Phylogenetic Characterization of Culturable Antibiotic Producing \textit{Streptomyces} from Rhizospheric Soils

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Abstract

\textit{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 \textit{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 \textit{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 \textit{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 \textit{Streptomyces} spp.

Keywords: \textit{Streptomyces}; Rhizosphere; Phylogenetic; Antimicrobial; 16S rDNA

Introduction

Soil is a habitat for microorganisms and also serves as reservoir for their metabolites [1]. The genus \textit{Streptomyces} is one of prominent soil inhabitant, comprising up to 90\% of actinomycetes isolated from soil samples. \textit{Streptomyces} is the largest and the most important genus in the order actinomycetales. The genus \textit{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 \textit{Streptomyces}, over two-thirds of all microbial antimicrobial agents are derived from them [4,5]. Many novel drugs have been developed from \textit{Streptomyces} spp. including \textit{S. griseus}, \textit{S. hygroscopicus}, \textit{S. coelicolor}, \textit{S. avermitilis}, \textit{S. rochei}, \textit{S. plicatus}, \textit{S. fungidicus}, \textit{S. flavus}, and \textit{S. globisporus}; belonging to different classes of antibiotics such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polynyes, 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]. \textit{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 \textit{Streptomyces}. It has been reported that only a fraction of the antibiotics produced by \textit{Streptomyces} strains have been discovered [15]. Some \textit{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 \textit{Streptomyces} from rhizospheric soils and evaluate their evolutionary lineage 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 km² 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 \textit{Streptomyces} from Rhizospheric Soils. Mol Biol S1: 001. doi:10.4172/2168-9547.S1-001

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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 37°C for 1 h. The tubes were mixed by inversion after 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 30 min. Following incubation, 250 µl of 7.5 M NaCl, 0.7 M NaCl) was added, and the tubes were heated in a 65°C water bath for 1 h to reconstitute the DNA, for immediate use or storage at -20°C.

**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 2300K model centrifuge; HERMLE Labortecnik Germany) for 5 min. The mycelial pellet was resuspended in 1 ml of 10 mM Tris-HCl–1 mM EDTA (pH 7.5) 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'-AGAGTTTGATCCTGCTCAAG-3') and rP2 (5'-ACGGCTACCCTTACGGACTT-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 MgCl₂, 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 Streptomyces 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 Streptomyces 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

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

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

| Isolate codes | Isolate codes |
|---------------|---------------|
| NWU4          | NWU14         |
| NWU49         | NWU91         |
| NWU100        | NWU110        |
| NWU195        | NWU204        |
| NWU233        | NWU339        |

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

| 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 4347     | -    | +     | +     | +     | +      | +      | +      | +      | +      | +      |
| Salmonella typhimurium ATCC 14208 | +    | +     | +     | +     | +      | +      | +      | +      | +      | +      |

**Activity: + = Activity; - = No activity**
The order
The unearthing of microbial secondary metabolites has a great
a source of bioactive compounds of industrial importance
plants or microorganisms [1]. Microorganisms have proven to be an
process to match unending demand for bioactive substances in order
to curtail the issues of infectious diseases. Nature has been a renowned

Overall, the high-level branching in the phylogenetic trees agrees
positions, confirmed by phylogenetic analyses (Figures 3 and S1-S4).

Phylogenetic analysis also revealed that the isolate NWU195 forms a
as its closest relative. This is further supported by its taxonomic
sequences has been proved to be a powerful method for phylogenetic
classification 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.

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/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
reported that root exudates stimulates the growth of
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 actinomyctes 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 described 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 lipolytic 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
classification of microorganisms [44]. It helps to elucidate the
evolutionary relationship among microorganisms. The phylogenetic
classification of the potent bacterial isolates to known Streptomyces spp.
was first estimated through a blast search of the GenBank database.

The 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]. Mutanlysin
was isolated from S. globisporus, it is a muralytic enzyme that cleaves
the β-N-acetylglucosaminyl-(1→4)-N-acetylglucosamine linkage of the

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

| 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 viridiosporus        | NR 0438575       | 99             | 0       |
| NWU91        | 1335                | Streptomyces rochei               | JF486442         | 100            | 0       |
| NWU100       | 1363                | Streptomyces hirsutus             | AB184844         | 89             | 0       |
| NWU110       | 1295                | Streptomyces arenentes            | NR 043869        | 98             | 0       |
| NWU195       | 1397                | Streptomyces hygroscopicus        | FJ406123         | 95             | 0       |
| NWU204       | 1308                | Streptomyces fungidicus           | AB184529         | 99             | 0       |
| NWU233       | 1373                | Streptomyces espinosus            | X08028           | 98             | 0       |
| NWU339       | 1362                | Streptomyces griseus              | AB184821         | 92             | 0       |

Discussion

Screening of nature for novel antimicrobial agents is a continuous
process to match unending demand for bioactive substances in order
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

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