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Article

Diversity and physiological properties of soil actinobacteria in Ulleung Island

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울릉도 유래 토양 방선균의 다양성과 생리활성

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Actinobacteria tolerating extreme conditions can be a rich source of bioactive compounds and enzymes. In this study filamentous actinobacteria were isolated from soils of Ulleung Island, and their physiological properties were examined. Soil samples were collected, serially diluted and spread on various agar media. The average viable counts of total bacteria were 1.28×10^7 CFU/g for soil sample 1 (ULS1) and 2.05×10^7 CFU/g for soil sample 2 (ULS2). As a result, 34 strains of actinobacteria were isolated and assigned to the genera *Streptomyces* (16 strains), *Isoptericola* (5 strains), *Rhodococcus* (4 strains), *Agromyces* (3 strains), *Micrococcus* (2 strains), *Arthrobacter* (1 strain), *Williamsia* (1 strain), *Microbacterium* (1 strain), and *Oerskovia* (1 strain) based on 16S rRNA gene sequence analysis. Enzyme activity and plant growth promoting potential were tested for representative isolates. Multiple strains of *Streptomyces* degraded starch, casein and Tween 80. As for plant growth promoting potential, strains of *Oerskovia*, *Williamsia*, *Isoptericola*, and *Streptomyces* solubilized phosphate, and those of *Agromyces*, *Oerskovia*, *Micrococcus*, *Rhodococcus*, *Streptomyces*, and Isoptericola produced 3-indole-acetic acid (IAA), respectively. Selected strains of *Streptomyces* exhibited strong antagonistic activity against *Staphylococcus aureus* and *Bacillus subtilis* as well as *Candida albicans*. This study confirms that actinobacteria from Ulleung Island can be a good source of novel bioactive compounds.

Keywords: *Streptomyces*, actinobacteria, antimicrobial activity, plant growth promoting potential, Ulleung Island

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinobacteria are an important group producing antibiotics of agricultural and medicinal importance. Filamentous bacteria within the class actinobacteria are aerobic Gram positive bacteria forming extensively branching mycelia, and widely distributed in both terrestrial ecosystems, mainly in soil, where they play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers derived from dead plants, animals and fungal materials (Goodfellow, 2012). Actinobacteria have been unceasingly reported as the most important producers of microbial bioactive secondary metabolites for potential agricultural, pharmaceutical and industrial applications (Gesheva and Gesheva, 2000; Balachandran *et al*., 2012; Dasari *et al*., 2012). More than two-thirds of commercially available active compounds have been originated from actinobacteria (Demain and Sanchez, 2009).

Ulleung Island is a dormant volcanic island, which is far

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from the mainland Korea, and has unique flora and fauna. The island is thus expected to have unique microbial communities, but little is known on the microbial diversity, in particular in soil environment. In this study, taxonomic diversity and physiological properties of filamentous actinobacteria isolated from Ulleung Island soils were examined, and the antimicrobial activities and hydrolysis of macromolecules were evaluated for the isolates.

Materials and Methods

Collection of soil samples

Two soil samples were collected from Ulleung Island, Republic of Korea in February 2016, and designated ULS1 (GPS 37° 29′ 19.7″ N, 130° 54′ 14.7″ E) and ULS2 (GPS 37° 28′ 44.4″ N, 130° 51′ 16.8″ E) (Fig. 1). The soil samples (2~5 cm depth) were then transported to the laboratory in sterile plastic bags under refrigerated conditions.

Soil pH measurement

Soil samples were suspended in sterile Ringer's solution (2.25 g NaCl, 0.12 g $CaCl_2$, 0.105 g KCl, 0.05 g $NaHCO_3$ per L distilled water) and rotated for 1 h. Hydrogen ion concentrations of each supernatant were measured by pH meter (ORION 3 STAR pH benchtop, Thermo Fisher Scientific).

Isolation and maintenance of isolates

One g of soil was suspended in Ringer's solution, serially diluted and inoculated on tryptic soy agar (TSA; BD), nutrient agar (NA; BD), Reasoner's 2A agar (R2A; BD), yeast extract-malt extract agar (ISP2; BD), starch casein agar (SCA; MB cell), and actinomycete isolation agar (AIA; MB cell). All media were supplemented with cycloheximide (50 μg/ml) as an antifungal agent. The inoculated plates were incubated at 28~30°C for 2 weeks. Actinobacterial colonies were selected on morphological basis (Shirling and Gottlieb, 1966) and purified on tryptic soy agar by restreaking under the same conditions. Isolates were preserved as 20% (v/v) glycerol suspension at -70°C.

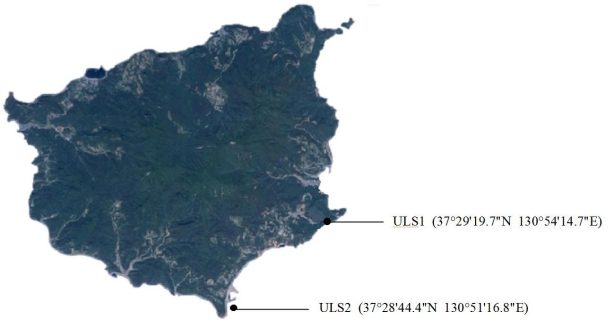

Fig. 1. Sampling sites (ULS1 and ULS2) in Ulleung Island.

DNA extraction, PCR amplification, and sequencing of 16S rDNA

The extraction of total genomic DNA from the isolated strains and PCR amplification were performed via the previously described procedures (Kim *et al*., 2014). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′). The PCR amplification conditions consisted of an initial denaturation step at 95°C for 3 min, which was followed by 30 cycles of 95°C for 1 min, 55°C for 40 sec and 72°C for 1 min. The purified PCR products were sequenced using the service of Macrogen. The 16S rRNA gene sequences of the isolates were compared with other sequences in the EzTaxon database (http://eztaxon-e.ezbiocloud.net; Kim *et al*., 2012) based on the pairwise alignment method.

Phylogenetic analysis

The sequences were proofread, edited and merged into full length sequences using the Ez-Editor program version 2.0. The sequences were aligned with those of reference taxa retrieved from public databases. Distance based phylogenetic trees were generated using the distance model of Jukes and Cantor (1969) and neighbor-joining algorithm (Saitou and Nei, 1987). The tree reliability was estimated by bootstrapping with 1,000 replicates.

Production of hydrolytic enzymes

Enzyme activities for degradation of macromolecules were tested. Cellulase activity was tested using the CMC medium of Criquet (2002), protease activity by casein degradation using

the skim milk agar (50 ml sterilized skimmed milk mixed with 50 ml of 1/5 tryptic soy agar, the final agar concentration adjusted to 2%), amylase activity using the soluble starch agar (BD), lipase activity using TSA with 1% Tween 80 or 1% tributyrin, and DNase activity using DNase agar (BD), respectively.

Plant growth promoting potential

Phosphate-solubilizing activity was tested using the chemically defined medium (NBRIP) of Sulbarán *et al.* (2009). Clearing zones were detected after incubation at 30°C for 7 days for all tests. The production of indole-3-acetic acid (IAA), a plant growth hormone, was determined using the Salkowski colorimetric technique developed by Glickmann and Dessaux (1995). Actinobacterial isolates were inoculated to tryptic soy broth containing 0.2% L-tryptophan and incubated at 30°C with shaking at 120 rpm for 2 weeks. Cultures were centrifuged at 12,000 rpm for 10 min. One ml of the supernatant was mixed with 2 ml of Salkowski reagent. Appearance of a pink color indicated IAA production. Optical density (OD) was recorded at 535 nm using a spectrophotometer. The IAA concentration was determined according to the standard curve with known concentrations of IAA (range of 0~30 mg/L with 5 mg/L intervals). The production of siderophore, a chelator of iron, was determined using the chrome azurol S (CAS) agar plate assay developed by Schwyn and Neilands (1997). On medium containing CAS-substrate, actinobacterial isolates were inoculated and incubated at 30°C for 10 days. The colonies with orange halos were considered as siderophore-producing isolates.

Antimicrobial activity

The antagonistic activities of the isolates were evaluated using the agar diffusion assay. Test organisms obtained from the Korea Collection for type Cultures (KCTC) and the Culture Collection of Antimicrobial Resistant Microbes (CCARM) were used. The isolates were challenged against three Gram-negative bacteria *Klebsiella pneumoniae* KCTC 2208, *Salmonella enterica* KCTC 12456, and *Pseudomonas aeruginosa* CCARM 0222, three Gram-positive bacteria, *Bacillus subtilis* CCARM 0003, *Micrococcus luteus* CCARM 0022, and *Staphylococcus aureus* CCARM 0022, and two yeasts, *Candida albicans* KCTC 7270 and *Candida krusei* CCARM 14017. On tryptic soy agar plates with lawns of test microbes, individual isolates were spotted and incubated for 7 days at 30°C. Antagonistic activity was observed by measuring the inhibition zone after 7 days. The results were presented as described by Kumar *et al*. (2014).

Results and Discussion

Soil characteristics and viable counts

The pH of ULS1 soil was 8.04, and that of ULS2 soil was 8.24. The viable counts of total bacteria were 8.00×10^5~2.23×10^7 CFU/g for ULS1 and 2.00×10^5 CFU/g~6.70×10^6 CFU/g for ULS2, respectively (Fig. 2). The viable count of ULS1 soil was highest in R2A medium and that of ULS2 soil was highest in tryptic soy agar medium, respectively. The number and morphological diversity of bacteria isolated from ULS1 soil were higher than from ULS2 soil. The viable counts were in the similar ranges with those recorded for various soil samples in previous studies (Park *et al*., 1998; Han *et al*., 2008; Han, 2015).

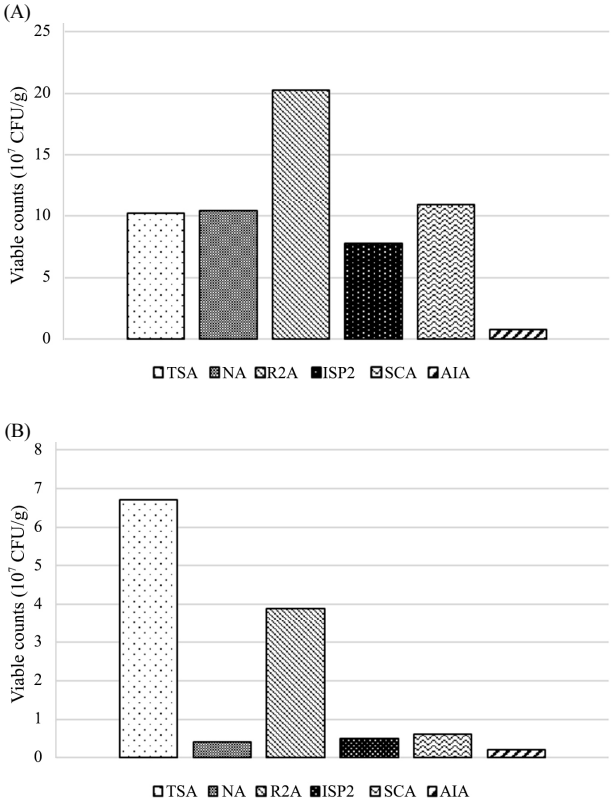

Fig. 2. Viable counts of total bacteria in Ulleung Island soils. (A) site ULS1; (B) site ULS2.

Phylogenetic analysis using 16S rRNA gene

From the two soil samples, 34 isolates of actinobacteria were obtained. The classification results of the isolates based on 16S rRNA gene sequences are shown in Figs. 3, 4, and 5. In the phylogenetic trees, the isolates fell into 22 distinctive phylogenetic clusters within the taxonomic variation encompassed 9 actinobacterial genera, and designated as Clusters 1–22. Each cluster potentially represented an independent species, and there were a number of candidates for novel species, as those formed independent phylogenetic lines in the trees. Clusters 1, 2, and 3 were assigned to the genus *Rhodococcus* and Cluster 4 was assigned to the genus *Williamsia* (Fig. 3). Clusters 5, 6, and 7 were assigned to the genus *Microbacterium* and Clusters 8 and 9 were assigned to the genus *Agromyces*, respectively. Cluster 10 was assigned to the genus *Micrococcus*, Clusters 11 and 12 were assigned to the genus *Oerskovia* and Clusters 13 and 14 were assigned to the genus *Ispotericola* (Fig. 4), respectively. Clusters 15~22 were assigned to the genus *Streptomyces* (Fig. 5).

In summary, all actinobacterial isolates were assigned to the genera *Streptomyces* (16 strains), *Isoptericola* (5 strains),

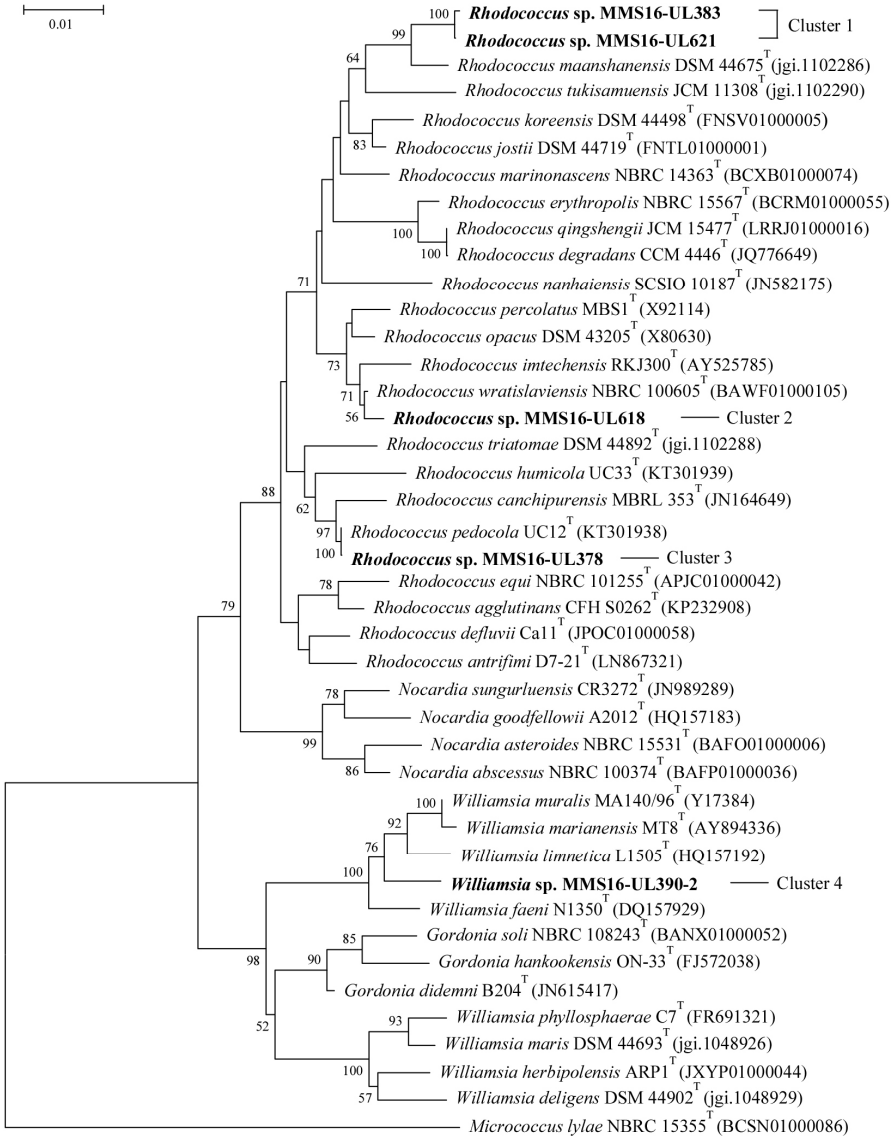

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships among isolates belonging to the order *Corynebacteriales* and related taxa. Numbers at nodes indicated bootstrap percentage (above 50%) based on 1,000 resampled data sets. Bar, 0.01 substitutions per nucleotide position.

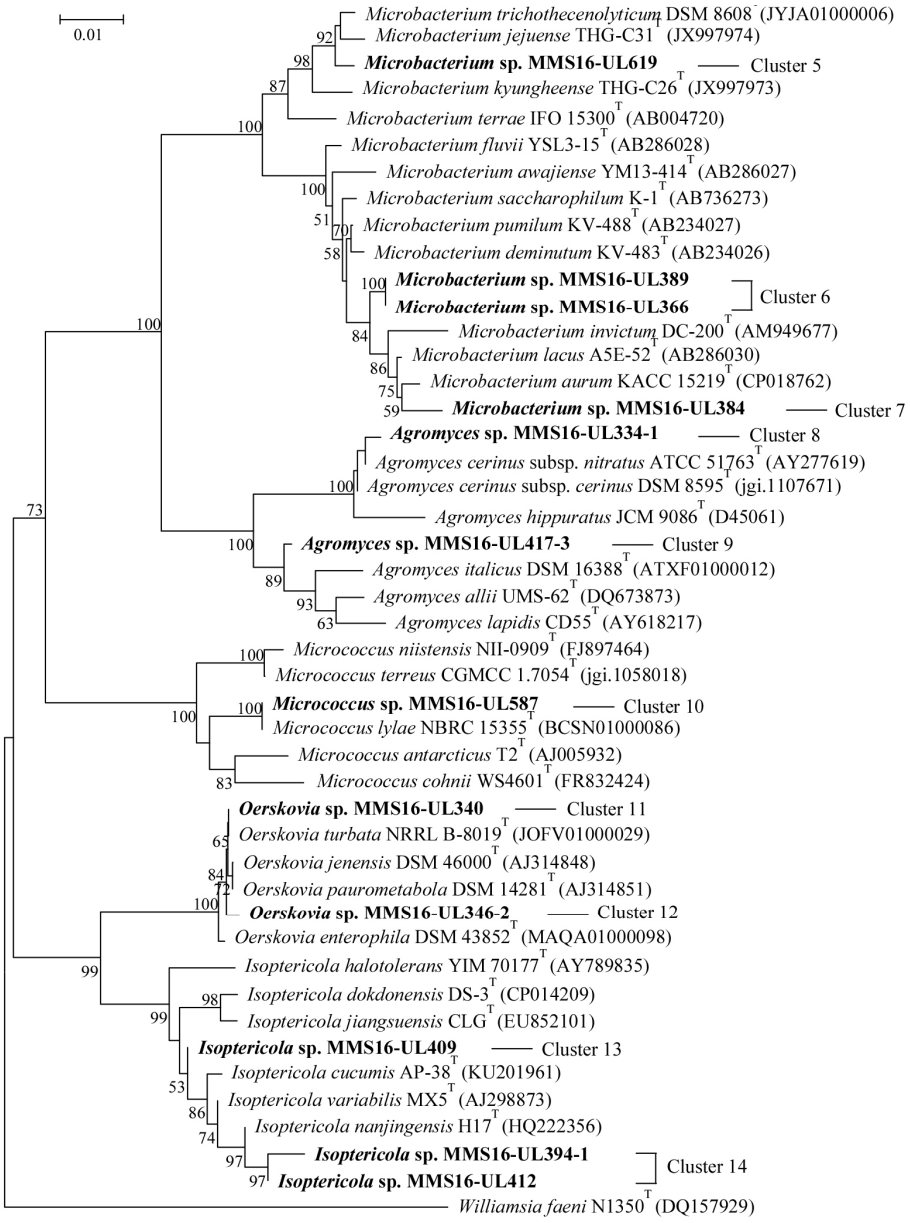

Fig. 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships among isolates belonging to the order *Micrococcales* and related taxa. Numbers at nodes indicated bootstrap percentage (above 50%) based on 1,000 resampled data sets. Bar, 0.01 substitutions per nucleotide position.

Rhodococcus (4 strains), *Agromyces* (3 strains), *Micrococcus* (2 strains), *Arthrobacter* (1 strain), *Williamsia* (1 strain), *Microbacterium* (1 strain), and *Oerskovia* (1 strain). Notably, most taxa were non-filamentous, pleomorphic groups except for *Streptomyces* and *Oerskovia* (Goodfellow, 2012). The results demonstrate that actinobacterial strains inhabiting Ulleung Island soil encompass unique and diverse taxonomic groups.

Physiological properties of the actinobacterial isolates

Most strains showed good hydrolytic enzyme activities for protease (58.8%), esterase for Tween 80 (79.4%), and DNase (61.8%) (Table 1). Sixteen strains (47.1% of the total isolates) exhibited phosphate solubilization activity, and 25 strains (73.5% of the total isolates) produced IAA, with the concentration in the range of 0.2~61 mg/L (Table 1). Among them, *Streptomyces* was the most dominant producers of IAA (32%), which was

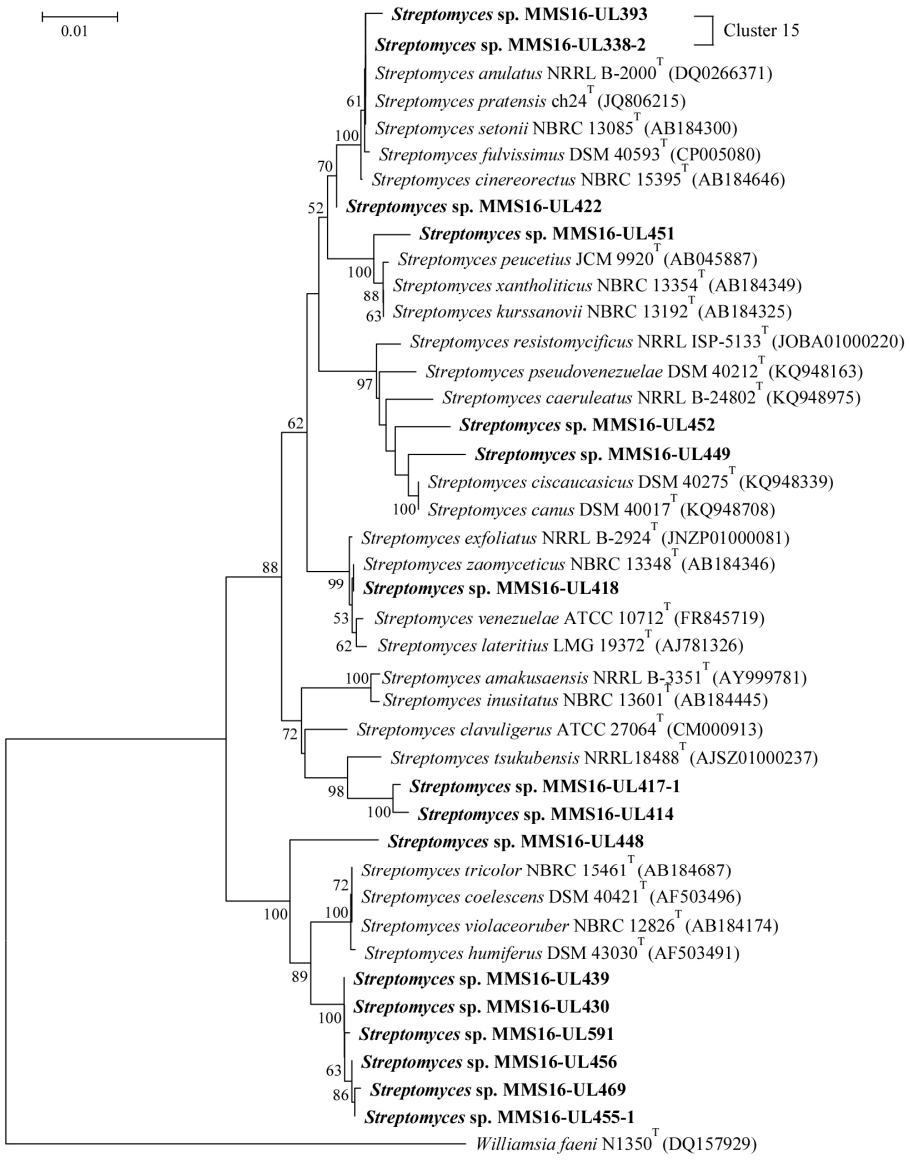

Fig. 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships among isolates belonging to the order *Streptomycetales* and related taxa. Numbers at nodes indicated bootstrap percentage (above 50%) based on 1,000 resampled data sets. Bar, 0.01 substitutions per nucleotide position.

then followed by *Agromyces* (12%), *Rhodococcus* (12%), *Isoptericola* (8%), *Micrococcus* (8%), *Microbacterium* (4%), *Oerskovia* (4%), and *Williamsia* (4%), respectively. *Micrococcus* sp. UL375-3 showed highest ability to produce IAA.

Siderophores are produced by diverse soil microbes to bind Fe^{3+} from the environment, transport it back to the microbial cell and make it available for growth. Microbial siderophores may also be utilized by plants as an iron source (Leong, 1996). Siderophore production was observed in 14 strains (41.2%) (Table 1). Among them, strains of *Isoptericola* were the most prominent producers (35.7%), followed by those of *Streptomyces* (28.6%), *Rhodococcus* (14.3%), *Agromyces* (14.3%), and *Micrococcus* (7.1%), respectively. It was notable that sierophore production from *Isoptericola* has never been reported before.

Antimicrobial activity of the actinobacterial isolates against pathogens

Six strains (17.6%) of actinobacterial isolates were active against at least one of the pathogenic bacteria or fungi. Two isolates, *Streptomyces* sp. UL338-2 and *Oerskovia* sp UL346-2

Table 1. Phylogenetic affiliation of total isolates from ULS1 and ULS2 soils based on 16S rRNA gene sequence similarity, hydrolysis of macromolecules, plant growth promoting activities, and antimicrobial activities

Strain	Closest match	Sim. (%)	Hydrolytic enzyme				Plant growth promoting activity*			Antimicrobial activity**					
			Amylase	Protease	Lipase	DNase	P	S	IAA (ppm)	1	2	3	4	5	6
UL378	*Rhodococcus canchipurensis* MBRL 353^T	99.31	-	-	+++	+	-	+	10.27	-	-	-	-	-	-
UL508	*Rhodococcus globerulus* DSM4954^T	99.93	-	+++	+	+	-	++	5.30	-	-	-	-	-	-
UL618	*Rhodococcus wratislaviensis* NBRC 100605^T	99.72	-	+	-	-	-	-	-	-	-	-	-	-	-
UL621	*Rhodococcus maanshanensis* DSM44675^T	99.14	-	-	+++	+	+	+	44.03	-	-	-	-	-	-
UL390-2	*Williamsia faeni* N1350^T	98.67	-	-	+++	-	+	+	5.30	-	-	-	-	-	-
UL334-1	*Agromyces cerinus subsp. cerinus* DSM 8595^T	99.60	-	-	+++	+	-	+	2.18	-	-	-	-	-	-
UL359	*Agromyces iriomotensis* IY07-20^T	99.52	-	++	-	+++	-	+	1.28	-	-	-	-	-	+
UL390-1	*Agromyces terreus* DS-10^T	99.51	-	+	+++	+	+	+	1.34	-	-	-	-	-	-
UL619	*Microbacterium trichothecenolyticum* DSM 8608^T	99.59	-	+	+++	+++	+	-	3.16	-	-	-	-	-	-
UL375-3	*Micrococcus yunnanensis* YIM 65004^T	99.70	-	+++	+	++	-	+	61.05	-	-	-	-	-	-
UL587	*Micrococcus lylae* DSM 20315^T	99.93	-	-	+	+++	-	+	5.30	-	-	-	-	-	-
UL346-2	*Oerskovia turbata* NRRL B-8019^T	99.65	-	+++	+++	+++	+	++	13.65	-	-	-	-	+	+
UL610	*Arthrobacter kerguelensis* KGN 15^T	99.37	-	-	-	-	-	-	-	-	-	-	-	-	-
UL394-1	*Isoptericola nanjingensis* H17^T	99.63	-	+	+++	++	+	++	2.24	-	-	-	-	-	-
UL396	*Isoptericola nanjingensis* H17^T	99.25	-	+	+++	++	+	++	3.16	-	-	-	-	-	-
UL409	*Isoptericola variabilis* MX5^T	99.56	-	+	+++	++	+	+	11.77	-	-	-	-	-	-
UL412	*Isoptericola nanjingensis* H17^T	100	-	-	++	++	+	+	17.35	-	-	-	-	-	-
UL461	*Isoptericola nanjingensis* H17^T	99.23	-	++	+++	++	+	++	6.83	-	-	-	-	-	-
UL338-2	*Streptomyces fulvissimus* DSM 40593^T	99.93	-	++	+++	+	+	+++	1.51	-	-	-	-	+	+
UL407	*Streptomyces ciscaucasicus* NBRC 12872^T	99.93	-	+++	+++	-	+	++	12.76	-	-	-	-	-	-
UL413	*Streptomyces anulatus* NRRL B-2000^T	100	+	++	++	-	-	-	-	-	-	-	-	-	-
UL414	*Streptomyces tsukubensis* NRRL 18488^T	99.30	+	-	-	-	-	-	-	-	-	+	+	+	-
UL417-1	*Streptomyces tsukubensis* NRRL 18488^T	99.28	-	+++	+++	+	-	+	2.44	-	+	-	-	-	-
UL418	*Streptomyces zaomyceticus* NBRC 13348^T	100	+	++	++	-	-	-	-	-	-	-	-	-	-
UL445-1	*Streptomyces coelescens* DSM 40421^T	100	-	-	+++	+	+	+	12.35	-	-	-	-	-	-
UL448	*Streptomyces thinghirensis* DSM 41919^T	99.51	+	-	+	-	-	-	-	-	-	-	-	-	-
UL449	*Streptomyces canus* NRRL B-1989^T	99.38	+	+	-	-	-	-	-	-	-	-	-	-	-
UL452	*Streptomyces canus* NRRL B-1989^T	99.93	+	-	-	-	-	-	-	-	-	-	-	-	-
UL456	*Streptomyces anthocyanicus* ATCC 19821^T	100	+	+	++	+	-	-	-	-	-	-	-	-	-
UL460	*Streptomyces ciscaucasicus* NBRC 12872^T	99.51	-	+++	++	-	-	+	5.24	-	-	-	-	-	-
UL468	*Streptomyces coelescens* DSM 40421^T	100	+	-	++	-	-	-	-	-	-	-	-	-	-
UL469	*Streptomyces tricolor* DSM 40421^T	100	-	+++	+	+	+	++	8.68	+	-	-	-	-	-
UL591	*Streptomyces coelescens* DSM 40421^T	99.93	-	-	-	-	-	-	-	-	-	-	-	-	-
UL617	*Streptomyces trurgidiscabies* ATCC 700248^T	98.51	-	+++	++	+	+	-	6.25	-	-	-	-	-	-

*P, phosphate solubilization; S, siderophore production.

**1, *Klebsiella pneumoniae* KCTC 2208; 2, *Pseudomonas aeruginosa* CCARM 0222; 3, *Bacillus subtilis* CCARM 0003; 4, *Staphylococcus aureus* CCARM 3089; 5, *Candida albicans* KCTC 7270; 6, *Candida krusei* CCARM 14017. For hydrolytic enzyme activities and siderophore production, +, 1-3 mm; ++, 4-6 mm; +++, ≥ 7 mm.

were antagonistic against *Candida albicans* KCTC 7270 and *Candida krusei* CCARM 14017. In addition, *Agromyces* sp. UL359 was antagonistic against *Candida krusei* CCARM. *Streptomyces* sp. UL414 was antagonistic against fungi and two Gram-positive pathogenic bacteria, *Candida albicans* KCTC 7270, *Bacillus subtilis* CCARM 0003 and *Staphylococcus aureus* CCARM 3089, respectively. *Streptomyces* sp. UL417-1 was antagonistic against *Pseudomonas aeruginosa* CCARM 0222. *Streptomyces* sp. UL469 was antagonistic against *Klebsiella pneumonia* KCTC 2208. Strains of *Oerskovia* and *Agromyces* were antagonistic against fungi or bacteria, although strains of *Streptomyces* formed the majority of isolates with antimicrobial

activities. More detailed investigation is required to demonstrate the potential of these organisms for the biocontrol of pathogenic fungi or bacteria.

Conclusion

This study is the first report on the culturable diversity of actinobacteria in Ulleung Island soils. High level of taxonomic diversity of actinobacteria, and also high activities of hydrolytic enzymes were confirmed through this study. Most strains which exhibited antimicrobial activities showed sequence variations from the type strains of closest known species, thus implying the possibility as producers of novel antimicrobial compounds.

적 요

본 연구에서는 경상북도 울릉군에서 분리한 토양 방선균에 대해 생리학적 특징과 다양성에 대해 연구하였다. ULS1 및 ULS2로 명명한 2개의 토양 시료를 채취하여 다양한 배지에 배양하여 분리하였으며, 평균 생균수는 ULS1 토양은 1.28×10^7 CFU/g, ULS2 토양은 2.05×10^7 CFU/g였다. 16S rRNA 유전자에 기반한 염기서열 분석 결과, 총 9개의 속에서 34개의 균주가 분리되었으며 해당 속은 *Streptomyces* (16 균주), *Isoptericola* (5 균주), *Rhodococcus* (4 균주), *Agromyces* (3 균주), *Micrococcus* (2 균주), *Arthrobacter* (1 균주), *Williamsia* (1 균주), *Microbacterium* (1 균주) 및 *Oerskovia* (1 균주)에 속하는 것을 알 수 있었다. 다양한 효소활성과 식물 생장 촉진 활성 측정 결과, 전체의 58.8%가 단백질 분해 활성을, 79.4%가 Tween 80 분해 활성을, 그리고 61.8%가 DNA 분해 활성을 각각 가지는 것으로 나타났다. *Oerskovia*, *Williamsia*, *Isoptericola* 및 *Streptomyces* 속에 속하는 분리주들로부터 인을 가용화시키는 능력을 확인할 수 있었으며, *Agromyces*, *Oerskovia*, *Micrococcus*, *Rhodococcus*, *Streptomyces* 및 *Isoptericola* 속에 속하는 분리주들은 식물호르몬인 3-indole-acetic acid (IAA)를 생산하는 것을 확인할 수 있었다. *Streptomyces* 속에 속하는 분리주들은 *Candida albicans* 뿐만 아니라 *Staphylococcus aureus*와 *Bacillus subtilis*에 항생활성을 나타내었다. 본 연구는 독특한 생태계를 구성하는 울릉도 지역의 토양 방선균 다양성 및 생리 활성에 대한 최초의 연구로서 의미를 가지며, 새로운 유용 생리 활성 물질의 좋은 원천이 될 것이라 사료된다.

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