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Novel insight into the role of thiamine for the growth of a lichen-associated Arctic bacterium, *Sphingomonas* sp., in the light

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Sphingomonas 속 세균의 명조건 생장에서 티아민의 필수적인 역할

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Bacteria in the polar region are under strong light and ultraviolet radiation. In this study, we investigated the effects of light on the growth of a psychrophilic bacterium, *Sphingomonas* sp. PAMC 26621, isolated from an Arctic lichen *Cetraria* sp. The growth of the strain in the light was lower than that in the dark. Surprisingly, thiamine increased the growth of *Sphingomonas* sp. PAMC 26621 in M9 minimal medium under light conditions. Thiamine increased the growth of the strain in a concentration-dependent manner along with ascorbic acid. N-acetylcysteine had no effect on the growth of the strain in the light. Thiamine and ascorbic acid also increased the activities of glucose-6-phosphate dehydrogenase and superoxide dismutase. The results of this study indicate that thiamine provided by the lichen symbiosis system plays an important role in light-induced oxidative stress in this Arctic bacterium as an antioxidant. Our study provide insight into the biochemistry and physiology of Arctic bacteria under strong light and ultraviolet radiation.

Keywords: *Sphingomonas* sp. PAMC 26621, ascorbic acid, light, NADPH, thiamine

Bacteria can survive the harsh environment of the Arctic and Antarctic regions, which include low temperatures (-34°C to 0°C in the winter and -10°C to 10°C in the summer), low available nutrients, long-term darkness (half of the year in darkness), and exposure to high ultraviolet (UV) radiation (Cary *et al.*, 2010). Polar bacteria cope with colder temperatures via physiological adaptations that include increased polyunsaturated fatty acid content, intracellular solute concentration, and lipopolysaccharide biosynthesis (De Maayer *et al.*, 2014). In addition to colder temperature, high radiation is another challenge that bacteria confront near the polar region. Among bacteria isolated from the Antarctic region, Gammaproteobacteria and Bacteroidetes are generally resistant to solar radiation (Dieser *et al.*, 2010; Musilova *et al.*, 2015), but Alphaproteobacteria and Roseobacter are sensitive to solar radiation (Alonso-Saez *et al.*, 2006).

The effects of light on bacterial growth have been extensively investigated, and UV light as well as white, blue, and red lights have been shown to affect bacterial growth (Northrop, 1957; Lipovsky *et al.*, 2010). Additionally, intensive blue light (wavelength of 415 nm) was lethal to *Staphylococcus aureus*, but a low intensity of the blue light resulted in proliferation of the bacterium (Lipovsky *et al.*, 2010). Moreover, the growth rate of bacteria in light differed depending on carbon sources such as pyruvate and glutamate (Hauruseu and Koblizek, 2012), while the growth rate of *Erythrobacter* sp. NAP1 in medium supplemented with pyruvate did not change, and its

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growth rate in medium supplemented with glutamate was slow (Hauruseu and Koblizek, 2012). However, the role that light plays in polar bacteria remains largely unknown.

In recent years, more psychrophilic microorganisms from the polar and alpine regions have been discovered and deposited into microbial culture collections (Lee *et al.*, 2014). In this study, we investigated the role of thiamine (vitamin B1) and ascorbic acid (vitamin C) in light-induced oxidative stress of a psychrophilic bacterium, *Sphingomonas* sp. PAMC 26621, which was isolated from an Arctic lichen, *Cetraria* sp. on Svalbard Islands (Lee *et al.*, 2012), and belong to the Alphaproteobacteria class. Sunlight affects the structures and diversity of bacterial communities in the lichen (Cardinale *et al.*, 2012; Hodkinson *et al.*, 2012). While the roles of antioxidants in bacterial metabolism are generally well known (Romine *et al.*, 2017), the role of antioxidants in polar bacteria under strong light and UV radiation is largely unknown.

Materials and Methods

Materials

Sphingomonas sp. PAMC 26621 was kindly provided by the Polar and Alpine Microbial Collection (PAMC) of Korea Polar Research Institute (Lee *et al.*, 2012). Reasoner's 2A (R2A) medium was purchased from Kisan Bio. Polyclonal antibody to glucose-6-phosphate dehydrogenase (G6PDH) was purchased from Rockland. Goat polyclonal antibody to rabbit IgG was purchased from Santa Cruz Biotechnology. Ascorbic acid, flavin-mononucleotide (FMN), glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP-Na), nitroblue tetrazolium (NBT), and thiamine were purchased from Sigma. All other reagents were obtained from Sigma, unless otherwise noted.

Bacterial growth

A single colony of *Sphingomonas* sp. PAMC 26621 grown on an R2A plate was suspended into 50 ml of R2A medium. After two days of growth at 15°C with shaking at 225 rpm (OD_{600} = 0.8~1.0), 1 ml culture was used to inoculate 100 ml of freshly prepared M9 medium (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 9.3 mM NH_4Cl, 0.2 M $MgSO_4$) or MT medium (60 mM K_2HPO_4, 37 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 0.017 mM $MgSO_4$, 15 μM thiamine). M9 and MT media are similar in composition, but MT contains thiamine. In all experiments, 0.5% glucose was added to the M9 medium and MT media as the sole carbon source. Bacterial cultures were grown in liquid medium at 15°C with shaking at 225 rpm as the growth of *Sphingomonas* sp. PAMC 26621 on both M9 and MT agar plates were very slow.

Effect of light on bacterial growth

Sphingomonas sp. PAMC 26621 was grown in M9 medium or MT medium under dark and light conditions. For dark conditions, bacteria were grown in an incubator (volume 150 L) without light, whereas light was provided by cool white light-emitting diodes (LED) (20 ml) in the incubator (the distance from the LED source to the top of the flask was 34 cm) with approximately 180 lux fluorescence light through the incubator window measured using a digital illuminance meter (TES). Cells were collected every 24 h and cell densities were measured using a Shimadzu UV-1800 spectrophotometer at a wavelength of 600 nm.

Effect of antioxidants on bacterial growth

Thiamine (10 μM) or ascorbic acid (10 μM) was added to M9 medium to evaluate the effects of antioxidants on the growth of *Sphingomonas* sp. PAMC 26621 under dark and light conditions, respectively, while M9 medium alone was used as a control. Cells were collected every 24 h and cell densities were determined based on the absorbance at 600 nm.

Preparation of crude extracts for enzyme activity

Cultures of *Sphingomonas* sp. PAMC 26621 grown in M9 and MT media were harvested at the mid-log phase and stationary phase by centrifugation at 10,000 rpm for 5 min at 4°C, respectively. After washing the cell pellet with phosphate buffered saline (10 mM Na_2HPO_4, pH 7.2, 137 mM NaCl, 2.7 mM KCl, and 1.8 mM KH_2PO_4), cells were resuspended and sonicated in sonication buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5% glycerol). The crude extracts were centrifuged at 10,000 × *g* for 15 min at 4°C, after which the supernatants were collected and assayed for both G6PDH and

superoxide dismutase (SOD) activities. Total protein concentration was measured by Bradford's method using gamma-globulin as a standard.

G6PDH activity

The G6PDH activity was measured to determine the formation of NADPH at 340 nm in 1 ml of reaction buffer (55 mM Tris-HCl, pH 7.0, 3.3 mM $MgCl_2$, 0.1 mM NADP, and 2 mM glucose 6-phosphate) by adding 150 μg of total cell extract. All assays were conducted at 30°C in triplicate. One unit of G6PDH was defined as the amount of enzyme forming 1.0 μmol of NADPH per min at 30°C.

Western blotting

Crude proteins (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V for 2.5 h. After electrophoresis, the gel was equilibrated for 30 min at room temperature with a transfer buffer (25 mM Tris-HCl, pH 8.5, 190 mM glycine, and 20% methanol) and proteins were transferred onto nitrocellulose membrane at 100 V for 1 h. Subsequently, the membrane was blocked with 3% bovine serum albumin in Tris buffered saline (150 mM Tris-HCl, pH 7.6, and 1.37 M NaCl) with 0.1% v/v Tween 20 at room temperature with gentle agitation for 1 h. The membrane was then incubated with polyclonal antibody to G6PDH (1: 1,000 dilution) overnight at 4°C with gentle agitation. After washing four times with Tris buffered saline containing 0.1% Tween 20, the membrane was incubated with goat anti-rabbit secondary antibody for 1 h at room temperature with gentle agitation. The membrane was washed with Tris buffered saline three times before reaction with 1 ml super signal west pico stable peroxidase solution and 1 ml luminol solution for 1 min, after which the signals were detected using X-ray film.

SOD activity

Crude proteins (30 μg/lane) were loaded into 10% native polyacrylamide gels, after which the samples were electrophoresed at 100 V for 4 h at 4°C. Subsequently, the gel was washed with distilled water three times. Samples were then stained for SOD activity by soaking the gel in 0.1% NBT solution for 20 min, followed by 15 min of immersion in FMN solution (28 μM FMN and 28 mM TEMED in 0.1 M potassium phosphate buffer, pH 7.0) in the dark at 4°C (Weissman *et al.*, 2005). The gel was then illuminated for 10~15 min at room temperature until white bands appeared in the blue background. Finally, SOD isoenzymes were identified by adding inhibitors to the FMN solution, 2 mM KCN to inhibit Cu/Zn-SOD or 8 mM H_2O_2 to inhibit Fe-SOD (Weissman *et al.*, 2005).

Results

Effects of light and antioxidants on the growth of *Sphingomonas* sp. PAMC 26621

To investigate the role of light on the growth of *Sphingomonas* sp. PAMC 26621, the strain was grown in M9 medium or in MT medium with glucose as a sole carbon source at 15°C. Under light conditions, the growth rate of the strain in M9 medium was slow (Fig. 1A), but surprisingly, the growth rate of the strain in the MT medium was significantly increased (Fig. 1B). The cell density of the strain in M9 medium was also lower than that in MT medium. However, under dark conditions, there were no changes in the growth rate of the strain in MT medium, whereas the growth rate of the strain in M9 medium was increased (Fig. 1B). The composition of M9 and MT medium were similar; however, MT medium has thiamine as an antioxidant. It is well known that thiamine and its active phosphate form (thiamine pyrophosphate or TPP) play important roles in metabolic processes (Charles and Peter, 2001; Tunc-Ozdemir *et al.*, 2009) and protecting cells against oxidative stress (Fu *et al.*, 2000; Ma *et al.*, 2012). However, *Sphingomonas* sp. PAMC 26621 is not a thiamine auxotroph because of its growth in M9 medium. To investigate the growth enhancement effect of MT medium on another strain, we measured the growth rate of *Pseudomonas mandelii* JR-1, a psychrotrophic bacterium isolated from natural mineral water (Jang *et al.*, 2012). However, *P. mandelii* JR-1 grown in MT medium showed similar growth rates under both dark and light conditions (Fig. 1C). These results indicate that thiamine plays an important role in the growth of *Sphingomonas* sp. PAMC 26621, possibly as an antioxidant or a ROS scavenger, preventing the light from suppressing cell growth. Thiamine serves as a cofactor in the form of thiamine pyrophosphate for pyruvate dehydrogenase

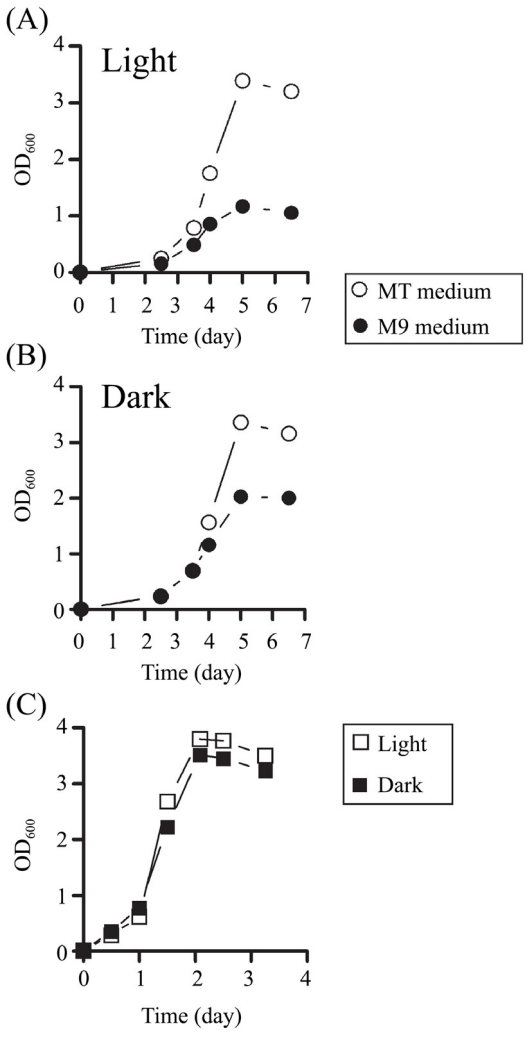

Fig. 1. The growth of *Sphingomonas* sp. PAMC 26621 in M9 medium and MT medium under light condition (A) and dark condition (B). The growth of *Pseudomonas mandellii* JR-1 in MT medium under dark and light conditions (C). Cultures were grown at 15°C and 225 rpm. Cells were collected every 24 h and the growth rate was measured using a Shimadzu UV-1800 spectrophotometer at 600 nm.

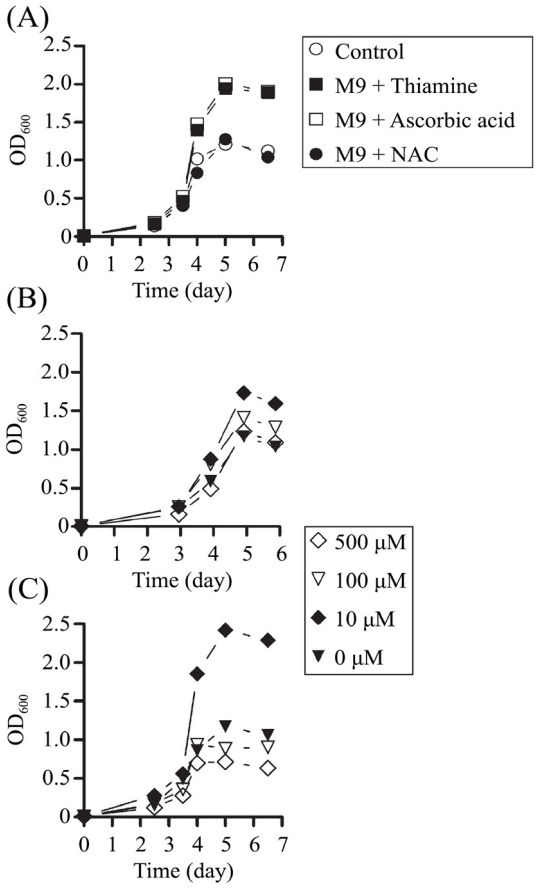

Fig. 2. The effects of antioxidants on the growth of *Sphingomonas* sp. PAMC 26621. Comparison of antioxidant activity of thiamine, ascorbic acid, and N-acethylcysteine (A). The effects of thiamine (B) and ascorbic acid (C) in increasing concentrations on the growth of the strain. Cultures were grown at 15°C at 225 rpm in M9 medium under light condition. Every 24 h, cells were collected and the growth rate was measured using a Shimadzu UV-1800 spectrometer at 600 nm.

(PDH) and α-ketoglutarate dehydrogenase (α-KDH) involved in carbohydrate metabolism (Arjunan *et al.*, 2002; Tretter and Adam-Vizi, 2005), and transketolase in pentose phosphate pathway for the production of NADPH (Schenk *et al.*, 1998). Both PDH and α-KDH are a highly integrated complex of three distinct enzymes having each own prosthetic group, including TPP, lipoamide and FAD. Supplementing lipoic acid to M9 medium instead of thiamine did not show an increase in growth rate under light condition (data not shown), supporting that thiamine worked as an antioxidant or a ROS scavenger.

To evaluate the role of antioxidants in the growth of the strain, ascorbic acid and N-acetyl cysteine (NAC) as well as thiamine were added separately to M9 medium. Ascorbic acid is a strong antioxidant that effectively scavenges a wide array of ROS and other oxidants (Frei, 1994; Halliwell, 1996; Flora, 2009). The strain exhibited increased growth rate when thiamine or ascorbic acid was added to the M9 medium (Fig. 2A), and these effects were concentration dependent (Fig. 2B and C). These results indicate that thiamine and ascorbic acid inhibit oxidative stresses induced by light. However, NAC is a thiol containing antioxidant in animals that reduces the formation of biofilm and antibacterial activity of aminoglycosides and erythromycin (Goswami and Jawali, 2010; Eroshenko *et al.*, 2017), but no noticeable changes were observed for the strain grown in M9 medium supplemented with NAC.

Effects of light and antioxidants on the activity of antioxidant enzymes

The reducing power in bacteria is generally provided by NADPH. G6PDH is an important regulatory enzyme in the oxidative pentose phosphate pathway that generates NADPH. We investigated the activity of G6PDH and SOD in *Sphingomonas* sp. PAMC 26621 grown with different antioxidants. The enzymatic activity of crude extracts of cells grown in MT medium, M9 medium supplemented with thiamine or ascorbic acid, or M9 medium without antioxidants under light conditions, as well as M9 medium without antioxidants under dark condition was measured. The G6PDH activities increased (1.2−1.4 fold in the mid-log phase and 1.2−1.8 fold in the stationary phase) in samples (MT medium as well as M9 medium with thiamine or ascorbic acid) compared with M9 medium under light conditions (25.9 U/mg in the mid-log phase and 36.7 U/mg in the stationary phase). The highest activities were observed in MT medium (35.9 U/mg in the mid-log phase and 65.6 U/mg in the stationary phase), followed by M9 medium with ascorbic acid, M9 medium with thiamine, and M9 medium under dark conditions (Fig. 3A). Western blot analysis showed that the expression of G6PDH increased in the presence of thiamine and ascorbic acid. Although the G6PDH activity in MT medium was highest, the expression of G6PDH was in the order of M9 medium with either thiamine or ascorbic acid > MT medium > M9 medium under light conditions > M9 medium under dark conditions (Fig. 3B). The same amount of protein was loaded onto a SDS gel. However, as there were no available antibodies to bacterial housekeeping proteins, no data were shown for the same protein loading upon Western blot analysis.

Native polyacrylamide gel analysis of SOD activity showed a single band in all samples. In nature, SOD have several isozymes depending on the metal cofactors: CN^--sensitive cytosolic and chloroplastic Cu/Zn-SOD, H_2O_2-sensitive chloroplastic Fe-SOD, and mitochondrial Mn-SOD. Treatment of cells with KCN or H_2O_2 did not inhibit SOD activity, suggesting that the SOD in *Sphingomonas* sp. PAMC 26621 is Mn-SOD isozyme. A deep band appeared in the M9 medium with thiamine and M9 medium with ascorbic acid, whereas only a faint band was observed in the MT medium and M9 medium under dark and light conditions (Fig. 4).

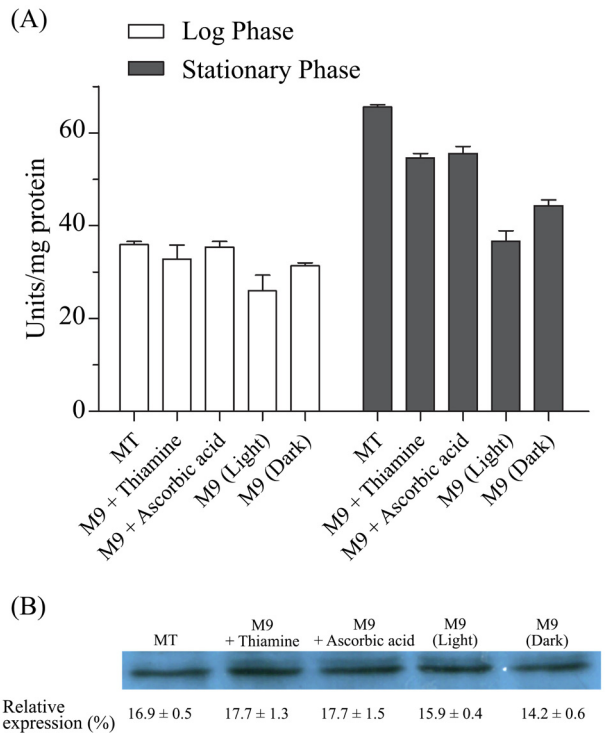

Fig. 3. Activities of G6PDH at mid-log and stationary phase on MT medium, M9 + thiamine, M9 + ascorbic acid, M9 (light), M9 (dark) (A). Enzyme activity was measured by following the rate of NADPH oxidation at 340 nm using glucose 6-phosphate as a substrate. The level of expression of G6PDH in each sample was shown on Western blot analysis and band intensities were analyzed using the ImageJ software (B).

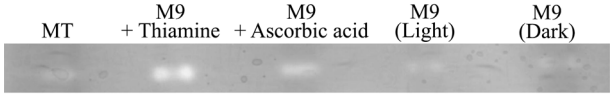

Fig. 4. Native polyacrylamide gel stained for SOD activity of samples from *Sphingomonas* sp. PAMC 26621. A total of 30 μg of crude extracted proteins were loaded into each lane.

Discussion

In this study, we investigated the effects of light and antioxidants on the growth and enzymatic activities of *Sphingomonas* sp. PAMC 26621, an aerobic bacterium isolated from the Arctic region. We showed that antioxidants (thiamine and ascorbic acid) increased the growth of the strain under both dark and light conditions as well as the activities of G6PDH and SOD. Without antioxidants, the activities of G6PDH and SOD were lower under both dark and light conditions.

The role of oxidative stress and defense mechanisms in bacteria have been extensively studied. The SOD activity was shown to increase under low to moderate stress conditions

(Foyer *et al.*, 1994). Reactive oxygen species such as hydroxyl radicals and hydrogen peroxide at high concentration inhibited and degraded SOD enzymes (Casano *et al.*, 1997). The hydroxyl and peroxyl radicals of ROS were irreversibly effective at inactivation of G6PDH enzyme as well (Ninfali *et al.*, 2001). A decrease in enzymatic activities of G6PDH and SOD with an increase of ROS in rehydration was reported for the lichen *R. lacera* (Weissman *et al.*, 2005). In contrast, as demonstrated in this study, the activity of G6PDH and SOD increased significantly in the presence of thiamine or ascorbic acid. This may have occurred because of the ROS scavenging activities of thiamine and ascorbic acid as previously reported (Foyer *et al.*, 1994; Frei, 1994; Charles and Peter, 2001; Okai *et al.*, 2007; Flora, 2009; Tunc-Ozdemir *et al.*, 2009). In the MT medium, G6PDH, and SOD showed different activities; therefore, additional studies to determine the reason for these differences are warranted.

The major role of NADPH is to reduce the disulfide form of glutathione to the sulfhydryl form (Salvemini *et al.*, 1999). Although participating indirectly, G6PDH, the key enzyme of the pentose phosphate pathway, plays an important role in the entire antioxidant system by providing NADPH (Zhang *et al.*, 2010; Stincone *et al.*, 2014). NADPH also protects catalase from oxidative damage by the reduction of oxidizing states and internal groups of catalase (Kirkman *et al.*, 1999). SOD does not use NADPH, but SOD-produced H_2O_2 is reduced by either catalase or glutathione peroxidase. ROS are normally formed during respiration and in response to exposure to light, but the production of ROS is also enhanced under stress conditions such as low nutrient concentrations or desiccation in the light (Frei, 1994; Kranner *et al.*, 2005; Weissman *et al.*, 2005). Cells have protection mechanisms to cope with the potential damaging effects of ROS. Many studies of the antioxidant activities of lichens have been published (Kranner *et al.*, 2005; Weissman *et al.*, 2005; Gulluce *et al.*, 2006; Kosanić *et al.*, 2011; Kim *et al.*, 2014), and they have been shown to contain several non-enzymatic antioxidants such as ascorbic acid, α-tocopherol (vitamin E), and phenolic compounds, as well as antioxidant enzymes, including catalase, superoxide dismutases, glutathione systems, and glucose 6-phosphate dehydrogenase (G6PDH) (Kranner *et al.*, 2005; Weissman *et al.*, 2005).

Interestingly, *Sphingomonas* sp. PAMC 26621 was isolated from the Arctic region (Lee *et al.*, 2012). As the polar regions reflect approximately 90% of the solar radiation that the earth receives, they receive less sunlight and radiation. These areas either receive midnight sun or shade polar night 24 h a day, which may explain the sensitivity of *Sphingomonas* sp. PAMC 26621 to light. The effects of light on growth of the strain may be associated with the antioxidant activities. In aerobic organisms, ROS (O_2^-, HO, and H_2O_2) are formed by redox mechanisms. In the presence of light, singlet oxygens ($^1O_2$), which cause damage to various biomacromolecules affecting the growth of cells, such as cellular membranes, lipoproteins, proteins, and DNA (Frei, 1994), can be formed from ground state triplet oxygen ($^3O_2$).

Lichen-associated bacteria such as *Sphingomonas* sp. PAMC 26621 have increasingly become a promising subject in microbial biotechnology, and bacteria associated with lichens isolated from extreme habitats of the Arctic and Antarctic are valuable sources to elucidate the functional and ecological roles of bacteria within lichens (Lee *et al.*, 2014). Lichen-associated bacteria are considered to supply and recycle nutrients to enhance the persistence of lichens in nutrient-poor, extreme and changing ecological conditions (Grube *et al.*, 2015; Sigurbjornsdottir *et al.*, 2015). Kono *et al.* (2017) reported that *Sphingomonas* sp. TZW2008 associated with lichen forming *Trebouxia* alga would help recycling ribitol from photobiont to mycobiont fungus. Bacterial strains from polar lichens ($\leq$ 97% sequence similarity) that are able to synthesize and secrete cold-adapted enzymes are of biotechnological and commercial interest (Lee *et al.*, 2014). The structures and diversity of bacterial communities in lichens may be affected by exposure to the sun. Cardinale *et al.* (2012) reported that the total number of bacteria in lichens grown under shaded conditions was higher than that of sun-exposed lichens. In this study, we also found that *Sphingomonas* sp. PAMC 26621 cultivated under dark conditions showed better growth than those grown under light conditions. The symbiotic partnership in lichens provides greater resistance to environmental stress than either partner alone. Kranner *et al.* (2005) reported that protective systems of algae and fungi were slow and ineffective when alone, but that they were up-regulated in lichens. To determine how bacteria interact with fungi and algae in symbiosis, bacteria may take an advantage of secondary metabolites, including antioxidants

that are of fungal origin, or they may contribute to the symbiotic partnership by synthesizing antioxidants. Secondary metabolites might cause selective pressure on the community structure of lichen-associated bacteria. In addition, extracts of bacteria isolated from the Arctic lichens *Ochrolechia* and *Stereocaulon* spp. were evaluated with a high amount of total phenolic and flavonoid contents (Kim *et al.*, 2013, 2014). By comparative omics, Grube *et al.* (2015) found the presence of enzymes involved in thiamine biosynthesis in a lichen-associated bacterium *Lobaria pulmonaria* (one hit in the metaproteome and 312 contigs in the metagenome). Further studies are needed to confirm the role of antioxidants in lichen symbiosis.

적 요

극지에 서식하는 세균은 강한 빛과 자외선을 받는다. 이 연구에서 우리는 북극에 서식하는 지의류 *Cetraria* sp.에서 분리한 호냉성 세균 *Sphingomonas* sp. PAMC 26621의 생장에 빛이 미치는 영향을 조사하였다. 이 세균은 암조건에서 명조건에서 보다 생장이 느렸다. 놀랍게도, 이 세균은 M9 최소배지에 티아민 혹은 아스코브르산을 첨가하면 명조건에서 생장이 증가하였지만, N-acetylcysteine을 첨가한 배지에서는 생장의 변화가 없었다. 첨가한 티아민과 아스코브르산은 포도당-6 인산 탈수소효소와 항산화 효소의 활성을 증가시켰다. 이 연구의 결과는 지의류와의 공생에서 제공된 티아민이 *Sphingomonas* sp. PAMC26621의 빛에 의한 산화적 스트레스를 완화시키는 항산화제 역할을 함을 의미한다. 이 연구는 강한 빛과 자외선이 만연한 북극에 서식하는 세균에 대한 생리적, 생화학적 관점에서 고찰할 점을 제시한다.

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