Full Paper

Characterization of extracellular matrix components from the desiccation-tolerant cyanobacterium Nostoc commune

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Kaori Inoue-Sakamoto,1 Yasunori Tanji,2 Minami Yamaba,2 Takumi Natsume,3 Takuya Masaura,2 Tomoy A solo,4,++ Takumi Nishiuchi,4 and Toshio Sakamoto2,3,*

1 Department of Applied Bioscience, College of Bioscience and Chemistry, Kanazawa Institute of Technology. Ohgaoka 7-1, No 901 921-8501, Japan
2 Division of Biological Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan
3 School of Natural System, College of Science and Engineering, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan
4 Division of Functional Genomics, Advanced Science Research Center, Kanazawa University, Takara, Kanazawa 920-0934, Japan

The terrestrial cyanobacterium Nostoc commune forms macroscopic colonies in its natural habitats, and these colonies consist of both cellular filaments and massive extracellular matrixes. In this study, the biochemical features of the extracellular matrix components were investigated. Naturally growing N. commune was tolerant to desiccation, and produced massive extracellular polysaccharides that contained both neutral sugars and glucuronic acid as constituent monosaccharides. The extracellular polysaccharide contents and desiccation tolerance were compared in laboratory culture strains of Nostoc species. The laboratory culture of N. commune strain KU002 was sensitive to desiccation and produced smaller amounts of extracellular polysaccharides, unlike the field-isolated naturally growing colonies. Nostoc punctiforme strain M-15, which is genetically close to N. commune, was able to tolerate desiccation, although the other Nostoc strains were desiccation-sensitive. A laboratory culture strain of the aquatic cyanobacterium Nostoc sphaer icum produced massive extracellular polysaccharides but was sensitive to desiccation, suggesting that extracellular matrix production is not enough to make this strain tolerant to desiccation. WspA (water stress protein) and SodF (superoxide dismutase) were found to be characteristic protein components of the extracellular matrix of N. commune. Because the WspA proteins were heterogeneous, the wspA genes were highly diverse among the different genotypes of N. commune, although the sodF gene was rather conservative. The heterogeneity of the WspA proteins suggests their complex roles in the environmental adaptation mechanism in N. commune.

Key Words: anhydrobiosis; desiccation tolerance; extracellular polysaccharide (EPS); superoxide dismutase (SOD); water stress protein (WspA)

Introduction

Desiccated organisms show little to no metabolic activity and are able to resume metabolism rapidly upon rehydration. This phenomenon is termed anhydrobiosis (Billi and Potts, 2002; Clegg, 2001; Crowe, 2002; Crowe et al., 1998). The terrestrial cyanobacterium Nostoc commune can remain viable for over 100 years in a desiccated state (Cameron, 1962; Lipman, 1941). Thus, N. commune is considered an anhydrobiotic microorganism with oxygenic photosynthetic capabilities. Because N. commune does not differentiate into akinetes (spores) (Potts, 2000), the molecular mechanism of extreme desiccation tolerance by this species is thought to involve multiple processes that include extracellular polysaccharide (EPS) production, compatible solute accumulation and antioxidative molecules.
We have studied the desiccation tolerance in *N. commune* in physiological and biochemical terms (Matsui et al., 2011, 2012; Morsy et al., 2008; Nazifi et al., 2013, 2015; Sakamoto et al., 2009, 2011; Tamaru et al., 2005; Yoshida and Sakamoto, 2009; Wada et al., 2013, 2015).

*N. commune* colonies are naturally subjected to regular cycles of desiccation and wetting. Their photosynthetic activity is maintained during desiccation and recovers rapidly upon rehydration (Sakamoto et al., 2009; Satoh et al., 2002; Scherer et al., 1984; Tamaru et al., 2005). During the desiccation of *N. commune* colonies, the photosynthetic activity decreases in a manner that is concomitant with water loss (Sakamoto et al., 2009). This cessation of photosynthetic electron transport is thought to be a requisite response to prevent the photodamage of the photosynthetic machinery (Fukuda et al., 2008; Hirai et al., 2004). However, the mechanism that regulates photosynthesis in response to desiccation still needs to be elucidated (Bar-Eyal et al., 2015; Raanan et al., 2016).

The production of EPS is widely known in cyanobacteria (Kehr and Dittmann, 2015; Pereira et al., 2009), and it is believed that EPS in cyanobacteria play a role in protecting cells from various stresses within severe habitats (Chakraborty and Pal, 2014; Ehling-Schulz et al., 1997; Yoshimura et al., 2007). In its natural habitats, *N. commune* forms visually conspicuous colonies that consist of an extracellular matrix with filamentous cells embedded inside (Helm and Potts, 2012; Potts, 2000). The EPS is a major component of *N. commune* colonies, and it accounts for approximately 80% of the dry mass (Morsy et al., 2008). The EPS of *N. commune* has been considered crucial for photosynthesis stress tolerance during desiccation and freeze-thawing (Tamaru et al., 2005). In addition to the accumulation of compatible solutes such as trehalose in response to desiccation (Hershkovitz et al., 1991; Higo et al., 2006; Sakamoto et al., 2009; Yoshida and Sakamoto, 2009), EPS is thought to play a protective role against desiccation to maintain the structures and functions of biological membranes (Chakraborty and Pal, 2014). Given its expected role in desiccation tolerance, the *N. commune* EPS shows a high water-absorbing capacity and a moisture-retention property (Li et al., 2011; Sakamoto et al., 2011; Shaw et al., 2003; Tamaru et al., 2005). However, studies on the involvement of EPS in the molecular mechanism of desiccation tolerance are limited (Hill et al., 1994, 1997). This could be due to the complexity of cyanobacterial EPS. As a characteristic feature, up to 12 different monosaccharides have been identified in cyanobacterial EPS (Pereira et al., 2009), and the partially characterized structures of *N. commune* EPS are highly complex (Brüll et al., 2000; Helm et al., 2000; Huang et al., 1998; Jensen et al., 2013; Kanekiyō et al., 2005).

The water stress protein, which is encoded by the *wspA* gene, is a 36-kDa protein that is present in the extracellular matrix of *N. commune* and is presumed to be relevant to the structure and/or function of the extracellular matrix (Helm and Potts, 2012; Morsy et al., 2008; Scherer and Potts, 1989; Wright et al., 2005). The WspA protein is known as the most abundant protein component in the extracellular matrix of *N. commune* and accounts for approximately 80% of the extracellular water-soluble proteins (Morsy et al., 2008; Sakamoto et al., 2011; Scherer and Potts, 1989; Wright et al., 2005). The SodF protein with superoxide dismutase activity is also abundant as an extracellular matrix protein (Shirkey et al., 2000; Wright et al., 2005). The heterogeneous forms of the WspA proteins are often found in naturally growing field-isolated colonies of *N. commune* (Helm and Potts, 2012; Sakamoto et al., 2011; Scherer and Potts, 1989; Wright et al., 2005), although the *wspA* gene is cloned as a single genomic copy in the laboratory-cultured *N. commune* strain known as DRH1 (Wright et al., 2005) and *N. commune* strain KU002 (Sakamoto et al., 2011). No *wspA* gene has been found in the genome of *Nostoc punctiforme* ATCC 29133 (Meeks et al., 2001), which is almost genetically indistinguishable from *N. commune* (Arima et al., 2012). Notably, the WspA protein is found in the extracellular matrix of the aquatic cyanobacterium *Nostoc verrucosum* (Sakamoto et al., 2011). *N. verrucosum* forms massive colonies with an extracellular matrix, the macroscopic and microscopic appearances of which are similar to those of *N. commune*, although *N. verrucosum* colonies always occur in streams and are sensitive to desiccation (Sakamoto et al., 2011). The *wspA* gene has been found in *N. commune* and *N. verrucosum*, which suggests that *Nostoc* species that form massive colonies with extracellular polysaccharides can be characterized by the occurrence of the *wspA* gene (Arima et al., 2012). The WspA protein of *N. commune* shows weak but heat-resistant β-galactosidase activity, which suggests contact between the EPS and the WspA protein (Morsy et al., 2008).

In this study, we assessed the EPS contents and monosaccharide composition of EPS in the terrestrial cyanobacterium *N. commune* compared to other *Nostoc* cyanobacteria. The protein components of the extracellular matrix were characterized in naturally growing field-isolated colonies of *N. commune*, and WspA protein heterogeneity was found in the different genotypes. We made a set of four laboratory culture strains of *N. commune* in which the genotypes are different. The *wspA* genes and the *sodF* genes were compared in the different *N. commune* genotypes using the laboratory culture strains. The occurrence of the WspA protein and *wspA* gene was tested in *Nostoc sphaerium* that form massive colonies with extracellular polysaccharides similarly to *N. commune* and *N. verrucosum*.

**Materials and Methods**

**Microorganisms.** Colonies of *N. commune* (Japanese vernacular name: Ishikurage) that were growing naturally in the field were collected, washed with tap water to remove the soil, air-dried, and stored at room temperature until use. Four *N. commune* genotypes that can be identified on the basis of the differences in their 16S rRNA genes (Arima et al., 2012) inhabit the Kakuma Campus of Kanazawa University (N 36.54, E 136.70). Because no obvious differences in their appearance can be observed by eye, we did not determine the genotype of the starting materials for the massive biochemical studies of the extracellular polysaccharide purification and the extracellular matrix protein analysis. For the WspA protein analysis, our stored...
samples with known genotypes were used.

The laboratory culture strain KU002 of *N. commune* (genotype A) has been isolated and maintained at Kanazawa University since 2002 (Tamaru et al., 2005). *N. commune* strain KU006 (genotype B) has been isolated recently (Nazifi et al., 2015). In this study, *N. commune* strain KU007 (genotype C) and *N. commune* strain KU008 (genotype D) were isolated from the Kakuma Campus at Kanazawa University and purified by streaking them onto agar plates. The nucleotide sequences of their 16S rRNA genes were determined to identify their genotypes (Arima et al., 2012). These laboratory culture strains of *N. commune* have been deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES-Collection) with the following collection numbers: NIES-2538 (*N. commune* strain KU002), NIES-3989 (*N. commune* strain KU006), NIES-3990 (*N. commune* strain KU007) and NIES-3991 (*N. commune* strain KU008).

*Nostoc muscorum* strain IAM M-14 and *Nostoc punctiforme* strain IAM M-15 (NIES-2108) were retrieved from the Institute of Molecular Biosciences at the University of Tokyo and have been maintained at Kanazawa University since 2004. *Nostoc* sp. strain PCC 7120 (also known as *Anabaena* sp. strain PCC 7120) was provided by Dr. A. Higo, Saitama University. The culture of *Nostoc sphaerium* strain MAC0910PER (Yamaguchi et al., 2015) and its dry powder were provided by Dr. H. Takenaka, Micro Algae Corporation (Gifu, Japan). To identify *N. sphaerium* strain MAC0910PER genetically, the marker gene sequences of its 16S rRNA gene, the *petH* gene and the *nrtP* gene were determined by PCR direct sequencing analysis according to Arima et al. (2012).

The cells were grown in aerated BG11 liquid medium with a nitrogen source of 1.5 g l–1 NaNO3 (Castenholz, 1988) buffered with 20 mM HEPES-NaOH (pH 7.5) at 30°C under constant illumination from fluorescent lamps (22–24 μmol m–2 s–1). To culture *N. commune* strain KU002, the culture media was buffered with 20 mM Tricine-NaOH (pH 8.5) under otherwise identical conditions. These cyanobacterial cultures were unialgal but not completely bacteria-free. Cells with the extracellular polysaccharides were harvested by centrifugation at 9,500 ¥ g at 4°C for 20 min, lyophilized and stored at –30°C as starting materials for the extraction and purification of extracellular polysaccharides.

**Desiccation or freeze-thaw stress treatments.** After 18 days, the concentration of dry materials in the cyanobacterial cultures reached a range of 0.3–1.6 mg ml–1. The culture of *Nostoc sphaerium* strain MAC0910PER provided by Dr. H. Takenaka contained dry materials at a density of 3.6 mg ml–1. The cells were harvested by centrifugation at 10,000 ¥ g at 4°C for 5 min, suspended in 25 mM HEPES-NaOH (pH 7.0), and adjusted to a cell density of 5 µg Chl a ml–1. After the initial level of the photosynthetic O2 evolution activity was measured, the cell suspension was subjected to desiccation or freeze-thaw treatments.

The cell suspension (15 ml) in a plastic vessel (14.5 cm W, 10.5 cm D, and 8 cm H) was desiccated in a chemical hood at room temperature for 2 days. The air-dried cells were rehydrated and suspended in distilled water (15 ml), and the restored photosynthetic activity was measured after 1 h of rehydration.

The cell suspension (15 ml) in a 50-ml plastic tube was frozen at –30°C, kept in a freezer overnight and thawed at room temperature. After the freezing and thawing treatment, its sustained photosynthetic activity was measured.

The photosynthetic O2 evolution was measured with an aqueous-phase Clark-type oxygen electrode (Rank Brothers Ltd., Cambridge, UK) at 30°C in 25 mM HEPES-NaOH (pH 7.0) containing 10 mM NaHCO3 as a final electron acceptor, under saturated actinic light (ca. 1 mmol m–2 s–1) from red light-emitting diode lamps (OPTILED LIGHTING, Tokyo, Japan). Chlorophyll a (Chl a) was extracted with 100% methanol, and the absorbance at 663 nm (A663) was measured after the subtraction of A750 to correct for light scattering. The Chl a concentration was calculated using an extinction coefficient of 74.5 l g–1 cm–1 (Mackinney, 1941).

**Visualization of extracellular polysaccharides.** The presence of extracellular polysaccharides was visualized by negative-staining with Indian ink and by positive-staining with Alcian blue. Cyanobacterial culture was mixed with Indian ink on a slide glass, and the prepared specimen was observed by light microscopy. Alcian blue reagent was prepared as follows. One g of Alcian Blue 8GS (Chroma-Gesellschaft) was added to 100 ml of 3% (v/v) acetic acid, stirred for 1 h and filtered. An equal volume of Alcian blue reagent was added to the cyanobacterial culture to stain acidic mucopolysaccharides. After 2 h of staining, the sample was observed by light microscopy.

**Measurement of neutral sugar content.** Lyophilized cyanobacterial powder was suspended in distilled water at a concentration of 1 mg ml–1 and an equal volume of 8 M HCl was added. After acid hydrolysis in 4 M HCl at 100°C for 2 h, the neutral sugars that were released were determined using the anthrone sulfuric acid method as described by Fales (1951), with modifications. The anthrone reagent consisted of 60 mg of anthrone, 2.4 ml of ethanol, 6 ml of distilled water and 30 ml of concentrated H2SO4, which were mixed under continuous cooling on ice. The acid-hydrolyzed sample solution (0.5 ml) was mixed with the anthrone reagent (3 ml) and heated in a boiling water bath for 10 min. After cooling, its absorbance was measured at 620 nm (A620). The sugar concentrations were determined from a standard curve constructed by using known amounts (5–25 µg) of D-glucose (Wako).

**Measurement of uronic acid content.** The amounts of uronic acids released by acid hydrolysis were determined using a modified carbazole sulfuric acid assay (Bitter and Muir, 1962). To prepare the sulfuric acid solution, 0.3 g of Na2B4O7·10H2O was added to 30 ml of H2SO4, which were mixed under continuous cooling on ice. The acid-hydrolyzed sample solution (0.5 ml) was mixed with the anthrone reagent (3 ml) and heated in a boiling water bath for 10 min. After the mixture cooled on ice, 0.1 ml of carbazole solution (1 mg ml–1 in ethanol) was added and the mixture was heated in a boiling water bath for 15 min. After it cooled, its absorbance at 530 nm (A530) was measured.
The uronic acid concentrations were determined from a standard curve that was constructed using known amounts (5–25 μg) of D-glucuronic acid (Sigma-Aldrich).

**Extraction and purification of EPS.** Extracellular polysaccharides (EPS) were extracted and purified as described previously (Sakamoto et al., 2011). Dry material (2 g) was rehydrated in distilled water (50 ml) at room temperature overnight. The total volume was adjusted to 100 ml using distilled water, and four volumes of acetone were added to yield 80% (v/v) acetone. The mixtures were stirred for 2 h at room temperature. The defatted samples were then collected by filtration and dried under a chemical hood for 1 h. Distilled water (150 ml) was added and the samples were heated in a boiling water bath for 2 h. After the samples cooled to room temperature, acetic acid was added to a final concentration of 1% (v/v), and the homogenates were heated in a boiling water bath for 2 h, cooled to room temperature, and centrifuged at 10,000 × g at 4°C for 10 min. Supernatants containing water-soluble polysaccharides were treated with 3 volumes of ethanol at 4°C overnight, and the precipitate was collected by centrifugation at 9,500 × g for 20 min at 4°C. After the pellets were washed in 70% (v/v) ethanol, their polysaccharide fraction was collected by centrifugation at 10,000 × g for 5 min at 4°C and lyophilized. Isolated polysaccharides were purified further as follows. Crude polysaccharide (0.5 g) was dissolved in distilled water (20 ml), and NaOH was added to reach 0.5 M. After the samples were incubated at 4°C overnight, their pH was adjusted to 7.8 using HCl and incubated at 4°C overnight with actinase E (10 mg; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan). After the samples had cooled on ice, trichloroacetic acid (5 ml; 40% (w/v)) was added, and the mixtures were incubated at 4°C overnight. After the mixtures were centrifuged at 10,000 × g at 4°C for 5 min, neutralized phenol (20 ml), chloroform and isoamyl alcohol (4 ml; 24:1 (v/v)) were added to the supernatant and mixed vigorously for phenol-chloroform extraction. The aqueous phase was recovered by centrifugation at 10,000 × g at 4°C for 5 min, and 3 volumes of ethanol were added. Following overnight incubation at 4°C, the precipitates were collected by centrifugation at 9,500 × g at 4°C for 20 min. The pellets were washed in 70% (v/v) ethanol, and purified polysaccharides were collected by centrifugation at 10,000 × g at 4°C for 5 min and lyophilized.

**Cellulose acetate membrane electrophoresis.** Purified EPS were characterized by electrophoresis on cellulose acetate membranes (Jokoh, Kawasaki, Japan) in 0.47 M formic acid-0.1 M pyridine buffer (pH 3) or 0.1 M barium acetate buffer, as described previously (Sakamoto et al., 2011). Polysaccharides were stained using Alcian blue (0.1% (w/v) in 50% (v/v) ethanol and 1% (v/v) acetic acid) and destained in 50% (v/v) ethanol and 1% (v/v) acetic acid. Dermatan sulfate (Seikagaku Corp.), chondroitin sulfate (Seikagaku Corp.), heparin (Wako), and hyaluronic acid (Nacalai) sodium salts were used as standards.

**Sugar composition analysis.** The sugar composition was determined by Toray Research Center, Inc. (Kamakura, Japan) largely as described before (Sakamoto et al., 2011). Purified EPS were hydrolyzed in 2 M trifluoroacetic acid at 100°C for 6 h. Neutral sugars were analyzed with an HPLC equipped with a TSK-gel Sugar AXG column (4.6 mm × 150 mm; Tosoh) at a temperature of 70°C. The mobile phase was 0.5 M borate buffer (pH 7.5) at a flow rate of 0.5 ml min⁻¹. The sugars were labeled with arginine (1% w/v) and borate (3% w/v) at 150°C and the emissions at 430 nm were detected using a spectrofluorescence detector (RF-10Axl; Shimadzu, Kyoto, Japan) with excitation at 320 nm. To analyze the uronic acids, a Shimpac ISA-07 column (4.6 mm × 250 mm; Shimadzu) was used with a 1 M borate buffer (pH 8.7) mobile phase at a flow rate of 0.8 ml min⁻¹. The sugars were identified by comparing their retention times with those of the standards, and their concentrations were determined using a standard curve constructed from known amounts of the following standard sugars: glucose, xylose, galactose, mannose, arabinose, ribose, rhamnose, fucose, glucuronic acid, and galacturonic acid.

**Characterization of N. commune extracellular matrix proteins by 2D gel electrophoresis.** Water-soluble proteins in the extracellular matrix were extracted as described previously (Morsy et al., 2008). Dry material (1.0 g) was rehydrated in distilled water (100 ml) at room temperature for 30 min. Thirty milliliters of 0.75 M potassium phosphate buffer (pH 7.0) was added to the water-absorbed colonies (11.2 g), and the colonies were homogenized in a blender three times for 10 s each. The homogenate was filtered through six layers of cheesecloth, and the filtrate was centrifuged at 10,000 × g for 10 min. The supernatant containing water-soluble proteins was collected and concentrated eight-fold using a Centriprep YM-10 filtering device with an ultrafiltration membrane (Amicon Inc., Beverly, MA, USA). The concentrated sample containing approximately 1.6 mg protein was obtained and subjected to two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was performed as described previously (Komatsubara et al., 2012). For the isoelectric focusing of the first dimension, the sample was loaded onto an immobilized pH gradient strip (Immobiline R-250. Two-dimensional gel electrophoresis was performed as described previously (Komatsubara et al., 2012). For the isoelectric focusing of the first dimension, the sample was loaded onto an immobilized pH gradient strip (Immobiline R-250.}

**Characterization of superoxide dismutase (SOD) from the N. commune extracellular matrix.** Dry colonies were rehydrated, suspended in 50 mM potassium phosphate buffer (pH 7.8), and homogenized in a blender. After being stirred overnight at room temperature, the homogenate was centrifuged at 6,000 × g for 10 min at 4°C. The supernatant, which retained the water-soluble extracellular matrix proteins, was recovered and fractionated by the ammonium sulfate precipitation method. Ammonium sulfate was added to 30% saturation and the precipitate was collected by centrifugation. The pellet was suspended in 50 mM potassium phosphate buffer (pH 7.8) and desalted by dialysis. After dialysis, the fraction was recovered as the 0–30% saturation fraction. Ammonium
sulfate was added to the 30%-supernatant to increase its saturation to 60%. After centrifugation, the pellet was suspended in 50 mM potassium phosphate buffer (pH 7.8) and dialyzed. The desalted fraction was recovered as the 30–60% saturation fraction. Ammonium sulfate was added to the 30–60% supernatant to increase its saturation to 90%. After centrifugation, the pellet was suspended in 50 mM potassium phosphate buffer (pH 7.8) and dialyzed. The desalted fraction was recovered as the 60–90% saturation fraction.

The SOD activity was measured using an XO/WST-1 system (Ukeda et al., 1999). To 1.024 μl of 50 mM sodium carbonate buffer (pH 9.4), 40 μl of 3 mM xanthine (Wako), 40 μl of 3 mM EDTA, 16 μl of a WST-1 solution (Cell Proliferation Reagent EST-1, Roche) and 40 μl of the sample solution containing SOD were added. Their reaction was initiated by adding 40 μl of an XO solution containing 60 mU ml⁻¹ xanthine oxidase from buttermilk (Wako). The reduction of WST-1 was monitored by measuring the absorbance at 438 nm (A438) for 5 min. One unit of SOD was defined as the amount of enzyme that inhibits the reduction reaction of WST-1 with superoxide anions by 50%. The inhibition curve was constructed on a dilution series of the sample solution, and the dilution ratio was determined at 50% inhibition.

The SOD in the polyacrylamide gel was detected by activity staining according to the method described by Beauchamp and Fridovich (1971). Non-denaturing PAGE was performed largely as described before (Morsy et al., 2008), except that the acrylamide concentration was 12%. Electrophoresis was performed at 4°C using vertical electrophoresis glass plates at a constant current of 10 mA per plate while the proteins were in the stacking gel, or 20 mA per plate while the proteins were in the separating gel. After electrophoresis, the gel was washed with distilled water for 30 s and soaked in 2.45 mM nitro blue tetrazolium for 20 min. In the dark, the gel was soaked in a solution containing 28 mM tetramethylethylenediamine, 28 μM riboflavin and 36 mM potassium phosphate buffer (pH 7.8) for 15 min. The gel was illuminated to develop the achromatic SOD band in a blue background.

Protein concentrations were determined using the CBB G-250 dye-binding method described by Bradford (1976), with bovine serum albumin (BSA) as a standard. SDS-PAGE was performed according to the Laemmli method (1970), except that the acrylamide concentration was 12%. The proteins were stained with CBB R-250. The molecular masses were estimated using a molecular size marker set (Dalton Mark VII-L, Sigma).

Comparison of the WspA proteins among four different genotypes of N. commune. The water-soluble proteins in the extracellular matrix were extracted mostly as described previously (Morsy et al., 2008). Dry powdered (0.1 g) N. commune colonies for which the genotype was identified were suspended in distilled water (5 ml) at room temperature for 20 min and centrifuged at 12,000 × g for 10 min. Two volumes of 0.75 M potassium phosphate buffer (pH 7.0) were added to the pellet, and the mixture was kept for 40 min with either rotary shaking or stirring. After centrifugation at 12,000 × g for 10 min, the supernatant containing water-soluble proteins was collected and concentrated four-to-seven-fold using an Ultra-15 filtering device with an ultrafiltration membrane (MWCO10000; Amicon, Merck Millipore Ltd., Tulagreen, Carriagtwihool, Co., Cork, IRL). The extracellular matrix proteins (20 μg) in a loading buffer were denatured at 100°C and immediately loaded on a 15% polyacrylamide gel for separation by SDS-PAGE. Commercial molecular size marker preparations (Precision Plus Protein unstained standards, Bio-Rad Laboratories, and BlueStar Prestained, Nippon Genetics Co., Ltd.) were used to determine the molecular sizes of the WspA proteins precisely.

The laboratory cultured thalli of N. sphaericum strain MAC0910PER were soaked in 0.75 M potassium phosphate buffer (pH 7.0), homogenized in a glass homogenizer and centrifuged at 17,500 × g for 15 min. The supernatant was collected as the extracellular fraction and concentrated using an Ultra-15 filtering device with an ultrafiltration membrane (MWCO10000; Amicon, Merck Millipore Ltd., Tulagreen, Carriagtwihoiull, Co., Cork, IRL). After the centrifugation, the pellet was collected and the cells were disrupted by freeze-thawing. The samples containing 1.5–2.0 μg of proteins were subjected to SDS-PAGE analysis.

Identification of proteins. After electrophoresis, the proteins were identified by in-gel trypsin digestion and tandem mass spectrometry (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA, USA) as described previously (Asano and Nishiuchi, 2011). Protein Pilot™ software was used to determine the amino acid sequences of peptides by fragmentation pattern analysis. Because wspA genes have been found in a limited number of Nostoc cyanobacteria species (Arima et al., 2012; Sakamoto et al., 2011; Wright et al., 2005) and the available sequence information is limited in the database, the WspA identities were confirmed manually by comparing their sequences with the amino acid sequences that were predicted from the nucleotide sequences of the wspA genes.

PCR amplification of the wspA gene. Genomic DNA was prepared from the cells of each laboratory culture strain, namely KU002, KU006, KU007 or KU008 of N. commune, strain MAC0910PER of N. sphaericum, or field-isolated naturally growing N. commune colonies, by using a DNasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The genomic DNA template was used to amplify the DNA fragments by PCR.

The wspA gene-specific primers were designed and synthesized on the basis of the nucleotide sequences of the wspA genes from N. commune strain KU002 (accession AB518000, Sakamoto et al., 2011) and strain DRH1 (DQ155425, Wright et al., 2005). A 1.1-kb DNA fragment was amplified using primers (forward 5'-TGTCTGTTAACAGCTTTTGAAGACA-3', and reverse 5'-TTTAACAGAGTTGAAGAAAGA-3'), and a 1.3-kb DNA fragment was amplified using other primers (forward 5'-TTAAGATAGTCCAATACACGTGTTAA and reverse 5'-CCTATCATATACCGCTTAGTAAGACA-3'). PCR was performed under the following conditions: an initial degradation at 98°C for 1 min, followed by 30 cycles of 98°C for 10 sec, 48–52°C for 30 s, and 68°C for 60–90 s with a final extension at 68°C for 7 min, using KOD-Plus-Neo DNA polymerase (TOYOBO). Alternatively, PCR was
performed under the following conditions: 35 cycles of 98°C for 10 s, 52–58°C for 15 s, and 72°C for 20 s, using PrimeSTAR MAX mix (TaKaRa).

A 0.8-kb DNA fragment containing the \( 	ext{wspA} \) gene was amplified by PCR from the genomic DNA template of \( N. \) sphaericum strain MAC9010PER using the following degenerated primers: forward primer (23-mer with 96-fold degeneracy) \( 5'\text{-TA(T/C) GGITAT(C/T/A) ACGTA(T/C/A)} \) and reverse primer (23-mer with 96-fold degeneracy) \( 5'\text{-TC (T/C)TG (A/G)TA ICC IT(T/C/A) TGAT(T/C)} \), which were designed and synthesized on the basis of the nucleotide sequence of the \( 	ext{sodF} \) gene from \( N. \) commune strain DRH1 (accession AF177945, Shirkey et al., 2000).

The DNA fragment was either subjected to direct sequencing or ligated into a EcoRV site of pBluescript KSII(-). The plasmid DNA containing insert DNA was prepared and subjected to sequencing analysis. Alternatively, the insert DNA fragment was amplified by PCR from the plasmid DNA template with the vector primers and used as the sequencing template.

**PCR amplification of the \( 	ext{sodF} \) gene.** A 0.6-kb DNA fragment containing the \( 	ext{sodF} \) gene was amplified by PCR using an \( N. \) commune genomic DNA template and gene-specific primers (the forward primer, \( 5'\text{-GCTTCGTAAATATGCTAAACTGG-3'} \) and the reverse primer, \( 5'\text{-GAACCTTTATAACTGACAGCTA-3'} \), which were designed and synthesized on the basis of the nucleotide sequence of the \( 	ext{sodF} \) gene from \( N. \) commune strain IAM M-14 (accession AJ177945, Shirkey et al., 2000). The PCR was performed according to the following profile: an initial denaturation for 30 s, followed by 35 cycles with denaturation at 98°C for 10 s, annealing at 44°C for 30 s, and extension at 72°C for 18 s with a final extension step at 72°C for 2 min. The 0.6-kb PCR product containing the \( 	ext{sodF} \) gene was purified and ligated into a \( 	ext{HincII} \) site in pUC19. The plasmid DNA with the 0.6-kb insert DNA was prepared and subjected to sequencing analysis.

**Accession numbers.** The nucleotide sequences determined in this study have been submitted to GenBank/EMBL/ DDBJ with the accession numbers listed in Table S1.

### Results and Discussion

#### Stress tolerance of \( Nostoc \) cyanobacteria

The terrestrial cyanobacterium \( Nostoc \) commune is tolerant to both desiccation and freeze-thawing (Tamaru et al., 2005), whereas the aquatic cyanobacterium \( Nostoc \) vettarosum is sensitive to desiccation but tolerant to freeze-thawing (Sakamoto et al., 2011). To assess the stress tolerance of \( Nostoc \) cyanobacteria, the restored \( \text{O}_2 \) evolution activity after the desiccation or freeze-thawing treatments was measured in laboratory culture strains of \( Nostoc \) species (Table 1). The photosynthetic \( \text{O}_2 \) evolution in \( N. \) commune colonies was highly tolerant to desiccation and freeze-thawing, and their \( \text{O}_2 \)-evolving capacity was almost undamaged after these stress treatments (Table 1), which confirms our previous reports (Sakamoto et al., 2011; Tamaru et al., 2005). The laboratory culture of \( N. \) commune strain KU002 was tolerant to freeze-thawing but sensitive to desiccation: approximately half of the activity remained after freeze-thawing, and no photosynthetic \( \text{O}_2 \) evolution was detected after desiccation (Table 1). Similar to \( N. \) commune strain KU002, \( Nostoc \) sp. strain PCC7120 and \( Nostoc \) sphaericum strain MAC9010PER were half-tolerant to freeze-thawing but sensitive to desiccation (Table 1). \( Nostoc \) punctiforme strain IAM M-15 showed stress tolerance against both desiccation and freeze-thawing, but the level of \( \text{O}_2 \) evolution was reduced after these stress treatments. \( Nostoc \) muscorum strain IAM M-14 was sensitive to desiccation and freeze-thawing; no \( \text{O}_2 \)-evolving capacity remained after the stress treatments (Table 1). These observations indicate that naturally growing \( N. \) commute colonies are highly tolerant to desiccation and freeze-thawing as a characteristic feature of this organism and that the laboratory culture strains of \( Nostoc \) cyanobacteria tested here are not as tolerant as the \( N. \) commute colonies.

#### Comparison of extracellular polysaccharides from \( Nostoc \) cyanobacteria

In naturally growing \( N. \) commute colonies, the cell filaments are surrounded by the extracellular matrix, and this characteristic architecture of \( N. \) commute colonies is thought to be relevant to the extreme desiccation tolerance of this organism (Tamaru et al., 2005; Wright et al., 2005). When examined microscopically, cell filaments that were embedded in the extracellular matrix were observed in field-isolated natural colonies of \( N. \) commute (Fig. S1). Indian ink does not permeate the extracellular matrix and Alcian blue reagent stains acidic mucopolysaccharides. These staining techniques emphasize the shape of the EPS. When stained with Alcian blue, the extracellular matrix of the \( N. \) commute colony appeared entirely blue (Fig. S1). Similar to the appearance of \( N. \) commute colonies, the cell filaments were embedded in the massive EPS in the laboratory culture of \( N. \) sphaericum strain.
Further, the uronic acid levels were low in these three strains, IAM M-14 and MAC0910PER, which suggests low amounts of EPS per cell. Whereas, the chlorophyll levels were high in the culture of N. punctiforme strain IAM M-15, EPS appeared to cover the filaments of cells and were associated with the cell surface (Fig. S1). The tightly associated EPS in cells from N. punctiforme strain IAM M-15 may be relevant to the desiccation tolerance of this strain (Table 1). N. sphaericum strain MAC0910PER produced massive EPS but was sensitive to desiccation (Table 1), and this finding was similar to that of our previous report in field-isolated naturally growing N. verrucosum colonies (Sakamoto et al., 2011).

Table 2 shows a comparison of the Nostoc cyanobacteria cultures in terms of their chlorophyll, neutral sugar and uronic acid contents in the dry materials. Consistent with the microscopic observation (Fig. S1), the neutral sugars and uronic acids that are components of the EPS were detected (Table 2). The chlorophyll levels were low in the field-isolated N. commune and in the culture of N. sphaericum strain MAC0910PER, which suggests high amounts of EPS per cell. Whereas, the chlorophyll levels were high in the culture of N. commune strain KU002, N. muscorum strain IAM M-14 and Nostoc sp. strain PCC7120, which suggests low amounts of EPS per cell. Moreover, the uronic acid levels were low in these three strains. These results suggest that N. commune strain KU002, N. muscorum strain IAM M-14, and Nostoc sp. strain PCC7120 contain low amounts of acidic exopolysaccharides.

Field-isolated natural colonies of N. commune had a high amount of EPS (Table 2, Fig. S1) and were highly tolerant to desiccation (Table 1). We previously reported that photosynthetic activity is damaged by desiccation when EPS is removed (Tamaru et al., 2005). The laboratory culture of N. commune strain KU002 produced smaller amounts of EPS but was sensitive to desiccation (Table 1), and this finding was similar to that of our previous report in field-isolated naturally growing N. verrucosum colonies (Sakamoto et al., 2011).

Table 2. Contents of chlorophyll a, neutral sugars and uronic acids in dry materials.

<table>
<thead>
<tr>
<th>Species</th>
<th>Chl a&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutral sugar&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Uronic acid&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Nostoc commune (field-isolated)</td>
<td>1.6 ± 0.2</td>
<td>276 ± 30</td>
<td>121 ± 26</td>
</tr>
<tr>
<td>Nostoc commune strain KU002</td>
<td>4.4 ± 1.3</td>
<td>158 ± 41</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Nostoc punctiforme strain IAM M-15</td>
<td>3.8 ± 0.5</td>
<td>245 ± 34</td>
<td>120 ± 11</td>
</tr>
<tr>
<td>Nostoc muscorum strain IAM M-14</td>
<td>9.6 ± 0.7</td>
<td>220 ± 24</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Nostoc sp. strain PCC7120</td>
<td>5.5 ± 0.6</td>
<td>267 ± 28</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>Nostoc sphaericum strain MAC0910PER</td>
<td>2.1 ± 1.0</td>
<td>281 ± 46</td>
<td>129 ± 10</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SD (n = 3).
<sup>a</sup>Chlorophyll a (Chl a) was extracted with methanol and determined photospectrometrically.
<sup>b</sup>Sugar content was determined by anthrone assay.
<sup>c</sup>Uronic acid content was determined by carbazole assay.

Extracellular polysaccharides were characterized by electrophoresis on cellulose acetate membranes in 0.47 M formic acid-0.1 M pyridine buffer (pH 3) (A) or 0.1 M barium acetate buffer (B). Dermatan sulfate (lane 1), chondroitin sulfate (lane 2), heparin (lane 3), and hyaluronic acid (lane 4), sodium salts were used as the standards. Four µg of the polysaccharides purified from naturally grown field-isolated colonies of Nostoc commune (lane 5) and laboratory cultures of Nostoc commune strain KU002 (lane 6), Nostoc punctiforme strain IAM M-15 (lane 7), Nostoc muscorum strain IAM M-14 (lane 8), Nostoc sp. strain PCC7120 (lane 9), and Nostoc sphaericum strain MAC0910PER (lane 10), were loaded. Polysaccharides were stained with Alcian blue. The arrows indicate the direction of electrophoresis. The purified polysaccharides from N. commune strain KU002 (lane 6), N. muscorum strain IAM M-14 (lane 8), Nostoc sp. strain PCC7120 (lane 9), and Nostoc sphaericum strain MAC0910PER (lane 10), were barely detectable by Alcian blue staining, probably because of their low uronic acid contents (Table S4).

EPS was extracted and purified from these Nostoc cyanobacteria, and their EPS yields are summarized in Table S3. High amounts of EPS were recovered from the
field-isolated *N. commune* colonies, *N. punctiforme* strain IAM M-15 and *N. sphaericum* strain MAC0910PER (Table S3), and the amounts of uronic acids were consistently high in these strains (Table 2). These results suggest that these three *Nostoc* cyanobacteria contain high amounts of acidic exopolysaccharides. Figure 1 shows the electrophoretic patterns of cellulose acetate membrane electrophoresis under 0.47 M formic acid-0.1 M pyridine buffer (pH 3) (Fig. 1A) and 0.1 M barium acetate buffer (Fig. 1B). EPS from the field-isolated *N. commune* colonies (Fig. 1, lane 5), *N. punctiforme* strain IAM M-15 (Fig. 1, lane 7) and *N. sphaericum* strain MAC0910PER (Fig. 1, lane 10) migrated as a single band, suggesting that they are homologous and similarly charged. Their electrical mobilities in 0.47 M formic acid-0.1 M pyridine buffer (pH 3) (Fig. 1A) were lower than those of the standards used here, suggesting low acidity. In 0.1 M barium acetate buffer (Fig. 1B), cyanobacterial EPS showed low electrical mobility, suggesting a high affinity for divalent cations. EPS from *N. commune* strain KU002 (lane 6), *N. muscorum* strain IAM M-14 (lane 8) and *Nostoc* sp. strain PCC 7120 (lane 9) were barely detectable by Alcian blue staining. Consistent with their low reactivity to Alcian blue staining, the uronic acid contents in these purified samples were low or less than the detection limit (Table S4), which suggests a low recovery of acidic exopolysaccharides from *N. commune* strain KU002, *N. muscorum* strain IAM M-14 and *Nostoc* sp. strain PCC 7120.

In the field-isolated *N. commune* colonies, *N. punctiforme* strain IAM M-15 and *N. sphaericum* strain MAC0910PER, massive or dense EPS-surrounded cells were observed microscopically (Fig. S1), and high amounts of EPS (Table 2, Table S3) that contained a high level of glucuronic acid (Table S4) were recovered. Interestingly, the cyanobacterial strains with the slimy EPS, such as *N. commune* strain KU002, *N. muscorum* strain IAM M-14, and *Nostoc* sp. strain PCC 7120 (Fig. S1), had low EPS yields (Table S3) and low levels of uronic acids (Table 2, Table S4). It is thought that acidic sugars are responsible for the polyanionic nature of cyanobacterial EPS, which form hydrated gels (Kehr and Dittmann, 2015). In *N. commune* strain KU002, *N. muscorum* strain IAM M-14, and *Nostoc* sp. strain PCC 7120, the cells are likely to be damaged during desiccation due to low amounts of acidic exopolysaccharides. At least 7 different monosaccharides were detected in the EPS from field-isolated *N. commune* colonies (Table S4), which suggest the complex structure of the *N. commune* EPS. Massive EPS production must be an important requisite for desiccation tolerance in *N. commune*; however, specific features relevant to its role in *N. commune* EPS remain to be elucidated.

### N. commune extracellular matrix proteins

The water-soluble extracellular matrix proteins were extracted from naturally growing *N. commune* colonies and separated by two-dimensional gel electrophoresis (Fig. 2) following identification by MALDI-TOF MS analysis (Table S5). The WspA protein was the most abundant protein in the extract, confirming previous reports (Helm and Potts, 2012; Morsy et al., 2008; Sakamoto et al., 2011; Wright et al., 2005). Consistent with the report by Helm and Potts (2012), proteins with anti-oxidative roles such as Mn-containing catalase, ferritin and Fe-superoxide dismutase (SodF) were identified (Table S5, Fig. S2).

SodF is known as the third-most abundant soluble protein in *N. commune* (Shirkey et al., 2000), and thus we characterized SOD in naturally growing *N. commune* colonies further. The enzymatic activity of SOD was detected in the water-soluble fraction from the extracellular matrix, and a single band with SOD activity was detected by native PAGE after activity staining for SOD (Fig. 3). The active SOD was successively fractionated using ammonium precipitation, and this protein was enriched in the 60–90% saturation fraction (Table S6, Fig. 3). The 23 kDa protein in the 60–90% saturation fraction was identified as the SodF protein (Fig. 3, Table S7, Fig. S3).

The sodF gene encoding Fe-SOD was isolated, and its nucleotide sequence was determined in the laboratory strains of *N. commune*. The nucleotide sequence of the sodF gene was identical in the strains of KU002 (A type), KU007 (C type), and KU008 (D type) (Table S1), and it is highly similar to that of *N. commune* strain DRH1 with 99% shared identity (accession no. AF177945; Shirkey et al., 2000). In *N. commune* strain KU006 (B type), a significantly similar but different sequence was found (Table S1), which shows a 97% shared sequence identity with that from the KU002 (A type), KU007 (C type), and KU008 (D type), strains. The SodF proteins were highly similar among the *N. commune* strains and their deduced amino acid sequences were >98% identical.

These results confirm previous reports on the extracellular protein components in *N. commune* (Helm and Potts, 2012; Shirkey et al., 2000; Wright et al., 2005). It is noteworthy that the *N. commune* colonies that were growing...
Extracellular matrix of *Nostoc* in the different habitats contain highly similar sets of extracellular proteins, and they could be a common feature of the extracellular matrix architecture of *N. commune*. The extracellular SOD and also the presence of catalase suggest their role in desiccation tolerance and/or functions during recovery upon rehydration, but the mechanism remains to be elucidated.

**WspA proteins from four different genotypes of *N. commune***

Reportedly, the WspA protein occurs exclusively in two *Nostoc* species, namely the terrestrial cyanobacterium *N. commune* and the aquatic cyanobacterium *N. verrucosum*, and both species form massive colonies with a large amount of extracellular matrix in natural habitats (Arima et al., 2012; Sakamoto et al., 2011). There are no detected *wspA* genes in *N. muscorum* strain IAM M-14, *N. punctiforme* strain IAM M-15, and *Nostoc* sp. PCC 7120 (Arima et al., 2012). The heterogeneous forms of the WspA proteins are known (Helm and Potts, 2012; Sakamoto et al., 2011; Scherer and Potts, 1989; Wright et al., 2005), and two obvious WspA spots were found in the extracellular matrix proteins in this study (Fig. 2). We have found four genotypes of *N. commune* (Arima et al., 2012); thus, the water-soluble extracellular proteins were extracted from the different genotypes of *N. commune* colonies and characterized by SDS-PAGE (Fig. 4). The WspA proteins were found in a range from 32 to 40 kDa, and they were the most abundant proteins in the water-soluble extracellular matrix proteins of the four *N. commune* genotypes (Fig. 4, Fig. S4). The heterogeneity of the WspA proteins was confirmed by a sequencing analysis of the *wspA* genes that were isolated from the different genotypes (Table S1). According to the genetic differences in *N. commune*, the *wspA* genes were highly diverse, as expected from the heterogeneity of the WspA proteins. At least two clones with the different nucleotide sequences of the *wspA* genes were obtained from each *N. commune* genotype (Table S1). Because the *N. commune* genotypes are hardly distinguishable morphologically, they are easily mixed together when

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**Fig. 3.** Identification of a superoxide dismutase (SOD) in extracellular matrix proteins.

(A) Water extract from field-isolated, naturally growing *Nostoc commune* colonies were separated by native PAGE, and the SOD was detected by activity staining (lane 1). The sample was denatured by heat treatment at 100°C for 1 h as a negative control (lane 2). The proteins were detected by staining with CBB (lane 3). (B) Extracellular matrix proteins (lane 1) were fractionated by ammonium sulfate precipitation (Table S7) and separated by SDS-PAGE (0–30% saturation, lane 2; 30–60% saturation, lane 3; and 60–90% saturation, lane 4). The proteins were detected by staining with CBB. The 23-kDa band (band III) was identified as an SOD by MALDI-TOF-MS analysis (Table S7, Fig. S3).

**Fig. 4.** Electrophoretic patterns of water-soluble extracellular matrix proteins from *Nostoc commune*.

The extracellular matrix proteins were extracted from genotype A (lane A), genotype B (lane B), genotype C (lane C), and genotype D (lane D). *N. commune* colonies, separated by SDS-PAGE and stained with CBB. The abundant proteins with molecular masses of 32 to 40 kDa were identified as the WspA by in-gel trypsin digestion and tandem mass spectrometry analysis. The identified fragments determined by MALDI-TOF-MS analysis are shown in Fig. S4.
sampling \textit{N. commune} colonies from the field. This mixing may increase the complexity of the WspA heterogeneity and was not considered in the previous report (Wright et al., 2005).

It has been proposed that the \textit{wspA} gene is a xenolog in \textit{N. commune}, that it is acquired through lateral gene transfer (Wright et al., 2005), and is thought to be a single copy gene in the genome (Arima et al., 2012; Sakamoto et al., 2011; Wright et al., 2005). However, the \textit{wspA} genes are likely to be multiple copy genes in each genotype of \textit{N. commune} (Table S1). The diversity of the WspA protein in \textit{N. commune} suggests their complex roles in the extracellular matrix architecture and may be crucial for adaptation to terrestrial environments.

The laboratory culture of \textit{N. sphaericum} strain MAC0910PER produced a massive extracellular matrix in which EPS was the primary constituent (Fig. S1, Table 2). Water-soluble extracellular proteins from \textit{N. sphaericum} strain MAC0910PER were examined to compare them with those from \textit{N. commune} (Fig. S5). In \textit{N. sphaericum} strain MAC0910PER, no obvious protein band at approximately 36 kDa was found. This result suggests that the WspA protein is not involved in the architecture of the extracellular matrix in \textit{N. sphaericum} strain MAC0910PER. In spite of the absence of the WspA protein in the extracellular matrix, \textit{wspA} genes were found in \textit{N. sphaericum} strain MAC0910PER, which were amplified by PCR using the degenerated primers designed from the characteristic amino acid sequences of the WspA protein (Arima et al., 2012; Sakamoto et al., 2011). Moreover, two different clones with the different nucleotide sequences from the \textit{wspA} genes were obtained from \textit{N. sphaericum} strain MAC0910PER (Table S1). The expression of the \textit{wspA} genes in \textit{N. sphaericum} strain MAC0910PER is suppressed under the laboratory culture conditions used here, and it is unknown whether the absence of the WspA proteins alters the architecture of the extracellular matrix and/or the physiological desiccation tolerance.

Concluding Remarks

We have characterized three \textit{Nostoc} cyanobacteria, namely \textit{N. commune}, \textit{N. verrucosum}, and \textit{N. sphaericum}, that form visible colonies in their habitats. These \textit{Nostoc} cyanobacteria produce massive extracellular matrices in which acidic exopolysaccharides are the primary constituents. Their macroscopic appearances and microscopic cellular filaments are similar. However, only \textit{N. commune} shows extreme desiccation tolerance and is adapted to terrestrial environments. We have isolated the laboratory-cultured strains of these \textit{Nostoc} cyanobacteria. Studies through comparative genomics using the laboratory-cultured strains will make their genetic differences clearer and will show the strategy needed to clarify the decisive features that are relevant to the extreme desiccation tolerance in \textit{N. commune}. The \textit{wspA} genes encoding a 36-kDa water stress protein are present in these \textit{Nostoc} cyanobacteria, and a diversity of WspA proteins is found in \textit{N. commune}. Further studies are necessary to elucidate the involvement of the WspA proteins in the architecture of the extracellular matrix and also in the desiccation tolerance mechanism of \textit{N. commune}.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/gjam).

References


