Molecular Biology: Open Access
ISSN: 2168-9547

Adegboye and Babalola, Mol Biol 2013, S1
DOI: 10.4172/2168-9547.S1-001

Research Article Open Access

Phylogenetic Characterization of Culturable Antibiotic Producing *Streptomyces* from Rhizospheric Soils

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Abstract

Streptomyces spp. were isolated from rhizospheric soils collected from Ngaka Modiri Molema District, North West Province, South Africa. Ten of these bacterial isolates were found to exhibit broad spectrum antimicrobial activity against test organisms with varying degree of activity. The cultural characteristics of the bacterial isolates were consistent with that of the members of the genus *Streptomyces*. Molecular identification of the potent bacterial isolates was carried out by amplifying the 16S rDNA gene; this gave the expected size of 1.5 kb and was sequenced. Further computational analysis including BLAST search and phylogenetic analysis were performed to correlate the bacterial isolates with other species of the genera in the database library. The computational analysis of the amplified 16S rDNA gene confirmed that the bacterial isolates are members of the genus *Streptomyces* with 89-100% sequence similarity. The phylogenetic analysis showed that the 10 bacterial isolates were divided into 3 major clusters with varying bootstrap values. The strain NWU195 formed a distinct phyletic line in the *Streptomyces* 16S rDNA gene tree suggesting a new strain. The 16S rDNA sequences of the bacterial isolates was submitted to the GenBank under the accession numbers JX284398-JX284407. The 16S rDNA gene sequence analysis is a significant tool for phylogenetic analysis of *Streptomyces* spp.

Keywords: *Streptomyces*; Rhizosphere; Phylogenetic; Antimicrobial; 16S rDNA

Introduction

Soil is a habitat for microorganisms and also serves as reservoir for their metabolites [1]. The genus *Streptomyces* is one of prominent soil inhabitant, comprising up to 90% of actinomycetes isolated from soil samples. *Streptomyces* is the largest and the most important genus in the order actinomycetales. The genus *Streptomyces* is prolific producers of bioactive secondary metabolites that have important applications both in medicine and agriculture [2,3]. In the history of drug discovery, majority of novel substances of microbial origin are isolated from *Streptomyces*, over two-thirds of all microbial antimicrobial agents are derived from them [4,5]. Many novel drugs have been developed from *Streptomyces* spp. including *S. griseus*, *S. hygroscopicus*, *S. coelicolor*, *S. avermitilis*, *S. rochei*, *S. plicatus*, *S. fungicidicus*, *S. flaveus*, and *S. globisporus*; belonging to different classes of antibiotics such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines [6-8]. These organisms are of high biotechnological and commercial values; and continue to be routinely screened for new bioactive compounds [9-11]. *Streptomyces* spp. are aerobic, filamentous, Gram positive soil dwelling bacteria with high G+C content in their genomic composition.

Pathogenic organisms are gaining resistance to existing clinical drugs either through acquire resistance (chromosomal mutation) or acquisition of genetic materials from other bacteria (vertical or horizontal transfer of genes) [12,13]. This uprising has rendered many onetime drug of choice ineffective against the pathogens [14]. Escalating numbers of antibiotic futile against pathogenic organisms is a worldwide scenario. There is a need to develop novel antibiotics with different mechanisms of action.

Various researches are taking place all over the world searching for antimicrobial agents to combat the menace of infectious disease agents. Despite the fact that soils have been continuously screened over 50 years for potent organisms there is still tendency of isolating novel antibiotic from terrestrial Streptomycetes. It has been reported that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered [15]. Some *Streptomyces* spp. possess more than 20 gene clusters devoted to the synthesis of secondary metabolites [16]. Determination of the nucleotide sequence of the 16S rDNA gene is a well established standard method for the identification and phylogenetic classification of unknown organisms up to the species level. Our investigation was aimed to isolate and screen antibiotic producing *Streptomyces* from rhizospheric soils and evaluate their evolutionary linage for possible novel antimicrobial agent(s).

Materials and Methods

Sampling area

The study area covered the Ngaka Modiri Molema District in North West Province of South Africa (Figure 1). The latitude and longitude of the district is 25°55'N and 25°50'E respectively. It covered a total of 28,206 km^2 area. Temperatures range from 17°C to 31°C (62° to 88°F) in the summer and from 3° to 21°C (37° to 70°F) in the winter. The average rain fall is 360 mm.

Isolation of actinomycetes

Isolation and enumeration of actinomycetes present in the soil sample was performed by serial dilution plate technique using starch casein agar, as described previously [17].

Screening for antibiotic producing actinomycetes

Determination of antimicrobial activities of pure actinomycetes

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Received November 12, 2012; **Accepted** December 19, 2012; **Published** December 21, 2012

Citation: Adegboye MF, Babalola OO (2013) Phylogenetic Characterization of Culturable Antibiotic Producing *Streptomyces* from Rhizospheric Soils. Mol Biol S1: 001. doi:10.4172/2168-9547.S1-001

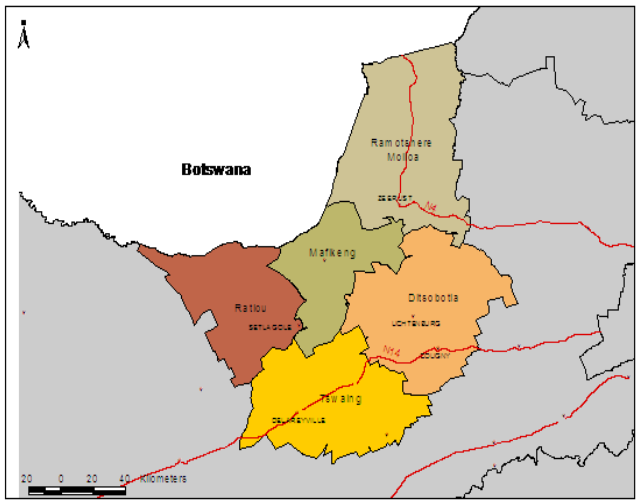

Figure 1: A map of Ngaka Modiri Molema district showing different localities where soil samples were collected.

cultures was performed by cross-streak method as described previously [18].

Test organisms

Staphylococcus aureus ATCC 29213, *Streptococcus pyogenes* ATCC 12344, *E. coli* ATCC 43478, *Bacillus subtilis* ATCC 11774, *B. cereus* ATCC 11778, *Proteus mirabilis* ATCC 49132, *Enterococcus faecalis* ATCC 14506, *Shigella boydii* ATCC 9207, *Klebsiella pneumoniae* ATCC 8308, *Pseudomonas aeruginosa* ATCC 10145 and *Salmonella typhimurium* ATCC 14208.

These typed cultures were obtained from the Microbial Biotechnology Group Culture Collection of the Biological Sciences Department, Faculty of Science, Agriculture and Technology, North-West University, South Africa.

Cultural characteristics

Pure bacterial isolates were characterized culturally following the protocol given by the international Streptomyces project (ISP) [19] growth on ISP-2 medium was recorded after incubation at 25°C for 14 days.

Isolation of genomic DNA

Actinomycete genomic DNA was isolated by a protocol previously described [20] with some modification. Cultures were grown in 10 ml of Luria Bertani broth (Merck) in McCartney bottles for 7 days and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik Germany) for 5 min. The mycelial pellet was resuspended in 500 µl of 5 M NaCl and transferred to a 2 ml Eppendorf tube. The cells were centrifuged at 10,000 rpm for 5 min, and the pellet was resuspended in 1 ml of 10 mM Tris-HCl–1 mM EDTA (pH 7.5) (TE) containing 20 mg of lysozyme/ml and 20 mg of RNase A/ml and incubated at 37°C for 30 min. Following incubation, 250 µl of 0.5 M EDTA, 250 µl of TE containing 5 mg of proteinase K/ml, and 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added, and the tubes were heated in a 65°C water bath for 30 min. Cellular debris was removed by centrifugation at 8,000 rpm for 5 and the supernatant solution was transferred to a new 2-ml microcentrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt. The supernatant was gently poured off and the pellets were dried under vacuum using Tomy Micro VacTM mv-100 (Tomy Medico, Japan) vacuum dryer. The DNA was resuspended in 50 µl of TE and incubated at 65°C for 1 h to reconstitute the DNA, for immediate use or storage at -20°C.

PCR amplification

The 16S rDNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primer fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') [21]. PCR was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The amplification reaction was performed with a DNA Engine DYAD Peltier thermal cycler (BioRad, USA). The thermal cycling condition used was an initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 96°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR amplicons were analysed by electrophoresis in 1% (w/v) agarose gel. The gel containing ethidium bromide (10 µg ml^{-1}) was view under Syngene Ingenius Bioimager (UK) to confirm the expected size of the product. The remaining mixture was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany).

Nucleotide sequence determination

PCR purified products of the 16S rDNA of the strains were analysed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Nucleotide sequence of the 16S rDNA of the strains were determined and compared for similarity level with the reference species of bacteria contained in genomic database banks, using the 'NCBI Blast' available at the ncbi-nlm-nih.gov website [22].

Molecular taxonomy determined by sequences and phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using softwares. Nucleotide sequences were analyzed and edited by using BioEdit software [23]. The partial 16S rDNA gene sequences were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions. Multiple alignments of the sequences were carried out by Mafft program 6.864 [24] against corresponding nucleotide sequences retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor [25]. Phylogenetic analyses was conducted using MEGA version 5.10 [26] and neighbour joining [27]; minimum evolution [28]; maximum likelihood; UPGMA and maximum parsimony [29] trees was constructed. The methods were used in order to expatiate on the phylogeny and for better comparison. The robustness of the tree topology was evaluated by bootstrap analysis [30] based on 1000 resamplings. Putative chimeric sequences were identified using the Chimera Buster 1.0 software. Manipulation and tree editing were carried out using TreeView [31].

Nucleotide sequence accession numbers

The 16S rDNA gene sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers indicated in parentheses, NWU4 (JX284398), NWU233 (JX284399), NWU49 (JX284400), NWU91 (JX284401), NWU110 (JX284402), NWU195 (JX284403), NWU204 (JX284404), NWU339 (JX284405), NWU100 (JX284406), and NWU14 (JX284407).

Results

Isolation of actinomycetes

Streptomyces spp. was isolated from the soil samples collected from different localities in Ngaka Modiri Molema District. The colonies of actinomycetes are recovered from different serial decimal dilution Petri dishes. The bacterial isolates have morphological characteristic that were consistent with members of the genus *Streptomyces*. The colonies appear dry, rough, coloured or not, adhering to the medium and presence of aerial and/or substrate mycelia. They are mended in the same isolation medium and incubated at 28°C during 7 days.

Cultural characterization

The *Streptomyces* isolates were studied for cultural characteristics (Table 1). Cultural characteristics of the bacterial isolates were derived on the basis of observations made after 21 days of incubation on ISP-2. All the isolates growth varied from good to few. The colours of the mycelia were varied from greyish to brown. These characteristic morphological properties strongly suggested that isolates belonged to the genus *Streptomyces*.

Screening of *Streptomyces* isolates

Among them, 10 isolates distinguished due to their antibacterial activity against test organisms. These *Streptomyces* isolates exhibited broad spectrum antimicrobial activities. The results were tabulated in table 2. The following percentage of *Streptomyces* isolates exhibited inhibitory effect against the test organisms including: *S. aureus* (100%), *S. pyogenes* (100%), *C. coli* (90%), *B. subtilis* (100%), *B. cereus* (90%), *P. mirabilis* (40%), *E. faecalis* (90%), *S. boydii* (70%), *K. pneumoniae* (40%), *P. aeruginosa* (30%) and *S. typhimurium* (50%). Three isolates exhibited high antibacterial activities against all the test organisms and appeared promising.

Molecular identification of *Streptomyces* isolates

A 1.5 kb fragment was amplified from the genomic DNA with the bacterial universal primers (F1R5) (Figure 2). Identification of the isolates was confirmed by computational analysis. The generic identification of Streptomycetes was performed by analysis of partial sequences of their 16S rDNA gene. The partial nucleotide sequences of the 16S rDNA gene of the isolates were compared with nucleotide database of NCBI web server through BLAST tool. The BLAST search inferred that the isolates were members of the GC-rich actinomycetales. The 16S rDNA gene sequence of different *Streptomyces species* was obtained by BLASTN search; however 24 strains of *Streptomyces* were selected based on high identity (%) with good E value. Table 3 results shows that query sequences were best pairwise aligned with 16S rDNA gene sequence of *Streptomyces* spp. with sequence similarity and identity ranged between 89-100%, with E value of 0.

Phylogenetic analysis and *Streptomyces* spp. diversity

The 10 potent *Streptomyces* isolates were subjected to sequencing and phylogenetic analysis. The 16S rDNA sequences of 10 isolates were aligned with 24 Streptomycetes sequences obtained from GenBank data library; and *Kitasatospora* spp. as the out-group. The phylogenetic position of the isolates was evaluated by constructing phylogenetic trees using neighbour-joining (NJ), minimum evolution

Isolate	Growth	Aerial mycelium	Substrate mycelium	Reverse colony colour	Pigmentation
NWU4	Good	Grey	Yellowish brown	Yellowish brown	Brown
NWU14	Good	Grey	Light brown	Brown	None
NWU49	Good	Dark green	Light brown	Green	None
NWU91	Moderate	White	Yellow	Orange	None
NWU100	Few	Grey	Greyish yellow	Brown	None
NWU110	Moderate	Greyish green	Dark brown	Brown	None
NWU195	Good	White	Yellowish grey	Reddish orange	None
NWU204	Good	Grey	Light brown	Brown	None
NWU233	Moderate	Cream	Yellow	Brown	None
NWU339	Few	Greyish green	Brown	Green	None

Table 1: Cultural characteristics of bacterial isolates on ISP-2 medium.

	Isolate codes									
™Test organisms	NWU4	NWU14	NWU49	NWU91	NWU100	NWU110	NWU195	NWU204	NWU233	NWU339
Pseudomonas aeruginosa ATCC 10145	-	+	-	+	-	-	-	+	-	-
Klebsiella pneumoniae ATCC 8308	-	+	-	+	-	-	-	+	-	+
Enterococcus faecalis ATCC 14506	-	+	+	+	+	+	+	+	+	+
Shigella boydii ATCC 9207	-	+	-	+	-	+	+	+	+	+
Proteus mirabilis ATCC 49132	-	-	-	+	-	-	-	+	+	+
Bacillus subtilis ATCC 11774	+	+	+	+	+	+	+	+	+	+
Bacillus cereus ATCC 11778	-	+	+	+	+	+	+	+	+	+
Streptococcus pyogenes ATCC 12344	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus ATCC 29213	+	+	+	+	+	+	+	+	+	+
Campylobacter coli ATCC 43478	+	+	+	+	-	+	+	+	+	+
Salmonella typhimurium ATCC 14208	+	+	-	+	-	+	-	+	-	+

+=Activity; -=No activity

Table 2: Antibacterial activity of potent actinomycetes isolates against pathogenic organisms.

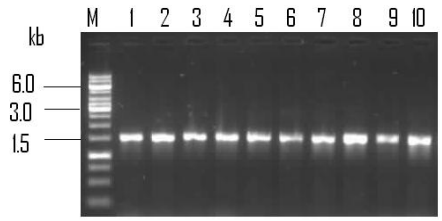

Figure 2: Amplified fragment of 16S rDNA gene of the potent bacterial isolates 1-10 (1.5 kb); M: 1 kb Marker.

Isolate code	Sequence length (bp)	Closest related strain in database	Accession number	Similarity (%)	E-value
NWU4	1338	*S. globisporus*	HQ995504	99	0
NWU14	1341	Uncultured *Streptomyces* spp.	JQ358574	95	0
NWU49	1306	*Streptomyces viridosporus*	NR 0438575	99	0
NWU91	1335	*Streptomyces rochei*	JF486442	100	0
NWU100	1363	*Streptomyces hirsutus*	AB184844	89	0
NWU110	1295	*Streptomyces emelensis*	NR 043869	98	0
NWU195	1397	*Streptomyces hygroscopicus*	FJ406123	95	0
NWU204	1308	*Streptomyces fungicidicus*	AB184529	99	0
NWU233	1373	*Streptomyces espinosus*	X80826	98	0
NWU339	1362	*Streptomyces griseus*	AB184821	92	0

Table 3: Results of 16S rDNA gene sequence similarities of Streptomyces isolates and GenBank accession numbers using BLASTN Algorithm.

(ME), maximum likelihood (ML), maximum parsimony (MP) and UPGMA methods (Figures 3 and S1-S4). These methods consistently placed the bacterial isolates in different clade encompassing members of the genus *Streptomyces* with bootstrap support. Bootstrap values based on 1000 replications were listed as percentages at the branching points. The results from the different approaches show completely resolved, well-supported phylogeny of the 10 bacterial isolates with high resolution of all inner branches. Bacterial isolate NWU100 shows a low sequence similarity (89%) with the closest related stain (*S. prasinopilosus*); this suggests that it's a novel *Streptomyces* spp. Phylogenetic analysis also revealed that the isolate NWU195 forms a distinct clade and the low sequence similarity values further suggest that NWU195 possibly belongs to a novel species with *S. hygroscopicus* as its closest relative. This is further supported by its taxonomic positions, confirmed by phylogenetic analyses (Figures 3 and S1-S4). Overall, the high-level branching in the phylogenetic trees agrees well with traditional systematic divisions, which group's organisms which belong taxonomically to the same family or the same genus into different species.

Discussion

Screening of nature for novel antimicrobial agents is a continuous process to match unending demand for bioactive substances in order to curtail the issues of infectious diseases. Nature has been a renowned source of bioactive compounds; this can be through screening of plants or microorganisms [1]. Microorganisms have proven to be an attractive source of bioactive compounds of industrial importance [3,6]. The unearthing of microbial secondary metabolites has a great capability in the development of industrial microbiology. The order actinomycetales, especially the genus *Streptomyces* are known to be inexhaustible producers of important microbial metabolites of medical and agricultural importance [32,33]. The terrestrial habitant is considered an excellent source for the exploration of *Streptomyces* with substantial potential. Several studies have previously reported on the isolation and diversity of *Streptomyces* from the terrestrial environment [34,35]. It was evident in this study that the genus *Streptomyces* is the dominant actinomycetes in rhizosphere. Similar studies carried out by other researchers' also show that *Streptomyces* spp. are predominant in the rhizosphere [36,37]. Previous research works have shown the number and diversity of the genus *Streptomyces* in the rhizospheric soils is in relation to the type and amount of exudates, and plant species [38]. It has been reported that root exudates stimulates the growth of actinomycetes in the rhizosphere [39]. The cultural characteristics of the bacterial isolates were similar to those describe by other researchers, this reveals that the isolates under study belong to the genus *Streptomyces* [34,40].

Majority of the novel antimicrobial agents are derived from soil borne actinomycetales. *Streptomyces* spp. are important group of organisms in the production of antimicrobial agents against pathogenic organisms. The bacterial isolates from the rhizospheric soils showed broad spectrum of antimicrobial activity against pathogenic organisms. The result of the screening reveals that most of the potent bacterial isolates were active against Gram positive organisms than Gram negative organisms. This can be attributed to the cell wall structure of the Gram negative organisms having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes [41]. The Gram positive organisms are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. The fact that the bacterial isolates exhibited broad spectrum of antimicrobial activity, this signify possible production of several antimicrobial compounds and/or production of compounds with multiple microbial targets. Several researchers have already reported *Streptomyces* to have biocontrol activity against pathogenic organisms [9,42]. This has been shown that the principle mechanism of this biological activity involved the production of secondary metabolites [6].

The identification of the bacterial isolates to the species level is vital since this provides informative insight about the organism, possible kind of bioactive compounds and if is novel or not [43]. The identification of the potent bacteria isolates in this study was based on 16S rDNA gene sequence analysis. The sequence comparison of the bacterial isolates showed 89-100% identification similarities with 16S rDNA gene sequence of the genus *Streptomyces*. Analysis of 16S rDNA gene sequences has been proved to be a powerful method for phylogenetic characterization of microorganisms [44]. It helps to elucidate the evolutionary relationship among microorganisms. The phylogenetic relationship of the potent bacterial isolates to known *Streptomyces* spp. was first estimated through a blast search of the GenBank database. For a more robust analysis, the closest related strains were chosen for pairwise sequence comparison and construction of the phylogenetic trees. The bacterial isolates were grouped in distinct branches from each other. It was reported that strains which are clustered in different groups might produce different antimicrobial agents [45].

All the closest strains to the bacterial isolates have been linked to the production of one bioactive compound or more. *S. globisporus* had been described as a soil dwelling Gram positive bacteria with antibacterial, antifungal and antitumor activities [46,47]. Mutanolysin was isolated from *S. globisporus*, it is a muralytic enzyme that cleaves the β-N-acetylmuramyl-(1→4)-N-acetylglucosamine linkage of the

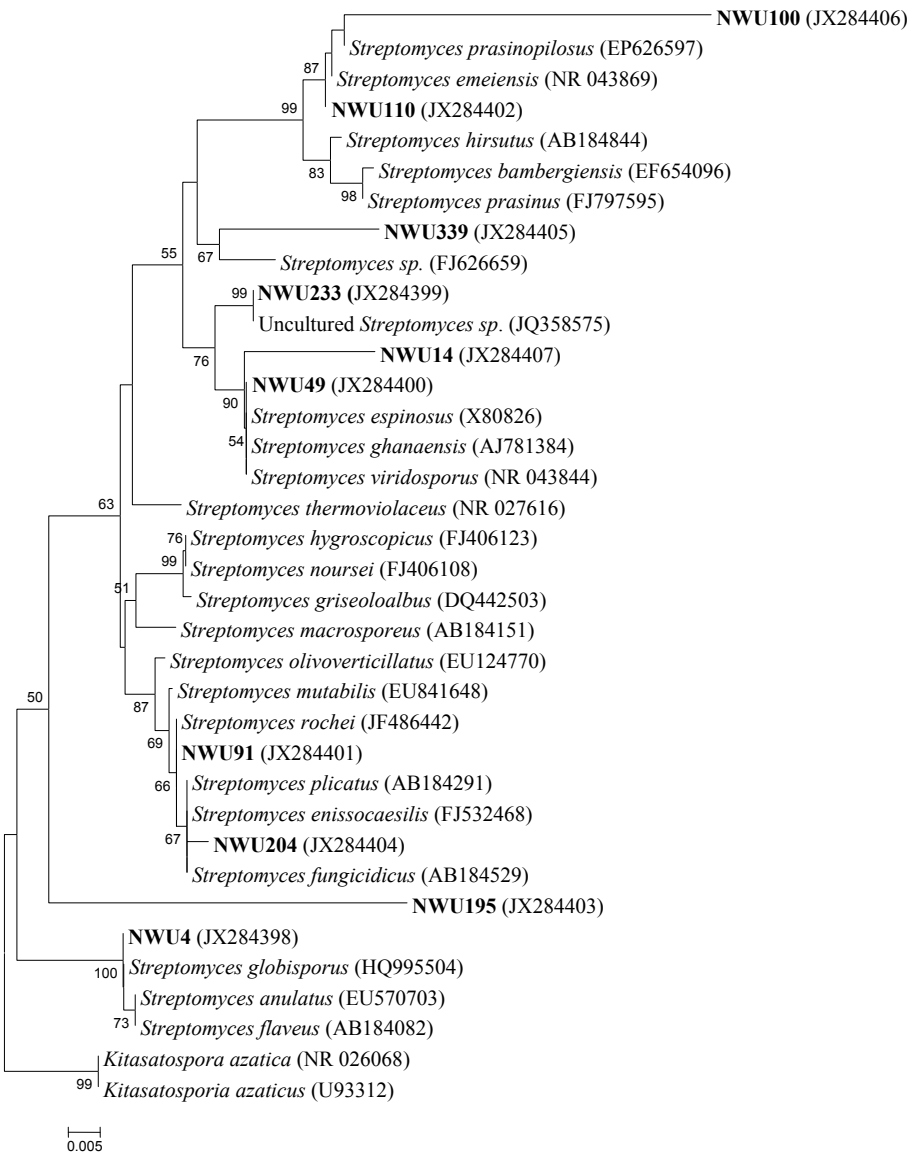

Figure 3: Neighbour-joining tree of the bacterial isolates and representative species of the genus *Streptomyces* based on partial 16S rRNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

bacterial cell wall polymer peptidoglycan-polysaccharide [48,49]. Volatiles from *S. globisporus* have been reported to act as antifungal agent against *Penicillium italicum* that cause blue mould infection on citrus [46].

A wide range of antimicrobial substances produced by *Streptomyces* spp. isolated from rhizosphere were been described, including *Streptomyces* spp. NRRL 30566 which bears 99% 16S rDNA sequence similarity to *S. griseus*. *Streptomyces* spp. NRRL 30566 was reported to produced a novel antibiotic, kakadumycins which are DNA intercalating antibiotics that act by inhibiting DNA directed enzymatic RNA synthesis [50]. A novel compound Faeriefungin (polyene macrolides), was isolated from *S. griseus*, it was reported to have antibacterial, antifungal and insecticidal activities [51]. Moenomycin A is a novel antibiotic produced by *S. ghanaensis*, is a direct inhibitor of the enzyme peptidoglycan glycosyltransferases (transglycosylases); thereby inhibiting cell wall synthesis [52].

Conclusion

Our results suggest that *Streptomyces* spp. in the rhizosphere are diverse and these strains are suitable for natural product screening. The current molecular techniques seem to be a powerful tool in the identification bacterial isolates based on the characterization of the rDNA genes. Computational analyses are effective and reliable tools use to envisage the relatedness between the bacterial isolates and those in the GenBank database towards identification of antimicrobial compounds. It can be concluded that the use of phylogenetic analysis gives a better picture of the evolutionary relationship in the species level identification.

Acknowledgements

M.F.A. gratefully acknowledges financial support through a North-West University Postgraduate Bursary. O.O.B received financial support from National Research Foundation, South Africa.

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