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The extracellular-matrix retaining cyanobacterium *Nostoc verrucosum* accumulates trehalose but is sensitive to desiccation

Running title: Trehalose and extracellular matrix in *Nostoc*

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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.

Materials and methods

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 $\mu g \cdot L^{-1}$), thiamine (2 $mg \cdot L^{-1}$), and cyanocobalamin (1 $\mu g \cdot L^{-1}$) (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* stain 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):

$$[\text{Chl a } (\mu g \cdot mL^{-1})] = (A_{665} - A_{750}) \times 13.9$$

$$[\text{Carotenoids } (\mu g \cdot mL^{-1})] = \{(A_{461} - A_{750}) - 0.046 \times (A_{665} - A_{750})\} \times 4$$

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 $mL \cdot min^{-1}$ 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 $L \cdot g^{-1} \cdot cm^{-1}$ (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 $L \cdot g^{-1} \cdot cm^{-1}$ (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).

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, Köngen, Germany) in 3% (w/w) acetic acid and observed by light microscopy (Tamaru *et al*., 2005).

Measurement of O_2 evolution

Photosynthetic O_2 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 $NaHCO_3$ as a final electron acceptor, under saturated actinic light of 1600 $\mu E\ m^{-2} \cdot s^{-1}$ at 30°C.

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).

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

M). 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 $mL \cdot min^{-1}$. 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-10Axl; Shimadzu, Kyoto, Japan) with excitation at 320 nm. For analysis of uronic acids, a Shimpac 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 $mL \cdot min^{-1}$. 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 $6000 \times g$ 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 cyanobacteia.

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.*

commune KU002. 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 $\mu g \cdot mg^{-1}$ 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.

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

	N. verrucosum	***N. commune***
Water content % (w/w)	99 ± 1 (*n* = 12)	98 ± 1 (*n* = 3)
Chl a[a] µg·mg^{-1} [DW]	3.4 ± 0.1 (*n* = 3)	1.6 ± 0.9 (*n* = 3)
Carotenoids[a] µg·mg^{-1} [DW]	0.7 ± 0.3 (*n* = 3)	0.5 ± 0.2 (*n* = 3)
MAA[b] µg·mg^{-1} [DW]	2.5 ± 0.4 (*n* = 3)	0.4 ± 0.03 (*n* = 3)
Scytonemin[b] µg·mg^{-1} [DW]	0.3 ± 0.1 (*n* = 3)	0.4 ± 0.03 (*n* = 3)
Uronic acid[c] µg·mg^{-1} [DW]	208 ± 97 (*n* = 3)	220 ± 86 (*n* = 3)

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

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

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

[c] 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*

Treatment	*N. verrucosum*	*N. commune*
	(% of control)	
Desiccation	ND (*n* = 12)	97 ± 8 (*n* = 6)
Freeze-thawing	87 ± 16 (*n* = 9)	115 ± 14 (*n* = 7)

Field-isolated, natural colonies retaining the extracellular matrix were examined. Initial O_2 evolution levels of wet colonies were determined. The normalized (100%) level in the untreated colonies was between 201 and 1381 μmol $O_2 \cdot g^{-1}$ [DW] h^{-1} for *N. verrucosum* and 79 to 499 μmol $O_2 \cdot g^{-1}$ [DW] h^{-1} for *N. commune*. Wet colonies were air-dried under ambient conditions or frozen at –30°C. After rehydration or thawing, O_2 evolution was measured using an aqueous-phase oxygen electrode with 10 mM $NaHCO_3$ 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*

	N. verrucosum		***N. commune***	
	Colonies	**Cultured cells of strain KU005**	**Colonies**	**Cultured cells of strain KU002**
	($\mu g\ mg^{-1}$ [DW])			
Hydrated	ND (n = 4)	ND (n = 5)	ND (n = 4)	ND (n = 5)
Desiccated[a]	2.3 ± 0.5 (n = 5)	3.1 ± 1.0 (n = 6)	1.3 ± 0.5 (n = 4)	4.2 ± 1.9 (n = 7)

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.

[a] Colonies and cells were air-dried under ambient conditions.

ND, not detected.

Fig. 1

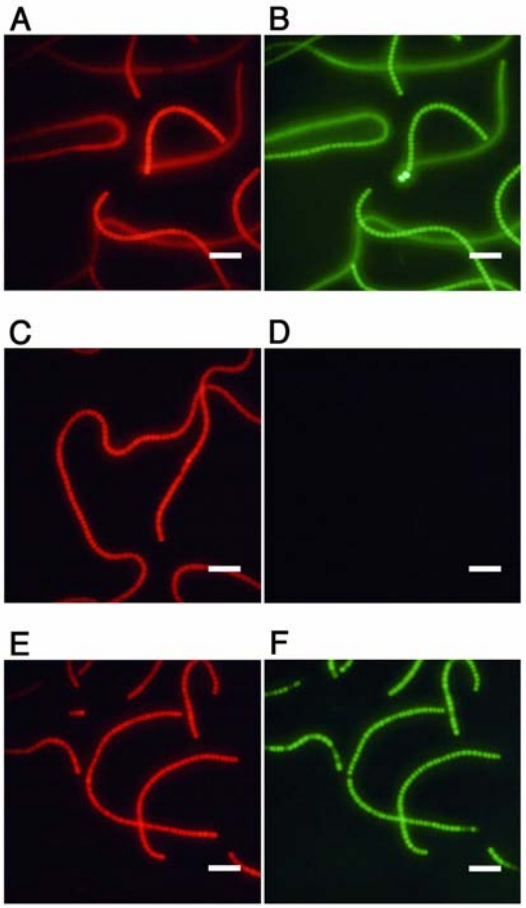

Fig. 1. Effects of desiccation and freeze-thawing on viability of cells in field isolated, naturally growing colonies of *Nostoc verrucosum*. Untreated controls (A and B). After desiccation treatment (C and D). After freeze-thawing treatment (E and F). Cells were visualized by red fluorescence from chlorophyll (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

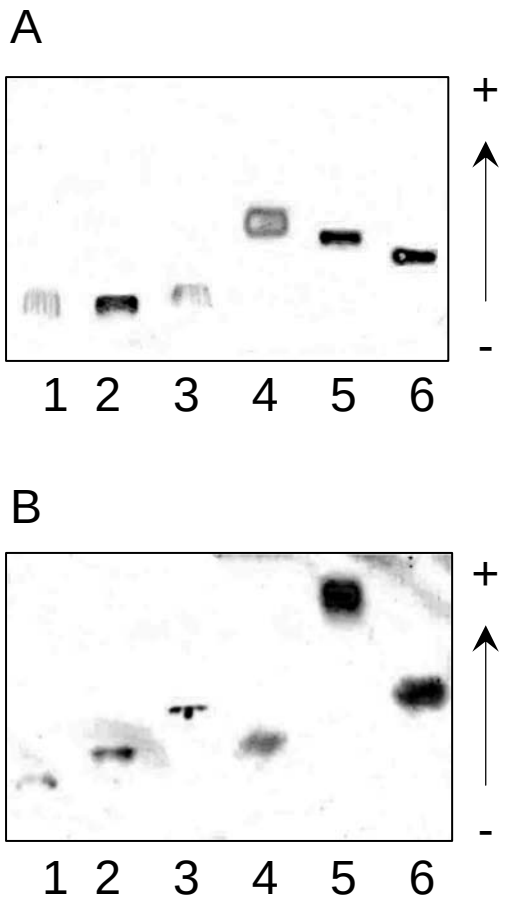

Fig. 2. Electrophoretic patterns of extracellular polysaccharides purified from *Nostoc verrucosum* and *Nostoc commune*. Purified extracellular polysaccharides of *N. verrucosum* (lane 1) and *N. commune* (lane 2) were characterized by electrophoresis on cellulose acetate membranes in 0.47 M formic 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

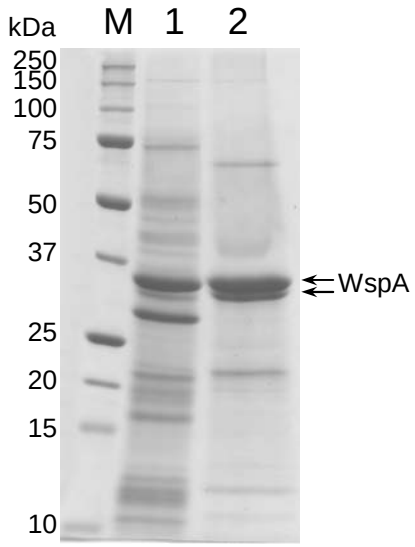

Fig. 3. Electrophoretic patterns of water-soluble extracellular matrix proteins of *Nostoc verrucosum* and *Nostoc commune*. Extracellular matrix proteins were extracted using 0.75 M phosphate buffer (pH 7.0) from of *N. verrucosum* (lane 1) or *N. commune* (lane 2) colonies, resolved by SDS-PAGE and 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

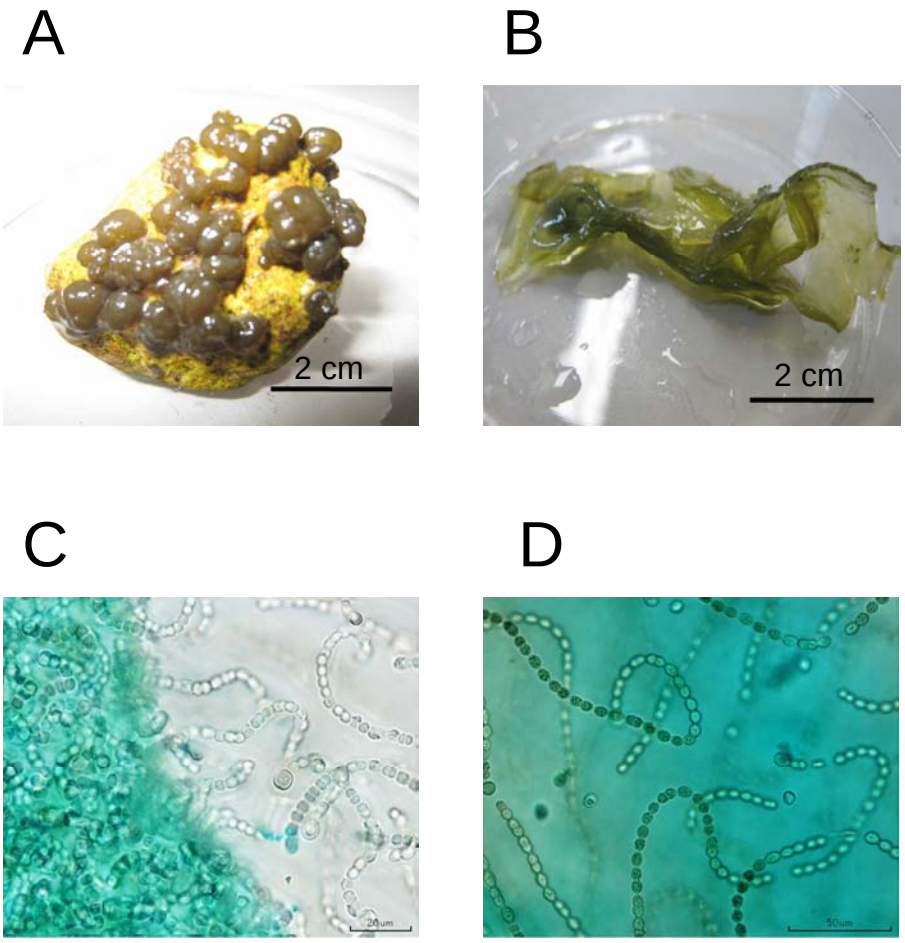

Fig. S1. Field-isolated, naturally growing colonies of *Nostoc verrucosum* (A) and *Nostoc commune* (B). Colonies of *N. verrucosum* (C) and *N. commune* (D) were stained by Alcian blue and observed by microscope. The extracellular matrix of both *Nostoc* species reacted similarly to Alcian blue reagent.

Fig. S2

A

Nostoc verrucosum 33-kDa WspA

MALFGYTIGEDRDQNASNGKQLDVYRFLIPNAPTTTPITPTGPVGTVTIA
GTVTTADLEGLGTNPTTGRVTAINETRVTGSTTPIDPTVFIVNNVDQPFA
PVNVPVVRTPSDLRRFGFESGADFGRDFSATSQPIVHYTIAGNRSASNGV
PVGSSLYKVTLTNPAISIVDNTPTTPERLGTQPTNPGKFADGLAIDNLTP
GRTRAFASDLSSTDGDGAQLYKVDLLTGQLSAPINLRNSSGQPLSVNSDS
GLAFSNVTGSTQRLFAWEETGRLYEITGFQDELNASGLTLGSTTGTNGAG
FATATLLATVNLPNASTGVVDYEGFTIANE

B

Nostoc commune 33-kDa WspA

MALYGYTIGEDRDQNPSNGKQLDVYRFLIPAAPGGTITPTLVGTVNLAGT
VTTADLEGLGTNPANPATERVGAVNEARVGGSTAIDPAPGAIVVNNVDQP
FAPATTSPTRTPSDLRRFGFDSGADFGRDYSAPGQPIVLYNISGNDDSSG
GVPVGSSLYKITTANPGVISLVDGFGAPTTAAQRQGTQPTNSQFADGLAI
DNLNPGRTRAFASDFSTVDGDGAQLYKVDLLTGELSAPITLRTPSGQALN
LNLDSGLSFTNSGSQRLIGWVETGAVYEITGYQDELTASGLGLGSATGSN
GAGFATATLLGNVNLPNAVSGVVDYEGFTIVNE

C

Nostoc commune 31-kDa WspA

MALYGYTIGEDRDQNPSNGKQLDVYRFLIPAAPGGTITPTLVGTVNLAGT
VTTADLEGLGTNPANPATERVGAVNEARVGGSTAIDPAPGAIVVNNVDQP
FAPATTSPTRTPSDLRRFGFDSGADFGRDYSAPGQPIVLYNISGNDDSSG
GVPVGSSLYKITTANPGVISLVDGFGAPTTAAQRQGTQPTNSQFADGLAI
DNLNPGRTRAFASDFSTVDGDGAQLYKVDLLTGELSAPITLRTPSGQALN
LNLDSGLSFTNSGSQRLIGWVETGAVYEITGYQDELTASGLGLGSATGSN
GAGFATATLLGNVNLPNAVSGVVDYEGFTIVNE

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).