Genotypic Identification of Bacterial Communities in a pretreated sample of Tigris River sediment

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Abstract
Streptomyces bacteria have a complex secondary metabolism, producing over two-thirds of the natural origin antibiotics. They reside in soil and river sediment environment and interact with other microorganisms, but little is known on their effects in soil microbial community. Studies reported that many bacterial strains affect soil microbial community. In this study, a soil microbial community was screened to identify the most prominent bacteria associated with Streptomyces by using the same culturing conditions. Tigris river sediment soil samples pretreated by heating and cultured in supplemented IPS4 media with NaCl and Tetracycline and Nystatin. Out of 5 samples, three Afipia spp., and one Sphingomonas genosp were isolated and confirmed by PCR using universal 16S rDNAs primer pair and partial sequencing, in addition to Streptomyces azureus. Phylogenetic along with the sequences of closely related reference organisms showed the distance between the tested isolates. This finding showed the constituents of bacterial species in soil community under the same condition with Streptomyces, which is a promising source of biocontrol agents and how Streptomyces affect soil microbial community.
Keywords: Streptomyces, soil sample community, Afpia, Sphingomonas.

1. Introduction

A million of bacterial species found in one gram of dry weight soils usually 109 to 1010 microorganisms (Gans et al., 2005). Small number of microbes has been cultivated and provides only limited information about their potential physiological ability and influence on soil ecosystems. Many bacteria which broadly distributed in soils but poorly appeared in culture and lack of genome sequences makes it difficult to realize the roles of specific microbes in soil environments (Eichorst et al., 2007). Streptomyces is the most important bacteria in soil have benefits to humans, either by secondary metabolites which used in production of human medicine and in promoting plant growth (Khan et al., 2011).
Recently, a new study has showed that Streptomycetes are not only free-living soil bacteria, but they live in symbiosis with plants, fungi and animals (Kaltenpoth, 2009). Streptomyces species produce secondary metabolites with high chemicals diversity as a direct result of important, most common and widespread interactions with other organisms which likely played a major role in the evolution of antibiotic biosynthesis abilities (Ryan et al, 2012). The aim of this study is to understand the interactions between Streptomyces spp. and other organisms present in soil environment to exploit using these capabilities in treatment of drug-resistant pathogenic bacteria.

2. Materials and Methods

Microbial source

Tigris river sediments soil samples at Baghdad \ Iraq were collected to isolate bacteria accompanied with Streptomyces in this community.

- Culture of pretreated soil sample

As reported by Al-rubaye et al. (2018), for Streptomyces isolation and identification, a stock suspension was made by adding one gram of dried sediment soil samples (pretreated soil sample by incubation at 70°C for 2 hours) to 99 ml of sterile distilled water and shook at 120 rpm for 30 minutes at room temperature. Serial dilutions were made from 10-1 to 10-3 and left at room temperature for 10 minutes. Each sample cultured in media supplemented with NaCl and Tetracycline 50 mg/L and Nystatin 50 mg/L). About 100-200 ul of each dilution was pipetted on the plates by a sterile swab to make a uniform distribution then incubated at 28°C for 7 to 14 days.

Secondary screening for isolates

The colonies which grew nearly to the suspected Streptomyces colony were sub-cultured in supplemented media mentioned above (specific media for Streptomyces isolation) by streaking to get a single colony. Isolates processed for microscopic examination by Gram stain, DNA extraction, PCR and sequencing.

DNA Extraction

Bacterial isolates from secondary screening were sub-cultured in 30 mL of supplemented ISP4 medium for 46 h with shaking at 28°. Cells were harvested by centrifugation (5 min, 4000× g), washed [2× 10 mL of 10% (w/v) sucrose] as described by (Stolz, 1999). Genomic DNA extraction and purification carried out according to the protocol of Wizard Genomic DNA Purification Kit (promega\ USA) for Gram negative and Gram-positive bacteria using separated protocol

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depending on the isolates gram stain result. DNA transfer to a clean tube containing 600µl of room temperature isopropanol, mixed, centrifuged for 2min at 13000 rpm and supernatant was decanted. Ethanol 70% at room temperature (600µl) were added then centrifuged for 2 minutes at 13,000 rpm. The pellet aspirated and air-dried by ethanol. DNA pellet was rehydrated in 100µl of Rehydration Solution for 1 hour at 65°C.

Quantitation of DNA

Quantus Florometer (Promega, USA) was used to detect the concentration of extracted DNA. For 1 µl of DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

DNA sequencing

The primers 27F: 5' AGAGTTTGATCTTGGCTCAG 3' and 1492R: 5' TACGGTTACCTTGTTACGACTT 3' were used for identification of bacterial species as reported by Aravena et al. (2020). PCR was performed in a 25 µl mixture containing 1× PCR buffer (10 mM Tris–HC l, 1.5 mM MgCl2, 50 mM KCl [H 9]) (P romega, USA), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Promega, USA), 10 pM each of forward and reverse primers, and 100 ng of template DNA. The program for PCR included an initial denaturation 95°C for 5min, 30-40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 45s, extension at 72°C for 1min and a final extension at 72 °C for 7min. The PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide (5ng/ml) using (OWL Electrophoresis System Thermo, USA). Band bands observed using a gel documentation system (Major Science, Taiwan). PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The obtained sequence was compared for similarity with sequences present in the genomic database banks, using the "NCBI Blast" program available at the ncbi. nl.nih.gov eb site and highest matching sequences downloaded.

3. Results

The Streptomyces were observed in addition to other microorganisms as mixed colonies after culturing the pretreated diluted soil sample on ISP4 agar (Figure 1). The taxonomic identity of Streptomyces and other bacterial species was ascertained by PCR and sequencing of 16S rRNA as reported by Senthilraj et al., 2016. About, 1300bp product was recovered by PCR using universal bacterial primer pair 27f/1492r to amplify the 16r RNA gene from the total genomic DNA of bacteria isolated from soil sediment, as shown in the electrophoresis pattern (Figure 2).

PCR products (1300 bp) of four different isolates and Streptomyces isolate were sequenced. The first isolate showed 99.92 % nucleotide identity with 16S rDNA of Sphingomonas aquatilis as represented in Figure (3a, b).

The other isolates which processed for sequencing, showed that 2 of them with the same clone with 100% 16S rDNAs identity to Afipia genosp (Fig 4, 5) and one was different clone with 99.95% Afipia genosp (Figure 6), while the Streptomyces isolate showed 95.74% identity to S. azureus (Figure 7). The partial sequence 16S rRNA gene for suspected Streptomyces in the culture showed 95.74% identity to Streptomyces azureus strain ATCC 14921 (Figure 7).

The 1300 bp sequences of the 16S rRNA were subjected to phylogenetic tree analysis using mega X (Figure 8), the isolates found into 2 groups. The phylographic grouping of isolates (1, 2 and 3) were strongly associated to the reference strain, while strain 4 is grouped with another cluster. The strains (1, 2, 3) accumulate cluster A, showed more genetic variation than cluster B.

4. Discussion

Suspected Streptomyces colonies were selected in accordance to their color (white to grey small colonies), colony diameter ranged from 1-10 mm and morphology showed smooth surface at the beginning, then became white to grey small, powdery, soft and granular by forming the aerial mycelium, the same results were reported by Al-rubaye et al. (2018). Other bacterial single colonies which grown on the same plates within Streptomyces, were selected for second screening by sub-culturing under the same condition to obtain pure culture. Pure Streptomyces isolates showed Gram positive characteristics while other isolates represented four different colonies with Gram negative characteristics by Gram staining. The single colony of clearly observed other than suspected Streptomyces isolate found within the culture may be due to presences of their spores in the soil or they were not killed by heating (Al-rubaye et al., 2018).

The taxonomic identity of Streptomyces and other bacterial species was ascertained by PCR and sequencing of 16S rRNA as reported by Senthilraj et al., 2016. About, 1300bp product was recovered by PCR using universal bacterial primer pair 27f/1492r to amplify the 16r RNA gene from the total genomic DNA of bacteria isolated from soil sediment, as shown in the electrophoresis pattern (Figure 2).

PCR and DNA sequence partially encompassing the 16S rRNA gene in species detection was also used by Al-Rubaye (2016), while Rosselli, et al., (2016) used direct sequencing of 16S rRNA without any primer- or PCR-dependent step.

The first isolate showed 99.92 % nucleotide identity with 16S rDNA of Sphingomonas aquatilis as represented in Figure (3a, b). The genus Sphingomonas isolate present as typical yellow colonies on the ISP4 medium at first screening during Streptomyces isolation. The presence of the Sphingomonas isolate within Streptomyces isolates may be due to streptomycin resistance which

produced from Streptomyces spp. and enhance the selection for these bacteria as reported by Leys, et al. (2004). Bacterial adaptation to various environmental conditions among soil community, explain the bacterial growth with the same temperature suitable for the growth of Streptomyces. Regarding their growth in a tetracycline (hydrophilic antibiotic) supplemented media, may be due to pumping tetracycline out of the cell (efflux), before it reaches the site of action (ribosomal binding site) by changing the permeability of the cell envelope or decreasing drug binding resulting in decrease of drug uptake, as shown by Tenover and McGowan, (2008). One of tetracycline resistance determinant is the tet (M) gene, which mediates resistance to many drugs under tetracyclin class like tetracycline doxycycline and minocycline as reported and explained by Chopra and Roberts (2001). The Sphingomonas has a cell surface more hydrophobic due to short carbohydrate moiety of glycosphingolipids than that of LPS of other Gram-negative bacteria which gives the ability to degrade hydrophobic polycyclic aromatic hydrocarbons and accounts antibiotics sensitivity to hydrophobic antibiotics (Balkwill et al., 2003). The other isolates which processed for sequencing, showed that 2 of them with the same clone with 100% 16S rDNAs identity to Afipia genosp (Fig 4, 5) and one was different clone with 99.95% Afipia genosp (Fig 6). Afipia is a genus of bacteria from the family of Nitrobacteraceae. It has been found in the atmosphere derive their energy from oxidizing ammonia to nitrite, or by oxidizing nitrite to nitrate. They are commonly found in freshwater and soil (Natasha et al., 2012), and the species Afipia felis is an important animal-associated bacteria, formerly thought to cause cat-scratch disease (Defeng, et al., 2008; Euzéby and Parte, 2021). This bacteria isolated from the lymph nodes of a cat-scratch patient in 1988 (Giladi et al., 1998). Also, some species of the sphingomonads play a role in human disease by causing an emerging opportunistic bacterial infection especially in bones and soft tissues either in immunocompetent or immunosuppressed individuals in the community or hospitals (El Beaino et al., 2012). Therefore, the relation between the presences of these bacteria in the environment and their role in causing human diseases makes it an important topic in this study. It recommend further studies about the relation considering their growth in the same bacterial community under the same condition, secondary metabolites evaluation which is very important in biological control and industrial and drug production and finally whole genomic sequence also important to evaluate the lifestyle genes of Iraqi isolates in the same community.

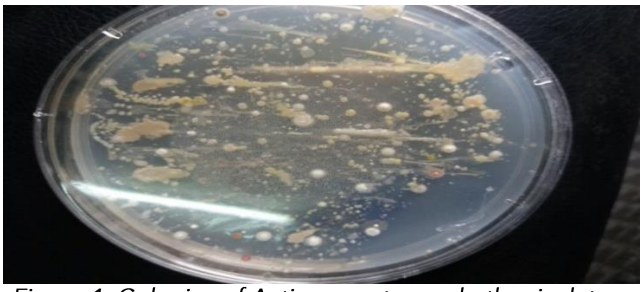


Figure 1: Colonies of Actinomycetes and other isolates from pretreated soil samples (dilution 10 -2) cultured in casein salt starch agar at 28°C for 10-14 days.

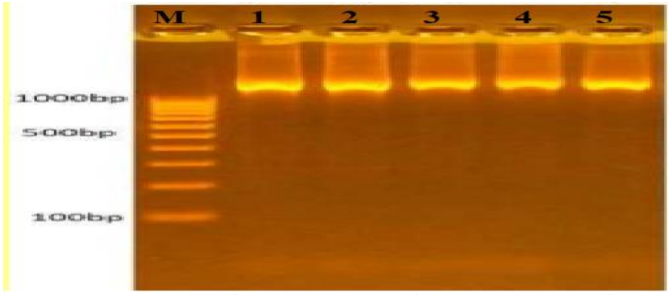


Figure 2: Results of the presence of 16s RNA gene of unknown bacterial species were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. Lane M:100bp DNA marker. Lane1,2,3,4 and 5: Bacterial isolates number.

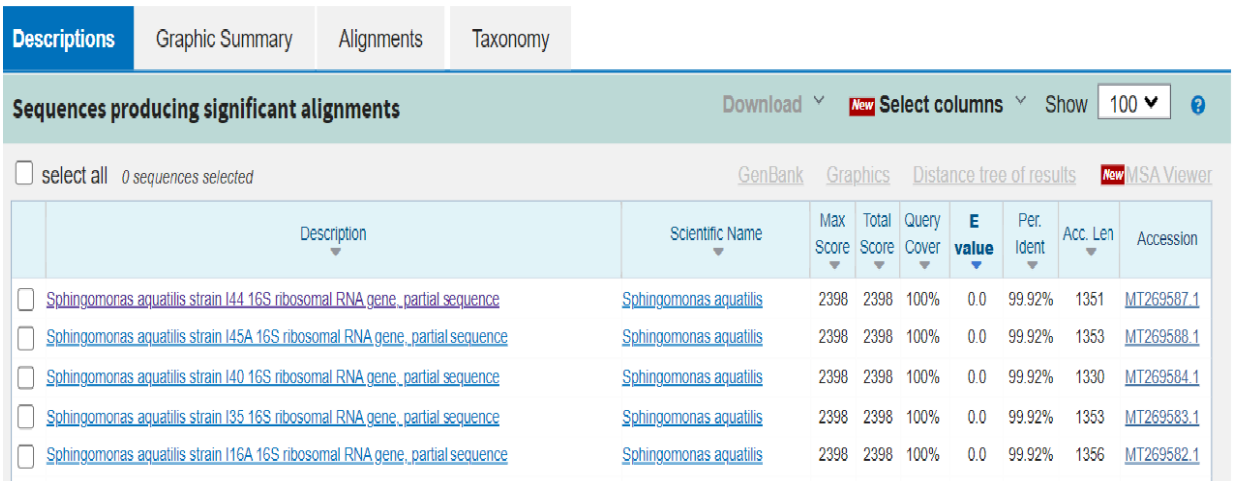
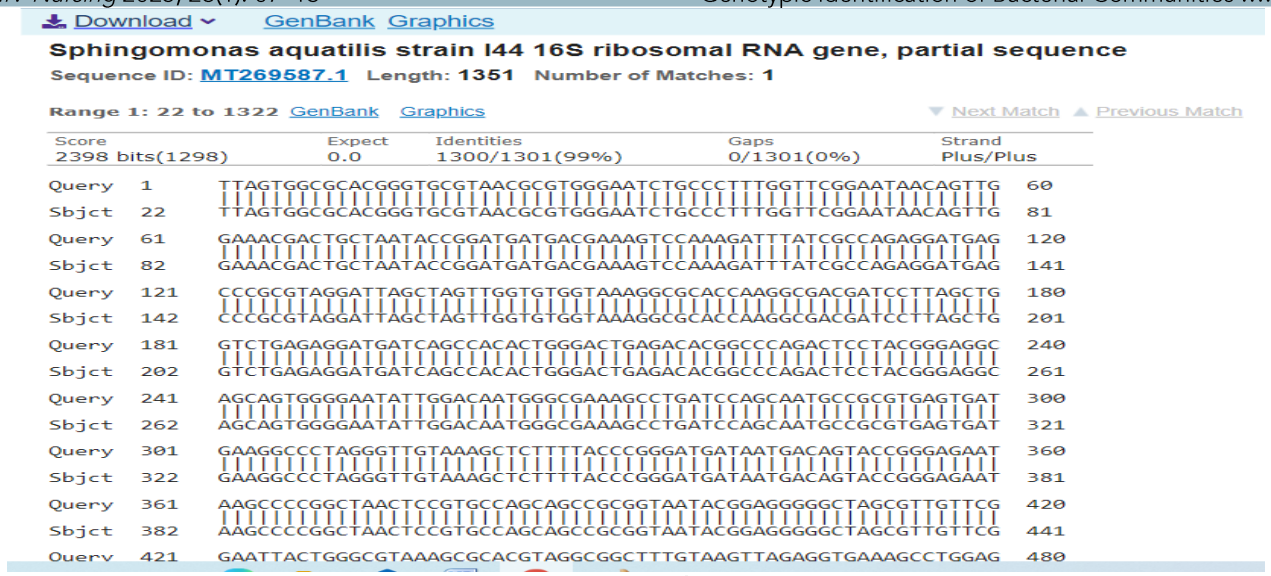


Figure 3 (a): Blast result for top sequence producing significant alignments, Sphingomonas aquatilis strain I44 16S rRNA gene, partial sequence 16S rRNA gene, (Sample 1).



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Sphingomonas aquatilis strain I44 16S ribosomal RNA gene, partial sequence

Sequence ID: MT269587.1 Length: 1351 Number of Matches: 1

Range 1: 22 to 1322 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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Sbjct  82   GAAACGACTGCTAATACCGGATGATGACGAAAGTCCAAAGATTTATCGCCAGAGGATGAG  141
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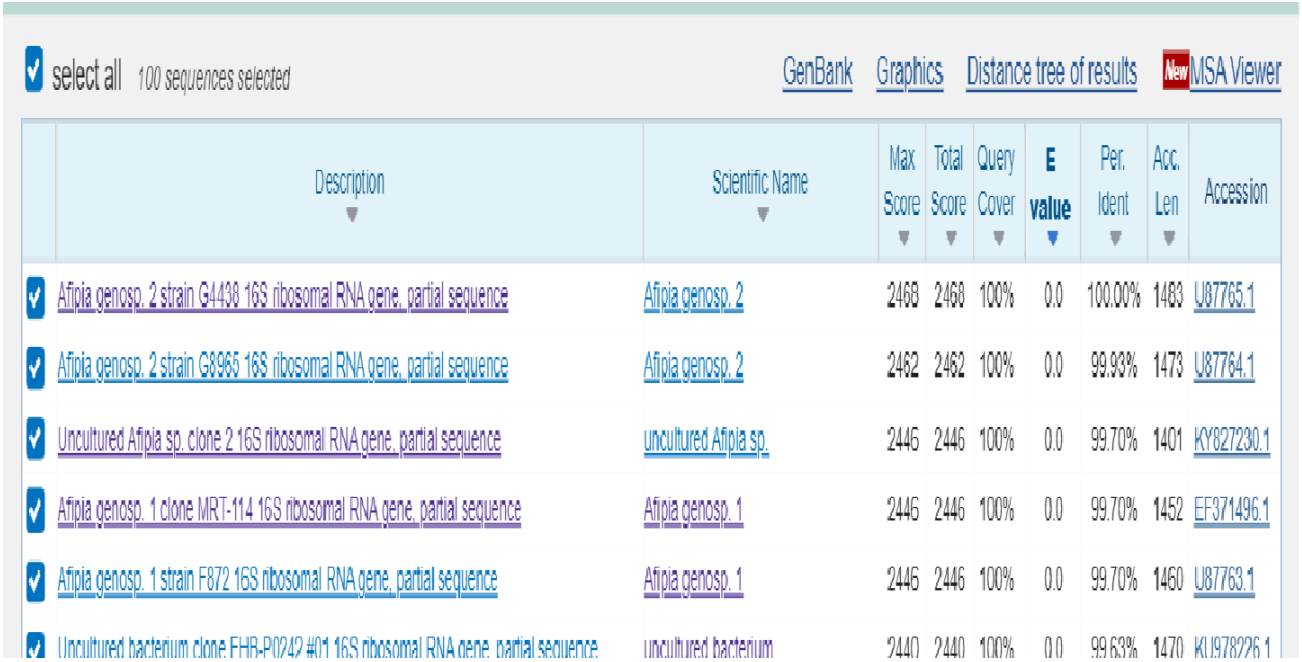
Figure 3 (b): partial sequence 16S ribosomal RNA gene of Sphingomonas aquatilis strain I44, Sequence ID: MT269587.1, Length: 1351. (Sample 1).



select all 100 sequences selected GenBank Graphics Distance tree of results New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Afipia genosp. 2 strain G4438 16S ribosomal RNA gene, partial sequence	Afipia genosp. 2	2468	2468	100%	0.0	100.00%	1483	U87765.1
Afipia genosp. 2 strain G8965 16S ribosomal RNA gene, partial sequence	Afipia genosp. 2	2462	2462	100%	0.0	99.93%	1473	U87764.1
Uncultured Afipia sp. clone 2 16S ribosomal RNA gene, partial sequence	uncultured Afipia sp.	2446	2446	100%	0.0	99.70%	1401	KY827230.1
Afipia genosp. 1 clone MRT-114 16S ribosomal RNA gene, partial sequence	Afipia genosp. 1	2446	2446	100%	0.0	99.70%	1452	EF371496.1
Afipia genosp. 1 strain F872 16S ribosomal RNA gene, partial sequence	Afipia genosp. 1	2446	2446	100%	0.0	99.70%	1460	U87763.1
Uncultured bacterium clone FHR-P0242 #01 16S ribosomal RNA gene, partial sequence	uncultured bacterium	2440	2440	100%	0.0	99.63%	1470	KU978726.1

Figure 4 (a): Blast result for top sequence producing significant alignments, Afpia genosp, 2strain G4438 16S rRNA gene, partial sequence 16S rRNA gene, (Sample 2).

Range 1: 60 to 1395 GenBank Graphics Next Match Previous Match

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Figure 4 (b): partial sequence 16S rRNA gene of Afipia genosp.2 strain G4438, Sequence ID: U87765.1, Length: 1483, (Sample 2).

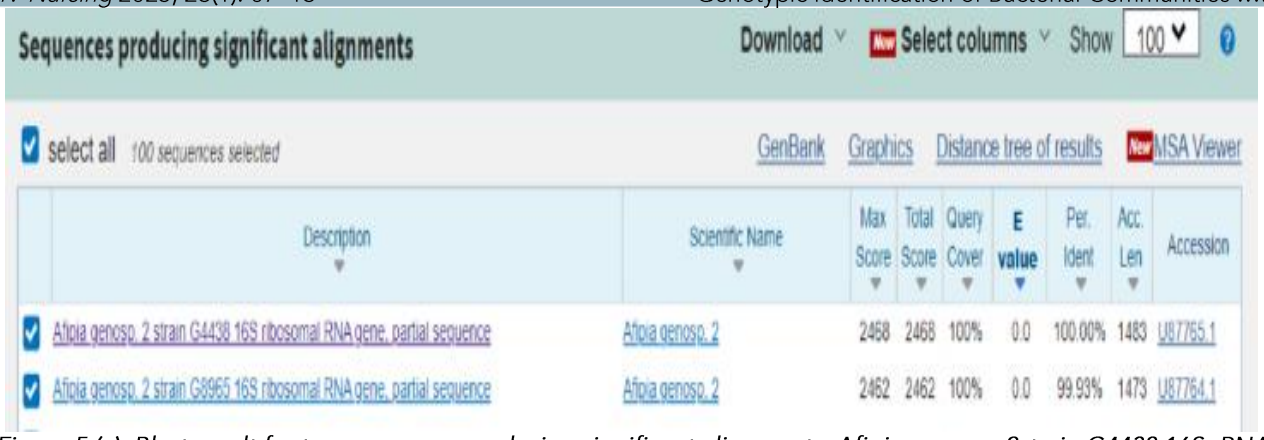

Figure 5 (a): Blast result for top sequence producing significant alignments, Afipia genosp, 2strain G4438 16S rRNA gene, partial sequence (Sample 3).

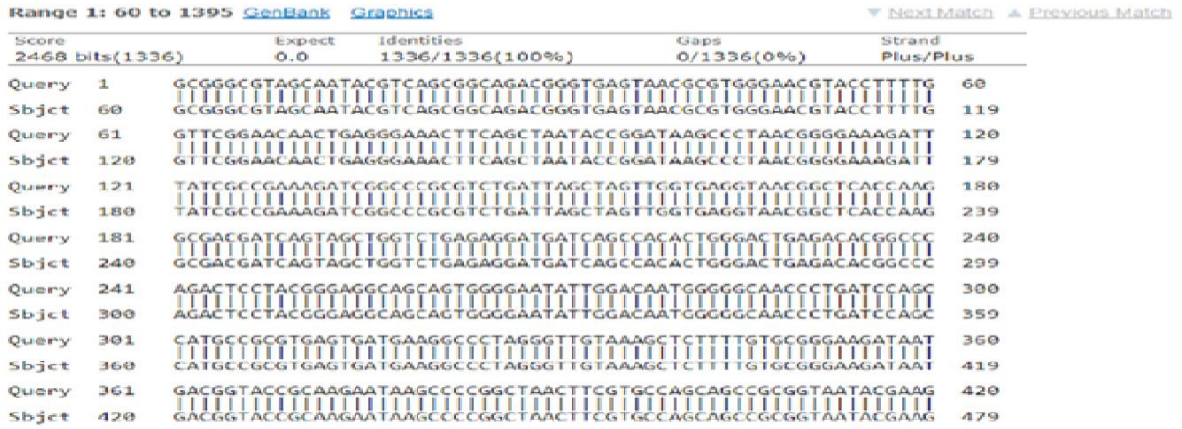

Figure 5 (b): partial sequence 16S rRNA gene of Afipia genosp.2 strain G4438, Sequence ID: U87765.1 Length: 1483, (Sample 3).

Figure 6 (a): Blast result for top sequence producing significant alignments, Afipia genosp.1 clone MRT-114, 16S rRNA gene, partial sequence, (Sample 4).

Figure 6 (b): partial sequence 16S rRNA gene of Afipia genosp.1 clone MRT-114, Sequence ID: EF371496.1, Length: 1452.

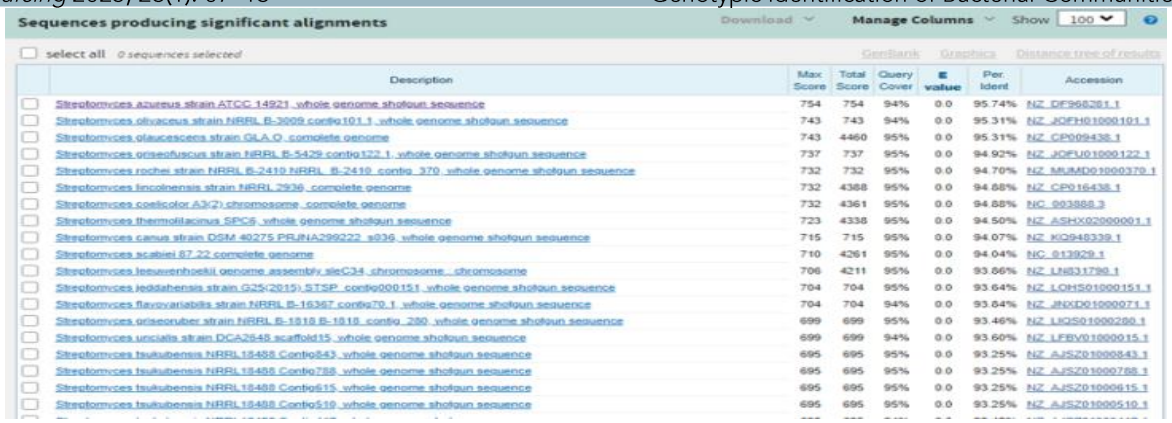

Figure (7): partial sequence 16S rRNA gene of Streptomyces azureus strain ATCC 14921.

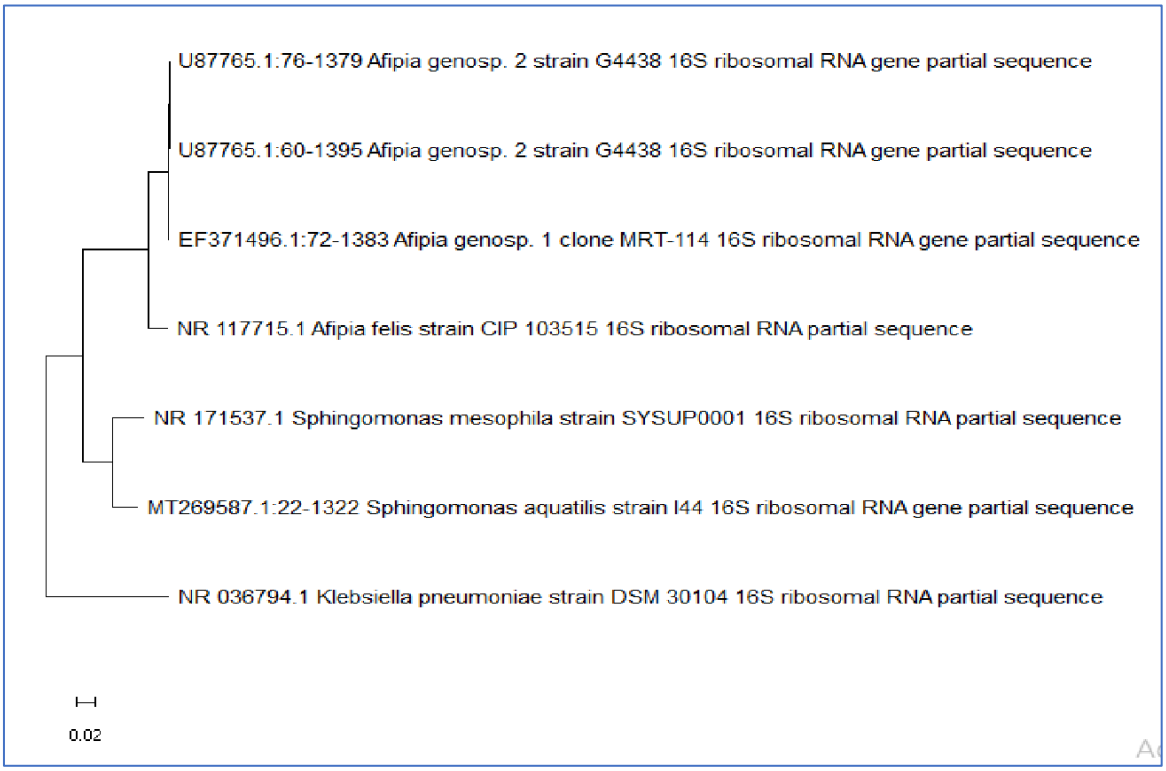

Figure (8): Neighbor – joining phylogenetic analysis based on the sequences of universal 16S rRNA. Isolates of Iraq and sequences of strains reported from different parts of the world available in public database GenBank (https://www. ncbi.nlm.nih.gov/genbank/).

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