**Introduction**

Lichens are symbiotic organisms that are composed of a fungus and an alga, and they can survive in extreme environments such as tropical, desert, and polar regions. Lichens and their natural products have been used as cosmetics, decorations, dyes, foods, and medicines (Oksanen, 2006). They have been attracting the attention of many researchers because of their diverse pharmaceutical potentials as shown in their antiviral, anti-proliferative, anti-inflammatory, anti-tumor, and antimycobacterial activities (Ingólfsdóttir et al., 1998; Lauterwein et al., 1995; Lawrey, 1989; Molnár and Farokh, 2010; Morita et al., 2009). Also, lichens are considered to be a source of natural antioxidants because of their inhibitory effects against microorganisms, their antimicrobial activities have also been widely investigated. In general, the natural products from lichens are their secondary metabolites, called "lichen substance", and most of these come from the fungal symbionts in lichens (Luo et al., 2011; Stocker-Wörgötter, 2008).

Recent molecular studies have suggested a variety of bacterial communities among lichens, including their structural and ecological contributions. For example, Gonzales et al. (2005) described the diversity of actinomycetes among lichens by DNA fingerprinting; Cardinale et al. (2006 and 2008) showed that diverse bacteria are present in lichens by the analysis of their ribosomal internal transcribed spacer polymorphism and by fluorescence in situ hybridization analysis. Grube et al. (2009) also analyzed and compared the structure and composition of associated bacterial communities of some lichen species using combined microscopic and molecular techniques; however, their biological activities have not been studied in detail. The biological activities and bacterial diversity of Arctic lichen *Ochrolechia* sp. are still unknown. In previous studies, we screened...
nine bacterial isolates from Arctic lichen Cladonia sp., Stereocaulon sp., Stereocaulon sp., and Umbilicaria sp. and evaluated their antibacterial and antioxidant activities. We found that bacterial isolates from lichen exhibited some antibacterial activities against Gram-positive and Gram-negative clinically isolated microorganisms. They also showed better antioxidant activity than the control ascorbic acid (vitamin C). Therefore, the aim of this study was to screen the microorganisms from lichen Ochrolechia sp., and evaluate their antibacterial potential against six test microorganisms and discern their antioxidant potential.

**Experimental**

**Materials and Methods**

**Collection and identification of lichen samples.** Lichen Ochrolechia sp. was collected in Ny-Ålesund, Svalbard, Dasan Korean Arctic Station (78, 91.140° N/ 011, 94.878° E) by the Korean Polar Research Institute (KOPRI). It was transferred at room temperature and stored at ~20°C until further use. Bacterial isolates were deposited in polar and alpine microbial collection (PAMC).

**Screening of microorganisms associated with lichen.** The isolation of microorganisms was performed by KOPRI. Fragments from lichen thalluses were separated by sterilized scissors or knife. Sterilized 0.85% NaCl solution was added and this was followed by vortexing for 10 min. The solution was then discarded, and the steps above were repeated three times. The tissue was subsequently broken with a mortar in sterilized 0.85% NaCl solution. After spreading the tissue on melt extract-yeast extract (MY) agar media and Reasoner's 2A (R2A) agar, it was incubated at 10°C for 15 to 21 days. To obtain pure single colonies, subculturing was repeated three times and it was preserved at ~80°C in 20% glycerol. The obtained bacterial isolates were identified by 16S rRNA gene sequences analysis. The 16S rRNA gene was amplified from a single colony of pure culture with two universal primers: 27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1492R (5’-GTT TAC CTT GTT ACG ACT T-3’) as described by Lane (1991). Polymerase chain reaction (PCR) was carried out with 25 µl reaction mixtures containing 1X PCR reaction buffer, 200 µM of dNTPs, 0.2 µM of each primer, a single colony as a template, and 1 unit of Taq DNA polymerase (In-Sung Science, Suwon, South Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min and 30 cycles of amplification (95°C for 30 s, 56°C for 30 s, and 72°C for 30 s) and a final extension step at 72°C for 5 min. PCR products were purified with an AccuPrep PCR Purification Kit (Bioneer, Daejeon, South Korea) and sequenced with the same primer used for PCR amplification. The sequence of the 16S rRNA gene was compared with that of type strains available in the database to find closely related species. All sample numbers were given by PAMC in KOPRI.

**Culture and extraction of bacterial isolates.** A total of five bacterial isolates were cultured in 50 ml of MY (4.0 g malt extract, 4.0 g yeast extract, 10.0 g glucose, and 1.0 L, pH 7.2 distilled water) and R2A (0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g dipotassium phosphate, 0.05 g magnesium sulfate-7H₂O, 0.3 g sodium pyruvate, and 1.0 L, pH 7.2 distilled water) liquid media at 15°C for 10 to 15 days. The culture broth was added to a double volume of ethyl acetate which was of analytical grade (Daejeung, Shihueung, South Korea). Extraction was performed individually on each solvent at room temperature and then the layer of culture media was discarded after 2 hours. The solvent layer was concentrated using a rotary evaporator, and the obtained dried crude extract was dissolved in 500 µl of ethyl acetate. The concentrations of extract ranged from 1.2 g/ml to 1/7 g/ml. Each extract was diluted to 1 mg/ml for further experiments.

**Evaluation of the antimicrobial activities of test microorganisms.** Ethyl acetate extracts were tested against the 3 Gram-positive bacteria Staphylococcus aureus (KCTC1927), Bacillus subtilis (KCTC1028), and Micrococcus luteus (KCTC9341), and 3 Gram-negative bacteria Escherichia coli (KCTC1682), Pseudomonas aeruginosa (KCTC1637), and Enterobacter cloacae (KCTC1685) used in this study. All of the six test strains are known as clinical isolates. They were purchased from the Korean Collection for Type Cultures (KCTC) and Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, South Korea). All bacterial isolates were kept on Luria-Bertani (LB; 10.0 g trypton, 5.0 g yeast extract, 10.0 g NaCl, and 1.0 L, pH 7.2 distilled water) at 4°C.

**Paper disk diffusion test.** The paper disk diffusion test was performed according to Bauer et al. (1966) with some modifications. All reagents were purchased from Difco (Franklin Lakes, USA). Bacterial cells were standardized to 0.5 McFarland and then mixed with soft agar (0.04 g/ml). 9 ml of this mixture was inoculated onto Mueller-Hinton agar plates. Afterwards, each extract was loaded into paper disks (6 mm in diameter, Advantec, Osaka, Japan) and transferred onto the plates inoculated with the bacterial strains. Disks loaded with ethyl acetate were used as a control. All inoculated culture plates were incubated at 37°C, and the inhibition zones of bacterial growth were measured after 12 to 18 h.

**Minimum inhibitory concentration (MIC) test.** MIC was determined by the broth dilution method.
All analyses were carried out according to the modified Benzie and Strain method (1996) while all of chemical reagents were purchased from Sigma-Aldrich (St. Louis, USA). The absorbance of reaction mixture was measured at 593 nm using a UV/Vis spectrophotometer. Temperature was maintained at 37°C. The readings at 30 min were selected for the calculation of FRAP values.

**Statistical analysis.** All analyses were carried out in triplicates. The experimental values are the mean ± standard deviation (SD). Statistical comparisons using one-way analysis of variance (ANOVA) with p < 0.05 were regarded as significant, with p < 0.01 being highly significant.

**Results and Discussion**

**Evaluation of antibacterial properties.** A total of five bacteria were isolated from the Arctic lichen *Ochrolechia* sp (Table I). To evaluate the antibacterial potential of extracts from our bacterial isolates, a paper disk diffusion test and an MIC test were carried out. In the paper disk diffusion test, all of the five bacterial isolates showed antibacterial activities against both Gram-positive (*S. aureus, B. subtilis, and M. luteus*) and Gram-negative (*E. cloacae, P. aeruginosa, and E. coli*) bacteria. No antibacterial activity was observed in the control (disk without extract, only ethyl acetate), while the antibacterial strengths of our extract were similar with the zone of inhibition diameter ranging from 8 to 12 mm (Table II). Also, while the MIC test was carried out, we used ampicillin as control antibiotics because all of the Gram-positive and Gram-negative tested organisms had sensitive resistance to ampicillin. The MIC for the extracts in the tested bacteria was 222.0 to >1000 μg/ml and all of the extracts inhibited the tested organism at higher concentrations compared to control ampicillin (from 0.31 to 0.58 μg/ml) (Table III). According to the 16S rRNA gene sequencing results, their sequence similarity with the closely related organisms and generated by Basic Local Alignment Search Tool (BLAST) search program (http://blast.ncbi.nlm.nih.gov/Blast).

### Table I

<table>
<thead>
<tr>
<th>PMAC No.</th>
<th>Isolation media</th>
<th>Bacterial species (Closest strain)</th>
<th>Similarity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26605</td>
<td>MY</td>
<td><em>Sphingomonas</em> sp.</td>
<td>98.888</td>
</tr>
<tr>
<td>26606</td>
<td>R2A</td>
<td><em>Burkholderia</em> sp.</td>
<td>98.401</td>
</tr>
<tr>
<td>26607</td>
<td>R2A</td>
<td><em>Burkholderia</em> sp.</td>
<td>99.296</td>
</tr>
<tr>
<td>26608</td>
<td>R2A</td>
<td><em>Burkholderia</em> sp.</td>
<td>99.763</td>
</tr>
<tr>
<td>26625</td>
<td>MY</td>
<td><em>Sphingomonas</em> sp.</td>
<td>99.763</td>
</tr>
</tbody>
</table>

* The value are expressed as sequence similarity with the closely related organisms.
similarities are almost the same and the species name of some of these are also the same. However, the morphological, chemical, and biological characteristics of the bacterial associates are somewhat different (data not shown). There are even differences in antibacterial activities among them. It is possible that media composition such as carbon source and/or nitrogen source may have affected the active compound of each extract. To search for new antibacterial sources from nature, a variety of lichen has been screened (Bhattarai et al., 2013; Celenza et al., 2013; Paudel et al., 2008 and 2010; Sultana and Afolayan, 2011). As for the lichen Ochrolechia species, however, there are few studies about their biological activities and active components (Millot et al., 2007; Ranković et al., 2010). Ranković et al. (2010) studied the antimicrobial activity of some lichens, including Ochrolechia androgyna, but not the bacterial associates of O. androgyna. Although antibacterial activities were lower than those of natural lichen O. adrogyna, the obtained results indicated that the bacterial isolates described in this study had the potential to be a source of treatment for various diseases caused by these clinical isolates or related microorganisms.

Evaluation of antioxidant properties. Many publications concerning the antioxidant activities of lichen have been reported (Behera et al., 2008; Gulluce et al., 2006; Kosanic et al., 2011; Luo et al., 2011; Stocker-Wörgötter, 2008). However, most of them used lichen or their fungal symbionts as their study resource. Although bacterial symbionts are also present in lichens and they contribute to lichens structurally and ecologically (Cardinale et al., 2006 and 2008; Gonzales et al., 2005; Grube et al., 2009), the biological activity of bacterial symbionts is still unexplored. Thus, the antioxidant potential of bacterial symbionts was evaluated. In general, antioxidant activity is dependent on phenolic contents and/or flavonoids contents (Hallqvorsen et al., 2002; Gardner et al., 2000; Pietta, 2000). Thus, TPC and TFC assays were carried out (Table IV). TPC values ranged from 1.07 (PAMC26607, B. sordidicola) to 10.44 (PAMC26625, Sphingomonas sp.) milligrams of GAE per gram of extract, and the TFC value ranged from 0.99 (PAMC26606, B. sordidicola) to 8.95 (PAMC26625, Sphingomonas sp.) milligrams of CE per gram of extract. PAMC26625 had the highest value of TPC and TFC among these extracts while Sphingomonas species had a higher amount of TPC and TFC than Burkholderia species in terms of bacterial species. Through these results, it was expected that PAMC26625 would have the strongest antioxidant activities. PAMC26625 showed 77.23% and 80.02% high, free-radical scavenging activity in the DPPH and ABTS assays (Table IV), respectively (control vitamin C was 29.31%). Extracts that have high amounts of phenolic and/or flavonoid content showed high antioxidant activities as expected. In the case of PAMC26607 and PAMC26605, however, the free radical scavenging activity of PAMC26605 showed stronger activity.
than that of PAMC26607-about 3.1 fold and 3.8 fold in both the DPPH and ABTS assays-while they have comparable values of TPC. It may be that all phenolics do not have same antioxidant activity and some of them may possess strong activity whereas others have weak activity. It is possible that there are synergistic or antagonistic interactions between phenolic compounds or different types of components such as carbohydrates and proteins (Rice-Evans et al., 1995). The FRAP assay was also carried out due to its simplicity and reproducibility (Table IV). The reducing activity of the extract can evaluate based on the theory that antioxidants act as reductants by performing the reduction of ferric ions to ferrous ions (Benzie and Strain, 1996). Therefore, the determination of the ferrous ion formation can be used to predict the reducing power of the samples. The FRAP value of the extract varied from 1.09 to 8.72 mM of ferrous ion per mg of extract, and the significantly higher (p < 0.05) amount of ferrous ion was detected in PAMC26625. Other extracts had a lower amount of ferrous ions than the ascorbic acid that was used as a control (6.81 ± 0.44 mM Fe(II)/mg extract). Finally, the results of this research suggest that the extract of PAMC26625 showed high antioxidant activity compared with control ascorbic acid. Moreover, our obtained results indicate that bacteria from lichen possess antioxidant activity and could be considered as potential sources of natural antioxidants.

### Conclusions

In this paper, the antibacterial and antioxidant potentials of five bacterial symbionts from the lichen *Ochrolechia sp.* were evaluated, with PAMC26625 finally showing the strongest antioxidant activities among these five bacterial isolates. Although the functional compounds were not confirmed, they have the potential to be a novel source of antibacterials and antioxidants.

Paudel et al. (2008) found that the antioxidant activity of crude extract from polar lichen was more effective than other lichen species from tropical and temperate regions. It is expected that bacterial symbionts will follow a similar pattern. Thus, the biological activities of the bacterial symbionts in lichen that can be found in extreme environments from polar to desert can be compared.

It appears that this is the first study investigating the biological activity of bacterial associates isolated from the Arctic lichen *Ochrolechia* sp. Since most identified lichen substances come from the fungal symbionts of lichen, it is a novel approach to search for new antibacterial and antioxidant compounds from nature. In addition to this, this kind of study may help illuminate the unique survival mechanisms of lichen in extreme conditions. Therefore, it is important to screen and isolate microorganisms from lichens and investigate their biological activities in detail.

### Acknowledgments

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### Literature


