomers of diaminopimelic acid and colour-grouping. Number of actinobacteria isolated from the Betpakdala, Usturt and Yungay environmental samples

Colonies presumptively identified as actinomycetes were distinguished from other bacterial colonies growing on the selective media based on morphology, their ability to form mycelial colonies, and in many cases aerial spore mass. One hundred forty nine presumptive actinomycetes representing different colony types were subcultered onto ISP2 medium **(Shirling and Gottlieb 1966**).

Table 3.2 Total viable counts of bacteria and numbers of presumptive actinomycetes per gram dry weight soil recorded on different media plates inoculated with 10^{-2} dilutions of environmental suspensions and incubated at 28°C for 21 days

Media	Average number of colonies per dilution		Average number of colonies per 200 mg of soil sample
	BKZ	UKZ	UKZ
HV agar with cyclohexemide (25 µg/ml)	23.5	2.6	96.0
HV agar with cyclohexemide (25 µg/ml), nystatin (25 µg/ml), naladixic acid (25 µg/ml)	18.0	1.4	11.2
SCA with cyclohexemide (25 µg/ml) and nystatin (25 µg/ml)	2.4	0.9	21.2
SCA with cyclohexemide (25 µg/ml), nystatin (25 µg/ml), oxytetracycline (25 µg/ml)	5.6	0.4	20.4

Legend: BKZ - (Betpakdala) plates with dry weight soil were covered with unwanted bacteria, UKZ - Usturt desert soil sample

It is evident in Table 3.2. that small number of actinomycete-like colonies were detected from the Batpakdala and the Usturt desert soil samples.
The highest presumptive actinomyces count, $2040x10^{-1}$ dilution, was from the Usturt environmental sample. Moreover, the highest number of presumptive actinomyces were recorded from the SCA agar with cyclohexemide, nystatin and oxytetracycline in 25 µg/ml concentration. A number of colonies data from the Betpakdala environmental sample are missing because plates were covered with unwanted bacteria's which made it impossible to count.

Assignment to colour-groups

One hundred and forty nine representative filemantous actinomycetes isolates were assigned to 19 multi- and 61 single-membered colour groups based on their ability to produce aerial spore mass, substrate mycelial and diffusible pigment colours after growth on oatmeal agar and melanin pigments production on peptone yeast extract-iron agar incubation at 28ºC for 14 and 4 days, respectively.

Classification of representative strains isolated from Betpakdala and Usturt environmental samples based on phylogenetic data

Phylogenetic analyses

The 12 representative strains isolated from the Betpakdala and Usturt deserts assigned to genera based on comparative 16S rRNA gene sequence analyses as shown below.
The genus *Pseudonocardia*. Isolate BA8 from the Betpakdala environmental sample and isolates UA7 and UA19 from the Usturt environmental sample were recovered within the *Pseudonocardia* 16S rRNA gene cluster (Fig. 3.1). The isolates formed a distinct branch with the type strains of *Pseudonocardia cypriaca* and *Pseudonocardia hierapolitana*, a relationship that was supported by the maximum-likelihood algorithm and by a 65% bootstrap value. Isolates BA8 and UA7 shared a 16S rRNA gene similarity of 99.6%, a value which corresponds to 5 nt differences at 1339 sites. Isolates BA8, UA7 and UA19 were most closely related to *P. cypriaca* KT2142^T sharing a 16S rRNA gene sequence similarity with the latter of 99.6%, 96.6% and 98.3% respectively, values equivalent to 5, 24 and 52 nt differences at 1338, 1404 and 1347 locations.
The genus *Streptomyces*. The remaining 22 representative strains isolated from the Betpakdala and Usturt environmental samples were recovered within the *Streptomyces* 16S rRNA gene cluster (data not shown) albeit in several subclades as shown below.
Streptomyces ambofaciens subclade. Isolates BB1 and BB3, representatives of colour group 16, were isolated from the Betpakdala environmental sample. The isolates formed a distinct branch in the *Streptomyces ambofaciens* 16S rRNA gene tree that was supported by all the tree-making algorithms and by a low bootstrap value of 50% (Fig. 3.2). The isolates shared a 16S rRNA gene similarity of 99.2%, a value which corresponds to a single nt difference at 1317 locations. The isolates were most closely related to *S. ambofaciens* ATCC 23877^T sharing a 16S rRNA similarity of 99.33% and 99.4%, respectively, values equal to 9 and 8 nt differences at 1348 and 1323 locations.

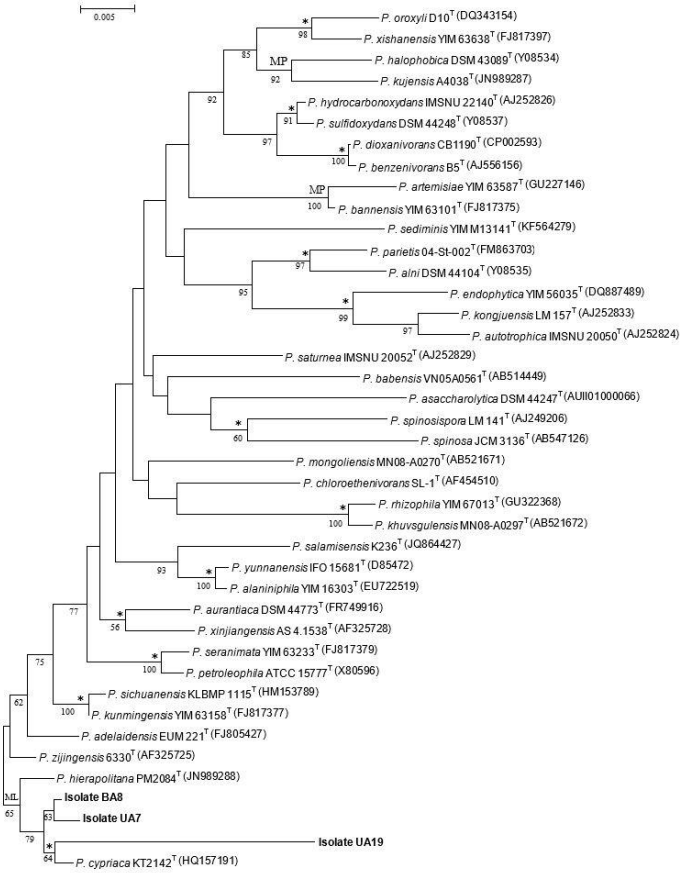

Figure 3.1 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BA8 from the Betpakdala environmental sample and isolates UA7 and UA19 from the Usturt environmental sample and relationships between them and the type strains of *Pseudonocardia* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were also supported by the maximum-likelihood method, while MP indicates branches of the tree that were supported by the maximum-parsimony method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

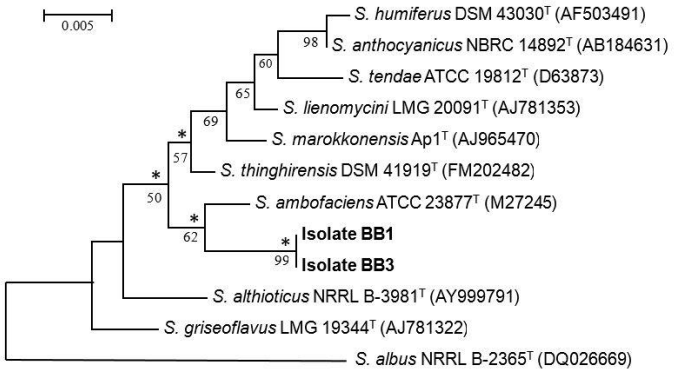

Figure 3.2 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between isolates BB1 and BB3 from the Betpakdala environmental sample and between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Streptomyces caelestis subclade. Strains BA3 and BC1, representatives of colour-group 4, were isolated from the Betpakdala environmental sample and recovered in the *Streptomyces caelestis* 16S rRNA subclade tree (Fig. 3.3). The isolates were closely related showing a 16S rRNA gene similarity of 99.9%, a value that is equal to a single nt difference at 1339 locations. They formed a distinct branch with the type strain *Streptomyces caelestis* that was supported by all of the tree-making algorithms and as 84% bootstrap value. Isolates BA3 and BC1 a 16S rRNA gene similarities with the *S. caelestis* NRRL 2418^T 99.78% and 99.7%, values equal to 3 and 4 nt differences at 1338 and 1354 locations.

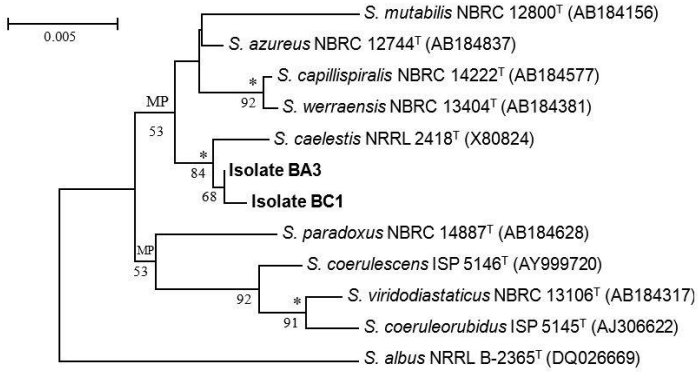

Figure 3.5 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between isolates BA3 and BC1 from the Betpakdala environmental sample and between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. MP indicates branches of the tree that were supported by the maximum-parsimony method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Streptomyces chattanoogensis subclade. Isolate BC21, a representative of colour group 39, was isolated from the Betpakdala environmental sample and assigned to the *Streptomyces chattanoogensis* 16S rRNA subclade, the integrity of latter was supported by the maximum-parsimony algorithm but not by a high bootstrap value (Fig. 3.4). The isolate was most closely related to the *Streptomyces sioyaensis* NRRL B-5408^T sharing similarity with the latter of 98.04%, a value corresponding to 26 nt differences at 1324 sites.

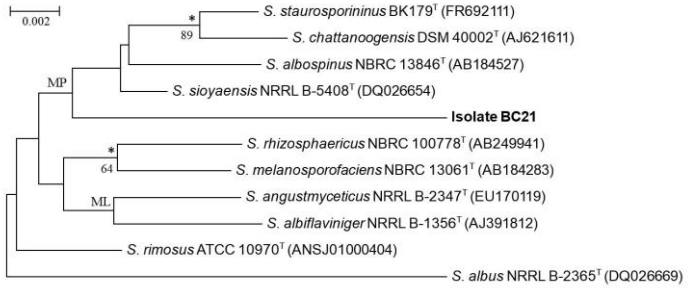

Figure 3.4 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BC21 from the Betpakdala environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with all of the tree-making methods. ML and MP indicates branches of the tree that were supported by the maximum-likelihood and by maximum-parsimony methods, respectively. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-samples datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nt position.

Streptomyces eurocidicus subclade. Isolate BD13, a representative of colour-group 21, was recovered in the *Streptomyces eurocidicus* 16S rRNA subclade, a relationship supported by an 85% bootstrap value and all of the tree-making algorithms (Fig. 3.5). The isolate was most closely related to *Streptomyces stramineus* NBRC 16131^T sharing a 16S rRNA gene sequence similarity with the latter of 98.7%, a value which corresponds to 18 nt differences at 1388 sites.

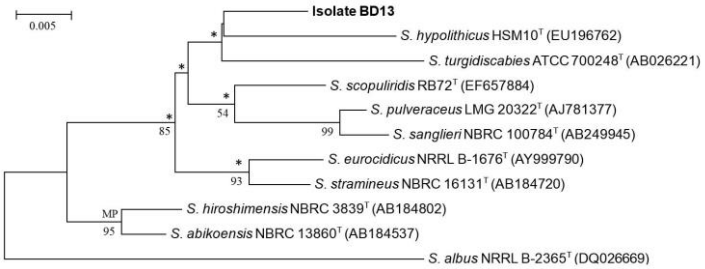

Figure 3.5 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BD13 from the Betpakdala environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with all of the tree-making methods. MP indicates branches of the tree that were supported by the maximum-parsimony method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Streptomyces flavovirens subclade. Isolate BA4, a representative of colour group 7, was recovered in the *Streptomyces flavovirens* 16S rRNA gene subclade, a loose assembly of strains supported by a 54% bootstrap value but not by the maximum-likelihood or maximum-parsimony algorithms (Fig. 3.6). Isolate shared an identical 16S rRNA gene sequence with Streptomyces *pratensis* ch24^T.

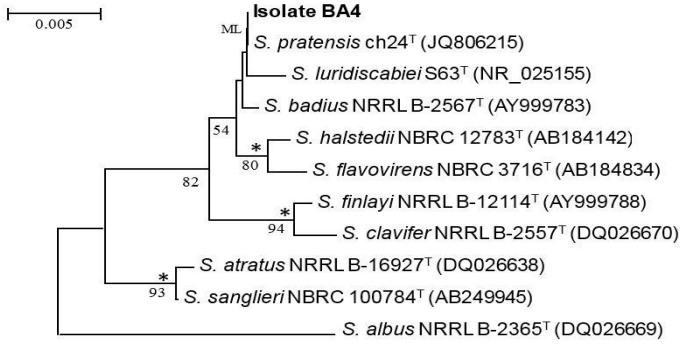

Figure 3.6 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BA4 from the Betpakdala environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. ML indicates branches of the tree that were also supported by the maximum-likelihood method. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

Streptomyces gobitricini subclade. Isolate BB10, a representative of colour-group 49 from the Betpakdala enviromental sample and isolate UB2, a representative of colour-group 29 from the Usturt enviromental sample were assigned to the *Streptomyces gobitiricini* 16S rRNA gene subclade, a relationship that was supported by a bootsrap value of 71% and by all of the tree-making algorithms (Fig. 3.7). The isolates shared a 16S rRNA gene similarity of 99.0%, a value equivalent to 13 nt differences at 1321 sites. Isolate BB10 was most closely related to *Streptomyces kurssanovii* NBRC 13192^T and *Streptomyces xantholiticus* NBRC 13354^T sharing a 16S rRNA sequence similarity with these strains of 98.26%, a value which corresponds to 23 nucleotide differences at 1322 sites. Isolate UB2 was also most closely related to *S. kurssanovii* NBRC 13192^T and *S. xantholiticus* NBRC 13354^T sharing a 16S rRNA sequence similarity with these organisms of 98.83%, a value which corresponds to 16 nt differences at 1370 sites.

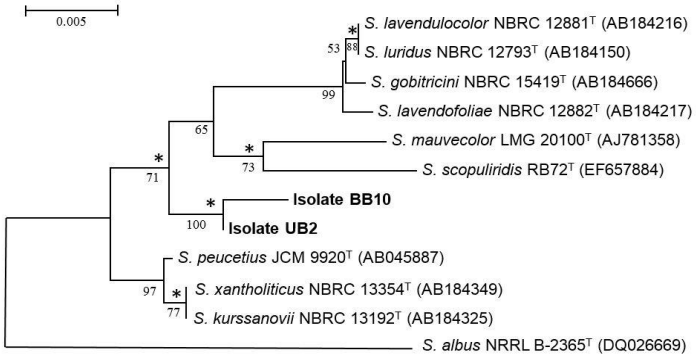

Figure 3.7 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BB10 from the Betpakdala environmental sample, and strain UB2 from the Usturt environmental sample and between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Streptomyces mutabilis subclade 1. Isolate BC22, a representative from colour group 2 and isolated from the Betpakdala environmental sample was recovered in the *Streptomyces mutabilis* 16S rRNA gene subclade, a relationship that was supported by all three tree-making algorithms but not by a high bootstrap value (Fig. 3.8). The isolate was most closely to *Streptomyces djakartensis* NBRC 15409^T, these strains shared a 16S rRNA sequence similarity of 99.47%, a value which corresponds to 7 nt differences at 1324 sites.

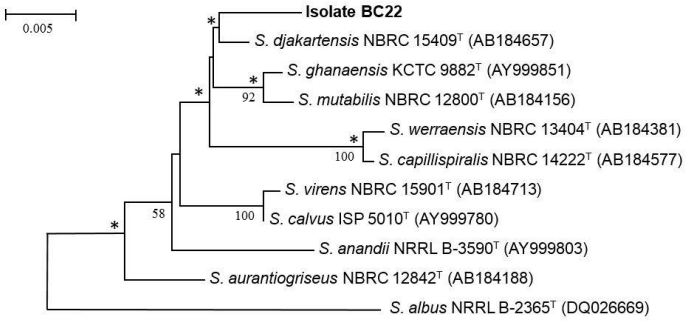

Figure 3.8 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BC22 from the Betpakdala environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Streptomyces mutabilis subclade 2. Isolates UD3 and UC28 representatives of colour-group 74 and 70, respectively were isolated from the Usturt environmental sample and isolate LB23 from the Lomas Bayas environmental sample. All three strains were recovered within *Streptomyces mutabilis* 16S rRNA subclade, relationship that was which was supported by the maximum-likelihood and neighbour joining methods and by a 69% bootstrap value (Fig. 3.9). These isolates shared a 16S rRNA similarity of 99.3%, a value that corresponds to a single nt difference at 1354 locations. Isolates UC28 and UD3 were closely related to *Streptomyces mutabilis* NBRC 12800^T sharing a 16S rRNA sequence similarity with the latter of 99.93% and 100%, respectively. Isolate LB23 also most closely related to the *Streptomyces mutabilis* NBRC 12800^T, these osrganisms shared a 16S rRNA sequence similarity of 99.78%, a value that corresponds to 3 nt differences at 1384 sites.

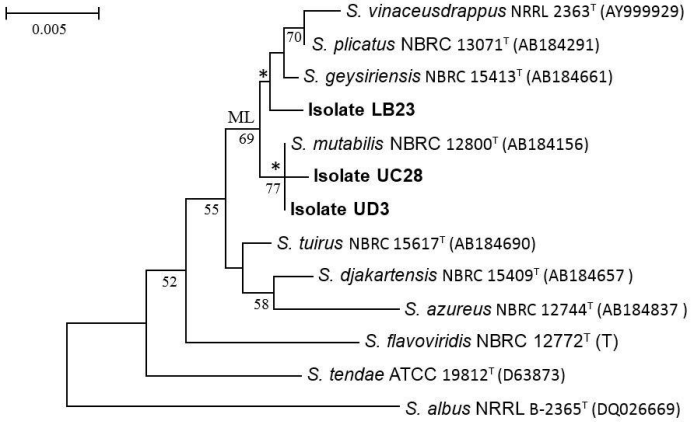

Figure 3.9 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains UD3 and UC28 from the Usturt environmental sample and isolate LB23 from the Lomas Bayas environmental sample and between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Streptomyces purpurascens subclade. Strains UB3 and UC25, representatives of colour group 19, were related from the Usturt environmental environmental sample formed a distinct phyletic line in the *Streptomyces purpurascens* 16S rRNA gene subclade; a taxon that was supported by the neighbour-joining and maximum-likelihood algorithms but not by a high bootstrap value (Fig. 3.10.). Isolates UB3 and UC25 were closely related sharing a 16S rRNA gene similarity of 99.8%, a value corresponding to 3 nt differences at 1328 sites. In turn, the two isolates shared a 16S rRNA sequence similarity with *Streptomyces coerulescens* ISP 5146^T of 99.3%, a value equivalent to 9 nt differences at 1360 and 1361 locations, respectively.

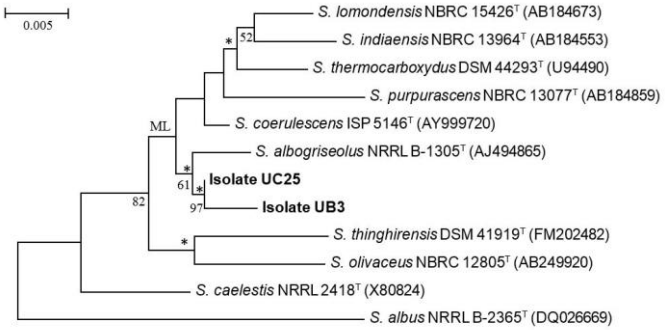

Figure 3.10 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between isolates UB3 and UC25 from the Usturt environmental sample and between them and the type strains of the most closely *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Streptomyces violaceus subclade. Isolates BC7 and BC14, representatives of colour-group 1 were related from the Betpakdala environmental sample and show to form a distinct branch in the *Streptomyces violaceus* 16S rRNA gene subclade (Fig. 3.11). The isolates shared a 16S rRNA gene similarity of 99.6%, which corresponds to 5 nt differences at 1302 locations. Isolate BC7 was closely related to *Streptomyces glebosus* CGMCC 4.1873^T, these strains shared a 16S rRNA sequence similarity of 98.1%, which corresponds to 26 nt differences at 1369 sites. Isolate BC14 was most closely related to *Streptomyces canifeus* NBRC 15389^T, *Streptomyces hawaiiensis* NBRC 12784^T, *Streptomyces janthinus* ISP5206^T and *Streptomyces violaceus* NBRC 13103^T, it shared a 16S rRNA similarity of 98.62% with all these organisms; which corresponds to 18 nt differences at 1302 sites.

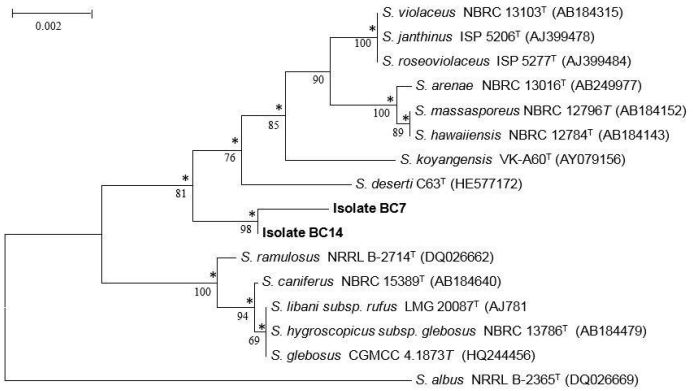

Figure 3.11 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strains BC7 and BC14 from the Betpakdala environmental sample and relationships between them and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

Streptomyces vinaceus subclade. Isolate BD2, a representative of colour-group 20 was isolated from the Betpakdala environmental sample and recovered in the *Streptomyces vinaceus* 16S rRNA subclade, a taxon supported by a 95% bootstrap value and by all of the tree-making algorithms (Fig. 3.12). Isolate BD2 formed a distinct branch in the subclade together with the type strain of *Streptomyces avidinii*, a relationship supported with all three tree-making methods and with 78% bootstrap value. The two strains shared a 16S rRNA sequence similarity 99.8%, a value which is corresponds to 3 nt differences at 1330 sites.

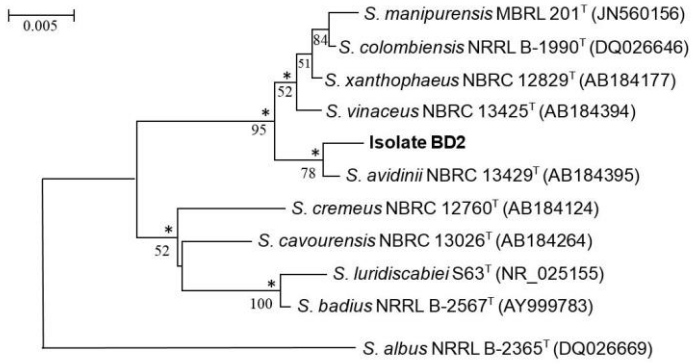

Figure 3.12 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing relationships between strain BD2 from the Betpakdala environmental sample and between it and the type strains of the most closely related *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making methods. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sample datasets; only values above 50% are shown. The scale bar indicates 0.005 substitutions per nt position.

Screening for antimicrobial activity

Plug assays

The results obtained for the 115 representative isolates screened for antimicrobial activity in the agar plug assays. Forty five of the isolates showed activity against *Bacillus subtilis* (39.1%), 8 against *Escherichia coli* (7.0%), 23 against *Pseudomonas fluorescence* (27.8%), 39 against *Staphylococcus aureus* (33.9%) and 36 against the *Saccharomyces cerevisiae* (31.3%). Isolates BD13, UC18, UC19, UC20 and UD2 gave zones of inhibition against all five wild type strains. In contrast, 52 (45.2%) isolates did not show any activity against the panel of strains, there 19 (16.5%) isolates from the Betpakdala desert, 26 (22.6%) from Usturt desert soil (Table 3.4.1).

Strains negative against all five wild type strains:

- Betpakdala desert - BA7, BA8, BA10, BB1, BB4, BB5, BB10, BC10, BC13, BC16, BC17, BC19, BC22, BC23, BC24, BD3, BD5, BD6, BD11.
- Usturt desert – UA1, UA5, UA6, UA7, UA8, UA12, UA13, UA17g, UA17r, UA18, UA19, UA20, UA21, UB1, UC2, UC8, UC22, UC23, UC31, UD5, UD6s, UD7, UD7s, UD8, UD9, UD10.

Plug assays with *Bacillus subtilis* reporter strains

The results obtained for the 61 isolates, which gave positive results against the panel of wild type strains, are shown in Table 3.4.2. Thirty-three (54%) of isolates showed false positive results with inhibition zones, five (8.2%) of isolates with false positive result, thirteen (21.3%) of isolates showed inhibition zone and seven (11.5%) of them gave negative results.

Strains negative against all six *Bacillus subtilis* reporter strains:

- Betpakdala desert – B7, BB9, BB2, BC14, BC15.
- Usturt desert – UA2s, UB4, UD6.

Fifty three strains gave false positive results, some showing inhibition zone without blue halos. Only reporter strains *YvqI* and *YvgS* showed visible inhibition zones.

DISCUSSSION

The primary objectives of this project was achieved as relatively large numbers of putatively novel actinomycetes were isolated from the Betpakdala and Usturt environmental samples and screened to produce bioactive compounds against a panel of wild microorganisms.

Low numbers of actinomycetes were isolated from the Betpakdala and Usturt sample, showing that the selective isolation procedures were effective. The highest number of actinobacteria was recorded from the Usturt environmental sample, 96.0x10-2 colony forming units per gram dry weight soils on HV agar plates. The lowest number was 0.4x10-2 cfu per gram dry weight soil on starch-casein agar modified with oxytetracycline antibiotic (25µg/ml) agar plates.

Similarly a number of actinomycetes was isolated from the Betpakdala environmental sample where the highest was 23.4x10-2; and the lowest 2.4x10-2, from starch-casein agar and HV agar, respectively.

Sixty one representative strains (54.8%) showed activity against one or more of the five strains used in the standard plug assays thereby providing further evidence that the use of dereplicated strains results in high hit rates **(Goodfellow and Fiedler 2010; Yuan *et al.* 2014**). Forty-five of the isolates showed activity against *Bacillus subtilis* (39.1%), 39 against *Staphylococcus aureus* (33.9%), 36 against *Saccharomyces cerevisiae* (31.3%), 23 against *Pseudomonas fluorescence* (27.8%) and 8 against *Escherichia coli* (7.0%). Isolates BD13, UC18, UC19, UC20 and UD2 gave zones of inhibition against all five wild type strains. In contrast, 52 (45.2%) isolates did not show any activity against the panel of strains: 19 (16.5%) isolates from the Betpakdala desert, 26 (22.6%) from Usturt desert soils and 3 (2.6%) from Yungay environmental samples.

Isolates that gave positive results against the panel of wild type strains were screened against *Bacillus subtilis* reporter strains. Thirty-three (54%) of isolates showed false positive results with inhibition zones, five (8.2%) of isolates gave false positive results, thirteen (21.3%) of isolates showed inhibition zone and 7 (11.5%) of them gave negative results. The majority of plugs coloured blue, which is a false positive result and might be interpreted as a presence of β-galactosidase gene in isolated strains. According to **Urban *et al.* (2007)** isolates are likely to be 30S or 50S protein inhibitors commonly produced by *Streptomyces* species and species related to them, which can be detected by *YheI* reporter strain.

The seventeen strains isolated from the Betpakdala and Usturt environmental samples were found to belong to the genera *Streptomyces*. In addition, it was identified that several *Pseudonocardia* strains were isolated, as a member of this rare genus has been found to predominate in the Atacama Desert soils back on culture independent studies (Idris, data not published).

The *Streptomyces* strains isolated from the Betpakdala and Usturt desert environmental sample were assigned to 6 multi- and 5 single-membered clusters. These results are similar to the reported hyper- and extreme hyper-arid Atacama Desert soils **(Okoro *et al.* 2009; Busarakam 2014)** and provide further evidence that arid desert soils contain a *Streptomyces* community that proves colour-groups are good indicators of *Streptomyces* species diversity **(Goodfellow and Fiedler 2010).**

It is difficult to assign actinomycetes from natural habitats to novel or putatively novel species phyletic branch on 16S rRNA gene sequence data. However, it has been commonly shown by **Meier-Kolthoff *et al.* (2013)** that actinomycetes that share a 16S rRNA gene sequence of less than 99.0% with their nearest neighbours can be putatively novel species. According that cut-off point, the five of the *Streptomyces* and two of the *Pseudonocardia* isolates from the Kazakhstan environmental samples are highly likely to be new species. Indeed, this is probably and underestimate as an additional 7 strains shared 16S rRNA gene similarity below 99.0%, they are BB10, BC7, BC14, BC21, UA7, UA19 and UB2. However, in each case the novelty of these isolates needs to be underpinned by approximate polyphase taxonomic studies and DNA:DNA pairing.

CONCLUSION

The Kazakhstan desert soils are a rich source of novel *Pseudonocardies* and *Streptomyces* strains that shows broadly it may to lead to drug leads. That will prove useful in the search for novel antibiotics for health care.

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