Bioactive compounds and molecular diversity of endophytic actinobacteria isolated from desert plants

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Abstract. With the aim to find endophytic actinomycetes that synthesize bioactive compounds over 800 strains were isolated from 53 desert plants of the Gobi-Sumber, Umnugobi, Dundgobi, Dornogobi, Bayankhongor, and Gobi-Altai provinces of Mongolia. The HPLC study of strains with high anti-quorum sensing and antibacterial activities revealed that they produced flavonoids and phenolic compounds. Molecular diversity evaluated with 16S rRNA gene sequences of 123 strains showed that they belonged to 12 genera: Streptomyces, Promicromonospora, Micromonospora, Streptosporangium, Kribbella, Pseudonocardia, Nocardia, Micromonospora, Saccharotherix, Friedmanniella, Actinocatenispora, and Geodermatophilus, the latter two genera were registered in Mongolia for the first time. Moreover, the genus Actinocatenispora was isolated from plants for the first time.

1. Introduction
Microbial resistance to current antibiotics needs new antimicrobials and improved strategies for treating bacterial infections, including the non-antibiotic-based approaches. The discovery that the quorum sensing (QS) systems of pathogenic bacteria control the production of virulence factors including biofilm formation led scientists to the recognition that compounds inhibiting the QS have great potential for use in the treatment of bacterial infectious diseases and to start searching for those compounds [1]. Furthermore, it has been found out that the use of antibiotics in combination with quorum sensing inhibitors (QSIs) increases the susceptibility of pathogenic bacteria to antibiotics [2]. This finding has been an additional stimulus for searching both activities.

For decades actinomycetes have been one of the most important sources for the discovery of new antibiotics, and many drugs and analogues were successfully introduced into the market and are still used today in clinical practice [3-5]. Besides antibiotics, actinomycetes can also synthesize different biologically active compounds such as anti-cancer substances, immunosuppressive agents, and other essential molecules [5-6].

Recently, intensive searching for new antimicrobials and QSIs has targeted previously unexplored marine and terrestrial extreme environments [7-10], and endophytic actinobacteria [11, 12].
Endophytic actinobacteria, which reside in the inner tissues of host plants, are gaining serious attention due to their capacity to produce a plethora of secondary metabolites possessing a wide variety of biological activity with diverse functions [12, 13]. Microorganisms, including actinobacteria, that live in changing extreme temperatures often evolve compounds or processes that help them survive in these harsh conditions [9].

Mongolia is a country rich in different extreme environments such as saline soils and lakes, high mountains, permafrost, and hot mineral springs. About 30% of its territory is occupied with the desert area including the northern part of the great Gobi Desert. Earlier, it was found out that the diversity of soil actinobacteria in extreme environments of Mongolia was quite rich, and most of the actinomycete strains isolated from soils and the rhizosphere of the Gobi Desert plants had antibacterial and enzymatic activities [14-17]. Furthermore, 41.2% of endophytic microscopic fungi isolated from the desert plants of Mongolia had antimicrobial activities showing that endophytes of the desert plants can be a promising source for screening of bioactive compounds [18]. This study was aimed at the isolation of endophytic actinobacteria from plants growing in the desert areas of Mongolia, identification of their molecular diversity, and bioactive compounds of strains having high anti-quorum sensing and antimicrobial activities. Here we present some preliminary results.

2. Material and Methods

Sample collection. Samples of 53 wild plants were collected from the areas of the Gobi-Sumber, Umnugobi, Dundgobi, Dornogobi, Bayankhongor, and Gobi-Altau provinces from June of 2017 to July of 2018.

Isolation of actinomycetes. The collected plants were disinfected by washing with 70% ethanol for 1 min, then rinsed with 1% sodium hypochlorite for 1 min, and again washed with 70% ethanol for 1 min, rinsed 3 times with sterile distilled water, and finally air-dried in a laminar flow chamber. Then the samples were grounded with a pestle and mortar. After that samples were mixed with 0.9% NaCl, transferred to Petri dishes with humic acid-vitamin (HV) agar [19] containing 25 µg·ml⁻¹ of nystatin, cycloheximide, and nalidixic acid and incubated at 28°C for 3 weeks. The actinomycete colonies grown were picked up, purified on ISP-2 medium [20], and used for further study.

The QSI assay and antibacterial activity. Each strain was tested for anti-quorum sensing and antibacterial activities. The antibacterial activity was tested by the paper disc diffusion method against the gram-positive bacterium Micrococcus luteus and gram-negative bacterium Chromobacterium violaceum CV026. The QSI activity was tested using Chromobacterium violaceum CV026 and the N-Acyl homoserine lactone auto-inducer. Extracts of active strains were loaded onto TLC plates in a narrow band and run using the system of chloroform: methanol (10:1, v/v), then active spots were visualized with the bioautography method.

Identification of bioactive compounds. The most active strains were grown in the liquid yeast extract-malt extract medium, then extracted using ethyl acetate and analysed by HPLC. The 1H-NMR analysis was employed using a 600 MHz Bruker Ascend 600 spectrometer (Bruker BioSpin, Rheinstetten, Germany), operating at a frequency of 600.30 MHz at a temperature of 299 K. For each sample, 64 scans were recorded using an acquisition time, a pulse width, and a relaxation delay parameters. The compound identification was aided by SciFinder online databases.

The PCR amplification and 16S rRNA gene sequence analysis. DNA isolation was performed using the protocol of Zhu et al. [21]. Universal primers 10F (5’-AGTTTGATCCTGCGCTC-3’) and 1541R (5’-AAGGAGGTGATCCAGGC-3’) were used for amplification of 16S rRNA gene fragments. PCR cycling conditions were as following: one cycle with 5 min at 95°C, 35 cycles with 10 sec at 98°C, 10 sec at 50°C, 2 min at 68°C, and 10 min at 68°C. The PCR products were purified and sequenced on the Wizard SV Gel and PCR Clean-Up System (Promega), Big Dye XTerminator (Applied Biosoystems), 3500 Genetic Analyzer (Applied Biosoystems).
3. Results and Discussion

3.1. Number and activity of isolates
Over 800 actinomycete strains were isolated from 53 different plants of the Mongolian Gobi Desert areas. Most strains (65.8%) were isolated from roots, 13.4% from leaves, 12.9% from stems, and 7.9% from flowers. Our result confirmed that using this method (see above) the majority of actinomycetes can be isolated from roots [12].

Results of screening of each strain for the QSI and antibacterial activities showed that 11.9% of our actinomycete strains had the anti-quorum sensing activity and 23.8% of strains were active against the gram-positive and negative bacteria tested. Furthermore, these strains also showed anti-QS and antibacterial activities with the bioautography assay (figure 1).

Figure 1. Antimicrobial and QSI activities of strain No. 2657 isolated from the root of Astragalus variabilis.

A: the first screening of endophytic actinomycetes using the agar blocks on ISP 2 medium;
B: QSI assay confirmed by the paper disk;
C: extract checked by bioautography assay (TLC, CHCl₃: MeOH, 10:1, v/v).

The 16 strains exhibiting the highest QSI and antimicrobial activities were analysed by HPLC. As a result, it was found out that all of them produced flavonoids and phenolic compounds (figure 2).

Figure 2. HPLC chromatograms of strains No. 3050 and No. 2556.
A – strain No. 3050 isolated from Spongiocarpella grubovitii: acidic, alkaline, and neutral extracts;
B – strain No. 2556 isolated from Astragalus variabilis: acidic extract.

Our results suggest that these actinomycetes could be potential candidates for the production of unique biologically active compounds.
3.2. Diversity of isolates

The molecular diversity of 123 strains was identified. Based on the partial 16S rRNA gene sequences they were assigned to 12 genera of 7 families of actinobacteria: *Streptomyces*, *Promicromonospora*, *Micromonospora*, *Streptosporangium*, *Kribbella*, *Pseudonocardia*, *Nocardia*, *Micromonospora*, *Saccharothrix*, *Friedmanniella*, *Actinocatenispora*, and *Geodermatophilus*, the latter two genera were registered in Mongolia for the first time. The genus *Actinocatenispora* was isolated for the first time from plants.

| Strain No. | Isolated plant name     | Plant part | Genus     | Similarity, % | Nucleotide, bp |
|------------|-------------------------|------------|-----------|---------------|----------------|
| 2205       | *Vincetoxicum sibiricum*| Root       | Nocardia sp. Acta 3026 | 97            | 347            |
| 2642       | *Astragalus variabilis*  | Root       | Streptomyces      | 95            | 237            |

Among isolates identified 2 strains exhibited low sequence similarities (<95-97%) with validly published species based on the partial 16S rRNA gene sequences demonstrating a high probability to be new taxa (table 1).

4. Conclusion

The results of isolation of actinobacteria from 53 plants of the desert and mountain areas of Mongolia revealed that they were rich in actinobacteria: over 800 strains were isolated. Strains isolated had anti-quorum sensing (11.9%) and antibacterial (23.8%) activities. Several strains with high activities studied by HPLC produced flavonoids and phenolic compounds suggesting that they could be potential candidates for further study. One hundred twenty-three strains studied were assigned to 11 genera. Among them, the genera *Actinocatenispora* and *Geodermatophilus* were found in Mongolia for the first time. Moreover, the genus *Actinocatenispora* was isolated for the first time from plants.

The investigation continues and we expect that new actinobacteria producing unique biologically active compounds could be found.

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