Diversity and antimicrobial activity of culturable actinobacteria isolated from the sediment of Sarıkum Lake

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ABSTRACT

In this study, actinobacteria isolated from Sarıkum Lake sediment were characterized (Sinop-Turkey). A total of 48 actinobacteria were isolated using ten selective media with the dilution-plating method. The phylogenetic analysis according to 16S rRNA gene sequencing showed that 40% of the isolated strains belong to the widely distributed genus of *Streptomyces*, 36% belong to genus of *Micromonospora*, 24% of the isolates belong to rare genera such as *Rhodococcus, Plantactinospora, Nonomuraea, Actinomadura* and *Streptosporangium*. Most of the isolated strains belong to the genus *Streptomyces* (40%) and two isolates may be new species. All of the isolates were tested for antimicrobial activity; only 12 isolates exhibited antimicrobial activity. Nevertheless, 11 isolates were active against gram-positive, 5 were potential against gram-negative and no isolates had any effect against pathogenic fungi. All of the 48 isolates were analysed for genes encoding nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). In all of the 48 isolates were detected NRPS sequences, PKS-I in 28 isolates and PKS-II in 22 isolates. PKS-I – PKS-II – NRPS genes were identified in 17 isolates.

Introduction

Actinobacteria are Gram-stain positive, filamentous, unicellular microorganisms and compose one of the greatest phyla within the kingdom Bacteria [1, 2]. These bacteria belong to the order actinomycetales and represent a group of gram-positive bacteria with high GC base pairs in their DNA [3, 4].

The first studies on actinobacteria from freshwater habitats were carried out in 1971. Willoughby [5] isolated *Micromonospora* and *Streptomyces* from the river and stream of Blelham Tarn. Subsequently, six different genera of actinobacteria (*Actinomadura, Microbispora, Micromonospora, Nocardia, Saccharopolyspora, Streptomyces*) were obtained from lake water and sediment of Middle Plateau, Yunnan, China [6].

Actinobacteria can be found in both terrestrial and aquatic habitats, and they are one of the most abundant taxa in freshwaters [7]. A number of antimicrobial compounds such as aminoglycosides, anthracyclines, beta-lactams, glycopeptides, macrolides, polyenes, phenazine and tetracyclines have already been isolated and characterized from actinobacteria [8]. However, most of these compounds are either nonribosomal peptides or polyketides which are synthesized by nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) respectively [4, 9].

The 16S rRNA gene is the most widely used marker for molecular identification and phylogenetic analysis of actinobacteria.

Sarıkum Lake is a natural water ecosystem. In 1991, Sarıkum Lake and its surroundings were registered as a Nature Conservation Area covering a 785-hectare complex of a marine, freshwater lake, sand, wetland, peatland, swamp and forest ecosystem. This complex ecosystem has contributed to the biodiversity of Sarıkum Lake [10]. Besides, riparian vegetation grows well around the lake. The bottom structure is composed of clay. It is close to the sea and it connects to the sea with a natural water channel. This situation makes the lake an appropriate habitat for birds and other aquatic organisms [11, 12].

To our knowledge, the biodiversity of actinobacteria of Sarıkum Lake (Sinop-Turkey) has not been studied so far. The aim of the present study was to detect the
biodiversity of actinobacteria of Sarıkum Lake (Sinop-Turkey) and the presence of the secondary metabolite genes in the isolates. Also, the antimicrobial potential of the isolates was examined against eight different pathogens.

Materials and methods

Collection of lake sediment sample

Sediment samples were collected from the three different stations of the Sarıkum Lake, Sinop, in July 2019 (Table 1). Sediment samples were randomly collected from three different stations of the lake. The labeled samples were placed in sterile tubes (5–10g), transported to the laboratory and were worked on immediately for the isolation of actinobacteria.

Selective isolation of actinobacterial strains

The collected samples were exposed to a preheated water bath (55°C for 20 min) to hinder the growth of fastgrowing bacteria and support the growth of actinobacteria [13]. Actinobacteria were isolated using the serial dilution method and the spread plate technique. The stock solution of the sample was prepared with 1 mL of sediment with lake water and 9 mL of Ringer’s solution in a test tube, and the solution was mixed for 40 min. The suspension was serially diluted by transferring 1 mL aliquots to a series of test tubes; each containing 9 mL of Ringer’s solution to prepare the final volumes of 10⁻¹, 10⁻² and 10⁻³, and the diluted suspension was spread over the surface of selective isolation agar. The most common media used for actinomycetes include humic acid-vitamin agar (HV), starch casein agar (SCA), nocardia agar (NA), SM1 agar, SM2 agar, SM3 agar, marine agar, R2A agar, M1 agar and oligotrophic agar. Certain concentrations of antibiotics (see Table 2) were added to selective isolation media to eliminate gram-negative bacteria and fungi. Diluted sediment samples (100 µL) were spread onto the surface of 10 different selective isolation media (Table 2). Isolation plates were incubated at 28°C for 30 days, and the colonies were observed periodically.

Maintenance and culture conditions

Bacterial colonies resembling the morphology of actinobacteria colonies were selected and inoculated on prepared yeast extract malt extract medium [14]. The inoculated plates were incubated for 15–21 days at 28°C. Suspensions of spores and mycelia were maintained on ISP 2 agar slopes at room temperature and as glycerol suspensions (20%, v/v) at −80°C.

DNA extraction

For molecular identification and phylogenetic analysis, genomic DNA was extracted and purified using a DNA extraction kit (Purelink Invitrogen) as described in past studies [15]. The DNA quantity was checked by the ratio of optical density 260/280 using a spectrophotometer (Thermo scientific Multiskan GO microplate reader).

Amplification and determination of 16S rRNA gene sequence

PCR was carried out on T100 (Bio-RAD) in a 25 µL volume. PCR mixtures (25 µL) included 0.5 µmol/L F (20 µmol/L), 0.5 µmol/L R (20 µM), Taq polymerase buffer (HotStarTaq®) and deoxynucleoside triphosphates mixture (Promega) (12.5 µmol/L). Taq polymerase (2.5 U, HotStarTaq®) and chromosomal DNA (50–300 ng) were added to the solution. The 16S rRNA genes were amplified using the universal primers 27F [5′-AGAGTTTGATC(AC)TGGCTCAG-3′] and 1492R [5′-ACGG(CT)TACCTTGTTACGACTT-3′] [16]. The PCR amplification (MyGenie-96 Gradient Thermal Cycler, Korea) included the following parameters: 95°C for 5 min (initial denaturation), 35 cycles of 95°C for 1 min (denaturation), 55°C for 2 min (annealing), and 72°C for 3 min (extension) and 72°C for 10 min (final extension). Then the amplification products were visualized by gel electrophoresis using 4 µL of PCR product in a 1% agarose gel (Merck) and were imaged with the Gene Genius Bioimaging system.

Sequence analysis

The PCR products of the 48 isolates were purified with QIAquick purification kit (Qiagen). PCR-mediated amplification and sequencing of the 16S rRNA gene were performed as described by Chun and Goodfellow [17].

Table 1. Locality and geographic coordinates of sediment samples.

| Locality             | Geographic coordinates |
|----------------------|------------------------|
| Mid-lake coastal area| 42° 01′ 00″ N          |
|                      | 34° 55′ 38″ E          |
| Sea front of the lake| 42° 01′ 25″ N          |
|                      | 34° 54′ 31″ E          |
| Lake coastline       | 42° 01′ 34″ N          |
|                      | 34° 54′ 34″ E          |
using an ABI PRISM 3730 XL automatic sequencer with the previously described oligonucleotide primers (Table 3). Chromatogram files in ABI format were converted to FASTA format using Chromas 1.7.5. An almost complete 16S rRNA gene sequences of the 48 isolates were compared to sequences of type strains in GenBank [18] and e zBioCloud [19] databases. Evolutionary trees were carried out using the neighbour-joining [20] algorithm drawn from MeGA version 7.0 software package [21]. Evolutionary distances were calculated using the Kimura two-parameter [22] and topologies of the resultant trees evaluated by bootstrap analyses [23] based on 1000 resamplings. The 16S rRNA gene sequences obtained in this study were deposited in GenBank (Table 4).

**Antimicrobial activity**

The antimicrobial activities of the 48 isolates to inhibit the growth of eight different pathogenic microorganisms (gram-positive bacteria: *Bacillus subtilis* ATCC 6633^T^, *Enterococcus faecalis* ATCC 29212^T^ and *Staphylococcus aureus* ATCC 25923^T^; gram-negative bacteria: *Escherichia coli* ATCC 25922^T^, *Klebsiella pneumoniae* ATCC 700603^T^ and *Pseudomonas aeruginosa* ATCC 27853^T^ and fungi: *Aspergillus niger* ATCC 16404^T^ and *Candida albicans* ATCC 10231^T^), was observed using an overlay technique described by Williams et al. [24]. Spot-inoculated colonies on modified Bennett’s agar plates were inverted over 2 mL chloroform for 40 min. Killed colonies were overlaid with 5–7 mL sloppy nutrient broth inoculated with the test organisms. Zones of inhibition were scored as positive results after 24 h at 37 °C.

**Amplification of NRPS, PKS I and II gene regions**

Non-ribosomal peptide synthetase (NRPS), polyketide synthase I and II gene regions of isolates were investigated with specific primers (Table 4). The amplified PCR products were observed in 1.5% agarose gel via

### Table 2. List of selective media used and codes of isolates.

| Number | Isolate codes | Name of medium | Antibiotic | References |
|--------|---------------|----------------|------------|------------|
| 1      | SL05, SL19, SL20, SL21, SL51, SL52, SL53, SL78, SL80, SL84, SL92, SL93 | Humic Acid-Vitamin Agar | Cycloheximide (50µg/mL), Nalidixic acid (10µg/mL) | [35] |
| 2      | SL30, SL31, SL32, SL34, SL41, SL42, SL44, SL54, SL62, SL65, SL66, SL86, SL87, SL88, SL97, SL109 | Starch-Casein Agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL) | [36] |
| 3      | SL13, SL27, SL49, SL98, SL100, SL101, SL102, SL103 | Nocardia Agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL) | [37] |
| 4      | – | SM1 Agar | Nystatin (50µg/mL), Nalidixic acid (10µg/mL), Neomycin sulfate (10µg/mL) | [38] |
| 5      | – | SM2 Agar | Nystatin (50µg/mL), Nalidixic acid (10µg/mL), Neomycin sulfate (10µg/mL) | [38] |
| 6      | – | SM3 Agar | Nystatin (50µg/mL), Rifampicin (5µg/mL) | [38] |
| 7      | SL35, SL48, SL59, SL77, SL105, SL108 | Marine agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL) | [39] |
| 8      | – | R2A Agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL) | [40] |
| 9      | SL67, SL68 | M1 Agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL), Rifampicin (5µg/mL) | [41] |
| 10     | SL37, SL38, SL39, SL64 | Oligotrophic Agar | Cycloheximide (50µg/mL), Nystatin (50µg/mL), Rifampicin (5µg/mL) | [42] |

**Table 3. Oligonucleotide primers used for 16S rDNA PCR amplification and sequencing.**

| Primer code | Sequences (5′–3′) | Base length | References |
|-------------|-------------------|-------------|------------|
| 27F         | AGAGTTTGATCACAC   | 21          | [16]       |
|             | TGGCCTACG         |             |            |
| S18F        | CCAGGACCCGGCCGAAT | 17          | [42]       |
| 800R        | TACCGAGGTATCTAATCC| 18          | [17]       |
| MGSF        | AAACCTAAAGGAAATTGACGG | 20        | [17]       |
| MG6F        | GATCAGTGATCGATGCCC | 19        | [17]       |
| 1492R       | ACGGCCT         | 21          | [16]       |
|             | TACCGTTGACGACTT   |             |            |
electrophoresis. Ethidium bromide (EtBr) was added to the gel before electrophoresis to a final concentration of 0.5 μg/mL, followed by separation at 100 V for 1 h.

Results

Morphological analysis

A total of 48 morphologically distinct actinobacterial isolates were obtained from sediment Sarıkum lake. Ten different selective isolation media were used. Sixteen strains were isolated on starch-casein agar, twelve strains from humic acid-vitamin (HV) agar, eight strains from nocardia agar, six strains from marine agar, four strains from oligotrophic agar, two strains from M1 agar and incubated at 28 °C for about 30 day. No improvement was observed on the other four types of agar (SM1, SM2, SM3 ve R2A agar).

These results clearly showed that starch-casein agar was the most suitable medium for the isolation of actinobacteria from lake sediments and provided 33% of the total isolates followed by humic acid-vitamin (HV) agar (25%) (Table 2). In total, 48 culturable actinobacterial isolates were isolated from the three different stations of the Sarikum Lake: 20 isolates from the first locality, 21 isolates from the second locality and 7 isolates from the third locality.

16s rRNA gene sequence analysis

The 16S rRNA genes of all 48 isolates were amplified using universal primers (Table 3). Most of the strains belonged to the genus Streptomyces (19 isolates) and to the genus Micromonospora (17 isolates). Other strains belonged to the genera Rhodococcus (5 isolates), Plantactinospora (3 isolates), Nonomuraea (2 isolates), Actinomadura (1 isolate) and Streptosporangium (1 isolate) (Table 5).

Antimicrobial potential

All 48 isolates were tested for antimicrobial activities against the pathogenic microorganisms Bacillus subtilis ATCC 6633T, Enterococcus faecalis ATCC 29212T, Staphylococcus aureus ATCC 25923T, Escherichia coli ATCC 25922T, Klebsiella pneumoniae ATCC 700603T, Pseudomonas aeruginosa ATCC 27853T, Aspergillus niger ATCC 16404T, Candida albicans ATCC 10231T. Twelve of the forty-six isolates (25%) exhibited activity against at least one of the pathogens tested. Activity against Micromonospora. Members of the genus Streptomyces and Micromonospora are dominant in sediments lake Sarikum (Figure 1).

Based on 16S rRNA gene sequence analysis, 19 isolates were identified as Streptomyces spp. The phylogenetic tree, according to the neighbor-joining algorithm, indicated that nineteen strains were members of the genus Streptomyces (Figure 1; Table 5). According to the 16S rRNA gene sequence analysis, 19 Streptomyces isolates showed close 16S rRNA gene sequence similarity with the type strain of Streptomyces, which are 100% and 98.41%.

The phylogenetic tree, according to the neighbor-joining algorithm indicated that 17 strains were members of the genus Micromonospora (Figure 2). Seventeen Micromonospora isolates showed close 16S rRNA gene sequence similarity with the type strain of Micromonospora, which are 100% and 99.10% (Table 5).

Five Rhodococcus isolates showed close 16S rRNA gene sequence similarity with the type strain of Rhodococcus which are 100% and 99.58%, three Plantactinospora isolates showed close 16S rRNA gene sequence similarity with the type strain of Plantactinospora which are 99.58% and 99.10%, two Nonomuraea isolates showed close 16S rRNA gene sequence similarity with the type strain of Nonomuraea, which are 100% and 99.65%, an Actinomadura isolate showed close 16S rRNA gene sequence similarity with the type strain of Actinomadura, which is 99.79%, and a Streptosporangium isolate showed close 16S rRNA gene sequence similarity with the type strain of Streptosporangium, which is 99.79% (Figure 3; Table 5).

Table 4. Primers used for amplification of NRPS, PKS-I and II gene regions.

| Target genes | Primers | Base length | Product length (bp) | References |
|--------------|---------|-------------|---------------------|------------|
| NRPS         | A3F (5′GCSTACAYSATSTACACCTCSCG3′) | 23          | 700–800            | [9]        |
|              | A7R (5′SASTGCVCCGTTSCGGTAS3′)     | 19          |                    |            |
| PKS-I        | K1F (5′TSAACTGCAAATCCGGCA3′)      | 19          | 1200–1400          | [9]        |
|              | M6R (5′CGCAGGTGTCGCTACAAGTA3′)    | 20          |                    |            |
| PKS-II       | KsαF (5′TSGCGTGCTGGAYGCSATC3′)    | 20          | 613                | [43]       |
|              | KsαR (5′TGGAANCCGCGGAABCCT3′)     | 20          |                    |            |
E. faecalis ATCC 29212T was clearly the most frequent (6 isolates (12.5%)). Activity against K. pneumoniae ATCC 700603T and B. subtilis ATCC 6633T were the least frequent (4.16%), while 10.4% and 6.25% of the isolates were active against S. aureus ATCC 25923T and P. aeruginosa ATCC 27853T, respectively. Two isolates were found to inhibit three pathogens, while two isolates could inhibit two pathogens. None of the isolates could inhibit the growth of E. coli ATCC 25922T, A. niger ATCC 16404T and C. albicans ATCC 10231T (Table 4). NRPS sequences were detected in 48 isolates (100%), while PKS-I and PKS-II sequences were detected in only 28 and 22 of the 48 strains (58.3% and 45.8%), respectively. Seventeen isolates gave positive amplification products with both the PKS-I, PKS-II and NRPS sequences (Figure 4; Table 7).

**Discussion**

Actinomycetes have been isolated from many different habitats so far. Among these habitats, aquatic environments attract more attention in terms of their new species hosting potential. Zothanpuia et al. [25] identified 84 actinobacterial isolates based on 16S rRNA gene sequence analysis in a study from two rivers and...
one lake sediment. They concluded that 84 actinobacterial isolates were separated into a common genus (*Streptomyces*) and eight rare genera (*Nocardiopsis*, *Saccharopolyspora*, *Rhodococcus*, *Prauserella*, *Amycolatopsis*, *Promicromonospora*, *Kocuria* and *Micrococcus*). In our study, 48 isolates were obtained

Figure 1. Neighbor-joining tree [20] based on 16S rRNA gene sequences showing the position of isolated *Streptomyces* strains among their phylogenetic neighbors. Numbers at the nodes demonstrate the levels of bootstrap support (%); only values ≥ 50% are shown. GenBank accession numbers are placed in parentheses. Bar, 0.005 substitutions per nucleotide position.
A. VEYISOGLU AND D. TATAR

as a result of the isolation we made from Sankum Lake. The 48 isolated strains were found to belong to Streptomyces, Micromonospora, Rhodococcus, Plantactinospora, Nonomuraea, Actinomadura and Streptosporangium.

According to a study conducted in 2019, 10 mangrove soil samples were taken from Futian and Maoweihai of China, and the culture-dependent method was employed to obtain actinobacteria [26]. A total of 539 cultivable actinobacteria were isolated and distributed in 39 genera affiliated to 18 families of 8 orders by comparison analysis of partial 16S rRNA gene sequences. The dominant genus was Streptomyces, followed by Microbacterium, Agromyces and Rhodococcus [26].

In another study carried out in 2020, a total of 32 isolates were isolated from soil samples of different forest locations of Bisle Ghat and Virjapet situated in Western Ghats of Karnataka, India. The isolates were identified as species of Streptomyces, Nocardiosis and Nocardioides by cultural, morphological, and molecular studies [27].

While members of the genus Micromonospora were the most frequently isolated actinobacteria from freshwater lakes in past studies, members of the genus Streptomyces were more frequently isolated in recent studies [5, 28].

Strains SL78 and SL84 may be new species that belong to the genus Streptomyces. Strain SL78 had the closest 16S rRNA gene sequence similarity with Streptomyces karpasiensis K413 T (98.62%) [29] and SL84
indicated the closest 16S rRNA gene sequence similarity with *Streptomyces haliclonae* DSM 41968 T (98.41%) [30]. All values are below the threshold of 98.65% for delineation of a novel species [31–33].

Rare actinobacteria are important sources in the discovery of novel antibiotics [34]. In this study, *Rhodococcus, Plantactinospora, Nonomuraea, Actinomadura* and *Streptosporangium* isolates were obtained as members of the rare actinobacteria.

**Conclusions**

Sarıkum Lake is very important in terms of microbial diversity since it is connected to the Black Sea by a natural water channel. Until now, there has been no study on the diversity of actinomycetes in Sarıkum.
In our study, the diversity and antimicrobial activity of cultivable actinobacteria from the sediment of Sarıkum Lake (Sinop-Tukey) were investigated. Overall, 48 isolated strains were found to belong to *Streptomyces*, *Micromonospora*, *Rhodococcus*, *Plantactinospora*, *Nonomuraea*, *Actinomadura* and *Streptosporangium* by the phylogenetic analysis based on 16S rRNA gene sequencing. *Streptomyces* sp. SL78 and *Streptomyces* sp. SL84 were considered as two different potential new species. Sarıkum Lake was shown to be a valuable source of Actinobacteria strains with a high proportion of putatively new and rare species. A relatively large number of strains showed antimicrobial activities and presence of secondary metabolite genes. Thus, Sarıkum lake has been found to contain many members of actinobacteria with secondary metabolic activity.

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**Disclosure of potential conflicts of interest**

There is no conflict between the authors.

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**Research involving human participants and/or animals**

The research does not involve human participants or animals.

**Informed consent**

I declare that all data in the study is correct. The work presented has not been published elsewhere.

**Data availability**

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

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