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Full Paper

Characterization of mycosporine-like amino acids in the cyanobacterium *Nostoc verrucosum*

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The aquatic cyanobacterium *Nostoc verrucosum* forms macroscopic colonies in streams, and its appearance is superficially similar to that of the terrestrial cyanobacterium *Nostoc commune*. *N. verrucosum* is sensitive to desiccation, unlike *N. commune*, although these *Nostoc* cyanobacterial species share physiological features, including massive extracellular polysaccharide production and trehalose accumulation capability. In this study, water-soluble sunscreen pigments of mycosporine-like amino acids (MAAs) were characterized in *N. verrucosum*, and the *mysABCD* genes responsible for MAA biosynthesis in *N. verrucosum* and *N. commune* were compared. *N. verrucosum* produced porphyra-334 and shinorine, with porphyra-334 accounting for >90% of the total MAAs. Interestingly, porphyra-334 is an atypical cyanobacteial MAA, whereas shinorine is known as a common and dominant MAA in cyanobacteria. Porphyra-334 from *N. verrucosum* showed little or no radical scavenging activity *in vitro*, although the glycosylated derivatives of porphyra-334 from *N. commune* are potent radical scavengers. The presence of the *mysABCD* gene cluster in *N. commune* strain KU002 (genotype A) supported its porphyra-334 producing capability via the *Nostoc*-type mechanism, although the genotype A of *N. commune* mainly produces the arabinose-bound porphyra-334. The *mysABC* gene cluster was conserved in *N. verrucosum*, but the *mysD* gene was not included in the cluster. These results suggest that the *mysABCD* gene products are involved in the biosynthesis of porphyra-334 commonly in these *Nostoc* species, and that the genotype A of *N. commune* additionally acquired the glycosylation of porphyra-334.

Key Words: antioxidant; environmental adaptation; mycosporine-like amino acid (MAA); *mys* genes

Introduction

Cyanobacteria are photoautotrophic prokaryotes that perform oxygen-evolving photosynthesis using water as the primary electron donor. Requiring only light, water, carbon dioxide, and inorganic salts, cyanobacteria occupy

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highly diverse ecological niches and are found in almost all terrestrial, freshwater and marine habitats. The genus *Nostoc* is a well-known cyanobacterial group, and species of *Nostoc* are capable of forming heterocysts to utilize atmospheric N_2 as a nitrogen source (Potts, 2000). The nitrogen-fixing activity allows them to grow in nutrient-poor environments, and *Nostoc* cyanobacteria supply fixed nitrogen sources to a wide range of terrestrial and aquatic ecosystems (Dodds et al., 1995). In spite of the ecological importance of the genus *Nostoc*, their taxonomic characters are limited. Thus, molecular genetic and chemotaxonomic markers are useful and convenient for the characterization of *Nostoc* species (Arima et al., 2012).

Nostoc commune (its Japanese vernacular name is *Ishikurage*) is a terrestrial species of the genus *Nostoc*, which has a cosmopolitan distribution from the tropics to the polar regions of the earth (Potts, 2000). *N. commune* is unique due to its extreme desiccation tolerance. The desiccated colonies show little to no metabolic activity, but they maintain the ability to grow for over 100 years (Cameron, 1962; Lipman, 1941). The molecular mechanism underlying the extreme desiccation tolerance of this species is thought to involve multiple processes that include extracellular polysaccharide (EPS) production, compatible solute accumulation, and antioxidants. We have studied physiologically and biochemically the desiccation tolerance in *N. commune* (Inoue-Sakamoto et al., 2018; Matsui et al., 2011, 2012; Morsy et al., 2008; Nazifi et al., 2013, 2015; Sakamoto et al., 2009, 2011; Tamaru et al., 2005; Yoshida and Sakamoto 2009).

The cyanobacterium *Nostoc verrucosum* (its Japanese vernacular name is *Ashitsuki*) forms macroscopic colonies that consist of trichomes of cells and extracellular matrix. Its appearance is similar to that of *N. commune*, although *N. verrucosum* colonies always occur in streams. *N. verrucosum* may require specific natural conditions, and the habitats suitable for *N. verrucosum* are limited. Thus, *N. verrucosum* is designated as an endangered species preserved by local governments in Japan. Being inspired by the morphological resemblance, we have investigated the physiological features of *N. verrucosum* and compared them with those of the anhydrobiotic cyanobacterium *N. commune* (Sakamoto et al., 2011). Despite its formation of macroscopic colonies with a massive extracellular matrix and the accumulation of trehalose in response to desiccation, the aquatic cyanobacterium *N. verrucosum* is sensitive to desiccation, in contrast to the terrestrial cyanobacterium *N. commune* (Sakamoto et al., 2011). The *wspA* gene encoding the water stress protein (Wsp), which is a 36 kDa protein present in the extracellular matrix (Helm and Potts, 2012; Morsy et al., 2008; Scherer and Potts, 1989), occurs in both *N. verrucosum* and *N. commune* (Sakamoto et al., 2011). The presence of WspA is a remarkable common feature in these *Nostoc* species (Arima et al. 2012; Sakamoto et al., 2011), which suggests the same, or a very similar, architecture of their extracellular matrix. Interacting with EPS and UV-absorbing pigments, namely mycosporine-like amino acids (MAAs) and scytonemin, WspA is thought to play a role in modulating the structure and function of the three-dimensional extracellular matrix in *N. commune* (Helm and Potts, 2012; Hill et al., 1994; Inoue-Sakamoto et al., 2018; Wright et al., 2005).

MAAs are water-soluble molecules that absorb UV-A and UV-B (Carreto and Carignan, 2011; Wada et al., 2015) and disperse the energy as heat (Conde et al., 2004). MAAs are thought to function as sunscreen compounds in a wide variety of organisms, including cyanobacteria (Carreto and Carignan, 2011; Castenholz and Garcia-Pichel, 2012; Leão et al., 2012; Wada et al., 2013, 2015). MAAs show great diversity in their molecular structures. They exhibit a range of molecular weights of 188 to 1050 Da, and their absorption maxima appear between 268 and 362 nm depending on their molecular structure. The MAAs consist of 3-aminocyclohexen-1-one or the 1,3-diaminocyclohexen chromophore as the scaffold. The substitution of different amino acids in the MAA core structure forms a variety of MAA species found in taxonomically diverse organisms (the reported MAA derivatives are surveyed in Wada et al. (2015)).

We have reported that *N. verrucosum* contains MAAs at levels similar to those in *N. commune* (Sakamoto et al., 2011), but the molecular species of the MAAs from *N. verrucosum* remain to be characterized. In this study, porphyra-334 was found to be a main MAA in *N. verrucosum*, and the *mysABC* gene cluster and the *mysD* gene were identified. The organization of the *mysABCD* genes in *N. verrucosum* and *N. commune* strain KU002 (genotype A), which is known as a glycosylated porphyra-334-producing *Nostoc* species (Nazifi et al., 2015), was compared.

Materials and Methods

Microorganisms. Colonies of *Nostoc verrucosum* growing naturally in a stream were collected from Shishiku Park, Hakusan-shi, Ishikawa, Japan (36.438737 N, 136.640761 E), washed with tap water, dried using a lyophilizer, and stored at –30°C until used. The laboratory strain KU005 of *N. verrucosum* was isolated by streaking and spreading on agar plates, and it has been maintained at Kanazawa University since 2009 (Sakamoto et al., 2011). The laboratory culture strain KU002 of *Nostoc commune* (genotype A) has been isolated and maintained at Kanazawa University since 2002 (Tamaru et al., 2005). The laboratory culture strains have been deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES-Collection) with the following collection numbers: NIES-2538 (*N. commune* strain KU002) and NIES-2539 (*N. verrucosum* strain KU005).

Cells of *N. verrucosum* strain KU005 were grown at 18°C under constant illumination from fluorescent lamps (2–4 μmol m^{-2} s^{-1}) on modified $BG11_0$ liquid medium (without $NaNO_3$ as an inorganic nitrogen source) or $BG11_0$ solid medium with 1.5% (w/v) agar supplemented with a vitamin mix at final concentrations of 1 μg l^{-1} biotin, 2 mg l^{-1} thiamin, and 1 μg l^{-1} cyanocobalamin (Castenholz, 1988) buffered with 20 mM HEPES-NaOH (pH 7.5). The appearances of field-isolated *N. verrucosum* colonies and its laboratory-grown colonies are shown in Fig. S1.

MAA standards. The known MAA standards, including mycosporine-glycine (CAS No. 65318-21-0), palythine

(67731-19-5), shinorine (73112-73-9) and porphyra-334 (70579-26-9), were used as authentic standards (Oyamada et al., 2008) for the comparison of retention times in HPLC analyses. Porphyra-334 from *N. commune* (Nazifi et al., 2015) was also used as a standard.

Spectroscopic methods. The UV-VIS spectra were recorded with a double-beam spectrophotometer (U-2800, Hitachi High-Technologies, Tokyo, Japan). The wavelength resolution was ±1 nm.

MS analysis. MALDI-TOF MS/MS analysis was performed at the Division of Functional Genomics, Advanced Science Research Center, Kanazawa University using a tandem mass spectrometer (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA, USA) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. The secondary mass spectrum was recorded when applicable. The expected resolution was less than ±0.2 *m/z*.

Measurement of MAA content. The MAA composition was analyzed by HPLC essentially as described previously (Nazifi et al., 2015). The dried materials (25–100 mg) were suspended in 30% (v/v) methanol (3–10 ml), and the debris was removed by centrifugation. The extract containing the MAAs was injected into an HPLC system with a pump (L-6000, Hitachi High-Technologies, Tokyo, Japan) and equipped with a reverse phase column (Cholester, 4.6 × 150 mm, Nacalai Tesque, Kyoto Japan). The mobile phase was 5% (v/v) methanol with 0.1% (v/v) acetic acid. The flow rate was 0.6 ml min^{-1}. The A_{330} was monitored with a UV-VIS detector (IRICA Σ873, Shiseido Irica Technology, Kyoto, Japan). The chromatograms were recorded with a recorder (D-2500 Chromato-Integrator, Hitachi High-Technologies, Tokyo, Japan). The MAA amounts were determined according to their peak areas on the HPLC chromatograms, and a standard curve was constructed with known amounts of porphyra-334 (170–1360 ng) and shinorine (6–47 ng).

Measurement of radical-scavenging activity. The radical-scavenging activity was measured using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate (Re et al., 1999). The decolorization of the ABTS radical cation at A_{734} was monitored spectrophotometrically for 1 h (Matsui et al., 2011). Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) was used as an artificial antioxidant standard, and the activities were calculated as the Trolox equivalent antioxidant capacity (TEAC).

Chromatographic separation of porphyra-334 and radical scavengers. *N. verrucosum* powder (500 mg) was suspended in distilled water (200 ml) and extracted at room temperature by stirring for 2 h. After centrifugation at 21,500 × *g* for 5 min at 4°C, the supernatant was vacuum-filtered with a Buchner sintered-glass filter funnel and then condensed to approximately 22 ml with a rotary evaporator under reduced pressure. Sufficient ethanol was added to the filtrate to yield a final concentration of 70% ethanol (v/v), and the mixture was kept at 4°C for 1 h to precipitate the 70% ethanol-insoluble materials. After centrifugation at 21,500 × *g* for 5 min at 4°C, the supernatant was vacuum-filtered with a Buchner sintered-glass filter funnel and condensed with a rotary evaporator under reduced pressure. After centrifugation at 21,500 × *g* for 10 min at 4°C, the supernatant was concentrated with a centrifugal concentrator (VC-360, TAITEC, Koshigaya, Japan) and filtered through a syringe driven Millex-GV filter (0.22 μm, PVDF, hydrophilic, Millipore). The concentrated water extract with 860 nmol TEAC was injected into an HPLC system with a Hitachi L-6200 pump and an L-4200 UV-VIS detector equipped with a reverse phase column (Wakosil 5C18, 4.6 × 250 mm; Wako, Osaka, Japan). The mobile phase changed stepwise from 0.025% acetic acid during the initial 23 min to 100% methanol during the next 29 min. The flow rate was kept at 0.5 ml min^{-1}, and a 1 ml fraction was collected every 2 min. The MAA-containing fractions with the radical-scavenging activity (approximately 72% of the total activity) were reinjected into another HPLC system with a Hitachi L-6200 pump and an L-4200 UV-VIS detector equipped with another reverse-phase column (Inertsil ODS-3, 4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan). The mobile phase was 0.2% TFA-ammonium solution (pH 3.26) for the initial 20 min and 100% methanol for the next 22 min. The flow rate was constant at 0.5 ml min^{-1}, and a 1 ml fraction was collected every 2 min. The MAA was detected by the A_{330}. Radical-scavenging activity was measured by the ABTS decolorization assay.

Identification of the mysABCD genes. Five micrograms of genomic DNA, which was extracted from cells of *N. verrucosum* strain KU005 or *N. commune* strain KU002, was treated with a Covaris S-2 sonicator (Covaris, Woburn, MA, USA) to produce DNA fragments with an average size of 300 bases. A DNA library was constructed using the NEBNext DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA), according to the manufacturer's protocol. The DNA library was sequenced using a massively parallel sequencer, GAIIx (Illumina KK, Tokyo, Japan), with the 100 base paired-end format. For *N. verrucosum* strain KU005, 1.45 Gb of sequence reads passing standard Illumina GA pipeline filters were retained. The read sequences were assembled *de novo* with the Velvet assembly program (Zerbino and Birney, 2008). Assembled contigs were annotated using the microbial genome annotation pipeline MiGAP (Sugawara et al., 2009). For *N. commune* strain KU002, 246 Mb of sequence reads were obtained by the GAIIx and were assembled by Velvet. The assembled contigs were annotated by MiGAP. These contigs were mapped on the genome sequence of the most closely related species, *Nostoc punctiforme* strain ATCC 29133/PCC 73102 (CP001037.1; Meeks et al., 2001), using the MUMmer sequence alignment package with default settings (Kurtz et al., 2004) to remove the contigs derived from minor contaminating microorganisms. A number of contigs with a total length of 3.6 Mb for *N. verrucosum* strain KU005 and 4.6 Mb for *N. commune* strain KU002, which showed high similarity to the genome of *N. punctiforme* strain ATCC 29133 (CP001037.1; Meeks et al., 2001), were obtained. Using the draft genome sequences of *N. verrucosum* strain KU005 and *N. commune* strain KU002, the putative *mys* gene clusters were searched using the BLAST.

Two overlapping DNA fragments, namely the 5′ 2.5 kb

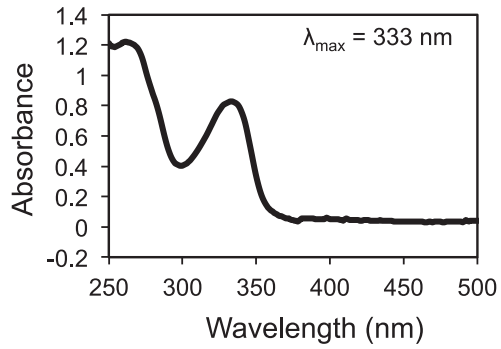

Fig. 1. A typical UV-VIS absorption spectrum of a water extract from field-isolated, naturally-growing colonies of *Nostoc verrucosum*.

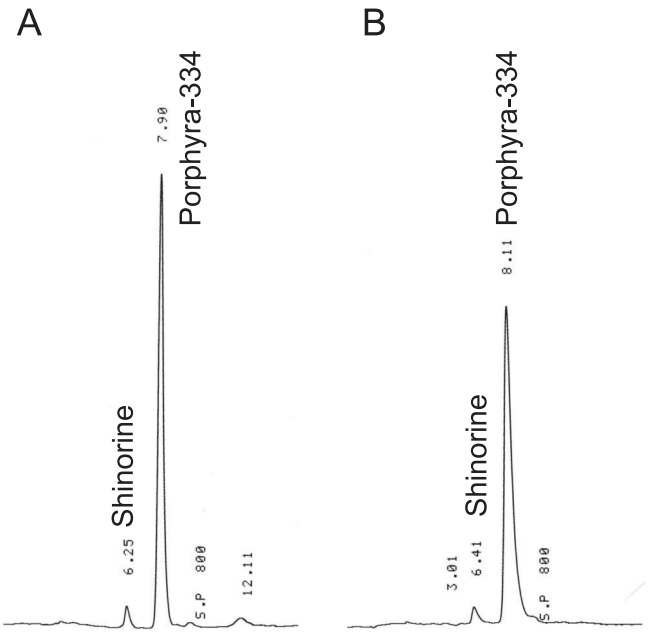

Fig. 2. Typical HPLC chromatograms of the MAAs from the cyanobacterium *Nostoc verrucosum*.

MAAs were extracted using 30% methanol from field-isolated, naturally-growing colonies of *N. verrucosum* (A) and the laboratory cultured strain KU005 of *N. verrucosum* (B). The extracts were fractionated by an HPLC system with a reverse-phase column (Cholester, 4.6 mm × 150 mm, Nacalai) using 5% methanol and 0.1% acetic acid at a flow rate of 0.6 ml min^{-1} as the mobile phase. MAAs were detected by the A_{330}. Shinorine and porphyra-334 were identified by comparison with the authentic standards.

fragment containing the *mysAB* genes and the 3′ 4.2 kb fragment containing the *mysBCD* genes, were amplified by PCR using an *N. commune* strain KU002 genomic DNA template and gene-specific primers (the forward primer, 5′-gtatattaaatactgagcag-3′ and the reverse primer, 5′-agaccaagcgtctaatcagg-3′ for the 5′-fragment; the forward primer, 5′-gacattaaagatgtttgtgc-3′ and the reverse primer, 5′-tatgtaatcctctgccggagcagtaccag-3′ for the 3′-fragment), which were designed and synthesized on the basis of the nucleotide sequence of the putative *mysABCD* gene cluster found in the assembled contig of *N. commune* strain KU002. The 2.5 kb PCR product and the 4.2 kb PCR product were sequenced to cover the 6.3 kb *mysABCD* gene cluster from *N. commune* strain KU002.

For the *mysABC* genes in *N. verrucosum*, a forward primer was designed and synthesized on the basis of the nucleotide sequence upstream of the putative *mysA* gene in the assembled contig of *N. verrucosum* strain KU005. Three PCR primers, initially designed to amplify the *mysABC* genes in *N. commune* strain KU002, were also used. A 2.0 kb DNA fragment containing the *mysAB* genes was amplified by PCR using an *N. verrucosum* strain KU005 genomic DNA template and primers (the forward primer, 5′-cgttattgggctgcgttgtggtaaatctga-3′ and the reverse primer, 5′-agaccaagcgtctaatcagg-3′), and a 2.1 kb DNA fragment containing the *mysBC* genes was amplified by PCR using the DNA template and primers (the forward primer, 5′-gacattaaagatgtttgtgc-3′ and the reverse primer, 5′-gtattctgatagtgcaggct-3′). The 2.0 kb PCR product and the 2.1 kb PCR product were sequenced.

Based on the determined nucleotide sequences of the overlapping DNA fragments from *N. verrucosum*, the nucleotide sequences of the *mysC* gene from *N. commune* strain KU002 (this study) and *N. punctiforme* strain ATCC 29133 (CP001037.1), the gene specific primers (forward primer, 5′-ttatacagtaccagcaccacaagacg-3′ and reverse primer, 5′-cctaattccacgagtttaccga-3′) were designed and synthesized for the amplification of the DNA fragment containing the *mysC* gene in *N. verrucosum*. The 1.0 kb PCR product to cover the 3′ region of the *mysC* gene was sequenced.

A 1.0 kb DNA fragment containing the *mysD* gene was amplified by PCR using an *N. verrucosum* genomic DNA template and the gene specific primers (the forward primer, 5′-atgccagtacttaatatccttcat-3′ and the reverse primer, 5′-gtttcattaatagcggttattaataa-3′), which were designed and synthesized based on the nucleotide sequence of the *mysD* genes from *N. commune* strain KU002 (LC102213) and *N. punctiforme* strain ATCC 29133 (CP001037.1). The 1.0 kb PCR product containing the *mysD* gene was sequenced.

The sequences were determined and deposited in GenBank/EMBL/DDBJ under the following accession numbers: LC102213 (the *mysABCD* gene cluster of 6278 b from *N. commune* strain KU002), LC205733 (the *mysABC* gene cluster of 3565 b from *N. verrucosum* strain KU005) and LC205734 (the *mysD* gene of 1011 b from *N. verrucosum* strain KU005).

Results

Identification of porphyra-334

Water-soluble UV-absorbing pigments from the cyanobacterium *Nostoc verrucosum* were characterized. The water extract of field-isolated natural colonies of *N. verrucosum* showed a UV-absorbing spectrum with a single absorption maximum at 333 nm (Fig. 1). The extract was examined by an HPLC system, and two different MAAs were detected (Fig. 2A). An HPLC chromatogram of the MAAs from the laboratory culture of *N. verrucosum* strain KU005 was very similar to that of field-isolated natural colonies of *N. verrucosum* (Fig. 2). The fraction containing the main MAA, which was estimated to be more than 90% of the total MAAs, was recovered and characterized further. The second MAA, which was estimated to be less than 10% of the total MAAs, was predicted to be

Table 1. MAA composition of the cyanobacterium *Nostoc verrucosum*.

	Porphyra-334		Shinorine	
	(mg [g DW]$^{-1}$)			
Field-isolated naturally-growing colonies	3.0 ± 0.5 (n = 3)	(93%)	0.23 ± 0.03 (n = 3)	(7%)
Cultured strain of KU005	0.48 ± 0.07 (n = 3)	(92%)	0.04 ± 0.004 (n = 3)	(8%)

Data are presented as the means ± SD and the relative amounts of the total MAAs.

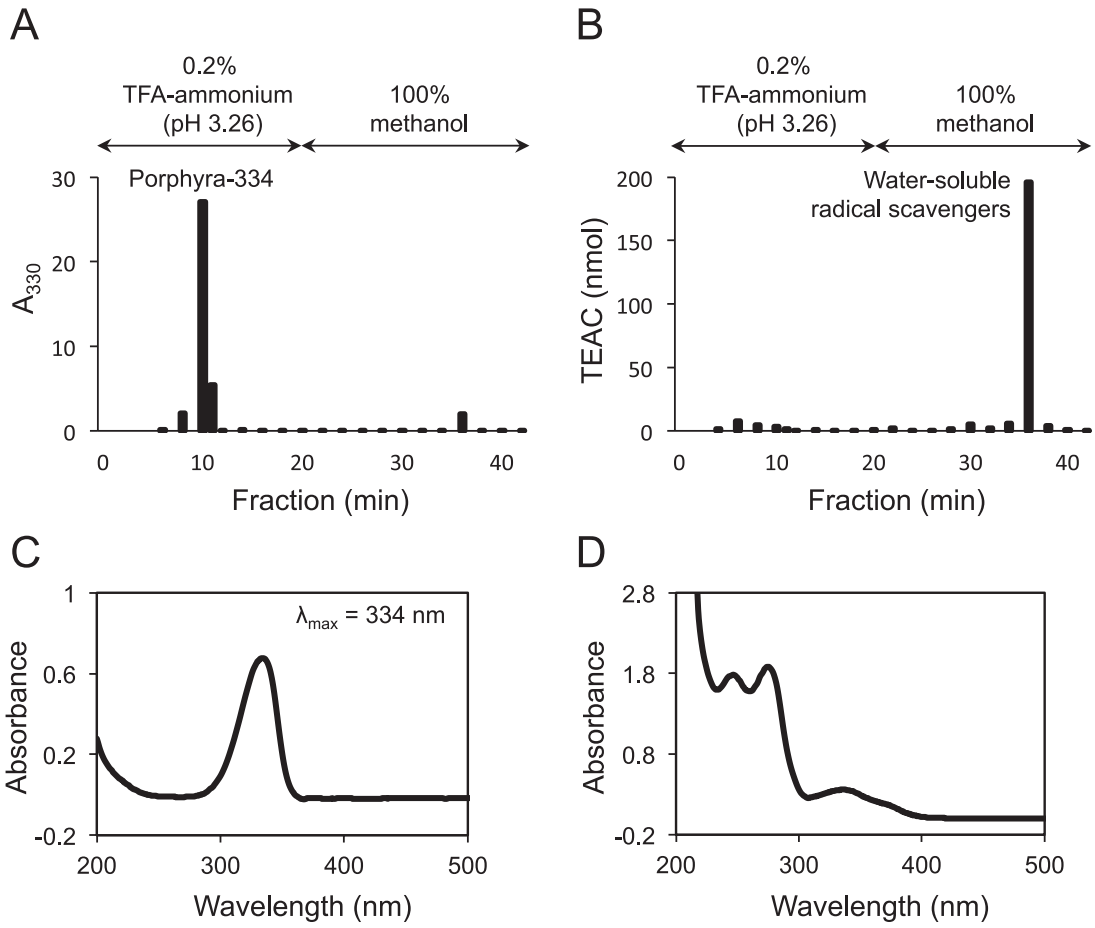

Fig. 3. Separation of porphyra-334 and water-soluble radical scavengers in a water extract of *Nostoc verrucosum*.

The MAA-containing fraction with radical-scavenging activities was fractionated with an HPLC system with a reverse-phase column (Inertsil ODS-3, 4.6 mm × 250 mm; GL Sciences Inc., Tokyo, Japan). A 1 ml fraction was collected every 2 min. The MAA was detected by the A_{330} (A). Radical-scavenging activity was measured by ABTS decolorization assays (B). A UV-VIS absorption spectrum of the fraction eluted at 10 min, which contained porphyra-334 (C). A UV-VIS absorption spectrum of the fraction eluted at 36 min, which contained uncharacterized water-soluble radical scavengers (D).

shinorine, because its retention time on the HPLC chromatograms was identical to that of the shinorine standard (Fig. S2).

The main MAA purified from *N. verrucosum* showed an absorption maximum at 334 nm. Its retention time was identical to the authentic standard of porphyra-334 on an HPLC analysis (Figs. S3A, B, and C). The molecular mass determined by MALDI-TOF-MS analysis was 346 Da, which is identical to that of porphyra-334 (Fig. S3D). The fragmentation patterns of MALDI-TOF-MS/MS analyses consistently supported that the parental molecular ion with m/z 347 was [porphyra-334+H$^+$] (Table S1). These results confirm that the cyanobacterium *N. verrucosum* produces porphyra-334 as the main water-soluble UV absorbing pigment.

Table 1 shows the MAA composition of *N. verrucosum* in the field-isolated natural colonies and the laboratory cultured strain of KU005. The total MAA contents of porphyra-334 and shinorine were higher in the field-isolated natural colonies. In both *Nostoc* samples, the content of porphyra-334 was more than 90% and the ratio of porphyra-334 to shinorine was similar. These results suggest that the growth conditions, e.g., the UV irradiation level in the light source, may affect the total MAA con-

tent, but the MAA composition of *N. verrucosum* is unchanged.

Chromatographic separation of porphyra-334 and radical scavengers

A water extract of *N. verrucosum* contained the ABTS radical-scavenging activity. The relation of porphyra-334 to the radical scavenger(s) was examined by an HPLC analysis. Figure 3 shows the elution profile of the second HPLC described in Section "Materials and Methods". The main MAA eluted at 10 min (Fig. 3A), and was confirmed as porphyra-334 by its absorption maximum at 334 nm (Fig. 3C). The 10-min fraction containing porphyra-334 showed very weak radical-scavenging activity, but strong radical-scavenging activity was detected in the fraction at 36 min (Fig. 3B). The activity in the 36-min fraction accounted for approximately 60% of the total recovered activity. The 36-min fraction showed UV absorption with maxima at 247, 275 and 336 nm, a spectrum that was different from those of the known MAAs (Fig. 3D). The molecular ion fragment with the *m/z* of 273 was detected as a main component in the 36-min fraction by MALDI-TOF MS analysis (Fig. S4, Table S2) and the molecular ion fragments with the *m/z* of 1157, *m/z* of 762, *m/z* of 730, *m/z* of 709, *m/z* of 563, *m/z* of 401 and *m/z* of 238 were also detected (Table S2). These compounds may be related to the ABTS radical-scavenging activity; however, further structural analysis could not be performed because sufficient amounts of the purified sample were not obtained. No ABTS radical-scavenging activity was detected in porphyra-334 purified from *N. verrucosum* using our assay conditions (data not shown). These results suggest that porphyra-334 is a major MAA in *N. verrucosum*, and it showed little or no radical-scavenging activity, whereas the uncharacterized water-soluble antioxidants contributed significantly to the total radical-scavenging capacity of *N. verrucosum*.

The mysABCD genes

The *mysABCD* genes, which are responsible for shinorine and porphyra-334 biosynthesis, were isolated from *Nostoc verrucosum* strain KU005 and *Nostoc commune* strain KU002. The *mys* gene products were similar to each other, and the amino acid sequence identities were 87–96% for those from *N. punctiforme* ATCC 29133, *N. commune* strain KU002 and *N. verrucosum* strain KU005 (Table S3).

Figure 4 shows the genomic organization of the *mys* genes of *N. punctiforme* ATCC 29133, *N. commune* strain KU002 and *N. verrucosum* strain KU005. The *mysABC* gene cluster was conserved among these *Nostoc* cyanobacteria as it is commonly found among the cyanobacterial species with MAA synthesizing capacity (Gao and Garcia-Pichel, 2011). In *N. commune* strain KU002, the *mysD* gene was located in the 3′ downstream region of the *mysABC* gene cluster with an anti-parallel orientation, and the organization of the *mysABCD* genes was similar to that in *N. punctiforme* ATCC 29133 (Fig. 4). Interestingly, the *mysD* gene in *N. verrucosum* strain KU005 is likely to be at a location separate from the *mysABC* cluster (Fig. 4). Further studies are necessary to determine the precise location of the *mysD* gene in the genome of *N. verrucosum* strain KU005.

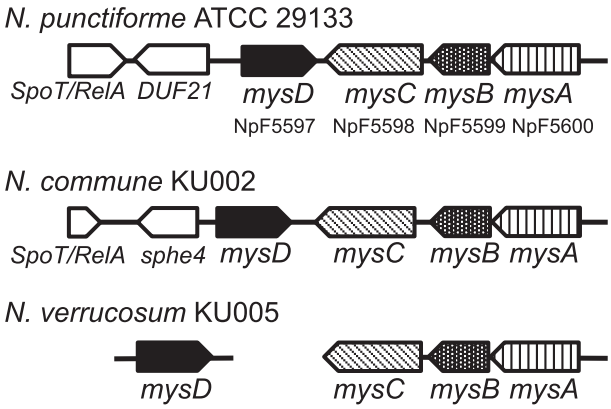

Fig. 4. Organization of the *mysABCD* genes of *Nostoc punctiforme* ATCC 29133 (Gao and Garcia-Pichel, 2011), *Nostoc commune* strain KU002 and *Nostoc verrucosum* strain KU005.

The nucleotide sequences have been deposited in GenBank/EMBL/DDBJ under the following accession numbers: LC102213 (the *mysABCD* gene cluster from *N. commune* strain KU002), LC205733 (the *mysABC* gene cluster from *N. verrucosum* strain KU005) and LC205734 (the *mysD* gene from *N. verrucosum* strain KU005).

Discussion

In this study, we demonstrated that prophyra-334 accounted for >90% of the total MAAs in *N. verrucosum* (Fig. 2, Table 1, Figs. S2 and S3, Table S1) and shinorine was detected as the minor MAA (Fig. 2, Table 1). The occurrence of porphyra-334 is rare in cyanobacteria and shinorine is a common and dominant cyanobacterial MAA (Sinha et al., 2003, 2007). It is noteworthy that *N. verrucosum* is an atypical cyanobacterial species which produces porphyra-334 dominantly. Certain *Nostoc* species mainly produce porphyra-334 (Richa and Sinha, 2015). Porphyra-334 is identified in three filamentous and heterocystous cyanobacterial strains of *Nodularia*, namely *N. baltica*, *N. harveyana* and *N. spumigena* (Sinha et al., 2003), and in the aquatic cyanobacterium *Aphanizomenon flos-aquae* (Torres et al., 2006), whereas 28 cyanobacteial strains are noted as shinorine producers (Sinha et al., 2007).

Figure S5 summarizes a plausible biosynthetic pathway for porphyra-334 and shinorine in *N. verrucosum* and the genes responsible for each reaction. The genes involved in MAA biosynthesis were elucidated in cyanobacteria recently (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011; Hu et al., 2015; Pope et al., 2015; Spence et al., 2012). The common precursor of the MAAs in cyanobacteria is 4-deoxygadusol, which is derived from the pentose phosphate pathway (Balskus and Walsh, 2010) and/or the shikimate pathway (Spence et al., 2012). The 4-deoxygadusol precursor is synthesized from sedoheptulose-7-phosphate, which is an intermediate of the pentose phosphate pathway. The *mysA* and *mysB* genes encode 2-epi-5-epi-valiolone synthase and *O*-methyltransferase, respectively, and these enzymes are involved in the conversion of sedoheptulose-7-phosphate to 4-deoxygadusol (Balskus and Walsh, 2010; Fig. S4). The 4-deoxygadusol

precursor is also synthesized from 3-deoxy-D-arabinoheptulosinate phosphate, which is derived from the shikimate pathway. In *Anabaena variabilis* ATCC 29413, both pathways function to synthesize the 4-deoxygadusol precursor (Spence et al., 2012), and the *O*-methyltransferase encoded by the *mysB* gene is involved in the formation of the 4-deoxygadusol precursor derived from both the pentose phosphate and the shikimate pathways (Pope et al., 2015). The *mysAB* genes are exclusively found in the MAA synthesizing cyanobacteria, and the MAA-producing capacity is thought to have evolved in a cyanobacterium and been transferred to other organisms by a lateral gene transfer (Singh et al., 2010). In the next step, the *mysC* gene product catalyzes the addition of glycine to 4-deoxygadusol to form mycosporine-glycine (Balskus and Walsh, 2010), which is a common intermediate in the formation of di-substituted (aminocyclohexene imine-type) MAAs, including shinorine and porphyra-334. There are two types of conjugation of mycosporine-glycine with a serine molecule to produce shinorine, namely, the Anabaena-type (Balskus and Walsh, 2010; D'Agostino et al., 2016) and the *Nostoc*-type (Gao and Garcia-Pichel, 2011; Miyamoto et al., 2014). In *A. variabilis* ATCC 29413, the last step of shinorine biosynthesis is catalyzed by a non-ribosomal peptide type ligase, which is encoded by Ava_3885 (Balskus and Walsh, 2010), whereas the addition of serine to mycosporine-glycine is catalyzed by an ATP-grasp type ligase, which is a different type of enzyme and is encoded by the *mysD* gene in *Nostoc punctiforme* ATCC 29133 (Gao and Garcia-Pichel, 2011). The *mysABCD* genes often form a gene cluster and are distributed in a variety of cyanobacterial species (Gao and Garcia-Pichel, 2011; Hu et al., 2015). In general, the presence of the *mys* genes is thought to relate to the MAA-producing capability in microorganisms (Katoch et al., 2016; Miyamoto et al., 2014).

There are the *mysABC* gene cluster and the *mysD* gene in *N. verrucosum* strain KU005, consistent with the presence of porphyra-334 and shinorine (Fig. 2, Table 1). It is noteworthy that the organization of the *mysABC* and the *mysD* in *N. verrucosum* strain KU005 is unique (Fig. 4), which may reflect the process of the *mys* gene transfer in this cyanobaterium. The presence of the *mysD* gene suggests that di-substituted MAAs are produced by the addition of an amino acid molecule to mycosporine-glycine via the *Nostoc*-type pathway using the ATP-grasp type ligase (Fig. S5), although prophyra-334, not shinorine, was the dominant MAA in *N. verrucosum* (Fig. 2, Table 1). Two important questions remain to be answered in future studies: What are (1) the biochemical mechanisms that produce porphyra-334, and (2) the ecophysiological roles of porphyra-334 and shinorine? Differences of the substrate specificity of the ATP-grasp type ligase and/or the composition of the cellular amino-acid pool may explain the preference for prophyra-334 production in *N. verrucosum*. It is likely to be determined genetically whether a cyanobacterial species is a porphyra-334 producer or a shinorine producer. However, it is unclear whether any differences in porphyra-334 and shinorine are selected in specific natural environments because of their similar structures and UV-absorbing capabilities.

It has been reported that the ABTS radical-scavenging capacity was found in the arabinose-bound porphyra-334 derivative from *N. commune* (Matsui et al., 2011; Nazifi et al., 2015), and its antioxidant property *in vitro* is as strong as that of ascorbic acid or Trolox, which is a water-soluble vitamin E derivative (Matsui et al., 2011; Nazifi et al., 2015). However, no ABTS radical-scavenging activity was detected in the purified porphyra-334 from *N. verrucosum* using our assay conditions (data not shown), and it is known that porphyra-334 displays little or no radical-scavenging activity, unlike the carbonyl-type MAAs, including mycosporine-glycine (Wada et al., 2015). Consistently, little radical-scavenging activity was detected in the porphyra-334 containing fractions from *N. verrucosum* extract (Figs. 3A and B), but significant activity independent of the MAAs was detected (Fig. 3). These results suggest the presence of uncharacterized water-soluble radical scavenger(s) in *N. verrucosum*. Moreover, glycosylation provides the radical-scavenging activity of porphyra-334 (Ishihara et al., 2017; Matsui et al., 2011; Nazifi et al., 2015); however, the structure-activity relationships in prophyra-334 derivatives remain to be elucidated in future studies.

The *mysABCD* gene cluster was found in *N. commune* strain KU002 (genotype A), consistent with the porphyra-334- and shinorine-producing capacity, although they are mostly glycosylated in this strain (Nazifi et al., 2015). We surveyed the 5′ and 3′ flanking regions of the *mysABCD* gene cluster, but there was no candidate gene for the MAA glycosylase in the draft genome sequence of *N. commune* stain KU002 (data not shown). It has been thought that the glycosylated MAA derivatives exclusively occur in the terrestrial species (Matsui et al., 2011; Nazifi et al., 2013, 2015; Volkmann and Gorbushina, 2006; Wada et al., 2015). However, fresh-water isolates of *Scytonema* cf. *crispum* produce hexose-bound derivatives of shinorine and palythine-serine (D'Agostino et al., 2016) and 13-*O*-(β-galactosyl)-porphyra-334 has been identified from the aquatic cyanobacterium *Nostoc sphaericum* recently (Ishihara et al., 2017). More surveys are needed to elucidate the distribution of MAA glycosylation.

The aquatic cyanobacterium *Nostoc verrucosum* is sensitive to desiccation (Sakamoto et al., 2011) despite its formation of macroscopic colonies with massive extracellular matrices apparently similar to those of the terrestrial cyanobacterium *Nostoc commune*. These species share physiological features, including trehalose accumulation in response to desiccation (Sakamoto et al., 2009, 2011). It can be postulated that the common ancestor of these *Nostoc* species had acquired the porphyra-334-producing capacity, and the ancestor of *N. commune* additionally acquired the glycosylation of porphyra-334 to adapt to terrestrial environments. This assumption will be evaluated in future studies through comparative genomics to understand the environmental adaptation mechanisms in *Nostoc* cyanobacteria.

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Supplementary Materials

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

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