Adıyaman University Journal of Science
https://dergipark.org.tr/en/pub/adyujsci
DergiPark AKADEMİK
ISSN 2147-1630
e-ISSN 2146-586X

# Bioactivity Features of Novel Actinobacteria Isolated from Lichen and Orchid Plant

Hilal ATEŞ[1], Hilal AY[2,*]

[1]*Ondokuz Mayıs University, Faculty of Science, Department of Biology, 55270, Samsun, Türkiye*
*hilal.ates@omu.edu.tr, ORCID: 0000-0002-5460-2360*
[*2]*Yildiz Technical University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, 34220, Istanbul, Türkiye*
*hilal.ay@yildiz.edu.tr, ORCID: 000-0002-8735-4703*

**Received:** 20.12.2022 **Accepted:** 26.09.2023 **Published:** 31.12.2023

## Abstract

A total of 10 actinobacterial strains were isolated from a lichen and an orchid species by employing a selective isolation procedure for actinobacteria. Antimicrobial activities of the isolates were evaluated against *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13311, *Staphylococcus aureus* ATCC 29213 and *Listeria monocytogenes* NCTC 5348. Out of the 10 isolates, eight showed antimicrobial activity against at least one pathogen. For molecular identification of the strains, 16S rRNA gene sequence analysis was performed. The pairwise comparison of the 16S rRNA gene sequences of the strains with the databases showed that the strains are members of the genus *Streptomyces* by sharing 98.9 – 100% gene sequence similarities. Phylogenetic relationships of the strains within the genus

* Corresponding Author DOI: 10.37094/adyujsci.1221660

*Streptomyces* were revealed by maximum likelihood and maximum parsimony phylogenetic trees. The results revealed that unexplored environmental habitats like lichens and plant tissues may represent a potential reservoir for novel actinobacteria with promising bioactivity features.

***Keywords:*** 16S rRNA Gene; Actinobacteria; Antimicrobial Activity; Lichen; Orchid Tuber.

## Liken ve Orkide Bitkisinden İzole Edilen Aktinobakterilerin Biyoaktivite Özellikleri

### Öz

Aktinobakteriler için seçici bir izolasyon prosedürü kullanılarak bir liken ve orkide türünden toplam 10 aktinobakteri izole edilmiştir. İzolatların antimikrobiyal aktiviteleri *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13311, *Staphylococcus aureus* ATCC 29213 ve *Listeria monocytogenes* NCTC 5348 patojenlerine karşı değerlendirilmiştir. On izolattan sekizi, en az bir patojene karşı antimikrobiyal aktivite göstermiştir. İzolatları moleküler olarak tanımlamak amacıyla 16S rRNA gen dizi analizi yapılmıştır. 16S rRNA geni karşılaştırmalı analizleri bütün izolatların *Streptomyces* cinsi üyesi olduklarını ve %98,9 – 100 gen dizi benzerliği taşıdıklarını göstermiştir. İzolatların *Streptomyces* cinsi içerisindeki filogenetik ilişkileri maksimum olabilirlik ve maksimum sıkılık filogenetik ağaçları ile ortaya çıkarılmıştır. Sonuçlar, likenler ve bitki dokuları gibi henüz çalışılmamış çevresel habitatların önemli biyoaktivite özelliklerine sahip yeni aktinobakteriler için potansiyel bir kaynak olabileceğini göstermektedir.

***Anahtar Kelimeler:*** 16S rRNA Geni; Aktinobakteriler; Antimikrobiyal Aktivite; Liken; Orkide Yumrusu.

## 1. Introduction

Novel drug leads, notably antimicrobial compounds, are needed to combat diseases such as increased antibiotic resistance and cancer that threaten human life [1, 2]. Although compounds with antimicrobial and therapeutic effects are produced by chemical synthesis, nature is still the richest and most important source for therapeutic compounds [3]. Microbial natural products with distinct biological activities are the most significant source for novel pharmaceutical compounds, including antimicrobial and antioxidant drugs. The numerous reports of the reappearance of

pathogenic microorganisms resistant to antibiotics increase the requirement for more powerful antimicrobials having new modes of action. Among the diverse resources, the advantageous chemical scaffolds and metabolic potential of actinobacteria have made it one of the most promising resources for bioprospecting. Roughly, more than half of the known antibiotics used in clinics today are produced by bacteria from the phylum *Actinomycetota*, formerly known as the phylum *Actinobacteria* [4, 5]. Notably, the genus *Streptomyces* alone constitutes 80% of the bioactive natural products produced by the members of the phylum *Actinomycetota* [6, 7].

The phylum *Actinomycetota*, also known as actinobacteria, comprises a diverse group of Gram-positive bacteria with high G+C content in their genomes. These bacteria, mostly producing substrate and aerial mycelia, are abundant in soil and have been isolated from various ecosystems, including alkaline soils [8], marine sponges [9], deep-sea sediments [10], hot springs [11], and medicinal plants [12]. Since most actinobacteria serve significant ecological functions, they have wide application potential in agriculture and environmental protection in addition to the production of antibiotics. However, the increasing emergence of new diseases and pathogens as well as antibiotic resistance has led to a reappearance of fascination for the discovery of new bioactive compounds for therapeutic applications. Therefore, the search for unexplored or extreme ecological habitats as sources of new actinobacteria with biological activity against pathogens has the utmost importance for discovering novel bioactive compounds with antimicrobial and/or anticancer activity. In this respect, lichens and plant tissues appear to have a good potential source for novel actinobacteria [13].

Lichens are the symbiotic communities of fungi and green algae or cyanobacteria. They have diverse morphologies and can be found in a wide variety of areas ranging from poles to tropical areas, especially in terrestrial areas [14, 15]. Most lichens are capable of producing bioactive substances with antioxidant, cytotoxic and antimicrobial activity [16].

Although the fungi and algae or cyanobacteria diversity in the symbiotic lichen community is often described, there is little information about the microorganisms found in the association of lichen as a bioactive secondary metabolite producer [17]. Recent reports have revealed that there is a large number of microorganisms in the lichen association [18, 19]. These lichen and related microorganisms form stable and specific populations, which represent a third form of lichen symbiosis as a whole [20].

Another promising source for the discovery of novel actinobacteria having bioactivity potential is plant tissues. Endophytic actinobacteria have great potential for the production of new natural compounds essential for commercial and medical use [21]. Currently, many studies have

focused on the isolation of novel actinobacteria producing bioactive secondary metabolites which have potential use in medicine, industry, and agriculture [22, 23].

In this study, our objectives were to isolate possible novel actinobacteria with antimicrobial and/or antibiofilm activities from unexploited sources of *Xanthoria* sp., a lichen collected from tree barks, and endophytic tissues of a *Serapias* sp., an orchid genus distributed in north Anatolia. The results underline novel *Actinobacteria* from plant and lichen samples have a high potential for bioactive secondary metabolites as well as the importance of exploitation of symbiotic actinobacteria from understudied sources for bioprospecting.

## 2. Materials and Methods

### 2.1. Isolation of actinobacteria

The lichen and orchid samples were collected from the campus of Ondokuz Mayıs University. The collected samples were delivered to the laboratory in sterile bags. In order to isolate actinobacteria from a lichen sample of *Xanthoria* sp., and an orchid species of *Serapias* sp., three selective media were prepared under aseptic conditions. The selective media used were the R2A medium [24], Czapex's Dox medium [25], and soil extract medium [26].

The lichen samples were brought to the laboratory and then incubated at 100 °C for an hour. Each lichen sample was then crushed in a sterile mortar and weighed 1 g and transferred to a 9-ml sterile Ringer's (Oxoid) solution under aseptic conditions. After good vortexing, the solutions were kept at 60 °C in a water bath for 15 min and then diluted to prepare $10^{-2}$ and $10^{-3}$ concentration tubes. For selective isolation of actinobacteria, 200 µl from each tube was spread onto the media. Both the rhizosphere and endophytic parts of the orchid tuber were used for actinobacteria isolation. The orchid tuber was kept in 100 ml sterile Ringer's solution (Oxoid) in a flask overnight. It was then transferred to a water bath at 60 °C and incubated for 20 min. After incubation, 200 µl Ringer's solution was spread onto selective isolation media. For isolation of endophytic actinobacteria, the orchid tuber removed from the Ringer's solution was crushed in sterile mortar and pestle and then transferred to a 9-ml sterile Ringer's solution. Then it was kept in a water bath for 15 min at 60 °C. After preparation of $10^{-2}$ and $10^{-3}$ concentration tubes, 200 µl of each tube was inoculated onto selective media. All plates were inoculated in triplicate and incubated at 28 °C for three weeks.

### 2.2. Antimicrobial activity test

Antimicrobial activity of the isolated actinobacteria was tested against *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13311, *Staphylococcus aureus* ATCC 29213, and *Listeria monocytogenes* NCTC 5348. The actinobacteria isolates were grown at 28 °C for a week on the ISP2 agar medium [27]. An amount of actinobacterial mycelia was taken and transferred into a 9-ml sterile Ringer's solution. A 7-µl of suspended bacterial solution was inoculated onto Bennett's Agar [28] without antibiotic supplementation and then incubated at 28 °C for 3 days. At the end of the incubation period, 1 ml chloroform was poured on the colonies which were developed under sterile conditions, and chloroform was expected to evaporate after 40 minutes. Subsequently, fresh pathogens developed on semi-solid nutrient agar were inoculated by spreading plates. After 48 hours of incubation under appropriate incubation conditions, the zone diameter of the region around the colonies was measured. Amphotericin B (5 mg $ml^{-1}$) was used for *A. niger,* and chloramphenicol (30 µg $ml^{-1}$) was used for other test pathogens as positive controls. The experiments were performed in duplicate, and the data are expressed as a mean value ± standard deviation.

### 2.3. Biofilm inhibition test

Antibiofilm activity of the isolates against pathogen *S. aureus* ATCC 29213 was evaluated in 96-well plates. The pathogen organism was incubated overnight in 100 ml LB medium. Test isolates were developed in ISP 2 medium, and supernatants were removed after centrifugation at 13000 rpm for 5 min. 100 µl of supernatants were placed in each well, and a volume of 100 µl of the pathogen grown in the LB medium was added to the wells and incubated at 37 °C for 18 hours. The bacteria–supernatant mixture was then removed from the wells and allowed to dry at room temperature for 15 min. Then, a volume of 200 µl of crystal violet dye (1%, w:v) was added to the wells and kept for 45 minutes. At the end of the period, the dye was removed, and the wells were washed with sterilized distilled water. Then, 200 µl of ethanol (95%, v:v) was added and the absorbance at 595 nm was measured [29].

### 2.4. Identification of the isolates and phylogenetic analysis

The actinobacteria with antimicrobial and/or antibiofilm activities were identified by 16S rRNA gene analysis. The genomic DNA extraction from the isolates was performed as described by Chun and Goodfellow [30]. The PCR amplifications of the 16S rRNA gene region of the isolates were performed using the 27F (5'AGAGTTTGATCTGGCTCAG3') and 1525R

(5'AAGGAGGTGWTCCARCC3') universal primers. The purified PCR products were sequenced using 518F (5'CCAGCAGCCGCGGTAATACG3'), 800R (5'TACCAGGGTATCTAATCC3') and MG5F (5′AAACTCAAAGGAATTGACGG3′) primers on an ABI PRISM 3730 XL automated sequencer in Macrogen Inc. (The Netherlands). The obtained sequences were assembled using the ChromasPro Version 1.7.6 (Technelysium Pty Ltd) program. The nearly complete 16S rRNA gene sequences were aligned with those of the closely related type strains downloaded from the EzBioCloud server [31]. The pairwise sequence similarities between the strains and their relatives within the genus *Streptomyces* were estimated by following the method described by Meier-Kolthoff et al. (2013) for the 16S rRNA gene on the GGDC web server (http://ggdc.dsmz.de/) [32]. The phylogenetic relationships between the strains and their close neighbours within the genus *Streptomyces* determined by pairwise gene comparisons were also inferred by the GGDC web server [32] by employing the DSMZ phylogenomics pipeline as described in previous works [33, 34]. Briefly, a multiple sequence alignment for the 16S rRNA gene sequences was built with MUSCLE [35]. The maximum likelihood (ML) and maximum parsimony (MP) phylogenetic trees were constructed from the alignment obtained through RAxML [36] and TNT algorithms [37], respectively. A rapid bootstrapping together with the autoMRE bootstrapping criterion [38] and a succeeding search for the best tree was employed to build the maximum likelihood tree. For the maximum parsimony tree, 1000 bootstrapping replications were applied combined with tree-bisection-and-reconnection branch swapping and ten arbitrary sequence addition replications. The aligned sequences were examined for compositional discrepancies by applying the test executed in PAUP* [39].

## 3. Results and Discussion

### 3.1. Antimicrobial activity

The antimicrobial properties of 10 isolates were tested against *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *S. aureus* ATCC 29213, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, *S. enterica* ATCC 13311, *A. niger* ATCC 16404, *C. albicans* ATCC 10231, *E. faecalis* ATCC 29212 and *L. monocytogenes* NCTC 5348 pathogens. It was determined that eight isolates showed antimicrobial activity against at least one pathogen. The results are given in Table 1 and show that actinobacteria obtained from lichen and orchid bulbs have a significant antimicrobial effect on pathogens.

**Table 1:** Inhibition zone diameters of actinobacteria obtained from lichen and orchid bulbs against the pathogens

| Strain No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **IC12A** | - | - | - | - | - | - | - | - | - | - |
| **IC12B** | 39.5±0.7 | 26±6.3 | 16.5±0.7 | - | 21.5±0.7 | 29±1.4 | 16.5±2.1 | 33.5±0.7 | 28.5±2.1 | - |
| **IC13** | 32.5±3.5 | - | 29±0 | - | - | 25.5±0.7 | - | 24±1.4 | 17±2.8 | - |
| **IC15A** | - | - | - | - | - | - | - | 15±1.4 | 13.5±2.1 | - |
| **IS21** | - | - | - | - | - | - | - | 17±4.2 | 17±4.2 | - |
| **IC21** | 36.5±6.3 | 24±1.4 | 30±1.4 | - | 13.5±2.1 | 25±5.6 | - | 30±5.6 | 27.5±3.5 | - |
| **SR31** | 14±1.4 | 32.5±9.6 | - | - | - | 47.5±9.6 | - | 43±4.2 | 46±5.6 | - |
| **SR22** | - | - | - | - | - | 32.5±3.5 | - | - | - | - |
| **SS31** | 33±2.8 | - | - | 15±1.4 | - | - | - | - | - | - |
| **SR32** | - | - | - | - | - | - | - | - | - | - |
| **Positive control** | 20.5±1.4 | 24±1.4 | 33.5±2.1 | 14.5±2.1 | 16.5±3.5 | 10±0 | 21.5±3.5 | 22.5±2.1 | 15±2.1 | 34.5±1.4 |

1; *C. albicans* ATCC 10231, 2; *B. subtilis* ATCC 6633, 3; *A. niger* ATCC 16404, 4; *E. faecalis* ATCC 29212, 5; *E. coli* ATCC 25922, 6; *S. aureus* ATCC 29213, 7; *K. pneumoniae* ATCC 700603, 8; *S. enterica* ATCC 13311, 9; *L. monocytogenes* NCTC 5348*, 10; P. aeruginosa* ATCC 27853.

The results show that strains IC12B, IC13 and IC21 exhibited high level of antimicrobial activity against *C. albicans*, *S. aureus*, *S. enterica* and *L. monocytogenes* pathogens while relatively lower against the *A. niger* pathogen. Strains IC12B and SR31 showed high antimicrobial activity against *B. subtilis* by forming an inhibition zone of 26 mm and 32.5 mm, respectively, compared to positive control (24 mm), while strain IC21 showed moderate antimicrobial activity. The strains SR22 and SR31 showed significantly higher antimicrobial activities against *S. aureus* compared to the positive control. Strain SR31 showed high antimicrobial activity against *B. subtilis*, *S. aureus*, *S. enterica* and *L. monocytogenes*. This strain was a highly bioactive microorganism against the pathogens *S. aureus*, *S. enterica* and *L. monocytogenes* by forming inhibition zones over 40 mm, which implies its bioactivity potential, particularly against foodborne infections. In addition, strain SR31 inhibited the growth of *C. albicans* moderately. On the other hand, strain SS31 showed higher inhibition activity against *C. albicans* and *E. faecalis* compared to the positive control. Strain IC12B exhibited high antimicrobial activity against *E. coli* and moderate activity against *K. pneumoniae,* while strains IC15A and IS21 exhibited moderate antimicrobial activity against *S. enterica* and *L. monocytogenes* by forming inhibition zones ranging from 13 – 17 mm in diameter. None of the isolates showed antimicrobial activity against *P. aeruginosa*. When the antimicrobial activity test results are evaluated in general, especially strain IC12B showed activity in a broad spectrum since

strain IC12B showed antimicrobial activity against all pathogens tested except *E. faecalis* and *P. aeruginosa*. Strain IC21 seemed to be effective as a promising source of antifungal agents.

### 3.2. Biofilm inhibition

Biofilm inhibition test was performed against the *S. aureus* ATCC 29213 pathogen for 10 isolates, and the results were evaluated with One-Way Anova program. Compared to the positive control group, strain IC12B significantly inhibited biofilm formation at the 95% significance level.

### 3.3. Phylogenetic characterization of actinobacteria

The strains were identified by the 16S rRNA gene pairwise sequence analysis. These strains were determined to be members of the genus *Streptomyces*. The most closely related type species according to the 16S rRNA gene pairwise sequence analysis were given in Table 2.

**Table 2:** The 16S rRNA gene sequence identity values to the closely related type species

| Isolates | GenBank accession number | Closest type strain | Identity (%) | Isolation source |
|---|---|---|---|---|
| **IC12A** | MK503625 | *Streptomyces aureus* NBRC 100912 | 99.93 | Endophytic tissues of *Serapias* sp. |
| **IC13** | MK503627 | *Streptomyces antimycoticus* NBRC 12839 | 100.00 | Endophytic tissues of *Serapias* sp. |
| **IC15A** | MK503628 | *Streptomyces aureocirculatus* NRRL ISP-5386 | 99.52 | Endophytic tissues of *Serapias* sp. |
| **IC21** | MK503629 | *Streptomyces antimycoticus* NBRC 12839 | 100.00 | Endophytic tissues of *Serapias* sp. |
| **IS21** | MK503630 | *Streptomyces aureocirculatus* NRRL ISP-5386 | 99.52 | Endophytic tissues of *Serapias* sp. |
| **IC12B** | MK503626 | Streptomyces antimycoticus NBRC 12839 | 100.00 | Endophytic tissues of *Serapias* sp. |
| **SR22** | MK503631 | *Streptomyces nanshensis* SCSIO 01066 | 99.57 | *Xanthoria* sp. |
| **SR31** | MK503632 | *Streptomyces decoyicus* NRRL 2666 | 100.00 | *Xanthoria* sp. |
| **SS31** | MK503634 | *Streptomyces daghestanicus* NRRL B-5418 | 99.58 | *Xanthoria* sp. |
| **SR32** | MK503633 | *Streptomyces nanshensis* SCSIO 01066 | 98.99 | *Xanthoria* sp. |

The phylogenetic tree based on comparative analysis of the 16S rRNA gene sequences of the strains and their close neighbours within the genus *Streptomyces* revealed that the strains were separated into six clades (Fig. 1). The strains IC12B, IC21 and IC13 were clustered together with *S. geldanamycininus* NRRL B-3602$^T$, *S. yatensis* NBRC 101000$^T$, *S. mordarskii* NRRL B-1346$^T$ as well as with *S. antimycoticus* NBRC 12839$^T$. All three strains were observed to have similar antimicrobial activity profiles by having strong inhibition against *C. albicans*, *A. niger*, *E. coli*, *S. aureus*, *S. enterica* and *L. monocytogenes* (Table 1). Similarly, their most closely related phylogenetic neighbour *S. antimycoticus* was reported to be used as a biocontrol agent [40] and a source organism for bioactive terpenoids napyradiomycins [41]. In addition, *S. yatensis* was also reported to produce bioactive metabolites with antifungal [42] and nematicidal activities [43]. Considering the bioactivity profiles of the *Streptomyces* species within the same clade as well as high antimicrobial activities, the strains can be considered as potential sources for new bioactive compounds. The close phylogenetic relationship of the strains with the type strain of *S. antimycoticus* was also confirmed by the phylogenetic tree. The pairwise comparison of the 16S rRNA gene sequences of the strains indicated that the strains have 100% identity with each other.

Strain SR31 formed a cluster with the type strains of *S. decoyicus* NRRL B-2666$^T$, *S. sioyaensis* NRRL B-5408$^T$, *S. caniferus* NBRC 15389$^T$, *S. platensis* JCM 4662$^T$ and *S. monumycini* NRRL B-24309$^T$. The antimicrobial activity tests showed that strain SR31 have strong inhibitory activity against *B. subtilis*, *S. aureus*, *S. enterica* and *L. monocytogenes* compared to the positive control. The type species in the same clade were also reported to produce antimicrobial and antitumor metabolites such as decoyinin, psicofuranine [44], siomycin [45] and caniferolide [46], which implies a high potential of strain SR31 to produce novel bioactive metabolites.

Strain SS31 was differentiated from the type species of *S. daghestanicus* NRRL B-5418$^T$, *S. albidoflavus* DSM 40455$^T$ and *S. violascens* ISP 5183$^T$ within the cluster they formed together. Considering the tree topology and antimicrobial activities against *C. albicans* and *E. faecalis*, strain SS31 may represent a novel species within the genus *Streptomyces*.

Strains SR22 and SR32 were clustered together with the type species of *S. nanshensis* SCSIO 01066$^T$. Although both strains shared the highest 16S rRNA gene sequence identity levels with the type species of *S. nanshensis*, they were differentiated from each other by 99.46% gene sequence identity level. Thus, both strains SR22 and SR32 may represent two novel species for the genus *Streptomyces*.

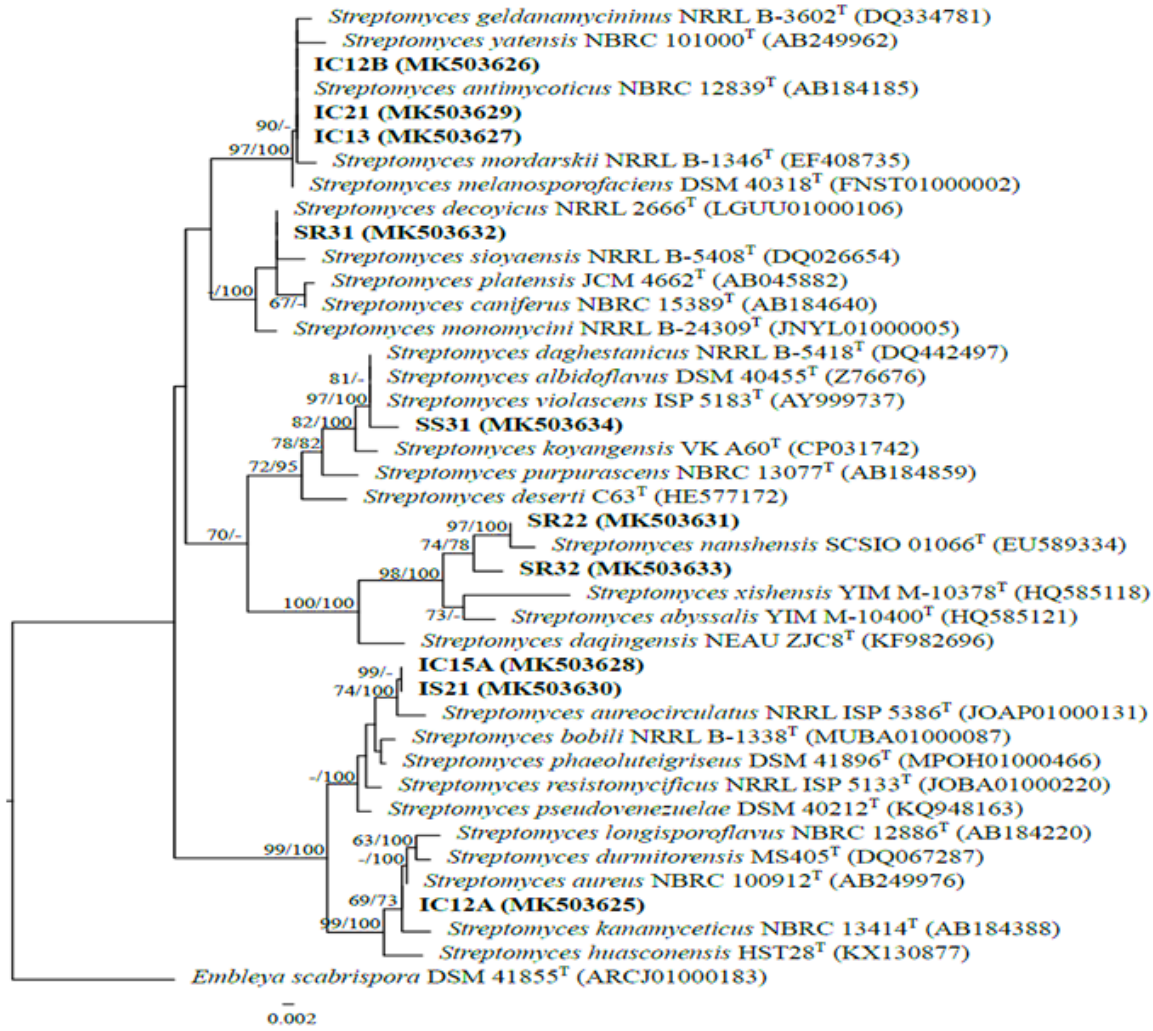


**Figure 1:** Phylogenetic tree of the *Streptomyces* strains based on the 16S rRNA gene sequence analysis. The tree was derived under the General Time Reversible with gamma distribution model and rooted through the midpoint. The scale indicates the expected number of nucleotide substitutions per site. The numbers above the branches are support levels when more than 60% bootstraps with maximum likelihood (left) and maximum parsimony (right). *Embleya scabrispora* DSM 41855$^T$ was used as an outgroup.

Strains IC15A and IS21 were clustered with their most closely related species *S. aureocirculatus* NRRL ISP 5386$^T$, while their 16S rRNA gene sequence identity level with each other was calculated as 100%. Therefore, strains IC15A and IS21 might be representatives of a novel species within the genus *Streptomyces*. Sahu et al. (2007) reported that *S. aureocirculatus* produced a bioactive sugar molecule that inhibits the growth of pathogens [47]. Consistently, strains IC15A and IS21 showed antimicrobial activity against *S. enterica* and *L. monocytogenes*. Considering phylogenetic positions on the tree as well as the relatively low level of 16S rRNA gene sequence similarity, strains IC15A and IS21 have the possibility to be novel species of the genus *Streptomyces*, and hence, their bioactivity might result from structurally new metabolites.

Strain IC12A formed a large cluster with the type strains of *S. aureus* NBRC 100912$^T$, *S. durmitorensis* MS405$^T$, *S. longisporoflavus* NBRC 12886$^T$, *S. kanamyceticus* NBRC 13414$^T$ and *S. huasconensis* HST28$^T$. Although the strain showed no antimicrobial activity against the pathogens tested, the tree topology may suggest that strain IC12A might represent a novel species within the genus *Streptomyces*.

As actinobacteria are well-known for their ability to produce a myriad of bioactive secondary metabolites, the exploration of novel actinobacteria from unexplored or underexplored habitats is considered to be the most effective strategy to discover novel species with distinct biochemistry. Lichens are one of the underexplored microbial communities in terms of actinobacterial biodiversity and hold high promise for novel actinobacteria, as revealed by the present study. Selbmann et al. [48] investigated culturable bacteria from four Antarctic lichen species, i.e. *Lecanora fuscobrunnea* Dodge & Baker, *Umbilicaria decussata* (Villars) Zahlbruckner, *Usnea antarctica* Du Rietz, *Xanthoria elegans* (Link) Th. Fries, and reported 30 morphologically distinct bacterial strains, 20 of which were actinobacteria. The bioactivity of actinobacteria isolated from lichen samples was also reported by previous studies [49, 50]. Davies et al. [49] and Williams et al. [50] extracted novel bioactive metabolites, i.e. uncialamycin with antibiotic activity and cladoniamides A−G with cytotoxicity, from *Streptomyces uncialis* isolated from the surface of a lichen *Cladonia uncialis* collected near Pitt River, British Columbia. Consistently, Parrot et al. [51] showed that the littoral lichens are important sources of novel bioactive actinobacteria. However, there is no report on *Streptomyces* spp. isolated from the lichen genus *Xanthoria* (Fr.) Th. Fr.; thus, the present study is the first report revealing putatively novel *Streptomyces* spp. with significant antimicrobial activities from this lichen genus.

The endophytic actinobacteria from plant species also have distinct bioactivity characteristics, such as the endophytes of orchid plants [52]. Alibrandi et al. [53] revealed that over 25% of microbial symbionts in *Serapias* sp. were members of the actinobacteria in their culture-independent study, but they did not conduct any bioactivity screening for those actinobacteria. On the other hand, Saikia et al. [54] investigated the phylogenetic affiliation of actinobacteria isolated from various orchid species belonging to the genera *Cymbidium* Sw., *Dendrobium* Sw., *Micropera* Lindl., *Renanthera* Lour., *Rhynchostylis* Blume and *Vanda* R. Br. They identified 51 morphologically distinct actinobacterial isolates with multiple plant growth-promoting activities as well as broad-spectrum antifungal properties against various plant pathogens. Although similar works about bioactive actinobacteria from orchids species are reported in the literature, the present study is the first to show the *Streptomyces* spp. isolated from *Serapias* sp. have antimicrobial activity against human pathogens.

## 4. Conclusion

Actinobacteria is a notable group of bacteria for researchers as they produce antibiotics and other therapeutically effective substances. In particular, the *Streptomyces* strains are known for their ability to produce new antibiotics and other biologically important compounds, including insecticides, herbicides, antiparasitics, immunosuppressants, and other compounds of industrial interest [55-57]. Thus, it is of utmost importance for the exploitation of this prolific group of bacteria isolated from unexplored or extreme habitats to discover novel bioactive metabolites.

In this study, the actinobacteria isolated from lichen and endophytic tissues of orchid tubers were revealed to have considerable antimicrobial activities against a number of human pathogens. From the phylogenetic point of view, moreover, these actinobacteria may represent novel species within the genus *Streptomyces*. However, a polyphasic approach involving morphological, biochemical, and phylogenetic characterization, as well as whole genome-based comparative analyses, must be employed to determine the taxonomic positions of these strains within the genus *Streptomyces*. Consequently, our present work on actinobacteria from lichen and orchid samples to search for promising natural sources for distinct bioactivity reveals that unexplored habitats are a real treasure for novel actinobacteria producing bioactive secondary metabolites waiting to be discovered. A comprehensive bioprospecting study supported by genome-based bioactivity estimation for these strains is required in the near future.

## References

[1] Payne, D.J., Gwynn, M.N., Holmes, D.J., Pompliano, D.L., *Drugs for bad bugs: confronting the challenges of antibacterial discovery,* Nature reviews Drug discovery, 6(1), 29-40, 2007.

[2] Olano, C., Méndez, C., Salas, J.A., *Molecular insights on the biosynthesis of antitumour compounds by actinomycetes,* Microbial Biotechnology, 4(2), 144-164, 2011.

[3] Manivasagan, P., Kang, K.H., Sivakumar, K., Li-Chan, E.C., Oh, H.M., Kim, S.K., *Marine actinobacteria: an important source of bioactive natural products,* Environmental toxicology and pharmacology, 38(1), 172-188, 2014.

[4] Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.P., Clément, C., Ouhdouch, Y., van Wezel, G.P., *Taxonomy, physiology, and natural products of Actinobacteria,* Microbiology and Molecular Biology Reviews, 80(1), 1-43, 2016.

[5] Lee, L.H., Goh, B.H., Chan, K.G., *Actinobacteria: Prolific producers of bioactive metabolites*, Frontiers Media SA, 1612, 2020.

[6] Kumar, N., Singh, R.K., Mishra, S., Singh, A., Pachouri, U.C., *Isolation and screening of soil Actinomycetes as source of antibiotics active against bacteria,* International Journal of Microbiology Research, 2(2), 12, 2010.

[7] Manivasagan, P., Venkatesan, J., Sivakumar, K., Kim, S.K., *Pharmaceutically active secondary metabolites of marine actinobacteria,* Microbiological research, 169(4), 262-278, 2014.

[8] Ay, H., Nouioui, I., del Carmen Montero-Calasanz, M., Carro, L., Klenk, H.P., Goodfellow, M., Igual, J.M., Çetin, D., Şahin, N., Işık, K., *Actinomadura alkaliterrae sp. nov., isolated from an alkaline soil,* Antonie van Leeuwenhoek, 110(6), 787-794, 2017.

[9] Khan, S.T., Tamura, T., Takagi, M., Shin-Ya, K., *Streptomyces tateyamensis sp. nov., Streptomyces marinus sp. nov. and Streptomyces haliclonae sp. nov., isolated from the marine sponge Haliclona sp.,* International journal of systematic and evolutionary microbiology, 60(12), 2775-2779, 2010.

[10] Xiao, J., Luo, Y., Xu, J., Xie, S., Xu, J., *Modestobacter marinus sp. nov., a psychrotolerant actinobacterium from deep-sea sediment, and emended description of the genus Modestobacter,* International journal of systematic and evolutionary microbiology, 61(7), 1710-1714, 2011.

[11] Duan, Y.Y., Ming, H., Dong, L., Yin, Y.R., Zhang, Y., Zhou, E.M., Liu, L., Nie, G.X., Li, W.J., *Streptomyces calidiresistens sp. nov., isolated from a hot spring sediment,* Antonie van Leeuwenhoek, 106(2), 189-196, 2014.

[12] Wang, Z., Tian, J., Li, X., Gan, L., He, L., Chu, Y., Tian, Y., *Streptomyces dioscori sp. nov., a Novel Endophytic Actinobacterium Isolated from Bulbil of Dioscorea bulbifera L.,* Current microbiology, 75(10), 1384-1390, 2018.

[13] Parrot, D., Legrave, N., Delmail, D., Grube, M., Suzuki, M., Tomasi, S., *Review–Lichen-associated bacteria as a hot spot of chemodiversity: focus on uncialamycin, a promising compound for future medicinal applications,* Planta Medica, 82(13), 1143-1152, 2016.

[14] Lutzoni, F., Miadlikowska, J., *Lichens.* Current Biology, 19(13), R502-R503, 2009.

[15] Asplund, J., Wardle, D.A., *How lichens impact on terrestrial community and ecosystem properties,* Biological Reviews, 92(3), 1720-1738, 2017.

[16] Boustie, J., Tomasi, S., Grube, M., *Bioactive lichen metabolites: alpine habitats as an untapped source,* Phytochemistry Reviews, 10(3), 287-307, 2011.

[17] Calcott, M.J., Ackerley, D.F., Knight, A., Keyzers, R.A., Owen, J.G., *Secondary metabolism in the lichen symbiosis,* Chemical Society Reviews, 47(5), 1730-1760, 2018.

[18] Muggia, L., Vancurova, L., Škaloud, P., Peksa, O., Wedin, M., Grube, M., *The symbiotic playground of lichen thalli–a highly flexible photobiont association in rock-inhabiting lichens,* FEMS Microbiology Ecology, 85(2), 313-323, 2013.

[19] Liu, C., Jiang, Y., Wang, X., Chen, D., Chen, X., Wang, L., Han, L., Huang, X., Jiang, C., *Diversity, antimicrobial activity, and biosynthetic potential of cultivable actinomycetes associated with lichen symbiosis,* Microbial ecology, 74(3), 570-584, 2017.

[20] Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., Wegner, U., Becher, D., Riedel, K., Sensen, C.W., Berg, G., *Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics,* The ISME journal, 9(2), 412-424, 2015.

[21] Liu, Y.H., Guo, J.W., Salam, N., Li, L., Zhang, Y.G., Han, J., Mohamad, O.A., Li, W.J., *Culturable endophytic bacteria associated with medicinal plant Ferula songorica: molecular phylogeny, distribution and screening for industrially important traits,* 3 Biotech, 6(2), 1-9, 2016.

[22] Li, J., Zhao, G.Z., Chen, H.H., Wang, H.B., Qin, S., Zhu, W.Y., Xu, L.H., Jiang, C.L., Li, W.J., *Antitumour and antimicrobial activities of endophytic streptomycetes from pharmaceutical plants in rainforest,* Letters in applied microbiology, 47(6), 574-580, 2008.

[23] Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., Rai, M., *Endophytic actinobacteria of medicinal plants: diversity and bioactivity,* Antonie Van Leeuwenhoek, 108(2), 267-289, 2015.

[24] Reasoner, D.J., Geldreich, E.E., *A new medium for the enumeration and subculture of bacteria from potable water,* Applied and environmental microbiology, 49(1), 1-7, 1985.

[25] Weyland, H., *Actinomycetes in North Sea and Atlantic ocean sediments,* Nature, 223(5208), 858-858, 1969.

[26] Hamaki, T., Suzuki, M., Fudou, R., Jojima, Y., Kajiura, T., Tabuchi, A., Sen, K., Shibai, H., *Isolation of novel bacteria and actinomycetes using soil-extract agar medium,* Journal of bioscience and bioengineering, 99(5), 485-492, 2005.

[27] Shirling, E.T., Gottlieb, D., *Methods for characterization of Streptomyces species,* International journal of systematic bacteriology, 16(3), 313-340, 1966.

[28] Jones, K.L., *Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic,* Journal of bacteriology, 57(2), 141-145, 1949.

[29] Sankar Ganesh, P., Rai Vittal, R., *In vitro antibiofilm activity of Murraya koenigii essential oil extracted using supercritical fluid $CO_2$ method against Pseudomonas aeruginosa PAO1,* Natural Product Research, 29(24), 2295-2298, 2015.

[30] Chun, J., Goodfellow, M., *A phylogenetic analysis of the genus Nocardia with 16S rRNA gene sequences,* International Journal of Systematic and Evolutionary Microbiology, 45(2), 240-245, 1995.

[31] Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J., *Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies,* International journal of systematic and evolutionary microbiology, 67(5), 1613, 2017.

[32] Meier-Kolthoff, J.P., Carbasse, J.S., Peinado-Olarte, R.L., Göker, M., *TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes,* Nucleic acids research, 50(D1), D801-D807, 2022.

[33] Ay, H., *Genomic insight into a novel actinobacterium, Actinomadura rubrisoli sp. nov., reveals high potential for bioactive metabolites,* Antonie van Leeuwenhoek, 114(2), 195-208, 2021.

[34] Meier-Kolthoff, J.P., Hahnke, R.L., Petersen, J., Scheuner, C., Michael, V., Fiebig, A., Rohde, C., Rohde, M., Fartmann, B., Goodwin, L.A., Chertkov, O., Reddy, T., Pati, A., Ivanova, N.N, Markowitz, V., Kyrpides, N.C., Woyke, T., Göker M., Klenk, H.P., *Complete genome sequence of DSM 30083T, the type strain (U5/41T) of Escherichia coli, and a proposal for delineating subspecies in microbial taxonomy*, Standards in Genomic Sciences, 9(2), 2014.

[35] Edgar, R.C., *MUSCLE: a multiple sequence alignment method with reduced time and space complexit,* BMC bioinformatics, 5(1), 1-19, 2004.

[36] Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies,* Bioinformatics, 30(9), 1312-1313, 2014.

[37] Goloboff, P.A., Farris, J.S., Nixon, K.C., *TNT, a free program for phylogenetic analysis,* Cladistics, 24(5), 774-786, 2008.

[38] Pattengale, N.D., Alipour, M., Bininda-Emonds, O.R., Moret, B.M., Stamatakis, A., *How many bootstrap replicates are necessary?* in *Annual International Conference on Research in Computational Molecular Biology*, pp.184-200, 2009.

[39] Swofford, D.L., *PAUP: phylogenetic analysis using parsimony, version 4.0 b10*, Sinauer Associates, Sunderland, MA, 2002.

[40] Koch, E., Löffler, I., *Partial characterization of the antimicrobial activity of Streptomyces antimycoticus FZB53,* Journal of Phytopathology, 157(4), 235-242, 2009.

[41] Motohashi, K., Sue, M., Furihata, K., Ito, S., Seto, H., *Terpenoids produced by actinomycetes: Napyradiomycins from Streptomyces antimycoticus NT17,* Journal of natural products, 71(4), 595-601, 2008.

[42] Benouagueni, S., Ranque, S., Kirane, D.G., *A non-polyenic antifungal produced by a Streptomyces yatensis strain isolated from Mellah Lake in El Kala, North-East of Algeria,* Journal de Mycologie Medicale, 25(1), 2-10, 2015.

[43] Park, E.J., Jang, H.J., Park, C.S., Lee, S.J., Lee, S., Kim, K.H., Yun, B.S., Lee, S.W., Rho, M.C., *Evaluation of Nematicidal Activity of Streptomyces yatensis KRA-28 against Meloidogyne incognita,* Journal of Microbiolog and Biotechnology, 30(5), 700-707, 2020.

[44] Kumar, Y., Goodfellow, M., *Reclassification of Streptomyces hygroscopicus strains as Streptomyces aldersoniae sp. nov., Streptomyces angustmyceticus sp. nov., comb. nov., Streptomyces ascomycinicus sp. nov., Streptomyces decoyicus sp. nov., comb. nov., Streptomyces milbemycinicus sp. nov. and Streptomyces wellingtoniae sp. nov.,* International journal of systematic and evolutionary microbiology, 60(4), 769-775, 2010.

[45] Tokura, K., Torı, K., Yoshımura, Y., Okabe, K., Otsuka, H., Matsushıta, K., Inagakı, F., Mıyazawa, T., *The structure of siomycin-D1, peptide antibiotic isolated from Streptomyces sioyaensis,* The Journal of Antibiotics, 33(12), 1563-1567, 1980.

[46] Alvariño, R., Alonso, E., Lacret, R., Oves-Costales, D., Genilloud, O., Reyes, F., Alfonso, A., Botana, L.M., *Caniferolide A, a macrolide from Streptomyces caniferus, attenuates neuroinflammation, oxidative stress, amyloid-beta, and tau pathology in vitro,* Molecular Pharmaceutics, 16(4), 1456-1466, 2019.

[47] zkumar Sahu, M., Murugan, M., Sivakumar, K., Thangaradjou, T., Kannan, L., *Occurrence and distribution of actinomycetes in marine environs and their antagonistic activity against bacteria that is pathogenic to shrimps,* Israeli Journal of Aquaculture-Bamidgeh, 59(3), 155-161, 2007.

[48] Selbmann, L., Zucconi, L., Ruisi, S., Grube, M., Cardinale, M., Onofri, S., *Culturable bacteria associated with Antarctic lichens: affiliation and psychrotolerance*, Polar biology, 33, 71-83, 2010.

[49] Davies, J., Wang, H., Taylor, T., Warabi, K., Huang, X.H., Andersen, R.J., *Uncialamycin, a new enediyne antibiotic*, Organic letters, 7(23), 5233-5236, 2005.

[50] Williams, D.E., Davies, J., Patrick, B.O., Bottriell, H., Tarling, T., Roberge, M., Andersen, R.J., *Cladoniamides A−G, tryptophan-derived alkaloids produced in culture by Streptomyces uncialis*, Organic letters, 10(16), 3501-3504, 2008.

[51] Parrot, D., Antony-Babu, S., Intertaglia, L., Grube, M., Tomasi, S., Suzuki, M.T., *Littoral lichens as a novel source of potentially bioactive Actinobacteria*, Scientific Reports, 5(1), 15839, 2015.

[52] Herrera, H., Fuentes, A., Soto, J., Valadares, R., Arriagada, C., *Orchid-associated bacteria and their plant growth promotion capabilities*, In Orchids phytochemistry, biology and horticulture: fundamentals and applications, 175-200, 2022.

[53] Alibrandi, P., Schnell, S., Perotto, S., Cardinale, M., *Diversity and structure of the endophytic bacterial communities associated with three terrestrial orchid species as revealed by 16S rRNA gene metabarcoding*, Frontiers in Microbiology, 11, 604964, 2020.

[54] Saikia, J., Mazumdar, R., Thakur, D., *Phylogenetic affiliation of endophytic actinobacteria associated with selected orchid species and their role in growth promotion and suppression of phytopathogens*, Frontiers in Plant Science, 13, 1058867, 2022.

[55] Chater, K.F., *Streptomyces inside-out: a new perspective on the bacteria that provide us with antibiotics,* Philosophical Transactions of the Royal Society B: Biological Sciences, 361(1469), 761-768, 2006.

[56] Bull, A.T., Stach, J.E., *Marine actinobacteria: new opportunities for natural product search and discovery,* Trends in microbiology, 15(11), 491-499, 2007.

[57] Sharma, S. B., Gupta, R., *Drug development from natural resource: a systematic approach,* Mini reviews in medicinal chemistry, 15(1), 52-57, 2015.