

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

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3 1 **The extracellular-matrix retaining cyanobacterium *Nostoc verrucosum* accumulates**
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6 2 **trehalose but is sensitive to desiccation**
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10 4 Running title: Trehalose and extracellular matrix in *Nostoc*
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3 1 **Abstract**
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5 2 The aquatic cyanobacterium *Nostoc verrucosum* forms macroscopic colonies, which consist
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7 3 of both cellular filaments and massive extracellular matrix material. In this study, the
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9 4 physiological features of *N. verrucosum* were investigated and compared with those of the
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11 5 anhydrobiotic cyanobacterium *Nostoc commune*. *N. verrucosum* cells were sensitive to
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13 6 desiccation but tolerant of freeze-thawing treatment in terms of both cell viability and
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15 7 photosynthetic O₂ evolution. Natural colonies of these cyanobacteria contained similar levels
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17 8 of chlorophyll a, carotenoids, the UV-absorbing pigments scytonemin and mycosporine-like
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19 9 amino acids (MAA), and uronic acid (a component of extracellular polysaccharides).
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21 10 Extracellular polysaccharides from both *N. verrucosum* and *N. commune* indicated low
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23 11 acidity and a high affinity for divalent cations, although their sugar compositions differed.
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25 12 The WspA protein, known to be a major component of the extracellular matrix of *N. commune*,
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27 13 was detected in *N. verrucosum*. Desiccation caused similarly high levels of trehalose
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29 14 accumulation in both cyanobacteria. Although previously considered relevant to
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31 15 anhydrobiosis in the terrestrial cyanobacterium *N. commune*, the data presented here suggest
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33 16 that extracellular matrix production and trehalose accumulation are not enough for standing
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35 17 extreme desiccation in *N. verrucosum*.
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48 20 Key words: compatible solute, stress tolerance, extracellular polysaccharide, water stress
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50 21 protein (WspA).
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1 Introduction

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

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

18 *N. commune* colonies are naturally subjected to regular cycles of desiccation and
19 wetting. Photosynthetic activity is maintained during desiccation and recovers rapidly upon
20 rehydration (Scherer *et al.*, 1984; Satoh *et al.*, 2002; Tamaru *et al.*, 2005; Sakamoto *et al.*,
21 2009). During desiccation of *N. commune* colonies, photosynthetic activity decreases
22 concomitant with water content (Sakamoto *et al.*, 2009). This cessation of photosynthetic
23 electron transport is thought to be an acclimatory response to desiccation (Hirai *et al.*, 2004;
24 Fukuda *et al.*, 2008); however, the mechanism regulating photosynthesis remains unknown.

25 Anhydrobiotic organisms accumulate trehalose (α -D-glucopyranosyl-[1,

1 1]- α -D-glucopyranoside) as a compatible solute. Trehalose protects biological membranes
2 and proteins against the deleterious effects of water removal by replacement of hydrating
3 water molecules and formation of amorphous glasses (vitrification) (Clegg, 2001). Trehalose
4 is synthesized from α (1,4)-linked glucose polymers by the TreY-TreZ pathway in
5 cyanobacteria of the genus *Nostoc* (Kato *et al.*, 2004; Higo *et al.*, 2006; Yoshida and
6 Sakamoto, 2009). Maltooligosyl trehalose synthase (Mts), encoded by the *treY* gene, converts
7 the terminal α (1,4)-linked residue of the glucose polymer to an α (1,1) linkage. Maltooligosyl
8 trehalose trehalohydrolase (Mth), encoded by the *treZ* gene, then produces free trehalose by
9 cleavage of the terminal disaccharide (De Smet *et al.*, 2000). Trehalose accumulation occurs
10 in response to water loss during desiccation (Sakamoto *et al.*, 2009; Yoshida and Sakamoto,
11 2009) and the trehalose accumulation capacity of *N. commune* is similar to that of the
12 drought-resistant cyanobacteria *Phormidium autumnale* and *Chroococcidiopsis* sp. when
13 exposed to matric or osmotic water stress (Hershkovitz *et al.*, 1991). Therefore, trehalose
14 accumulation is thought to be relevant to cyanobacterial desiccation tolerance.

15 The water stress protein, encoded by the *wspA* gene, is a 36-kDa protein present in
16 the extracellular matrix of *N. commune* and is presumed to be relevant to the structure and/or
17 function of the extracellular matrix (Scherer & Potts, 1989; Wright *et al.*, 2005; Morsy *et al.*,
18 2008). No *wspA* gene has been found in the genome of *Nostoc punctiforme* ATCC 29133
19 (Meeks *et al.*, 2001) and the *wspA* gene of *N. commune* may be a xenolog, acquired through
20 lateral gene transfer (Wright *et al.*, 2005).

21 The cyanobacterium *Nostoc verrucosum* forms macroscopic colonies with an
22 extracellular matrix the appearance of which is superficially similar to those of *N. commune*,
23 although *N. verrucosum* colonies always occur in streams. As such colonies may be subjected
24 to periodic drying in their natural habitat when the water level in the stream decreases, *N.*
25 *verrucosum* may also be tolerant to desiccation but little is known about the physiological

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1 features of this cyanobacterium. In this study, we assessed (i) the desiccation tolerance of *N.*
2 *verrucosum* as compared to the terrestrial cyanobacterium *N. commune* (ii) components of the
3 extracellular matrix and (iii) the capacity to accumulate trehalose in response to desiccation.
4 Unlike *N. commune*, *N. verrucosum* was sensitive to desiccation, despite increased trehalose
5 levels within dry colonies.

6 **Materials and methods**

7 **Microorganisms**

8 Colonies of *N. verrucosum* (Japanese vernacular name: *Ashitsuki*) growing naturally in the
9 stream were collected from Shishiku Park, Hakusan-shi, Ishikawa, Japan (36°26'8"N,
10 136°38'37"E). The laboratory strain KU005 of *N. verrucosum* was isolated by streaking and
11 spreading on agar plates (Castenholz, 1988) and cultured at 18°C under fluorescent light in
12 modified BG11₀ (without nitrate) medium or BG11₀ agar supplemented with biotin (1 µg·L⁻¹),
13 thiamine (2 mg·L⁻¹), and cyanocobalamin (1 µg·L⁻¹) (Castenholz, 1988) buffered with
14 HEPES-NaOH (20 mM; pH 7.5). *N. verrucosum* strain KU005 has been deposited in
15 Microbial Culture Collection at National Institute for Environmental Studies
16 (NIES-Collection) and its 16S rRNA gene sequence has been submitted to
17 GenBank/EMBL/DDBJ under the accession number AB494996.

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

1 **Measurement of water content**

2 After determination of fresh weight (FW), colonies were dried under ambient conditions and
3 their dry weight (DW) was determined. Water content was calculated as follows: (water
4 content %) = $(FW - DW)/FW \times 100\%$.

5 **Measurement of chlorophyll and total carotenoids**

6 Chlorophyll a (Chl a) and carotenoids were extracted with 100% methanol and concentrations
7 were determined spectrophotometrically (Tandeau de Marsac & Houmard, 1988; Hirschberg
8 & Chamovitz, 1994). Chl a and carotenoid concentrations were calculated using the following
9 equations (A_{750} was subtracted to correct for light scattering):

$$10 \quad [\text{Chl a } (\mu\text{g}\cdot\text{mL}^{-1})] = (A_{665} - A_{750}) \times 13.9$$

$$11 \quad [\text{Carotenoids } (\mu\text{g}\cdot\text{mL}^{-1})] = \{(A_{461} - A_{750}) - 0.046 \times (A_{665} - A_{750})\} \times 4$$

12 **Measurement of UV-absorbing pigments**

13 The UV-absorbing pigments mycosporine-like amino acid (MAA) and scytonemin were
14 extracted in 100% methanol and analyzed by high-performance liquid chromatography
15 (HPLC) with a UV-VIS detector. The methanol-soluble fraction was separated on a reverse
16 phase column (Wakosil 5C18, 4.6 mm \times 250 mm; Wako, Osaka, Japan) using 100% methanol
17 at a flow rate of 1 mL \cdot min⁻¹ as the mobile phase. MAA was detected by A_{330} and the
18 concentration was determined using a standard curve, itself calculated from A_{330} with an
19 extinction coefficient of 120 L \cdot g⁻¹ \cdot cm⁻¹ (Garcia-Pichel & Castenholz, 1993). Scytonemin was
20 detected by A_{380} and its concentration was determined using a standard curve, itself calculated
21 from A_{380} with an extinction coefficient of 112.6 L \cdot g⁻¹ \cdot cm⁻¹ (Ehling-Shulz *et al.*, 1997).

22 **Measurement of uronic acid content**

23 The amounts of uronic acid released from natural colonies by acid hydrolysis were

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3 1 determined using the carbazole assay (Dische, 1947) as described previously (Tamaru *et al.*,
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5 2005; Morsy *et al.*, 2008).
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9 **Visualization of extracellular polysaccharides**

10 To stain acidic mucopolysaccharides, *Nostoc* colonies were placed in Alcian blue reagent (pH
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12 2.5) containing 0.33% (w/w) Alcian Blue 8GS (Chroma-Gesellschaft, Köngen, Germany) in
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14 3% (w/w) acetic acid and observed by light microscopy (Tamaru *et al.*, 2005).
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19 **Measurement of O₂ evolution**

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22 8 Photosynthetic O₂ evolution was measured with an aqueous-phase Clark-type oxygen
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24 9 electrode (Rank Brothers Ltd., Cambridge, UK) in HEPES-NaOH (25 mM; pH 7.0),
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26 10 containing 10 mM NaHCO₃ as a final electron acceptor, under saturated actinic light of 1600
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28 11 $\mu\text{E m}^{-2}\cdot\text{s}^{-1}$ at 30°C.
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33 **Vital staining of cells with fluorescein diacetate (FDA)**

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36 13 After desiccation or freeze-thawing, FDA was used to stain live cells. Live cells emitting
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38 14 green fluorescence were observed by fluorescence microscopy (Tamaru *et al.*, 2005).
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43 **Extraction and purification of EPS**

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45 16 Air-dried colonies of *N. commune* (20 g) were rehydrated in distilled water. After weighing,
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47 17 four volumes of acetone were added and mixtures were stirred for 2 h at room temperature.
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49 18 The defatted colonies were then collected, dried under a chemical hood for 1 h, distilled water
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51 19 (1000 mL) was added, and samples were heated in a boiling water bath for 2 h. After cooling
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53 20 to room temperature, the swelled colonies were homogenized in a blender four times at
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55 21 maximum speed for 30 s each time. Homogenates were then passed through a stainless steel
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57 22 mesh (pore diameter ~1.5 mm). The blender was rinsed using 600 mL of water to collect the
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59 23 rest and the rinsate was mixed with the homogenate. Acetic acid was added to a final
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1 concentration of 1% (pH 4) and homogenates were heated in a boiling water bath for 2 h,
2 cooled to room temperature, and centrifuged at $9000 \times g$ for 20 min. Supernatants containing
3 water-soluble polysaccharides were treated with 2.5 volumes of ethanol at 4°C overnight and
4 the precipitate was collected by centrifugation at $9500 \times g$ at 4°C for 20 min. After washing in
5 70% (w/v) ethanol, the polysaccharide fraction in pellets was collected by centrifugation at
6 $7100 \times g$ for 10 min and lyophilized (Freeze Dryer FD-80; Eyela). A yield of 4 g of crude
7 polysaccharide was obtained from 20 g of dried *N. commune* colonies. Using the same
8 method, 2 g of polysaccharide was isolated from 10 g of lyophilized *N. verrucosum* colonies.

9 Isolated polysaccharides were purified further as follows. Crude polysaccharide (1 g)
10 was dissolved in 0.4 M NaOH (20 ml) and incubated at 4°C overnight. Samples were adjusted
11 to pH 7.8 using HCl and incubated at 37°C overnight with actinase E (10 mg; Kaken
12 Pharmaceutical Co., Ltd., Tokyo, Japan). After cooling on ice, trichloroacetic acid (5 mL;
13 40% (w/v)) was added and polysaccharides were precipitated at 4°C overnight. After
14 centrifugation at $13000 \times g$ at 4°C for 10 min, neutralized phenol (20 mL), chloroform and
15 isoamyl alcohol (4 mL; 24:1 (v/v)) were added, and samples were mixed vigorously for
16 phenol-chloroform extraction. The aqueous phase was recovered by centrifugation at $13000 \times$
17 g at 4°C for 10 min and 2.5 volumes of ethanol were added. After incubation for 10 min at
18 -30°C , precipitates were collected by centrifugation at $13000 \times g$ at 4°C for 10 min. Pellets
19 were washed in 70% (w/v) ethanol, and purified polysaccharides were collected by
20 centrifugation at $13000 \times g$ at 4°C for 10 min and lyophilized (Freeze Dryer FD-80; Eyela,
21 Tokyo, Japan).

22 Cellulose acetate membrane electrophoresis

23 Purified EPS were characterized by electrophoresis on cellulose acetate membranes (Jokoh,
24 Kawasaki, Japan) in formic acid-pyridine buffer (0.47 M; pH 3) or barium acetate buffer (0.1

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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.

5 Sugar composition analysis

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

19 Isolation of the *wspA* gene

20 A 0.8 kb DNA fragment containing the *wspA* gene was amplified by PCR from *N. commune*
21 KU002 or *N. verrucosum* KU005 genomic DNA using the following degenerate primers:
22 forward (23-mer with 96-fold degeneracy) 5'-TA(T/C) GGI TA(T/C) ACI AT(T/C/A) GGI
23 GA(A/G) GA-3', derived from the amino acid sequence YGYTIGED; and reverse (23-mer
24 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',

1 derived from the amino acid sequence YEITGYQD. PCR was performed under the following
2 conditions: initial denaturation at 94°C for 1 min, followed by 40 cycles of 94°C for 1 min,
3 50°C for 2 min, and 72°C for 3 min, with a final extension step at 72°C for 5 min. Specific
4 primers for PCR amplification of the entire *wspA* gene were designed based on the nucleotide
5 sequences of the PCR product, the *wspA* gene of *N. commune* DRH1 (accession no.
6 DQ155425), and the *Nostoc punctiforme* ATCC 29133 genome (Meeks *et al.*, 2001). These
7 were: for *N. commune* KU002 (forward: 5'-TAATAGCGATCGCCTGCGAA-3', and reverse:
8 5'-CCATTAACGGATGCTTGAG-3') and for *N. verrucosum* KU005 (forward:
9 5'-ATTCTTGATTCTCCATTAAC-3', and reverse: 5'-AGATGAGTACATTGAAGGTG-3').
10 The *wspA* gene sequences were determined and submitted to GenBank/EMBL/DDBJ under
11 accession numbers AB518000 (*N. commune* strain KU002) and AB509258 (*N. verrucosum*
12 strain KU005).

13 **Extraction and characterization of extracellular matrix water-soluble proteins**

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

24 Protein concentrations were determined using the Coomassie Brilliant Blue (CBB)
25 G-250 dye-binding method (Coomassie® Protein Assay Reagent; Pierce Biotechnology Inc.,

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1 Rockford, IL) as described by Bradford (1976) using BSA as a standard. SDS-PAGE was
2 performed according to the method of Laemmli (1970) using vertical electrophoresis glass
3 plates. Gels were stained using CBB R-250. Molecular masses were estimated using a
4 molecular size marker set (Precision Plus Protein unstained standards; Bio-Rad Laboratories,
5 Hercules, CA). The relative intensities of protein bands were determined using the public
6 domain NIH Image program (developed at the U.S. National Institutes of Health and
7 available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

8 Proteins were identified by in-gel trypsin digestion and tandem mass spectrometry
9 (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA) (Asano &
10 Nishiuchi, 2010). Protein Pilot™ software was used to determine the amino acid sequences of
11 peptides by fragmentation pattern analysis. Peptide identities were confirmed manually by
12 comparison with the amino acid sequences predicted from the nucleotide sequences of the *N.*
13 *verrucosum* and *N. commune* *wspA* genes.

14 Determination of trehalose

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

1 **Results and discussion**

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

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

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

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

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1 **Stress tolerance of *N. verrucosum* and *N. commune***

2 Restoration of O₂ evolution after stress was measured to determine stress tolerance (Table 2).
3 Photosynthetic O₂ evolution in *N. verrucosum* colonies was sensitive to desiccation; no
4 O₂-evolving capacity remained after air-drying (Table 2). Consistent with this loss of
5 photosynthetic O₂ evolution, no cells were detected by FDA staining after air-drying (Fig. 1D),
6 indicating that cells of *N. verrucosum* was fatally damaged by desiccation. Although cells of
7 *N. verrucosum* were sensitive to desiccation treatment, O₂ evolution was unaffected by
8 freeze-thawing (Table 2). Cell viability was confirmed by vital staining, and almost all cells
9 survived after freeze-thawing (Fig. 1F). These observations suggest that *N. verrucosum* cells
10 are sensitive to desiccation but tolerant to freeze-thawing, although the terrestrial
11 cyanobacterium *N. commune* is tolerant to both desiccation and freeze-thawing (Table 2;
12 Tamaru *et al.*, 2005).

13 **Comparison of the EPS**

14 EPS of *N. verrucosum* and *N. commune* were isolated and purified. Figure 2 shows the
15 electrophoretic patterns generated by the EPS upon cellulose acetate membrane
16 electrophoresis using 0.47 M formic acid-pyridine buffer (pH 3) (Fig. 2A) and 0.1 M barium
17 acetate buffer (Fig. 2B). EPS from both *N. verrucosum* (Fig. 2, lane 1) and *N. commune* (Fig.
18 2, lane 2) migrated as a single band, suggesting that they are similarly charged. The electrical
19 mobilities of both EPS in 0.47 M formic acid-pyridine buffer (pH 3) (Fig. 2A) were similar to
20 that of hyaluronic acid (lane 3) and lower than those of heparin (lane 4), chondroitin sulfate
21 (lane 5), or dermatan sulfate (lane 6), suggesting low acidity. In 0.1 M barium acetate buffer
22 (Fig. 2B), cyanobacterial EPS showed low electrical mobility, suggesting a high affinity for
23 divalent cations.

24 EPS purified from *N. verrucosum* contained glucose, mannose, xylose, and
25 glucuronic acid in a molar ratio of 5:5:2:1. EPS from *N. commune* contained glucose, xylose,

1 galactose, mannose, and glucuronic acid in a molar ratio of 5:5:3:2:1. The neutral sugars
2 detected in *N. commune* EPS were similar to those of nostoflan, a polysaccharide isolated and
3 characterized from the terrestrial cyanobacterium *Nostoc flagelliforme* (Kanekiyo *et al.*,
4 2005). Further studies are required to determine the structure of EPS from *N. verrucosum* to
5 compare with that of *N. commune* EPS partially determined (Helm *et al.*, 2000).

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

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

14 Degenerate primers were designed according to the deduced amino acid sequence of WspA of
15 *N. commune* DRH1 (Wright *et al.*, 2005). A DNA fragment of 0.8 kb containing part of the
16 *wspA* gene was successfully amplified from *N. commune* KU002 genomic DNA. Using the
17 same primers, the *wspA* gene was amplified and identified in *N. verrucosum* KU005. DNA
18 fragments containing the entire *wspA* gene were isolated and sequenced. The *wspA* gene of *N.*
19 *commune* KU002 (accession no. AB518000) encoded a polypeptide of 333 amino acids with
20 77% sequence identity to WspA of *N. commune* DRH1 (Wright *et al.*, 2005). The WspA of *N.*
21 *verrucosum* KU005 (AB509258) contained 330 amino acids and showed 73% sequence
22 identity to that of *N. commune* KU002 (AB518000), indicating that *N. verrucosum* possesses
23 the *wspA* gene. It has been thought that *N. commune* is the only cyanobacterial species to
24 possess the *wspA* gene, and no *wspA* gene has been found in the genome of *N. punctiforme*
25 ATCC 29133 (Meeks *et al.*, 2001) despite 97% sequence identity of 16S rRNA to *N.*

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1 *commune* KU002. The presence of *wspA* in the aquatic cyanobacterium *N. verrucosum* raises
2 a new question of the origin of the *wspA* gene in cyanobacteria.

3 4 **Comparison of water-soluble proteins in the extracellular matrix**

5 Water-soluble extracellular matrix proteins of *N. verrucosum* and *N. commune* were prepared
6 using mild extraction conditions so as not to disrupt the cells (Fig. 3). In *N. verrucosum*, a
7 33-kDa protein predominated (Fig. 3 lane 1); its molecular mass was similar to that predicted
8 for the *N. verrucosum* *wspA* gene product. This 33-kDa protein was identified as WspA by
9 MALDI-TOF tandem mass spectrometry analysis. Peptide fragments with an identical amino
10 acid sequence to WspA of *N. verrucosum* KU005 were detected with 63% coverage (Fig. S2).
11 These data suggest that WspA is a major component of the extracellular matrix of *N.*
12 *verrucosum*, although whether WspA has identical or similar functions in *Nostoc* species
13 remains unknown.

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

24 **Trehalose accumulation in response to desiccation**

25 Trehalose levels in *N. verrucosum* and *N. commune* colonies and *N. verrucosum* KU005 and

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3 1 *N. commune* KU002 laboratory cultures were examined (Table 3). Trehalose was not detected
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6 2 in wet colonies of either cyanobacterial species or in wet cells cultured in the laboratory.
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8 3 When induced by air-drying, trehalose was detected at concentrations of 1.3 to 4.2 $\mu\text{g}\cdot\text{mg}^{-1}$
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10 4 DW (Table 3). These values were essentially equivalent to those reported previously for both
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12 5 *N. commune* (Sakamoto *et al.*, 2009) and laboratory cultures of *Nostoc punctiforme* strain
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14 6 IAM M-15 (Yoshida and Sakamoto, 2009). Trehalose accumulation in cyanobacteria is
15
16 7 thought to be related to desiccation tolerance as both the terrestrial cyanobacterium *N.*
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18 8 *commune* (Sakamoto *et al.*, 2009) and the desert-isolated drought-resistant cyanobacteria
19
20 9 *Phormidium autumnale* and *Chroococcidiopsis* sp. (Herskovitz *et al.*, 1991) accumulate
21
22 10 trehalose at high levels compared to desiccation-sensitive cyanobacteria, such as *Nostoc* sp.
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24 11 PCC 7120 (Higo *et al.*, 2006) and *Spirulina platensis* (Ohmori *et al.*, 2009). As trehalose
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26 12 levels in air-dried *N. verrucosum* cells were similar to those of *N. commune* (Table 3), the
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28 13 capacity of trehalose production is not linked to desiccation tolerance in *N. verrucosum* (Fig.
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30 14 1, Table 2).

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36 15 Trehalose accumulation appears to be triggered by desiccation; the level of trehalose
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38 16 increases markedly when water content decreases below a critical value (Yoshida and
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40 17 Sakamoto, 2009; Sakamoto *et al.*, 2009). It has been suggested that control of trehalase plays
41
42 18 an important role in trehalose accumulation; the rate of trehalose production exceeds that of
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44 19 hydrolysis. This is mediated by specific inactivation of trehalase under conditions of water
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46 20 stress characterized by increased cellular solute concentrations (Yoshida and Sakamoto,
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48 21 2009). The details of the regulation of trehalase activity in response to cellular solute
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50 22 concentration remain to be elucidated.

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55 23 Sucrose is a stress-inducible, non-reducing disaccharide that accumulates in
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57 24 cyanobacterial cells (Herskovitz *et al.*, 1991; Higo *et al.*, 2006; Yoshida and Sakamoto,
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59 25 2009). In untreated *N. verrucosum* KU005 and *N. commune* KU002 cells, sucrose

1 concentrations ranged from 1 to 3 $\mu\text{g}\cdot\text{mg}^{-1}$ DW. The sucrose concentration of air-dried *N.*
2 *verrucosum* KU005 cells was approximately 5.3 $\mu\text{g}\cdot\text{mg}^{-1}$ DW, which was around three times
3 higher than that in untreated cells, indicating an increase in response to desiccation. In
4 contrast, the sucrose level in desiccated *N. commune* KU002 cells was approximately 1
5 $\mu\text{g}\cdot\text{mg}^{-1}$ DW, which was essentially identical to that in untreated cells. Consistent with these
6 observations, sucrose accumulation in response to desiccation is not evident in natural *N.*
7 *commune* colonies (Sakamoto *et al.*, 2009), suggesting that regulation of sucrose metabolism
8 may be different in these *Nostoc* species. The levels of sucrose detected were similar to those
9 of trehalose but the relative contributions of these non-reducing sugars to stress responses are
10 unknown.

11 **Concluding remarks**

12 The aquatic cyanobacterium *Nostoc verrucosum* was sensitive to desiccation, despite its
13 formation of macroscopic colonies with massive extracellular matrix apparently similar to
14 that of the terrestrial cyanobacterium *Nostoc commune*. In response to desiccation, *N.*
15 *verrucosum* accumulated trehalose at a similar level to *N. commune*. Extracellular matrix
16 production and trehalose accumulation have previously been considered relevant to
17 desiccation tolerance; however, they are not always linked to extreme desiccation tolerance.
18 An important question remains to be answered in future studies; which factor is missing in *N.*
19 *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

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

Field-isolated, natural colonies retaining the extracellular matrix were examined. Initial O₂ evolution levels of wet colonies were determined. The normalized (100%) level in the untreated colonies was between 201 and 1381 μmol O₂·g⁻¹ [DW] h⁻¹ for *N. verrucosum* and 79 to 499 μmol O₂·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, O₂ evolution was measured using an aqueous-phase oxygen electrode with 10 mM NaHCO₃ 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*

	<i>N. verrucosum</i>		<i>N. commune</i>	
	Colonies	Cultured cells of strain KU005	Colonies	Cultured cells of strain KU002
	($\mu\text{g mg}^{-1}$ [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 $0.2 \mu\text{g trehalose mg}^{-1}$ [DW]. Data are presented as means \pm SD.

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

ND, not detected.

Fig. 1

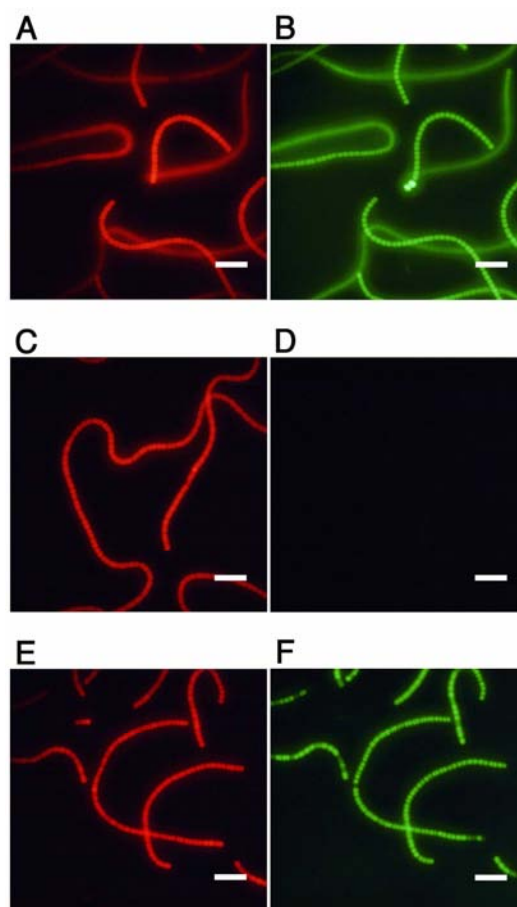


Fig. 1. Effects of desiccation and freeze-thawing on viability of cells in field isolated, naturally growing colonies of *Nostoc verrucosum*. Untreated controls (A and B). After desiccation treatment (C and D). After freeze-thawing treatment (E and F). Cells were visualized by red fluorescence from chlorophyll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

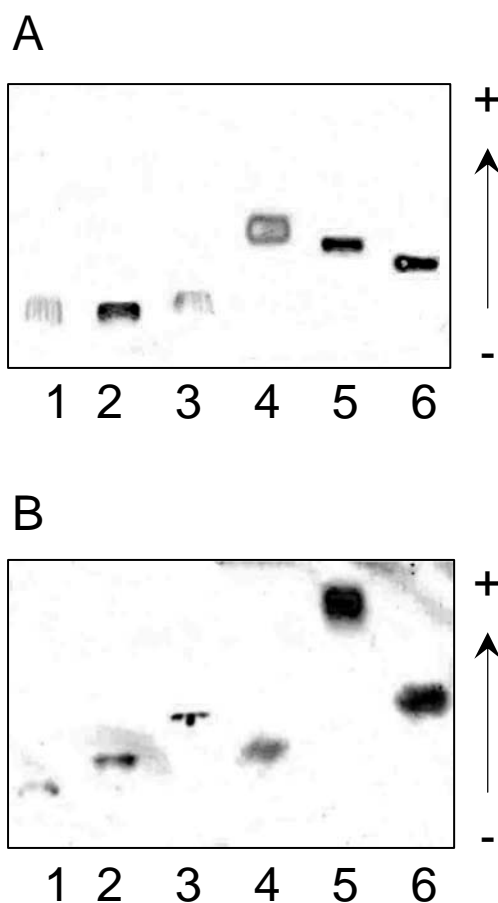


Fig. 2. Electrophoretic patterns of extracellular polysaccharides purified from *Nostoc verrucosum* and *Nostoc commune*. Purified extracellular polysaccharides of *N. verrucosum* (lane 1) and *N. commune* (lane 2) were characterized by electrophoresis on cellulose acetate membranes in 0.47 M formic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

Fig. 3

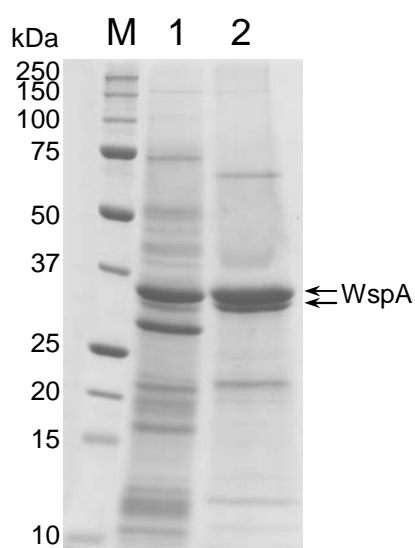


Fig. 3. Electrophoretic patterns of water-soluble extracellular matrix proteins of *Nostoc verrucosum* and *Nostoc commune*. Extracellular matrix proteins were extracted using 0.75 M phosphate buffer (pH 7.0) from of *N. verrucosum* (lane 1) or *N. commune* (lane 2) colonies, resolved by SDS-PAGE and stained with CBB. Each lane contained 20 μ g 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

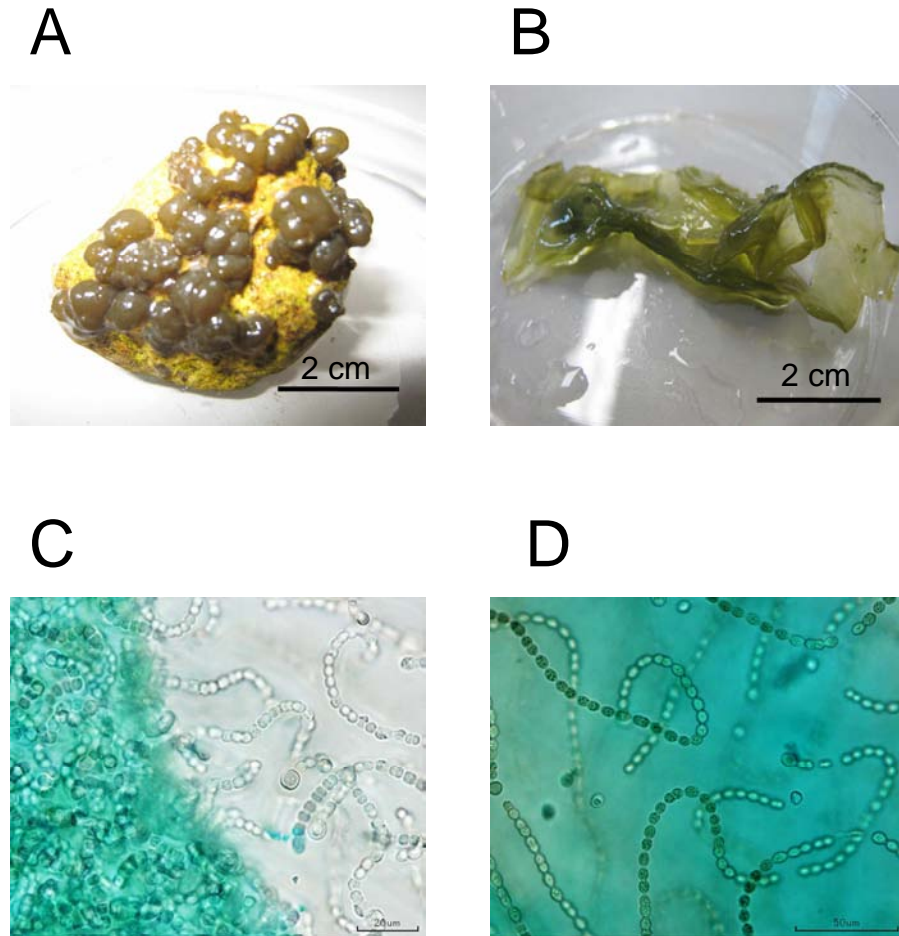


Fig. S1. Field-isolated, naturally growing colonies of *Nostoc verrucosum* (A) and *Nostoc commune* (B). Colonies of *N. verrucosum* (C) and *N. commune* (D) were stained by Alcian blue and observed by microscope. The extracellular matrix of both *Nostoc* species reacted similarly to Alcian blue reagent.

Fig. S2

A

Nostoc verrucosum 33-kDa WspA

MALFGYTI**GEDRDQNASNGKQL**DVYRFLIPNAPTTTPITPTGPVGTVTIA
 GTVTTADLEGLGTNPTTGRVTAINETR**VTGSTTPIDPTVFIVNNVDQPFA**
PVNVPPVVRTPSDLRRFGFESGADFGRDFSATSQPIVHYTIAGNRSASNGV
PVGSSLYKVTLTNPAISIVDNTPTTPERLGTQPTNPGKFADGLAIDNLTP
GRTRAFASDLSSTDGDGAQLYK**VDLLTGQLSAPINLRNSSGQPLSVNSDS**
GLAFSNVTGSTQRLFAWEETGRLYEITGFQDELNASGLTLGSTTGTNGAG
 FATATLLATVNLPNASTGVVDYEGFTIANE

B

Nostoc commune 33-kDa WspA

MALYGYTI**GEDRDQNP**SNGKQLDVYRFLIPAAPGGTITPTLVGTVNLAGT
 VTTADLEGLGTNPANPATERVAVNEARVGGSTAIDPAPGAIVVNNVDQP
 FAPATTSPTRTPSDLRRFGFDSGADFGRDYSAPGQPIVLYNISGNDSSG
 GVPVGSSLYKITANPGVISLVDGFGAPTAAQR**QGTQPTNSQFADGLAI**
DNLNPGRTRAFASDFSTVDGDGAQLYK**VDLLTGELSAPITLR**TPSGQALN
 LNLDSGLSFTNSGSQRLIGWVETGAVYEITGYQDELTASGLGLGSATGSN
 GAGFATATLLGNVNLPNVAVSGVVDYEGFTIVNE

C

Nostoc commune 31-kDa WspA

MALYGYTI**GEDRDQNP**SNGKQLDVYRFLIPAAPGGTITPTLVGTVNLAGT
 VTTADLEGLGTNPANPATERVAVNEARVGGSTAIDPAPGAIVVNNVDQP
 FAPATTSPTRTPSDLRR**FGFDSGADFGRDYSAPGQPIVLYNISGNDSSG**
 GVPVGSSLYKITANPGVISLVDGFGAPTAAQR**QGTQPTNSQFADGLAI**
DNLNPGRTRAFASDFSTVDGDGAQLYK**VDLLTGELSAPITLR**TPSGQALN
LNLDSGLSFTNSGSQRLIGWVETGAVYEITGYQDELTASGLGLGSATGSN
 GAGFATATLLGNVNLPNVAVSGVVDYEGFTIVNE

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).