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Molecular and Phenotypic Characterization of Novel *Streptomyces* Species Isolated from Kurdistan Soil and its Antibacterial Activity Against Human Pathogens

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Abstract

The rise in antibiotic resistance globally has expedited the search for novel antibiotics. Streptomycetaceae are the producer of more than 70% of clinical antibiotics; researchers have been shedding light on the genus *Streptomyces* in hope of discovering novel species with the ability to produce effective and efficient molecules against superbugs. This study aims to investigate different sources of Kurdistan soil for the existence of novel *Streptomyces* species that possess bioactive compounds. So, twenty soil samples were obtained from agricultural soil, house garden soil, cave soil, and soil contaminated with petroleum. Selective media combined with morphological characterisation, biochemical tests and molecular techniques were used for species identification. Only fifty-eight bacterial samples were given a positive PCR product in which thirty-one 16S rDNA sequences were compared with previously existed prokaryotic DNA sequences using the EzTaxon database. Twenty-nine out of thirty-one samples showed >99% similarity to previously cultured *Streptomyces* spp. and two isolates from house garden soil samples were candidates to be novel species, and they have shown antibacterial activity against *E. coli* (ATCC 25218) and *Staphylococcus aureus* (ATCC 25923) by inhibiting their growth on Mueller-Hinton agar plate using cross streak method.

Keywords: *Streptomyces*, Soil, 16S rDNA, Phylogenetic tree

1. Introduction

The genus *Streptomyces* is well-known for producing plenty of bioactive specialized metabolites with advantageous applications in clinical, veterinary, and agriculture settings (Li *et al.,* 2019), such as antifungal, antibacterial, anticancer, and anthelmintic drugs (Janardhan *et al.,* 2014; Chen *et al.,* 2018).

This enormous resource of diverse compounds puts *Streptomyces* at the top of medically important microbial genera (George *et al.,* 2010). In addition, it has a number of important functions, including degradation/ decomposition of all sorts of organic substances such as cellulose, polysaccharides, protein fats, and organic acids, they have a great role in the subsequent decomposing of humus (resistant material) in soil (Anandan *et al.,* 2016). It is also responsible for the distinctive earthy odor of freshly ploughed soil caused by geosmin production (Adegboye and Babalola, 2012).

The rapid emergence of antimicrobial resistance in bacterial and fungal pathogens is a public health crisis (Chevrette *et al.,* 2019). For instance, the clinical bacterial strain methicillin-resistant *Staphylococcus aureus* (MRSA) has been designated as one of the major hazardous pathogens associated with the development of antimicrobial resistance (AMR), along with the other clinical strain vancomycin-resistant *Enterococcus faecium* (VRE) (Yücel and Yamaç, 2010; Walker *et al.,* 2019) and *Pseudomonas aeruginosa* that are resistant to even last resort antibiotics (Murray *et al.,* 2019). Novel antibacterial molecules are necessary to fight against pathogens that have advanced resistance against current antibiotics (Fatima *et al.,* 2019; Sottorff *et al.,* 2019). So, researchers are eagerly searching for a novel, sustainable, potent, and broad-spectrum antimicrobial compounds from various sources, including microbes in natural soil habitats. Many known species of *Streptomyces* with antibacterial and antifungal activities were identified in the past decade during Iraqi soil investigation for novel species of actinomycetes (Al-Hulu *et al.,* 2011; Laidi *et al.,* 2013). However, to the best of our knowledge, no one targeted Kurdistan soil to isolate *Streptomyces* spp. So, based on that and considering, *Streptomyces* as prolific producers of useful bioactive compounds (Singh *et al.,* 2016), this study was aimed to isolate and identify new species of *Streptomyces* from the soil of Kurdistan Region in Iraq then test its secondary metabolites activities toward exemplary of gram positive and negative pathogenic bacteria.

2. Materials and Methods

2.1. Sample Collection

Several diverse habitats in Kurdistan Region-Iraq (36.4103°N, 44.3872°E) were chosen to increase the chances of finding new species. Between October and November 2018 twenty samples from each of the

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following locations were collected; Jgila-Kirkuk (35.869°N, 44.552°E)/ agricultural soil, Taqtaq-Irbil (35.915°N, 44.490°E)/ house garden soil, Kanylala (35.886°N, 44.583°E)/ cave soil, and Barhushtr-Irbil (36.350°N, 43.888°E)/ petroleum soil. The samples were collected from down to 15 cm depth after discarding 3.0 cm of soil surface. Polyethylene bags were used to collect about 5gm of the soil samples then sealed and immediately transported to the laboratory. Then, the soil was kept at 4°C until the time of processing. To provide a pH condition similar to the original habitat of the taken samples, the soil's pH was measured as follows; 50 gm of soil from each location was suspended in 100 ml of distilled water and vortexed and incubated for 30 min at 20°C then filtrated using Whatman filter paper No. 1. Then the supernatant pH was measured by pH meter (HI 22 11 Ph/ ORP Meter, Italy) (Massadeh and Mahmoud., 2019).

2.1.1. Isolation of Streptomyces spp.

The collected soil samples were prepared as follows: 5 gm of soil from each sample was added to 45 ml distilled water in a 250 ml Pyrex bottle under sterile condition and incubated at room temperature for 30 min with shaking (120 rpm) (shaker incubator GFL, Germany). 1 ml from each sample supernatant was serially diluted up to 10^{-3} dilutions, then aliquot of 100 µl from each dilution was spread over International *Streptomyces* project medium No. 4 (ISP4) agar plates contain ampicillin and nystatin at a final concentration of 25 µg/ml and 50 µg/ml, respectively (Chen *et al.,* 2018). All the cultured plates were incubated at 28°C in aerobic condition for 5 days. After incubation, *Streptomyces* look alike colonies were selected according to their phenotype and subjected to pure culture technique (George *et al.,* 2010; Maleki *et al.,* 2013).

2.1.2. Cultural Purification and Spore Stocks Preparation:

A single colony from each grown plate in section 2.1.1 was streaked on ISP4 agar plates and incubated at 28°C for 5 days. Then the single isolated colonies were characterised based on the colony pigmentation and *Streptomyces* morphological appearance (Arifuzzaman *et al.,* 2010). To store the bacterial strains for long term and be used when needed, a Mannitol Soya Flour medium (MSF) was used for this purpose. Then 20% glycerol spore stocks of suspected *Streptomyces* spp. were prepared according to Kim *et al.* (2015) and stored at -80°C which can remain viable for several years even after multiple freeze-thaw cycles (Shepherd *et al.,* 2010).

2.1.3. Gram Stain and Biochemical Tests

Standard Gram stain was carried out on the bacterial isolates and visualized under a compound microscope at X100. At the same time, they were subjected to the following biochemical tests: catalase test, citrate utilization test, indole production test, and melanin production. *Streptomyces coelicolor* M15 was used as a positive control.

2.1.4. Phenotypic Characterisation

40 ml of Yeast Extract-Malt Extract medium (YEME) supplemented with 50 µg/ml ampicillin in 250 ml Pyrex bottle was inoculated with the bacterial isolate to be examined; all the inoculated bottles were incubated for 3 to 5 days with shaking at 220 rpm 20 µl from each cultured strain was spotted onto Tryptone Soya Agar (TSA). All plates were incubated for 5 days before being photographed at this point every 24 hrs for the next 120 hrs. Morphological observations through macromorphology were based on the growth pattern on the TSA medium. The colour of *Streptomyces* colonies and soluble pigment was observed by the naked eye.

2.2. Molecular identification

2.2.1. Strain Preparation for Genomic DNA Extraction

To extract genomic DNA from each bacterial isolate individually, 40 ml of YEME supplemented with 50 µg/ml ampicillin in a Pyrex bottle (250 ml volume) was inoculated with an appropriate amount of certain *Streptomyces* spores. The Pyrex bottle was fitted with glass beads for better aeration and to break up mycelium clump. All bottles were incubated at 28°C with shaking at 220 rpm for 72 hrs or up to 96 hrs for the slow growing isolate (Minas *et al.,* 2000).

After the incubation period, the 40 ml culture was transferred into a 50 ml Falcon tube, centrifuged at 13000 rpm (Cooling Centrifuge 3-30K Sigma 147101, Germany) for 10 min The cell pellet was washed with 10 ml sterilised D.W and re-harvested then subjected to DNA extraction according to Romero *et al.* (2014). The cell pellet was re-suspended in 10 ml of 1 M TE buffer (pH 8) containing 20 mg/ml lysozyme then incubated at 37°C for 1 hr. SDS and NaCl were added to final concentrations of 0.5% [w/v] and 150 mM, respectively. The tube was added to a boiling-water bath for 1 min after brief vortexing and then placed in ice to cool down. An equal volume of phenol pH 8 (buffer-saturated) was added, and the mixture was vortexed. The cell debris was separated from the cell lysate at room temperature by centrifugation at 13000 rpm for 10 min For further extraction as described for the phenol, the supernatant was moved to a new Eppendorf tube containing an equal volume of phenol (pH 8): chloroform: isoamyl alcohol (25:24:1) extraction. The extraction was then repeated using chloroform: isoamyl alcohol (49:1). The resulting aqueous phase was transferred to new tubes and a 2.5 volume of absolute ethanol was added to each. Sodium chloride was added to its final concentration (150 mM) and the tube was incubated for 30 min at -20°C. The precipitate genomic DNA was collected at 4°C by centrifugation for 30 min at 13000 rpm The harvested pellet was washed with 70% (v/v) ethanol then re-suspended in nuclease free D.W to be stored at -20°C.

2.2.2. Standard PCR Amplification of 16S rRNA Genes

Partial amplification of 16S rRNA genes was performed using Prime Taq premix PCR Master Mix (2X) kit. All amplification reactions were performed in a final volume of 40 µl of PCR reaction mixture which included 20 µl of 2X prime Taq premix, 10 pmol (2 µl) of forward (FWD) and reverse (REV) primers. StrepB (FWD) and StrepF (REV) primers were used to amplify the 16S rRNA gene partially with an end product size of 1074bp (see Table 1), 100 ng (1 µl) template DNA, and 15 µl DEPC treated D.W or nuclease-free water were added for each reaction. The PCR process was performed using BIO RAD T100TM Thermal Cycler (UK) and programmed as follows: 5 min of initial denaturation at 98°C, followed by 25 cycles of reaction with 30s of denaturing at 98°C, 30s of annealing at 59°C, 45s of extension at 72°C, and the final extension was performed for 5 min at 72°C.

Table 1. Names, target gene, sequences, size, binding site, and annealing temperature of primers used in this study.

Primer Name	Target Gene	Sequence (5' >>>>>> 3') *	Size (bp)	Position**	Tm
StrepB (FWD)	16S rRNA	ACAAGCCCTGGAAACGGGGT	1074	139–158	58
StrepF (REV)		ACGTGTGCAGCCCAAGACA		1194–1212	

* The 16S RNA gene primers sequence were taken from (Rintala *et al.,* 2001). ** Reference to the 16s rRNA genes in *Streptomyces coelicolor*.

2.2.3. Gel Electrophoresis Analysis

To confirm that a correct size of the targeted gene was amplified, an aliquot of 2 µl of PCR reaction products was electrophoresed on a 1% agarose gel containing ethidium bromide (0.5 µg/ml) along with 100bp DNA marker (Amresco DNA MW Marker 100bp) and ran in 1X TBE buffer at 85 V for 1:15 hrs. After the course of running, the DNA bands were visualized and photographed using (UV Gel Imager SynGene 1409) (Abdullah *et al.,* 2017).

2.3. Sequencing of The 16S rRNA Gene Amplicons

The resulted PCR products were sent out for sequencing, after size confirmation. The sequencing was carried out by (Macrogen Inc, a South Korean company) using StrepF for partial 16S rRNA gene sequencing.

2.3.1. Sequence Quality and Length

Sequence analysis and editing were performed using DNA Baser Assembler. In order to perform quality trimming, the start and end of the sequences were trimmed when more than 80% good bases in a 20 bases window were found. After trimming, the sample was counted as low quality when less than 90% of the bases with less than 25 quality values (QV) were detected. The sequence samples were considered good when over 90% of the bases have QV over 25. When the size of the remaining DNA segment after trimming was shorter than 600bp, the sample was discarded.

2.3.2. Novel Species Identification

To identify the isolated bacterial samples individually, the 16S rRNA genes query sequence was compared with previously existed prokaryotic DNA sequences using the EzTaxon database (Yoon *et al.,* 2017). In order to detect novel strains of *Streptomyces* the following criteria were applied: candidate for uncultured species (similarity threshold between 98.7% and 99.0%), genera (95.3-90.0%) or family (<90.0%), >99% similarity considered as same species (Stackebrandt and Ebers, 2006; Schlaberg *et al.,* 2012). For strains with no similar sequences, the data were deposited in the GenBank database using the following website "http://www.ncbi.nlm.nih.gov/BankIt".

2.4. Phylogenetic Analysis

The sequences of new *Streptomyces* spp. candidate were aligned against the sequence of all species of *Streptomyces* that came up after comparison search using Clustal W (Thompson *et al.,* 1994). The Neighbor-joining (NJ) method was used to find out the phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA-X) software (Saitou and Nei, 1987; Tamura *et al.,* 2011).

2.5. Antibacterial Activity of Secondary Metabolites

In order to screen the antibacterial activities of *Streptomyces* spp. secondary metabolites, a straight line was drawn by a loopful of certain *Streptomyces* bacterial colony from one side of Mueller-Hinton agar plate to the other side across the center (Alabi *et al.,* 2019). After seven days of incubation at 28°C, one side of the grown line was inoculated by a single streak of *E. coli* (ATCC 25218) and the other side was inoculated by *Staphylococcus aureus* (ATCC 25923) at a 90-degree angle toward the grown line of *Streptomyces* spp. then the plates were incubated at 37°C for 24 hrs. For comparison, positive and negative controls were set up using *Streptomyces coelicolor* L646 strain and *Streptomyces* spp. free Mueller-Hinton agar plate inoculated with *E. coil* and *Staphylococcus aureus*, respectively. This experiment was performed in triplicate and the antimicrobial activity was observed by the naked eye.

3. Results and Discussion

3.1. Sample Collection, Bacterial Isolation and Purification

In total, 68 bacterial entities were detected from 80 soil samples that were collected from different areas of Kurdistan Region-Iraq. The soil samples were collected specifically from agricultural and house garden soil where a great component of roots organic compounds exudate is available, which in turn can promote differential recruitment of actinomycetes (Massenssini *et al.,* 2014), cave soil with manure where the composition of manure such as carbon and other organic materials increases the rate of soil respiration and microbial activity which lead to increase the functional diversity of microbial biomass (Adebola *et al.,* 2017) and petroleum soil where petroleum inhibits and reduces species member of a microbial community; thus few of them govern the community such as *Pseudomonas* and *Streptomyces* (Xu *et al.,* 2018).

The pH of the soil was between (7.5 – 7.8) for all the locations that have been examined. As known, soil provides a suitable environment for many bacterial genus and fungi to grow, so in order to inhibit their growth ISP4 media was used which is a selective media supplemented with nystatin that inhibits fungal growth and ampicillin that suppresses the growth of a wide range of bacteria (Awad and Germoush, 2017).

In general, *Streptomyces* colonies show powdery consistency and stick firmly to the ISP4 agar surface, producing hyphae and conidia/ sporangia-like fungi (Anandan *et al.,* 2016). Colonies were relatively smooth surfaced, but later they developed a weft of aerial mycelium that appeared floccose, granular, powdery, and

velvety coloured white or grey with a white ring (Ambarwati *et al.*, 2012).

Although every grown single colony on ISP4 may be counted as *Streptomyces*, only those that expressed typical *Streptomyces* morphology have been isolated from the collected samples and subjected to pure culture technique (Hasani *et al.*, 2014). So, a colony with *Streptomyces* look alike morphology was re-streaked out on the ISP4 agar plate contains nystatin and ampicillin. After 5 days of incubation, they produced a wide variety of pigments such as white, grey with a white ring, grey, red, yellow, blue, purple, and pink, which are responsible for the colour of the vegetative and aerial mycelia (see Lane A in Figures 1, 2, 3 and 4) (Flärdh and Buttner, 2009). The spores were grown on MSF with different colour characteristics such as grey with various shades, white, dark green, pink, yellow, brown, purple, purple-red, a black centered colony with a white edge, and pale blue after five days of incubation at 28°C (see Lane B in Figures 1, 2, 3 and 4). Some strains had slow growth properties that took more than 10 days to produce spores and pigmentation; those were AS18-3, HGS5-3, HGS5-5, HGS7-2, HGS8-3, HGS13-2, HGS14-1, CS12, PS5-2, and PS13. Observed growth status appeared to be in the scope of *Streptomyces* slow growing properties with their limited resource of nutrition (Westhoff *et al.*, 2020). For future work, these strains can be compared with the reported from the literature to check if this behavior (slow growth) is common. The most dominant colours in the population of sporulation process have been assigned to two groups: grey with different shades and brown; however, unique pigments were detected as well such as green, blue and purple. *Streptomyces* spp. spore pigmentation is the result of polyketide synthesis regulated by *whi* genes that are responsible for the production of polyketide type II components such as tetracenomycin, granaticin, oxytetracycline, and actinorhodin (Kelemen *et al.*, 1998; Salerno *et al.*, 2013).

3.2. Bacterial Identification

3.2.1. Microscopic Characterisation and Biochemical Tests

Streptomyces isolates were found as long filamentous gram-positive bacteria when examined by compound microscope at X1000 magnification. According to the biochemical tests that were carried out to identify the isolated strains, all the isolates including *S. coelicolor* M145 were found to be positive for catalase and citrate utilization tests and negative for indole and melanin productions.

3.2.2. Phenotypic Characteristics

Streptomyces is well known for its ability to produce a wide range of pigments that colour the aerial spore mass and vegetative and aerial mycelia. This ability has been used to identify the genus of *Streptomyces*, and in fact, it was the only character used in many early descriptions (Al-Saadi *et al.*, 2013). So, in this study pigment production during isolation (on ISP4), sporulation, and vegetative stage were adopted as a primitive method to determine the phenotype of certain bacterial strains after being cultured on MSF agar for sporulation and TSA agar for mycelial growth. Spore's colour was determined during spore stock preparation on MSF, which was mentioned previously.

Actinomycetes produce many types of antibiotics that have pigments and can be detected in artificial media; these pigments are commonly defined in different colours (Kheiralla *et al.*, 2016). The colours and nature of the pigments are varied depending on the type of nitrogen and carbon sources present in the media (Reddy and Umamaheshwara, 2016) and are also affected by incubation temperature, aeration, and initial medium pH (Kheiralla *et al.*, 2016). In order to examine the ability of purified bacterial samples to produce secondary metabolite (SM), 20 µl of fresh bacterial culture in YEME was spotted as a patch on TSA agar in triplicate and incubated for five days at 28°C. The bacterial isolates were assigned to six colour series: white, pink, yellow, grey, pale grey, and brown. Surprisingly, a group of four bacterial isolates did not grow on TSA agar plates which can be due to the limitation of some crucial nutrients and elements or inadequate incubation temperatures or pH conditions or the growth might have been inhibited by antibacterial substances present within the medium. For future work, we will try to change these conditions and check for growth (see Lane C in Figures 1, 2, 3 and 4) (Vartoukian *et al.*, 2010).

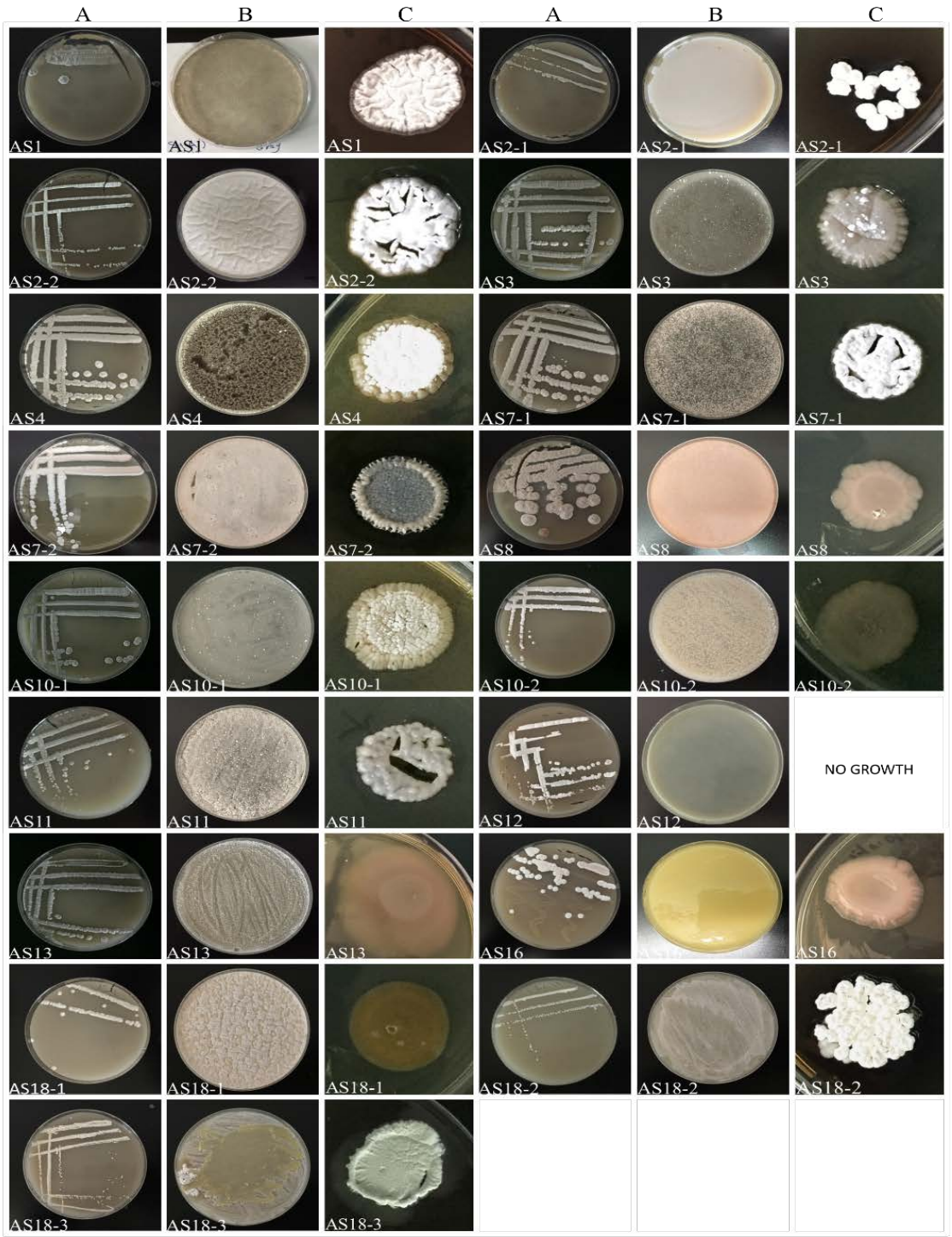

Figure 1. Colour and morphology of *Streptomyces* spp. that are isolated from agricultural soil. The names have been abbreviated to include A (agriculture) S (soil) followed by the sample number. Lane A shows a pure culture process. A single bacterial colony from the original soil sample was streaked on ISP4 agar plates. Lane B shows spore formation on MSF agar plates. Lane C shows patches of colour for each bacterial isolate on TSA plates. All the plates were incubated at 28°C for five days.

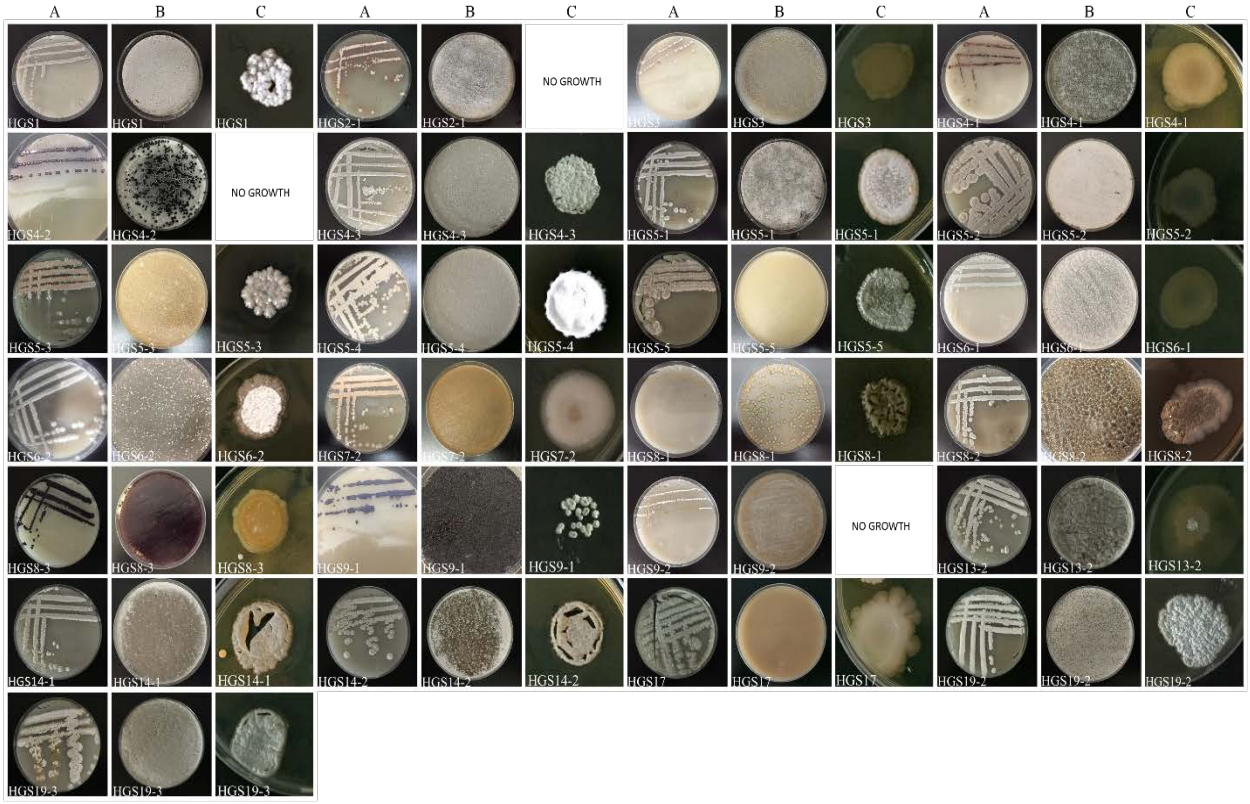

Figure 2. Colour and morphology of *Streptomyces* spp. that are isolated from house garden soil. The names have been abbreviated to include HG (house garden) S (soil) followed by the sample number. Lane A shows a pure culture process. A single bacterial colony from the original soil sample was streaked on ISP4 agar plates. Lane B shows spore formation on MSF agar plates. Lane C shows patches of colour for each bacterial isolate on TSA plates. All the plates were incubated at 28°C for five days.

Figure 3. Colour and morphology of *Streptomyces* spp. that isolated are from cave soil. The names have been abbreviated to include C (cave) S (soil) followed by the sample number. Lane A shows a pure culture process. A single bacterial colony from the original soil sample was streaked on ISP4 agar plates. Lane B shows spore formation on MSF agar plates. Lane C shows patches of colour for each bacterial isolate on TSA plates. All the plates were incubated at 28°C for five days.

Figure 4. Colour and morphology of *Streptomyces* spp. that are isolated from petroleum contaminated soil. The names have been abbreviated to include P (petroleum) S (soil) followed by the sample number. Lane A shows a pure culture process. A single bacterial colony from the original soil sample was streaked on ISP4 agar plates. Lane B shows spore formation on MSF agar plates. Lane C shows patches of colour for each bacterial isolate on TSA plates. All the plates were incubated at 28°C for five days.

3.2.3. Molecular Characteristics

The expected size of DNA fragment (1074bp) was amplified successfully from template DNA isolated from the positive control *Streptomyces coelicolor* M145 and 58 out of 68 bacterial isolates. No PCR products were detected in the negative control and the remaining 10 bacterial samples (see Figure 5).

To track down each bacterial isolate to its exact species, the 16S rRNA gene PCR amplicons of each isolate were sent out for sequencing using the reverse primer StrepF.

3.3. Partial Sequencing of 16S rRNA Gene and its Quality

Based on the validity of the DNA sequencing, just 31 samples were considered for further investigation. The samples were AS1, AS3, AS7-1, AS7-2, AS10-1, AS10-2, AS11, AS12, AS13, AS18-1, AS18-3, HGS3, HGS4-1, HGS4-3, HGS5-1, HGS5-3, HGS5-5, HGS6-1, HGS9-1, HGS14-1, HGS19-3, CS2, CS7-1, CS8-1, CS12, CS13-1, CS16-4, CS17, CS19, PS5-2, and PS13.

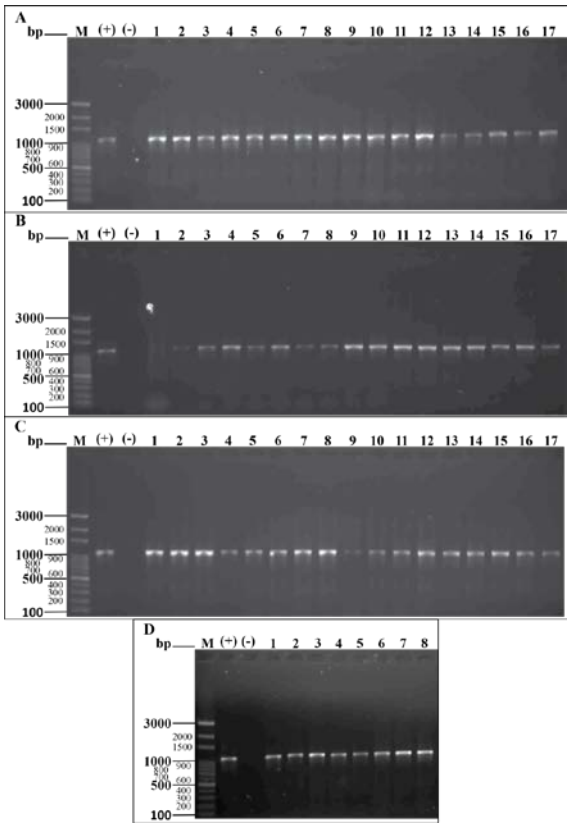

Figure 5. Genomic DNA amplification using StrepB and StrepF primer pairs that target 16S rRNA gene (1074bp). Lanes M, +V, and –V corresponds to the 100bp DNA ladder (Amresco DNA MW Marker 100bp), positive control that contained DNA template from *Streptomyces coelicolor* M145 and negative control that has been run without any DNA template, respectively. The bands are showing 1074bp of PCR amplicons. **Panel A** Lanes 1 to 17 contain PCR products from AS1, AS2-2, AS3, AS4, AS7-1, AS7-2, AS10-1, AS10-2, AS11, AS12, AS13, AS18-1, AS18-3, HGS1, HGS3, HGS4-1, and HGS4-3, respectively. **Panel B** Lanes 2 to 17 contain PCR products from HGS5-1, HGS5-2, HGS5-3, HGS5-4, HGS5-5, HGS6-1, HGS6-2, HGS7-2, HGS8-1, HGS8-2, HGS8-3, HGS9-1, HGS13-2, HGS14-1, HGS14-2, and HGS19-2, respectively. **Panel C** Lanes 1 to 17 contain PCR products from HGS19-3, CS1, CS2, CS3, CS5-1, CS5-2, CS6, CS7-1, CS8-1, CS8-2, CS9, CS11, CS12, CS13-1, CS13-2, CS14, and CS15, respectively. **Panel D** Lanes 1 to 8 contain PCR products from CS16-2, CS16-4, CS17, CS19, PS5-1, PS5-2, PS13, and PS18, respectively.

3.4. Uncultured Species

To analyse the taxonomic position of the 31 good quality 16S rDNA samples, individual automated alignment was conducted using EZBioCloud (Yoon *et al.*, 2017) against available bacterial 16S rDNA sequences. The samples were identified through sequence pairwise-similarity based on the criteria mentioned in section (2.3.2). A total of 29 samples showed >99% similarity to previously cultured *Streptomyces* spp., so no further investigation was conducted on them. The remaining two isolates from house garden soil HGS6-1 and HGS19-3 showed 98.8% and 98.9% similarity to their top hit, which were *Streptomyces nigra* and *Streptomyces albogriseolus*, respectively. This percentage of similarity and mismatches candidate HGS6-1 and HGS19-3 isolates as uncultured species because the sequence identity value of their shared gene is located between 98.7% and 99% (Stackebrandt and

Ebers, 2006). So, HGS6-1 and HGS19-3 isolates are considered to serve as novel species for which the name *Streptomyces nigra* strain BA1 and *Streptomyces albogriseolus* strain BA2 proposed, respectively.

Online multiple alignments (Huang and Miller, 1991) between *S. nigra* strain BA1 and *S. nigra* showed one gap and 7 mismatches at nucleotide positions 155, 166, 17, 193, 202, 08, 328, and 502 based on *S. nigra* 16S rDNA sequence (Data not shown). The same approach of multiple alignments was applied to *S. albogriseolus* strain BA2 and *S. albogriseolus* which showed 9 nt mismatches located at potions 185, 193, 199, 202, 249, 308, 328, 522, and 930 based on the 16S rDNA sequence of *S. albogriseolus* (Data not shown).

The sequence identity of the two proposed novel species *S. nigra* strain BA1 and *S. albogriseolus* strain BA2 fell within the specified cutoff of 98.7%–99.0%, in which *S. nigra* strain BA1 showed 98.8% similarity to *S. nigra 452* and *S. albogriseolus* strain BA2 showed 98.9% similarity to *S. albogriseolus* NRRL B-1305 based on the query sequence length during comparison against other prokaryotic species in EzBiocloud. In addition to molecular evidence above, *S. nigra* strain BA1 (HGS6-1) can be differentiated from its closely related species *S. nigra* by morphological characteristics in which it gave pale blue pigmented spores on MSF and yellow pigmented mycelia on TSA against greyish blue spores and white mycelia for *S. nigra*. On the other hand, *S. albogriseolus* strain BA2 (HGS19-3) showed pale brown spores on MSF and grey mycelia on TSA compared with its closely related species *S. albogriseolus* which gives grey spores on MSF and white mycelia on TSA. Both proposed new species were isolated from house garden soil and incubated for 5 days at 28°C during morphological characteristics.

3.5. GenBank accession number

The nucleotide sequence of *S. nigra* strain BA1 and *S. albogriseolus* strain BA2 were deposited in GenBank with accession numbers MT239403 and MT239401, respectively.

3.6. Phylogenetic tree analysis

To coin a phylogenetic tree of *Streptomyces nigra* strain BA1, 16S rRNA gene sequence was aligned with homologous of fifty different species of *Streptomyces* using multiple sequence alignment command in MEGA X software. The resulting file was used to build up a neighbor-joining tree (see Figure 6).

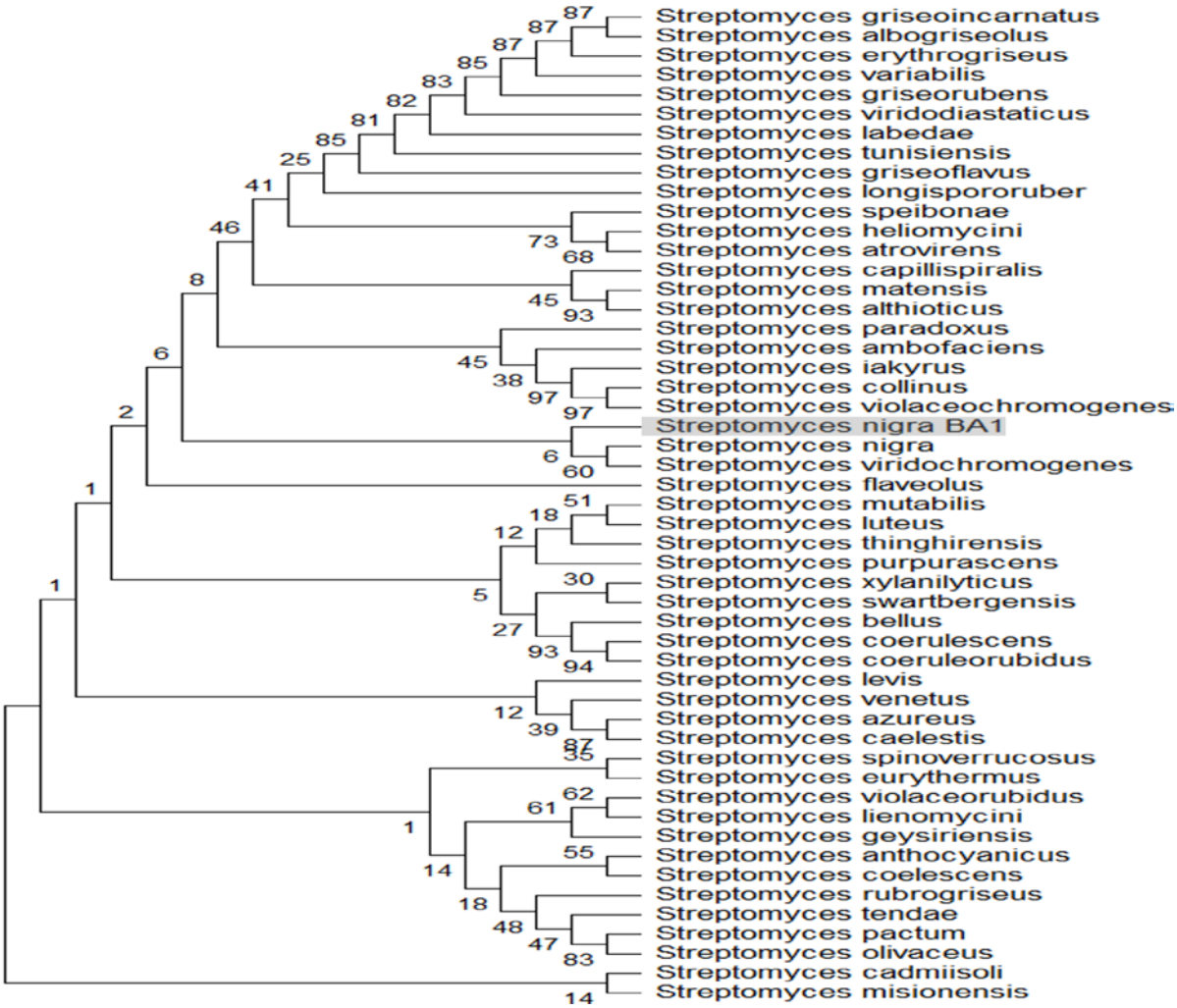

Figure 6. Phylogenetic tree represents relationships among fifty *Streptomyces* spp. and *Streptomyces nigra* strain BA1 (highlighted in grey) with based on 682bp nucleotide of 16S rRNA gene that positioned 154-836 based on *S. nigra* 16s rRNA. Numbers above nodes represent bootstrap values, the greater values give stronger support for the nodes. The proposed new species are highlighted in grey.

The partial sequence of the 16S rRNA gene of *S. nigra* strain BA1 was found to be reasonably in strong relation with *S. nigra* and *S. viridochromogenes* which was supported by 60% bootstrap replicates. These results support what has been detected from the 16S-based ID database (Yoon *et al.,* 2017), which revealed that the closest species to *S. nigra* strain BA1 is *S. nigra* in terms of nucleotide sequence similarity (98.8%) followed by *S. viridochromogenes* with 98.5% similarity. *S. nigra* strain BA1 has seven unique nucleotides at the following positions of its 16S rDNA sequence compared to *S. nigra*: 155 (A→gap), 166 (C→T), 173 (A→G), 193 (C→T), 202 (C→T), 308 (C→G), 328 (C→T) and 502 (A→C). A weaker relationship was found between *S. nigra* strain BA1 and the last thirteen species which were *S. coelescens*, *S. atrovirens*, *S. speibonae*, *S. thinghirensis*, *S. luteus*, *S. xylanilyticus*, *S. venetus*, *S. geysiriensis*, *S. heliomycini*, *S. anthocyanicus*, *S. mutabilis* and *S. capillispiralis* with 97.5% similarity to *S. nigra* strain BA1 16S rDNA sequence.

A neighbor-joining tree was carried out between *Streptomyces albogriseolus* strain BA2 and homologous of fifty different species of *Streptomyces* using MEGA X software to obtain the taxonomic position of *S. albogriseolus* strain BA2 (Figure 7).

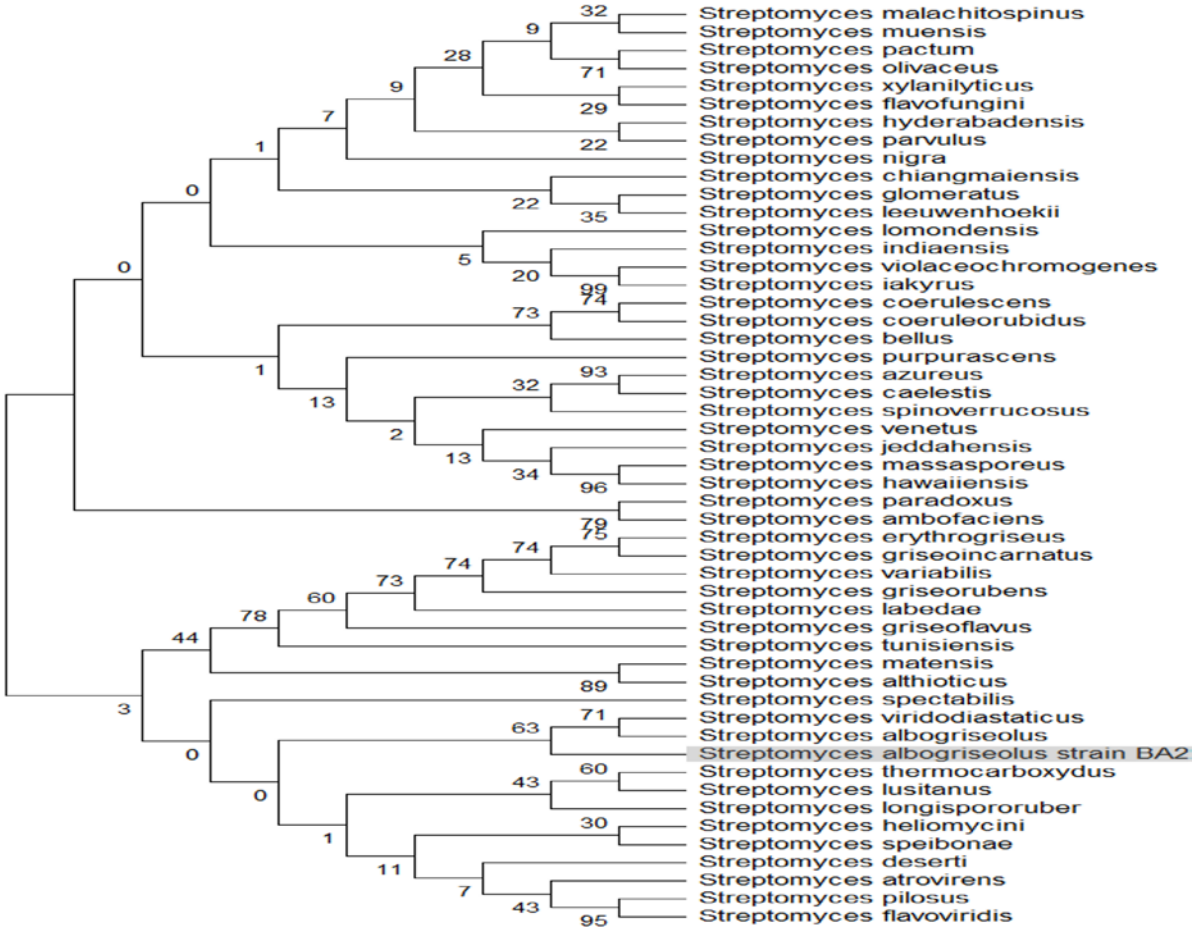

Figure 7. Phylogenetic tree shows relationships among fifty *Streptomyces* spp. and *Streptomyces albogriseolus* strain BA2 (highlighted in grey) based on 856bp nucleotide of 16S rRNA gene that positioned 167-1022 based on *S. albogriseolus* 16S rRNA. Numbers above nodes represent bootstrap values, the greater values give stronger support for the nodes.

It was found through comparing the 16S rDNA partial sequence of *S. albogriseolus* strain BA2 with its homologous that the strongest relation formed with *S. albogriseolus* and *S. viridodiastaticus* supported by 63% bootstrap. A weaker association was shaped with *S. caelestis*, *S. azureus*, *S. malachitospinus*, *S. chiangmaiensis*, *S. jeddahensis* and *S. paradoxus* with a bootstrap of 0%. These results are in line with what has been detected from the 16S-based ID database (Yoon *et al.*, 2017), which revealed that the closest species to *S. albogriseolus* strain BA2 is *S. albogriseolus* and *S. viridodiastaticus* with 98.9% similarity and less similarity (97%) was found with last six *Streptomyces* spp. that mentioned above. *S. albogriseolus* strain BA2 has nine unique nucleotides at the following positions compared to the closest species of *Streptomyces* (*S. albogriseolus*) based on the phylogenetic tree position and percentage of 16s rDNA similarity: 185 (A→C), 193 (C→T), 199 (C→T), 202 (C→T), 249 (A→T), 308 (C→G), 328 (C→T), 522 (C→T) and 930 (C→T).

3.7. Antibacterial Activity of S. nigra strain BA1 and S. albogriseolus strain BA2 Secondary Metabolites

Secondary metabolites of *S. nigra* strain BA1 and *S. albogriseolus* strain BA2 showed antimicrobial activity against *staphylococcus aureus* and *E.coli* (Figure 8, Panels C and D); however, *S. coelicolor* strain L646 showed less inhibition ability against *E.coli* (Figure 8, Panel A).

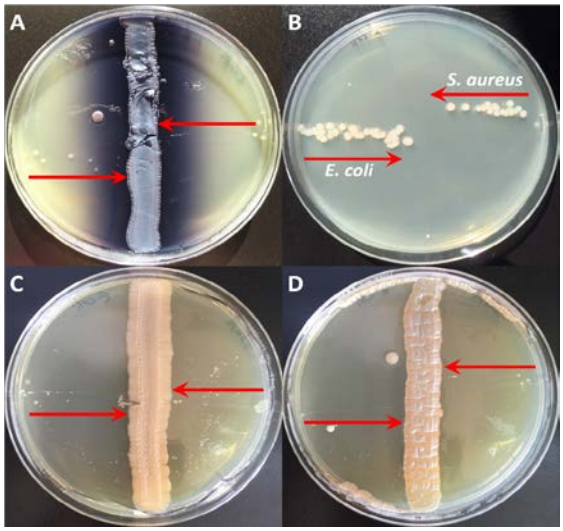

Figure 8. Antibacterial activity of *Streptomyces* spp. Panels A and B serve as positive and negative controls, respectively. *S. coelicolor* strain L646 was used in panel A and no *Streptomyces* spp. were used in panel B, it just streaked out with the tested bacteria as shown. **Panels C and D** represent the antibacterial activity of *S. nigra* strain BA1 and *S. albogriseolus* strain BA2 against *E. coli* (left-hand side) and *Staphylococcus aureus* (right-hand side), respectively. The red arrows indicate the direction of inoculated bacteria toward the midline of grown *Streptomyces* spp.

The antibacterial inhibition activities of current *Streptomyces* spp. isolates can be identified by doing mode of action studies on purified or semi-purified extracts (Imai *et al.*, 2015).

4. Conclusion

Morphological characterisation, biochemical test, partial 16S rDNA sequencing and phylogenic analysis were pronounced as two new candidate species of *Streptomyces* which were distinctive from their most closely related species. Therefore, HGS6-1 and HGS19-3 isolates are considered to serve as novel species for which the name *Streptomyces nigra* strain BA1 and *Streptomyces albogriseolus* strain BA2 proposed, respectively. Inhibition of *E. coli* and *Staphylococcus aureus* growth by secondary metabolites produced by the proposed novel *Streptomyces* species introduced strong evidence that they possess small molecules of medically important activity. Further work needs to be done on these two proposed novel species to identify their bioactive compounds then use them in the development of new therapeutic agents.

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