KURAに登録されているコンテンツの著作権は、執筆者、出版社（学協会）などが有します。KURAに登録されているコンテンツの利用については、著作権法に規定されている私的使用や引用などの範囲内で行ってください。著作権法に規定されている私的使用や引用などの範囲を超える利用を行う場合には、著作権者の許諾を得てください。ただし、著作権者から著作権等管理事業者（学術著作権協会、日本著作出版権管理システムなど）に権利委託されているコンテンツの利用手続については、各著作権等管理事業者に確認してください。

<table>
<thead>
<tr>
<th>項目</th>
<th>内容</th>
</tr>
</thead>
<tbody>
<tr>
<td>タイトル</td>
<td>The extracellular-matrix-retaining cyanobacterium Nostoc verrucosum accumulates trehalose, but is sensitive to desiccation</td>
</tr>
<tr>
<td>著者</td>
<td>Sakamoto, Toshio; Kumihashi, Keisuke; Kunita, Shinpei; Masaura, Takuya; Inoue-Sakamoto, Kaori; Yamaguchi, Masaaki</td>
</tr>
<tr>
<td>言及</td>
<td>FEMS Microbiology Ecology, 77(2): 385-394</td>
</tr>
<tr>
<td>発行日</td>
<td>2011-08</td>
</tr>
<tr>
<td>型式</td>
<td>Journal Article</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2297/27840">http://hdl.handle.net/2297/27840</a></td>
</tr>
<tr>
<td>右</td>
<td><a href="http://dspace.lib.kanazawa-u.ac.jp/dspace/">http://dspace.lib.kanazawa-u.ac.jp/dspace/</a></td>
</tr>
</tbody>
</table>
The extracellular-matrix retaining cyanobacterium *Nostoc verrucosum* accumulates trehalose but is sensitive to desiccation

Running title: Trehalose and extracellular matrix in *Nostoc*

Toshio Sakamoto¹, *, Keisuke Kumihashi¹, Shinpei Kunita¹, Takuya Masaura¹, Kaori Inoue-Sakamoto² and Masaaki Yamaguchi¹

¹Division of Biological Sciences, Graduate School of Natural Science and Technology, Kanazawa University. Kakuma, Kanazawa 920-1192, Japan.

²Department of Applied Bioscience, College of Bioscience and Chemistry, Kanazawa Institute of Technology. Ohgigaoka 7-1, Nonoichi-machi 921-8501, Japan.

*Address for correspondence:*

Dr. Toshio Sakamoto

School of Natural System, College of Science and Engineering, Kanazawa University.

Kakuma, Kanazawa 920-1192, Japan.

Phone: +81-76-264-6227

Fax: +81-76-264-6215

E-mail: sakamot@kenroku.kanazawa-u.ac.jp
Abstract

The aquatic cyanobacterium *Nostoc verrucosum* forms macroscopic colonies, which consist of both cellular filaments and massive extracellular matrix material. In this study, the physiological features of *N. verrucosum* were investigated and compared with those of the anhydrobiotic cyanobacterium *Nostoc commune*. *N. verrucosum* cells were sensitive to desiccation but tolerant of freeze-thawing treatment in terms of both cell viability and photosynthetic O$_2$ evolution. Natural colonies of these cyanobacteria contained similar levels of chlorophyll a, carotenoids, the UV-absorbing pigments scytonemin and mycosporine-like amino acids (MAA), and uronic acid (a component of extracellular polysaccharides). Extracellular polysaccharides from both *N. verrucosum* and *N. commune* indicated low acidity and a high affinity for divalent cations, although their sugar compositions differed. The WspA protein, known to be a major component of the extracellular matrix of *N. commune*, was detected in *N. verrucosum*. Desiccation caused similarly high levels of trehalose accumulation in both cyanobacteria. Although previously considered relevant to anhydrobiosis in the terrestrial cyanobacterium *N. commune*, the data presented here suggest that extracellular matrix production and trehalose accumulation are not enough for standing extreme desiccation in *N. verrucosum*.

Key words: compatible solute, stress tolerance, extracellular polysaccharide, water stress protein (WspA).
Introduction

Desiccated organisms show little to no metabolic activity and are able to rapidly resume metabolism upon rehydration—a phenomenon termed “anhydrobiosis” (Crowe et al., 1998; Clegg, 2001; Potts, 2001; Crowe, 2002; Billi & Potts, 2002). The terrestrial cyanobacterium Nostoc commune can retain viability for over 100 years upon desiccation (Lipman, 1941; Cameron, 1962). Thus, N. commune is considered an anhydrobiotic microorganism with oxygenic photosynthetic capabilities. As N. commune does not differentiate into akinetes (spores), the mechanism of its extreme desiccation tolerance most likely involves multiple processes (Potts, 1994; 1999; 2000).

In natural habits, N. commune forms visible colonies that consist of biochemically complex extracellular matrices and cellular filaments embedded within extracellular polysaccharides (EPS), accounting for 60%–80% of the dry mass (Hill et al., 1994a; 1997). The production of EPS is widely known in cyanobacteria (Bertocchi et al., 1990; Gloaguen et al., 1995) and it is believed that EPS in cyanobacteria play a major role in protecting cells from various stresses in severe habitats. As removal of EPS causes a significant reduction in photosynthetic activity, EPS of N. commune has been considered crucial for stress tolerance of photosynthesis during desiccation and freeze-thawing (Tamaru et al., 2005).

N. commune colonies are naturally subjected to regular cycles of desiccation and wetting. Photosynthetic activity is maintained during desiccation and recovers rapidly upon rehydration (Scherer et al., 1984; Satoh et al., 2002; Tamaru et al., 2005; Sakamoto et al., 2009). During desiccation of N. commune colonies, photosynthetic activity decreases concomitant with water content (Sakamoto et al., 2009). This cessation of photosynthetic electron transport is thought to be an acclimatory response to desiccation (Hirai et al., 2004; Fukuda et al., 2008); however, the mechanism regulating photosynthesis remains unknown.

Anhydrobiotic organisms accumulate trehalose (α-D-glucopyranosyl-[1,
1]-α-D-glucopyranoside) as a compatible solute. Trehalose protects biological membranes and proteins against the deleterious effects of water removal by replacement of hydrating water molecules and formation of amorphous glasses (vitrification) (Clegg, 2001). Trehalose is synthesized from α(1,4)-linked glucose polymers by the TreY-TreZ pathway in cyanobacteria of the genus Nostoc (Katoh et al., 2004; Higo et al., 2006; Yoshida and Sakamoto, 2009). Maltooligosyl trehalose synthase (Mts), encoded by the treY gene, converts the terminal α(1,4)-linked residue of the glucose polymer to an α(1,1) linkage. Maltooligosyl trehalose trehalohydrolase (Mth), encoded by the treZ gene, then produces free trehalose by cleavage of the terminal disaccharide (De Smet et al., 2000). Trehalose accumulation occurs in response to water loss during desiccation (Sakamoto et al., 2009; Yoshida and Sakamoto, 2009) and the trehalose accumulation capacity of N. commune is similar to that of the drought-resistant cyanobacteria Phormidium autumnale and Chroococcidiopsis sp. when exposed to matric or osmotic water stress (Hershkovitz et al., 1991). Therefore, trehalose accumulation is thought to be relevant to cyanobacterial desiccation tolerance.

The water stress protein, encoded by the wspA gene, is a 36-kDa protein present in the extracellular matrix of N. commune and is presumed to be relevant to the structure and/or function of the extracellular matrix (Scherer & Potts, 1989; Wright et al., 2005; Morsy et al., 2008). No wspA gene has been found in the genome of Nostoc punctiforme ATCC 29133 (Meeks et al., 2001) and the wspA gene of N. commune may be a xenolog, acquired through lateral gene transfer (Wright et al., 2005).

The cyanobacterium Nostoc verrucosum forms macroscopic colonies with an extracellular matrix the appearance of which is superficially similar to those of N. commune, although N. verrucosum colonies always occur in streams. As such colonies may be subjected to periodic drying in their natural habitat when the water level in the stream decreases, N. verrucosum may also be tolerant to desiccation but little is known about the physiological
features of this cyanobacterium. In this study, we assessed (i) the desiccation tolerance of \( N. verrucosum \) as compared to the terrestrial cyanobacterium \( N. commune \) (ii) components of the extracellular matrix and (iii) the capacity to accumulate trehalose in response to desiccation. Unlike \( N. commune \), \( N. verrucosum \) was sensitive to desiccation, despite increased trehalose levels within dry colonies.

6 Materials and methods

7 Microorganisms

Colonies of \( N. verrucosum \) (Japanese vernacular name: Ashitsuki) growing naturally in the stream were collected from Shishiku Park, Hakusan-shi, Ishikawa, Japan (36°26'8"N, 136°38'37"E). The laboratory strain KU005 of \( N. verrucosum \) was isolated by streaking and spreading on agar plates (Castenholz, 1988) and cultured at 18°C under fluorescent light in modified BG11\( _0 \) (without nitrate) medium or BG11\( _0 \) agar supplemented with biotin (1 \( \mu \)g·L\(^{-1}\)), thiamine (2 mg·L\(^{-1}\)), and cyanocobalamin (1 \( \mu \)g·L\(^{-1}\)) (Castenholz, 1988) buffered with HEPES-NaOH (20 mM; pH 7.5). \( N. verrucosum \) strain KU005 has been deposited in Microbial Culture Collection at National Institute for Environmental Studies (NIES-Collection) and its 16S rRNA gene sequence has been submitted to GenBank/EMBL/DDBJ under the accession number AB494996.

Colonies of \( N. commune \) (Japanese vernacular name: Ishikurage) growing naturally in the field were collected, washed with tap water to remove soil, air-dried, and stored at room temperature until required. \( N. commune \) strain KU002, isolated from Kakuma Campus of Kanazawa University, was cultured at 25°C under the same conditions as described above. \( N. commune \) strain KU002 has been deposited in NIES-Collection and its 16S rRNA gene sequence has been submitted to GenBank/EMBL/DDBJ under the accession number AB088375.
Measurement of water content

After determination of fresh weight (FW), colonies were dried under ambient conditions and their dry weight (DW) was determined. Water content was calculated as follows: (water content %) = (FW – DW)/FW × 100%.

Measurement of chlorophyll and total carotenoids

Chlorophyll a (Chl a) and carotenoids were extracted with 100% methanol and concentrations were determined spectrophotometrically (Tandeau de Marsac & Houmard, 1988; Hirschberg & Chamovitz, 1994). Chl a and carotenoid concentrations were calculated using the following equations (A_750 was subtracted to correct for light scattering):

\[
\text{[Chl a (µg·mL}^{-1}\text{)] = (A}_{665} - A_{750} \times 13.9
\]

\[
\text{[Carotenoids (µg·mL}^{-1}\text{)] = } \{(A}_{461} - A_{750} - 0.046 \times (A}_{665} - A_{750} \}\times 4
\]

Measurement of UV-absorbing pigments

The UV-absorbing pigments mycosporine-like amino acid (MAA) and scytonemin were extracted in 100% methanol and analyzed by high-performance liquid chromatography (HPLC) with a UV-VIS detector. The methanol-soluble fraction was separated on a reverse phase column (Wakosil 5C18, 4.6 mm × 250 mm; Wako, Osaka, Japan) using 100% methanol at a flow rate of 1 mL·min\(^{-1}\) as the mobile phase. MAA was detected by A_330 and the concentration was determined using a standard curve, itself calculated from A_330 with an extinction coefficient of 120 L·g\(^{-1}\)·cm\(^{-1}\) (Garcia-Pichel & Castenholz, 1993). Scytonemin was detected by A_380 and its concentration was determined using a standard curve, itself calculated from A_380 with an extinction coefficient of 112.6 L·g\(^{-1}\)·cm\(^{-1}\) (Ehling-Shulz et al., 1997).

Measurement of uronic acid content

The amounts of uronic acid released from natural colonies by acid hydrolysis were
determined using the carbazole assay (Dische, 1947) as described previously (Tamaru et al., 2005; Morsy et al., 2008).

3 Visualization of extracellular polysaccharides

To stain acidic mucopolysaccharides, Nostoc colonies were placed in Alcian blue reagent (pH 2.5) containing 0.33% (w/w) Alcian Blue 8GS (Chroma-Gesellschaft, Köggen, Germany) in 3% (w/w) acetic acid and observed by light microscopy (Tamaru et al., 2005).

7 Measurement of O₂ evolution

Photosynthetic O₂ evolution was measured with an aqueous-phase Clark-type oxygen electrode (Rank Brothers Ltd., Cambridge, UK) in HEPES-NaOH (25 mM; pH 7.0), containing 10 mM NaHCO₃ as a final electron acceptor, under saturated actinic light of 1600 µE m⁻²·s⁻¹ at 30°C.

12 Vital staining of cells with fluorescein diacetate (FDA)

After desiccation or freeze-thawing, FDA was used to stain live cells. Live cells emitting green fluorescence were observed by fluorescence microscopy (Tamaru et al., 2005).

15 Extraction and purification of EPS

Air-dried colonies of N. commune (20 g) were rehydrated in distilled water. After weighing, four volumes of acetone were added and mixtures were stirred for 2 h at room temperature. The defatted colonies were then collected, dried under a chemical hood for 1 h, distilled water (1000 mL) was added, and samples were heated in a boiling water bath for 2 h. After cooling to room temperature, the swelled colonies were homogenized in a blender four times at maximum speed for 30 s each time. Homogenates were then passed through a stainless steel mesh (pore diameter ~1.5 mm). The blender was rinsed using 600 mL of water to collect the rest and the rinsate was mixed with the homogenate. Acetic acid was added to a final
concentration of 1% (pH 4) and homogenates were heated in a boiling water bath for 2 h, cooled to room temperature, and centrifuged at 9000 × g for 20 min. Supernatants containing water-soluble polysaccharides were treated with 2.5 volumes of ethanol at 4°C overnight and the precipitate was collected by centrifugation at 9500 × g at 4°C for 20 min. After washing in 70% (w/v) ethanol, the polysaccharide fraction in pellets was collected by centrifugation at 7100 × g for 10 min and lyophilized (Freeze Dryer FD-80; Eyela). A yield of 4 g of crude polysaccharide was obtained from 20 g of dried N. commune colonies. Using the same method, 2 g of polysaccharide was isolated from 10 g of lyophilized N. verrucosum colonies.

Isolated polysaccharides were purified further as follows. Crude polysaccharide (1 g) was dissolved in 0.4 M NaOH (20 ml) and incubated at 4°C overnight. Samples were adjusted to pH 7.8 using HCl and incubated at 37°C overnight with actinase E (10 mg; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan). After cooling on ice, trichloroacetic acid (5 mL; 40% (w/v)) was added and polysaccharides were precipitated at 4°C overnight. After centrifugation at 13000 × g at 4°C for 10 min, neutralized phenol (20 mL), chloroform and isoamyl alcohol (4 mL; 24:1 (v/v)) were added, and samples were mixed vigorously for phenol-chloroform extraction. The aqueous phase was recovered by centrifugation at 13000 × g at 4°C for 10 min and 2.5 volumes of ethanol were added. After incubation for 10 min at –30°C, precipitates were collected by centrifugation at 13000 × g at 4°C for 10 min. Pellets were washed in 70% (w/v) ethanol, and purified polysaccharides were collected by centrifugation at 13000 × g at 4°C for 10 min and lyophilized (Freeze Dryer FD-80; Eyela, Tokyo, Japan).

Cellulose acetate membrane electrophoresis

Purified EPS were characterized by electrophoresis on cellulose acetate membranes (Jokoh, Kawasaki, Japan) in formic acid-pyridine buffer (0.47 M; pH 3) or barium acetate buffer (0.1
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. Hyaluronic acid (Nacalai, Kyoto, Japan), heparin (Wako), chondroitin sulfate (Seikagaku Corp., Tokyo, Japan), and dermatan sulfate (Seikagaku Corp.) sodium salts were used as standards.

Sugar composition analysis

Sugar composition was determined by Toray Research Center, Inc. (Kamakura, Japan) as follows. Purified EPS (2 mg) were hydrolyzed in 2 M trifluoroacetic acid at 100°C for 6 h. The hydrolysate neutral sugars were analyzed by HPLC equipped with a TSK-gel Sugar AXG column (4.6 mm × 150 mm; Tosoh) and an oven temperature of 70°C. The mobile phase was borate buffer (0.5 M; pH 8.7) at a flow rate of 0.4 mL·min⁻¹. The sugars were labeled with arginine (1% w/v) and borate (3% w/v) at 150°C and emission at 430 nm was detected using a spectrofluorescence detector (RF-10AxI; Shimadzu, Kyoto, Japan) with excitation at 320 nm. For analysis of uronic acids, a Shimpack ISA-07 column (4.6 mm × 250 mm; Shimadzu) was used with a borate buffer (0.5 M; pH 8.7) mobile phase at a flow rate of 0.8 mL·min⁻¹. Sugars were identified by comparison of their retention times with those of the standards and their concentrations were determined using a standard curve constructed using known amounts of the standard sugars: rhamnose, ribose, mannose, arabinose, galactose, xylose, glucose, galacturonic acid, and glucuronic acid.

Isolation of the wspA gene

A 0.8 kb DNA fragment containing the wspA gene was amplified by PCR from N. commune KU002 or N. verrucosum KU005 genomic DNA using the following degenerate primers: forward (23-mer with 96-fold degeneracy) 5'-TA(T/C) GGI TA(T/C) ACI AT(T/C/A) GGI GA(A/G) GA-3', derived from the amino acid sequence YGYTIGED; and reverse (23-mer with 96-fold degeneracy) 5'-TC (T/C) TG (A/G) TA ICC IGT (A/G/T) AT (T/C) TC (A/G) TA-3',
derived from the amino acid sequence YEITGYQD. PCR was performed under the following conditions: initial denaturation at 94°C for 1 min, followed by 40 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, with a final extension step at 72°C for 5 min. Specific primers for PCR amplification of the entire wspA gene were designed based on the nucleotide sequences of the PCR product, the wspA gene of N. commune DRH1 (accession no. DQ155425), and the Nostoc punctiforme ATCC 29133 genome (Meeks et al., 2001). These were: for N. commune KU002 (forward: 5'-TAATAGCGATCGCCTGCGAA-3', and reverse: 5'-CCATTAAACGGATGCTTGAG-3') and for N. verrucosum KU005 (forward: 5'-ATTCTTGATTCTCCATTAAC-3', and reverse: 5'-AGATGAGTACATTGAAGGTG-3'). The wspA gene sequences were determined and submitted to GenBank/EMBL/DDBJ under accession numbers AB518000 (N. commune strain KU002) and AB509258 (N. verrucosum strain KU005).

Extraction and characterization of extracellular matrix water-soluble proteins

Water-soluble proteins from the extracellular matrix of N. verrucosum and N. commune colonies were prepared as described previously (Morsy et al., 2008). Frozen N. verrucosum colonies (32 g FW) were thawed, suspended in potassium phosphate buffer (64 mL, 0.75 M, pH 7.0) and homogenized in a blender three times at medium speed for 10 s each time. After stirring for 1 h at room temperature, the suspension was further homogenized in a blender three times at medium speed for 10 s each time, and left to stand for 10 min at room temperature. The upper EPS layer was removed and the lower aqueous layer was centrifuged at 6000 × g for 10 min at 20°C. Supernatants containing the water-soluble fraction were concentrated using Centriprep-10 or Centriprep-30 ultrafiltration units (Amicon® Bioseparations; Millipore, Bedford, MA).

Protein concentrations were determined using the Coomassie Brilliant Blue (CBB) G-250 dye-binding method (Coomassie® Protein Assay Reagent; Pierce Biotechnology Inc.,
Rockford, IL) as described by Bradford (1976) using BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (1970) using vertical electrophoresis glass plates. Gels were stained using CBB R-250. Molecular masses were estimated using a molecular size marker set (Precision Plus Protein unstained standards; Bio-Rad Laboratories, Hercules, CA). The relative intensities of protein bands were determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Proteins were identified by in-gel trypsin digestion and tandem mass spectrometry (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA) (Asano & Nishiuchi, 2010). Protein Pilot™ software was used to determine the amino acid sequences of peptides by fragmentation pattern analysis. Peptide identities were confirmed manually by comparison with the amino acid sequences predicted from the nucleotide sequences of the *N. verrucosum* and *N. commune wspA* genes.

Determination of trehalose

Trehalose levels were assayed by HPLC equipped with an evaporative light-scattering detector (ELSD, Model 300S; SofTA Corp., Westminster, CO) as described previously (Sakamoto *et al.*, 2009). *Nostoc* samples (ca. 10 mg DW) were suspended in 0.2 to 0.5 mL of ultrapure water and heated in a boiling water bath for 25 min. After centrifugation at 21500 × g for 5 min, acetonitrile was added to a final concentration of 75% (v/v). Insoluble materials were removed by centrifugation at 21500 × g for 5 min and the supernatant (50 µL) was separated on an Asahipak NH2P-50 4E column (4.6 mm × 250 mm; Shodex, Tokyo, Japan). The mobile phase was acetonitrile and water (75:25 (v/v)) at a flow rate of 1 mL·min⁻¹. Trehalose concentrations were determined using a standard curve (90–905 ng trehalose). Sucrose concentrations were also determined using a standard curve (250–2500 ng sucrose).
Results and discussion

Comparison of field-isolated, natural colonies of *N. verrucosum* and *N. commune*

Both *N. verrucosum* and *N. commune* form extracellular matrix-containing macroscopic colonies (Fig. S1), although there are marked differences between the colonies of these two *Nostoc* species; the aquatic cyanobacterium *N. verrucosum* grows submerged in water, while the terrestrial cyanobacterium *N. commune* are regularly subjected to desiccation and re-wetting. When examined microscopically, filaments of cells embedded in extracellular matrix were observed in colonies of both cyanobacteria. The extracellular matrix of both species reacted similarly to Alcian blue reagent (Fig. S1), which is known to stain acidic mucopolysaccharides. The 16S rDNA sequence of *N. verrucosum* strain KU005 showed 95% identity to that of *N. commune* strain KU002, suggesting that they are genetically so close but different species of cyanobacteria.

Table 1 shows a comparison of *N. verrucosum* and *N. commune* colonies in terms of their water content, pigments, and uronic acid contents. Levels of chlorophyll and MAA were slightly higher in *N. verrucosum* than in *N. commune*, but the two cyanobacteria were similar with respect to all other parameters measured. Consistent with the results of Alcian blue staining, uronic acid accounted for approximately 20% of dry mass (Table 1), indicating that these compounds are components of the extracellular matrix in both cyanobacteria.

MAAs have diverse chemical structures (Cockell & Knowland, 1999; Shick & Dunlap, 2002). We noted that the MAA retention time of *N. verrucosum* as determined by HPLC was different from that of *N. commune* and that the UV-VIS absorption spectra of these MAAs were different, suggesting that the chemical structures of MAAs are different in *N. verrucosum* and *N. commune*. Determination of chemical structures is currently underway and will be reported at a later date.
Stress tolerance of *N. verrucosum* and *N. commune*

Restoration of O$_2$ evolution after stress was measured to determine stress tolerance (Table 2). Photosynthetic O$_2$ evolution in *N. verrucosum* colonies was sensitive to desiccation; no O$_2$-evolving capacity remained after air-drying (Table 2). Consistent with this loss of photosynthetic O$_2$ evolution, no cells were detected by FDA staining after air-drying (Fig. 1D), indicating that cells of *N. verrucosum* was fatally damaged by desiccation. Although cells of *N. verrucosum* were sensitive to desiccation treatment, O$_2$ evolution was unaffected by freeze-thawing (Table 2). Cell viability was confirmed by vital staining, and almost all cells survived after freeze-thawing (Fig. 1F). These observations suggest that *N. verrucosum* cells are sensitive to desiccation but tolerant to freeze-thawing, although the terrestrial cyanobacterium *N. commune* is tolerant to both desiccation and freeze-thawing (Table 2; Tamaru *et al.*, 2005).

Comparison of the EPS

EPS of *N. verrucosum* and *N. commune* were isolated and purified. Figure 2 shows the electrophoretic patterns generated by the EPS upon cellulose acetate membrane electrophoresis using 0.47 M formic acid-pyridine buffer (pH 3) (Fig. 2A) and 0.1 M barium acetate buffer (Fig. 2B). EPS from both *N. verrucosum* (Fig. 2, lane 1) and *N. commune* (Fig. 2, lane 2) migrated as a single band, suggesting that they are similarly charged. The electrical mobilities of both EPS in 0.47 M formic acid-pyridine buffer (pH 3) (Fig. 2A) were similar to that of hyaluronic acid (lane 3) and lower than those of heparin (lane 4), chondroitin sulfate (lane 5), or dermatan sulfate (lane 6), suggesting low acidity. In 0.1 M barium acetate buffer (Fig. 2B), cyanobacterial EPS showed low electrical mobility, suggesting a high affinity for divalent cations.

EPS purified from *N. verrucosum* contained glucose, mannose, xylose, and glucuronic acid in a molar ratio of 5:5:2:1. EPS from *N. commune* contained glucose, xylose,
galactose, mannose, and glucuronic acid in a molar ratio of 5:5:3:2:1. The neutral sugars detected in *N. commune* EPS were similar to those of nostoflan, a polysaccharide isolated and characterized from the terrestrial cyanobacterium *Nostoc flagelliforme* (Kanekiyo *et al*., 2005). Further studies are required to determine the structure of EPS from *N. verrucosum* to compare with that of *N. commune* EPS partially determined (Helm *et al*., 2000).

A unique uronic acid, 3-O-[(R)-1-carboxyethyl]-D-glucuronic acid, designated nosturonic acid, has previously been reported to be a component of *N. commune* EPS (Helm *et al*., 2000). During our analysis of EPS sugar composition, an unidentified uronic acid was detected in *N. commune*, which may have been nosturonic acid. This component was not detected in *N. verrucosum* EPS, and so may be unique to *N. commune*. An important question remains to be answered whether nosturonic acid contributes to the extreme desiccation tolerance of *N. commune*.

### Isolation of the wspA gene from *N. verrucosum*

Degenerate primers were designed according to the deduced amino acid sequence of WspA of *N. commune* DRH1 (Wright *et al*., 2005). A DNA fragment of 0.8 kb containing part of the wspA gene was successfully amplified from *N. commune* KU002 genomic DNA. Using the same primers, the wspA gene was amplified and identified in *N. verrucosum* KU005. DNA fragments containing the entire wspA gene were isolated and sequenced. The wspA gene of *N. commune* KU002 (accession no. AB518000) encoded a polypeptide of 333 amino acids with 77% sequence identity to WspA of *N. commune* DRH1 (Wright *et al*., 2005). The WspA of *N. verrucosum* KU005 (AB509258) contained 330 amino acids and showed 73% sequence identity to that of *N. commune* KU002 (AB518000), indicating that *N. verrucosum* possesses the wspA gene. It has been thought that *N. commune* is the only cyanobacterial species to possess the wspA gene, and no wspA gene has been found in the genome of *N. punctiforme* ATCC 29133 (Meeks *et al*., 2001) despite 97% sequence identity of 16S rRNA to *N.
The presence of wspA in the aquatic cyanobacterium *N. verrucosum* raises a new question of the origin of the wspA gene in cyanobacteria.

**Comparison of water-soluble proteins in the extracellular matrix**

Water-soluble extracellular matrix proteins of *N. verrucosum* and *N. commune* were prepared using mild extraction conditions so as not to disrupt the cells (Fig. 3). In *N. verrucosum*, a 33-kDa protein predominated (Fig. 3 lane 1); its molecular mass was similar to that predicted for the *N. verrucosum* wspA gene product. This 33-kDa protein was identified as WspA by MALDI-TOF tandem mass spectrometry analysis. Peptide fragments with an identical amino acid sequence to WspA of *N. verrucosum* KU005 were detected with 63% coverage (Fig. S2). These data suggest that WspA is a major component of the extracellular matrix of *N. verrucosum*, although whether WspA has identical or similar functions in *Nostoc* species remains unknown.

In *N. commune*, two proteins of 33 kDa and 31 kDa predominated (Fig. 3 lane 2). Both were identified as WspA by MALDI-TOF analysis (Fig. S2). Based on band intensity analysis, WspA was estimated to account for 53% (33 kDa) and 20% (31 kDa) of total extracellular matrix protein mass. The heterogeneity of *N. commune* WspA was consistent with previous reports (Scherer and Potts, 1989; Hill *et al.*, 1994b; Wright *et al.*, 2005). WspA of *N. commune* possesses β-D-galactosidase activity (Morsy *et al.*, 2008) and has been associated with 1,4-β-D-xyanxylanohydrolase activity (Hill *et al.*, 1994b), suggesting a role in EPS modulation. As WspA is thought to function via interaction with EPS, it will be necessary to identify the specific sugar moieties with which WspA reacts in vitro to determine its function.

**Trehalose accumulation in response to desiccation**

Trehalose levels in *N. verrucosum* and *N. commune* colonies and *N. verrucosum* KU005 and
N. commune KU002 laboratory cultures were examined (Table 3). Trehalose was not detected in wet colonies of either cyanobacterial species or in wet cells cultured in the laboratory. When induced by air-drying, trehalose was detected at concentrations of 1.3 to 4.2 µg·mg⁻¹ DW (Table 3). These values were essentially equivalent to those reported previously for both N. commune (Sakamoto et al., 2009) and laboratory cultures of Nostoc punctiforme strain IAM M-15 (Yoshida and Sakamoto, 2009). Trehalose accumulation in cyanobacteria is thought to be related to desiccation tolerance as both the terrestrial cyanobacterium N. commune (Sakamoto et al., 2009) and the desert-isolated drought-resistant cyanobacteria Phormidium autumnale and Chroococcidiopsis sp. (Hershkovitz et al., 1991) accumulate trehalose at high levels compared to desiccation-sensitive cyanobacteria, such as Nostoc sp. PCC 7120 (Higo et al., 2006) and Spirulina platensis (Ohmori et al., 2009). As trehalose levels in air-dried N. verrucosum cells were similar to those of N. commune (Table 3), the capacity of trehalose production is not linked to desiccation tolerance in N. verrucosum (Fig. 1, Table 2).

Trehalose accumulation appears to be triggered by desiccation; the level of trehalose increases markedly when water content decreases below a critical value (Yoshida and Sakamoto, 2009; Sakamoto et al., 2009). It has been suggested that control of trehalase plays an important role in trehalose accumulation; the rate of trehalose production exceeds that of hydrolysis. This is mediated by specific inactivation of trehalase under conditions of water stress characterized by increased cellular solute concentrations (Yoshida and Sakamoto, 2009). The details of the regulation of trehalase activity in response to cellular solute concentration remain to be elucidated.

Sucrose is a stress-inducible, non-reducing disaccharide that accumulates in cyanobacterial cells (Hershkovitz et al., 1991; Higo et al., 2006; Yoshida and Sakamoto, 2009). In untreated N. verrucosum KU005 and N. commune KU002 cells, sucrose
concentrations ranged from 1 to 3 µg·mg⁻¹ DW. The sucrose concentration of air-dried *N. verrucosum* KU005 cells was approximately 5.3 µg·mg⁻¹ DW, which was around three times higher than that in untreated cells, indicating an increase in response to desiccation. In contrast, the sucrose level in desiccated *N. commune* KU002 cells was approximately 1 µg·mg⁻¹ DW, which was essentially identical to that in untreated cells. Consistent with these observations, sucrose accumulation in response to desiccation is not evident in natural *N. commune* colonies (Sakamoto *et al.*, 2009), suggesting that regulation of sucrose metabolism may be different in these *Nostoc* species. The levels of sucrose detected were similar to those of trehalose but the relative contributions of these non-reducing sugars to stress responses are unknown.

### Concluding remarks

The aquatic cyanobacterium *Nostoc verrucosum* was sensitive to desiccation, despite its formation of macroscopic colonies with massive extracellular matrix apparently similar to that of the terrestrial cyanobacterium *Nostoc commune*. In response to desiccation, *N. verrucosum* accumulated trehalose at a similar level to *N. commune*. Extracellular matrix production and trehalose accumulation have previously been considered relevant to desiccation tolerance; however, they are not always linked to extreme desiccation tolerance. An important question remains to be answered in future studies; which factor is missing in *N. verrucosum* to be anhydrobiotic.

### Acknowledgement

We thank Dr. Ishida K for helpful information about *Nostoc verrucosum*, Dr. Sasayama Y and Mr. Mizuno H for generous assistance with microscopic analysis, Drs. Nishiuchi T and Asano T for MS-based protein identification, Drs. Ohta T and Katoh H for NMR analysis of MAA and EPS, Ms. Arima H and Mr. Horiguchi N for 16S rDNA analysis, Dr. Morsy FM, Ms.
Andoh Y, Ms. Hiratani E, Ms. Matsumura S, and Mr. Miyachi Y for technical assistance, and Dr. Yoshida T for helpful discussion. This work was supported by JSPS KAKENHI (22510227), SUNBOR Grant and HABA Laboratories Inc.
References


Garcia-Pichel F & Castenholz RW (1993) Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity.
Gloaguen V, Morvan H & Hoffmann L (1995) Released capsular polysaccharides of
characterization of the released polysaccharide of desiccation-tolerant Nostoc commune
Hershkovitz N, Oren A & Cohen Y (1991) Accumulation of trehalose and sucrose in
trehalose metabolism in dehydration tolerance of the filamentous cyanobacterium
Anabaena sp. PCC 7120. Microbiology 152: 979-987.
Hill DR, Peat A & Potts M (1994a) Biochemistry and structure of the glycan secreted by
(cyanobacteria) are secreted with UV-A/B-absorbing pigments and associate with
polysaccharide of Nostoc commune (cyanobacteria) inhibits fusion of membrane
Deactivation of photosynthetic activities is triggered by loss of a small amount of water
in a desiccation-tolerant cyanobacterium, Nostoc commune. Plant Cell Physiol 45:
872-878.
Hirschberg J & Chamovitz D (1994) Carotenoids in cyanobacteria. The Molecular Biology of
Netherlands.
Isolation of an antiviral polysaccharides, nostoflan, from a terrestrial cyanobacterium,
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of
Lipman CB (1941) The successful revival of Nostoc commune from a herbarium specimen


Table 1. Comparison of naturally growing colonies of *Nostoc verrucosum* and *Nostoc commune* in terms of water content, pigment, and uronic acid level

<table>
<thead>
<tr>
<th></th>
<th><em>N. verrucosum</em></th>
<th><em>N. commune</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content (%)</td>
<td>99 ± 1 (<em>n</em> = 12)</td>
<td>98 ± 1 (<em>n</em> = 3)</td>
</tr>
<tr>
<td>Chl a µg·mg⁻¹ [DW]</td>
<td>3.4 ± 0.1 (<em>n</em> = 3)</td>
<td>1.6 ± 0.9 (<em>n</em> = 3)</td>
</tr>
<tr>
<td>Carotenoids µg·mg⁻¹ [DW]</td>
<td>0.7 ± 0.3 (<em>n</em> = 3)</td>
<td>0.5 ± 0.2 (<em>n</em> = 3)</td>
</tr>
<tr>
<td>MAA µg·mg⁻¹ [DW]</td>
<td>2.5 ± 0.4 (<em>n</em> = 3)</td>
<td>0.4 ± 0.03 (<em>n</em> = 3)</td>
</tr>
<tr>
<td>Scytonemin µg·mg⁻¹ [DW]</td>
<td>0.3 ± 0.1 (<em>n</em> = 3)</td>
<td>0.4 ± 0.03 (<em>n</em> = 3)</td>
</tr>
<tr>
<td>Uronic acid µg·mg⁻¹ [DW]</td>
<td>208 ± 97 (<em>n</em> = 3)</td>
<td>220 ± 86 (<em>n</em> = 3)</td>
</tr>
</tbody>
</table>

Colonies of *N. verrucosum* and *N. commune* naturally grown in the field were examined. Data are presented as means ± SD.

* Chlorophyll a (Chl a) and carotenoids were extracted with methanol and determined spectrophotometrically.

* Mycosporine-like amino acids (MAA) and scytonemin were extracted with methanol and determined by HPLC as described in Materials and Methods.

* Uronic acid was determined by carbazole assay.
Table 2. Effects of desiccation and freeze-thawing on photosynthetic activity of naturally growing colonies of *Nostoc verrucosum* and *Nostoc commune*

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>N. verrucosum</em></th>
<th><em>N. commune</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Desiccation</td>
<td>ND (n = 12)</td>
<td>97 ± 8 (n = 6)</td>
</tr>
<tr>
<td>Freeze-thawing</td>
<td>87 ± 16 (n = 9)</td>
<td>115 ± 14 (n = 7)</td>
</tr>
</tbody>
</table>

Field-isolated, natural colonies retaining the extracellular matrix were examined. Initial O\textsubscript{2} evolution levels of wet colonies were determined. The normalized (100%) level in the untreated colonies was between 201 and 1381 µmol O\textsubscript{2}·g\textsuperscript{-1} [DW] h\textsuperscript{-1} for *N. verrucosum* and 79 to 499 µmol O\textsubscript{2}·g\textsuperscript{-1} [DW] h\textsuperscript{-1} for *N. commune*. Wet colonies were air-dried under ambient conditions or frozen at –30°C. After rehydration or thawing, O\textsubscript{2} evolution was measured using an aqueous-phase oxygen electrode with 10 mM NaHCO\textsubscript{3} as a final electron acceptor. Data are presented as means ± SD.

ND, not detected.
Table 3. Effects of desiccation on trehalose accumulation by *Nostoc verrucosum* and *Nostoc commune*

<table>
<thead>
<tr>
<th></th>
<th><em>N. verrucosum</em></th>
<th><em>N. commune</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colonies</td>
<td>Cultured cells of strain KU005</td>
</tr>
<tr>
<td>Hydrated</td>
<td>ND (n = 4)</td>
<td>ND (n = 5)</td>
</tr>
<tr>
<td>Desiccated*</td>
<td>2.3 ± 0.5 (n = 5)</td>
<td>3.1 ± 1.0 (n = 6)</td>
</tr>
</tbody>
</table>

Trehalose levels in field-isolated natural colonies of *N. verrucosum* and *N. commune* and laboratory cultured cells, *N. verrucosum* strain KU005 and *N. commune* strain KU002, were determined by HPLC as described in Materials and Methods. The detection limit of this assay was approximately 0.2 µg trehalose mg⁻¹ [DW]. Data are presented as means ± SD.

*Colonies and cells were air-dried under ambient conditions.

ND, not detected.
Fig. 1

Fig. 1. Effects of desiccation and freeze-thawing on viability of cells in field isolated, naturally growing colonies of *Nostoc verrucosum*. Untreated controls (A and B). After desiccation treatment (C and D). After freeze-thawing treatment (E and F). Cells were visualized by red fluorescence from chlorophyll (A, C, and E) and live cells emitting green fluorescence from fluorescein were detected by vital staining (B, D, and F). Scale bar = 20 µm.
Fig. 2. Electrophoretic patterns of extracellular polysaccharides purified from *Nostoc verrucosum* and *Nostoc commune*. Purified extracellular polysaccharides of *N. verrucosum* (lane 1) and *N. commune* (lane 2) were characterized by electrophoresis on cellulose acetate membranes in 0.47 M formic acid-pyridine buffer (pH 3) (A) or 0.1 M barium acetate buffer (B). Polysaccharides were stained with Alcian blue. Hyaluronic acid (lane 3), heparin (lane 4), chondroitin sulfate (lane 5), and dermatan sulfate (lane 6) sodium salts were used for comparison. Each lane contained 1 µg of sample. Arrows indicate the direction of electrophoresis.
Fig. 3. Electrophoretic patterns of water-soluble extracellular matrix proteins of *Nostoc verrucosum* and *Nostoc commune*. Extracellular matrix proteins were extracted using 0.75 M phosphate buffer (pH 7.0) from of *N. verrucosum* (lane 1) or *N. commune* (lane 2) colonies, resolved by SDS-PAGE and stained with CBB. Each lane contained 20 µg of protein. A commercial molecular size marker preparation (Precision Plus Protein unstained standards; Bio-Rad Laboratories, Hercules, CA) was used as a standard.
Fig. S1. Field-isolated, naturally growing colonies of *Nostoc verrucosum* (A) and *Nostoc commune* (B). Colonies of *N. verrucosum* (C) and *N. commune* (D) were stained by Alcian blue and observed by microscope. The extracellular matrix of both *Nostoc* species reacted similarly to Alcian blue reagent.
Fig. S2

A

Nostoc verrucosum 33-kDa WspA
MALFGTYGEDRDQNASNGKQLDVYRFLIPNAPTPTTPTPGVGTVTIA
GTVVTALEGLTNPTGTGRTAINERVTGSSPIDPFTIVNVNPDQPA
PVNVPVVRTPSDLRFQFEGSGADGRDFSATSQPIVHYTIAGNRSASNGV
PVGSSSLKYKTLNPAISIVDNTPTTPERLGTQPTNPGKADGLAIDNLTP
GRTRAFASDLSTDDGDQALYKVDTLTQALSAPANLRSNQHQLSVDNDS
GLAFSNVTGSTQRLFAWEETGRLYEITGFQDELNASGLTLGSTGNGAG
FATATLAVLNPASTGVDYEGFTIANE

B

Nostoc commune 33-kDa WspA
MALGYTGEDRDQNPNSGQQLDVYRFLIPAAPGTITPTLVTGVTNLAGT
VTTADLEGLGNPANPATERTVGAVNEARVGSTAIDAPGAIVVINNVDQP
FAPATTSPRTPSLDLRFFGDFSGADGRDYSAPQYIVLNYISNGDSSG
GVVGSYLLKITTANPGVISLVDGFGAPTQAQRQPGTPTNSQFADGLAI
DNLNPGRTRAFSDFSTEVDGDQALYKVDTLTGELSAPITLRTPQALN
LNLDGLSFTNSQGLRIGWETGAAYEITGYQDELASGLGLGSATGSN
GAGFATATLNGVNPNASGVVDYEGFTIVNE

C

Nostoc commune 31-kDa WspA
MALGYTGEDRDQNPNSGQQLDVYRFLIPAAPGTITPTLVTGVTNLAGT
VTTADLEGLGNPANPATERTVGAVNEARVGSTAIDAPGAIVVINNVDQP
FAPATTSPRTPSLDLRFFGDFSGADGRDYSAPQYIVLNYISNGDSSG
GVVGSYLLKITTANPGVISLVDGFGAPTQAQRQPGTPTNSQFADGLAI
DNLNPGRTRAFSDFSTEVDGDQALYKVDTLTGELSAPITLRTPQALN
LNLDGLSFTNSQGLRIGWETGAAYEITGYQDELASGLGLGSATGSN
GAGFATATLNGVNPNASGVVDYEGFTIVNE

Fig. S2. Summary of WspA identification by MALDI-TOF tandem mass spectrometry analysis. (A) The obtained peptide fragments from N. verrucosum 33-kDa WspA protein are marked by red on the deduced amino-acid sequence of the wspA gene of N. verrucosum strain KU005 (AB509258). The obtained peptide fragments from N. commune 33-kDa WspA protein (B) and N. commune 31-kDa WspA protein (C) are marked by red on the deduced amino-acid sequence of the wspA gene of N. commune strain KU002 (AB518000).