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Characterization of the locus of genes encoding enzymes producing heptadepsipeptide micropeptin in the unicellular cyanobacterium *Microcystis*

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The gene cluster involved in producing the cyclic heptadepsipeptide micropeptin was cloned from the genome of the unicellular cyanobacterium *Microcystis aeruginosa* K-139. Sequencing revealed four genes encoding non-ribosomal peptide synthetases (NRPSs) that are highly similar to the gene cluster involved in cyanopeptolins biosynthesis. According to predictions based on the non-ribosomal consensus code, the order of the *mcnABCE* NPRS modules was well consistent with that of the biosynthetic assembly of cyclic peptides. The biochemical analysis of a $McnB_{K-139}$ adenylation domain and the knock-out of *mcnC* in a micropeptin-producing strain, *M. viridis* S-70, revealed that the *mcn* gene clusters were responsible for the production of heptadepsipeptide micropeptins. A detailed comparison of nucleotide sequences also showed that the regions between the *mcnC* and *mcnE* genes of *M. aeruginosa* K-139 retained short stretches of DNA homologous to halogenase genes involved in the synthesis of halogenated cyclic peptides of the cyanopeptolin class including anabaenopeptilides. This suggests that the *mcn* clusters of *M. aeruginosa* K-139 have lost the halogenase genes during evolution. Finally, a comparative bioinformatics analysis of the congenial gene cluster for depsipetide biosynthesis suggested the diversification and propagation of the NRPS genes in cyanobacteria.

Keywords: cyanobacteria/halogenase/*Microcystis*/micropeptin/non-ribosomal peptide synthetase.

Abbreviations: ACP, acyl carrier protein; Ahp, 3-amino-6-hydroxy-2-piperidone; HA, hexanoic acid; NRPS, non-ribosomal peptide synthetase; OA, octanoic acid; PKS, polyketide synthase; PPi, [^{32}P]pyrophosphate.

Planktonic bloom-forming cyanobacteria belonging to the genera *Anabaena*, *Microcystis, Nostoc* and *Planktothrix* produce structurally diverse peptides as secondary metabolites. The best known are the heptatoxic cyclic peptides, microcystin and nodularin. Cyanobacterial depsipeptides designated cyanopeptolins are cyclic peptides in which one amide bond is replaced by an ester bond and make up the majority of cyanobacterial peptides (*1–4*). The cyanopeptolin class including the subclasses anabaenopeptilide and micropeptin has been found in variant forms composed of six to nine L-amino acids including the residue 3-amino-6-hydroxy-2-piperidone (Ahp) (*5*, *6*), and halogenated cyanopeptolins have been reported along with non-halogenated ones (*7*). Cyanobacterial depsipeptides were shown to inhibit trypsin and chymotrypsin (*8–10*). The crystal structure of the cyanobacterial depsipeptide A90720A, a potent serine proteinase inhibitor, has also been reported (*11*).

Many bacterial peptides contain novel non-protein amino acids, suggesting that they are produced by non-ribosomal peptide synthetases (NRPSs) (*12*). Several cyanobacterial NRPS and hybrid NRPS/modular polyketide synthase (PKS) clusters have been reported. Microcystins are synthesized on large NRPS/PKS enzyme complexes (*13–17*). Nishizawa *et al.* (*18*) and Noguchi *et al.* (*19*) reported that the general arrangement of the microcystin biosynthetic (*mcy*) gene cluster was highly conserved among the genus *Microcystis*, but the diversification of the *mcyB* gene and the deletion of the *mcyI* gene were also reported (*19*, *20*). Additionally, the NRPS gene cluster responsible for depsipeptide anabaenopeptilide biosynthesis (*apd*) in the heterocyst *Anabaena* sp. strain 90 was confirmed with the *apd* gene knockout (*21*) and the NRPS genes for cyanopeptolin biosynthesis (*mcn*/*oci*) in *Microcystis* cf. *wesenbergii* NIVA-CYA 172/5 (N-C 172/5), *M. aeruginosa* PCC 7806 and NIES-843, and *Planktothrix agardhii* N-C 116 were cloned and sequenced (*7*, *22–24*). However, a gene knockout of *mcn* has not been created. Meanwhile, we have investigated the gene cluster for NRPS in *M. aeruginosa* K-139 and confirmed that this strain possesses NPRS modules in addition to the NRPSs of *mcy* by means of polymerase chain reaction (PCR) utilizing

specific primer (*13*). In the cells of *M. aeruginosa* K-139, non-halogenated depsipeptide, micropeptin K139 (*25*) (Fig. 1) and tetrapeptide, aeruginosin K139 (*26*), were identified to date.

Herein, we cloned and sequenced a gene cluster of seven NRPS modules and a putative transporter, involved in the biosynthesis of micropeptins, in *M. aeruginosa* K-139. Furthermore, we provided genetic and biochemical evidence that the *mcn* gene is responsible for micropeptin biosynthesis. In addition, a comparison of how the genes for depsipeptide biosynthesis are organized among the genus *Microcystis* was carried out in this study. According to a detail comparison analysis of the non-coding region between *mcnC* and *mcnE*, we revealed that the evolution of the *mcn* gene clusters in relation to the halogenase gene.

Materials and Methods

Bacterial strains and growth conditions

All NIES strains were obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Japan). Strains of *M. aeruginosa* B-19, B-35, B-47, K-81, K-139 and S-77 and *M. viridis* S-70 were isolated from Lake Kasumigaura (*27–29*). Strains were grown in CB or MA medium at 30°C with continuous illumination under fluorescent (cool white) lights (35 mol/m/s) (*30*).

DNA extraction and manipulation

Total cyanobacterial DNA was isolated from cells grown to the late logarithmic phase using a previously described procedure (*13*, *31*). *Escherichia coli* DH5αMCR (Cosmo Bio., Tokyo, Japan) and JM109 (Toyobo, Tokyo, Japan) were used as a host for recombinant plasmids and grown at 37°C in 2× YT medium. Media were supplemented with ampicillin at 75 μg/ml, if necessary. pUC119 (Toyobo) and pBluescript KSII (+) (Toyobo) were used for cloning. Other molecular cloning and PCR procedures were carried out using standard techniques (*32*).

Isolation and sequencing of the micropeptin biosynthetic gene cluster

A genomic library was prepared by the cloning of a partially digested (HindIII) *M. aeruginosa* K-139 genome into the cosmid Lorist 6 vector (*13*). To isolate NRPS genes involved in the biosynthesis of micropeptin, NRPS-core fragments of TN10 and TN12 (*13*) were used to screen approximately 1,000 clones of the cosmid library by colony hybridization. Two overlapping cosmids (pCOTnβ4 and β6) that hybridized to TN10 and TN12 were isolated and sequenced further. To obtain the rest of the flanking regions of peptide synthetase genes, several shotgun clonings were performed by colony hybridization. Four positive clones were obtained and designated pSMU, pSMT4, pSMDe and pPURM. pSMU included a 1.8-kb HincII fragment containing the NRPS of the A-domain of the *mcnA* gene. pSMT4 possessed a 1.9-kb HindIII fragment containing the NRPS of the C-domain of *mcnA*. pPURM had a 2.5-kb HincII fragment containing the *mcnF* gene. pSMDe included an 8.1-kb XbaI fragment containing the NRPS of the A-domain, T-domain and TE-domain of *mcnE*. These cloned inserts were analysed by restriction digestion with BamHI, ClaI, HincII and XbaI fragments, and subcloned into pUC119 and sequenced.

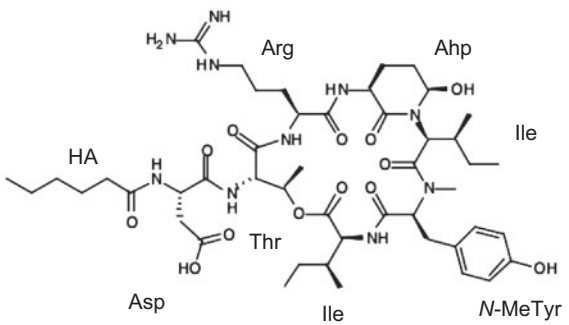

Fig. 1 Structure of depsipeptide micropeptin K139. Ahp, 3-amion-6-hydroxy-2-piperidone; HA, hexanoic acid.

DNA sequencing reactions were carried out using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster, CA, USA) according to the protocol of the manufacturer and analysed on a PE Applied Biosystems Automated DNA Sequencer (model 310). The DNA sequences were assembled and analysed using Genetyx-MAC software (Genetyx Co., Tokyo, Japan).

Southern hybridization and PCR amplification for identification of the genes for micropeptin biosynthesis

The HindIII-digested cyanobacterial DNA was separated on 0.8 % agarose gels and transferred to Hybond-NX membranes (GE-Healthcare Bio-Sciences, Buckinghamshire, UK) described as previously (*13*). Probe DNA fragments were labelled using the ECL random prime labeling Kit (GE-Healthcare Bio-Sciences). Southern hybridization was carried out at 60°C for 12 h and detection was performed as described in the instruction manual of the kit. Amplification of the non-coding region between *mcnC* and *mcnE* of *Microcystis* sp. was performed using the 3′-mcnC end primer (5′-TTTCTCGCACCTCGTAC-3′) and 5′-mcnE end primer (5′-A AGGAGAAAGCAATCGC-3′). The reactions were performed in a TaKaRa PCR thermal cycler Dice (Takara Bio., Ostu, Japan). PCR was performed at an annealing temperature of 58°C for 30 s and for 25 cycles.

Overexpression of McnB/Module 2 corresponding to the adenylation and thiolation domains

The pQE70-pREP4 overexpression system (Qiagen, GmbH, Germany) was used to construct pQE-MCNB2$_{AT}$, which was designed for overexpression of the adenylation and thiolation domains of McnB. The corresponding coding region of *M. aeruginosa* K-139 DNA was amplified by PCR using Takara LA *Taq* Polymerase (Takara Bio.), with the primers: mcnB-SphI (5′-ATGCATGCATT TTCAAACCTTACTGGAGGGAATTGTC-3′) and mcnB-BglII (5′-TAAGATCTTTTTCTTTTTCTTGGGAAAATGGG-3′). SphI and BglII sites (underlined) were introduced by the oligo DNAs. The amplified DNA fragment and backbone vector DNA of pQE70 were digested by SphI and BglII, and ligated, resulting in pQE-MCNB2$_{AT}$. The primers QE/F2 (5′-TTGCTTTGTGAGCGG ATAAC-3′) and QE/R1 (5′-CATTACTGGATCTATCAA CAGG-3′) were used to confirm the sequence of the cloning sites.

Escherichia coli M15[pREP4] was transformed with pQE-MCNB2$_{AT}$ and cultured at 30°C in 2× YT medium supplemented with ampicillin (75 μg/ml), neomycin (20 μg/ml) and magnesium chloride (10 mM). Overexpression of the truncated AT-domain in the *mcnB* gene was induced by adding isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) when the OD_{600} reached 0.8, and allowing it to grow further for an additional 7–10 h at 20°C. Purification of the His_6-tagged protein was carried out at 4°C except for the Ni^{2+} chromatography, which was performed at room temperature. Cells were collected by centrifugation for 20 min at 4,000*g*, and resuspended in sonication buffer; 50 mM sodium phosphate (pH 8.0), 300 mM sodium chloride and 10 mM imidazole (pH 7.0). Subsequently, a lysozyme solution was added to the cell suspension (final concentration, 1 mg/ml). After freeze–thawing (three times), sonication using a TOMY UD-201SONIC (TOMY, Tokyo, Japan) at output 4.5 was carried out (six 10-s sonications at 10-s intervals). The supernatant was separated by centrifugation (10,000*g*, 25 min) and applied directly to an open Ni^{2+}-charged chelating column, equilibrated with sonication buffer in advance. The column was washed once with wash buffer, containing 50 mM sodium phosphate (pH 8.0), 300 mM sodium chloride and 20 mM imidazole (pH 7.0), and then the His_6-tagged protein was eluted by applying 250 mM imidazole in the sonication buffer. After sodium dodecyl sulphate–polyacrylamide gel electrophoresis (10%), appropriate fractions were pooled, and dialysed against assay buffer [50 mM Tris–HCl (pH 8.0), 10 mM magnesium chloride, 0.5 mM DTT, 1 mM EDTA and 40% glycerol]. After dialysis, protein concentrations were measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc. Hercules, CA, USA).

ATP-PPi exchange assay

The ATP-[^{32}P]pyrophosphate (PPi) exchange activity (*33*) was measured according to the method of Hori and Kurotsu (*34*) with some modifications. The reaction mixture contained 50 pmol of enzyme, 50 mM Tris–HCl (pH 8.0), 2 mM magnesium chloride, 1.5 mM specified amino acid, 1.5 mM ATP, 1.5 mM DTT, 1.5 mM potassium fluoride, 1.5 mM tetrasodium pyrophosphate and 0.1 μCi of [^{32}P] pyrophosphate (NEN Life Science Products Inc., Boston, MA, USA) and had a total volume of 200 μl. After 15 min at 30°C, the reaction was stopped by adding 360 μl of 10% TCA solution, and 20 mg of active charcoal was added to capture free ATPs. The charcoal suspension was stirred for 30 s with a vortex mixer and centrifuged at 2,000*g* for 5 min. The precipitated charcoal was washed once with 500 μl of distilled water. Finally, the ATPs adsorbed on the charcoal were eluted with 1.0 ml of 0.3 M ammonia in 50% ethanol. The radioactivity was measured with a 2300TR liquid scintillation analyser (Packard, Downers Grove, IL, USA). The control value was determined from the reaction without amino acids.

Disruption of mcnC

Plasmids for the gene disruption of *mcnC*/Module 6 by homologous recombination were constructed as follows. The 2.3-kb HindIII fragment containing coding sequences for the adenylation domain and the *N*-methylation domain of *mcnC* from pCOTnβ6 was cloned into the HindIII site of pUC119, generating pMHF2308. The 1.2-kb HincII fragment containing the *Cm*^{r} gene cassette from pR107, which is derived from pR107XH (*13*), was inserted into the HpaI site within the region corresponding to the *mcnC*/*N*-methyltransferase domain of pMHF2308, generating pMHF2308/CM. After both the vector and insert DNAs were treated with the Klenow fragment, the 3.5-kb HindIII fragment from pMHF2308/CM was inserted into the KpnI-BamHI site of the pDXS7 vector (*13*), resulting in pMS2. The pSM2 in *E. coli* S17-1 was introduced into *Microcystis* cells by conjugation method. In brief, 50 ml aliquots of log-phase *Microcystis* culture and overnight culture of *E. coli* S17-1 containing the conjugative plasmid were centrifuged, respectively. Cell pellet washed with 25 ml of sterilized MilliQ water and then resuspended in 0.2 ml of the water. Aliquots of *Microcystis* and *E. coli* cell suspensions (50 μl) were mixed on sterilized Millipore membrane filters (HAWP 02500, Bedford, MA, USA) and then placed on CB agarose plates without antibiotics. The plates were incubated at 30°C for 20–24 h in the dark. Membrane filters were transferred to 50 ml of CB medium and incubated at 30°C under continuous illumination (35 mol/m/s). After 2 days, chloramphenicol-resistant (chloramphenicol 8 μg/ml) conjugants were selected on CB agarose plates.

HPLC of peptides from Microcystis *sp*

Lyophilized *Microcystis* cells (100 mg) were extracted three times with 5% acetic acid (aq.) (10 ml) for 40 min while stirring (*25*). The combined extracts were centrifuged at 3,500*g* for 10 min, and the supernatant was applied to a preconditioned ODS silica gel cartridge (0.5 g, Varian BOND ELUT C_{18} Varian; Palo Alto, CA, USA) after filtration through a glass microfiber GF/C filter (Whatman, Kent, UK). The cartridge was rinsed with water (5 ml) and 20% methanol (aq.) (5 ml), and then eluted with 90% methanol (aq.) and methanol (each 5 ml) until the desired peptide fraction was reached. The separation of peptides was performed on a COSMOSIL column (150 × 4.6 mm i.d.; Nacalai, Kyoto, Japan) with a constant temperature of 40°C. This temperature was maintained using a TOSOH HPLC system (TOSOH Corp., Tokyo, Japan) composed of a pump (TOSOH CCPD), detector (TOSOH UV-800), controller (TOSOH PX-8010), degasser (TOSOH SD-8022), column oven (TOSOH RE-8000) and mixer (TOSOH MX-8000). Detection was done with UV at 238 nm for microcystins and at 280 nm for micropeptins.

Nucleotide sequence accession number

The nucleotide sequences in this report have been submitted to DDBJ under the following accession numbers: AB481215 (gene cluster for micropeptin biosynthesis in *M. aeruginosa* K-139), AB481216-AB481222 (*mcnC-mcnE* region in *Microcystis* strains).

Results

Comparison of mcn *gene clusters and arrangement of the micropeptin synthetase gene cluster*

The gene cluster involved in micropeptin biosynthesis (*mcn*) was cloned from the genomic DNA of *M. aeruginosa* K-139 with colony hybridization using the TN10 and TN12 probes (*13*). A total of 33,711 bp was sequenced along both strands to reveal eight open reading frames (ORFs) predicted to be responsible for the assembly and transport of cyclic peptides (Fig. 2). The *mcnA-mcnB-mcnC-mcnE* genes, which are transcribed in the same direction and encode NRPSs, are organized as a putative operon of 26 kb. Sequencing of *mcnA*, *mcnB*, *mcnC* and *mcnE* revealed the typical modular structure for NRPS genes (*12*). McnA and McnB have one module, containing a condensation (C), an adenylation (A) and a thiolation (T) domain. McnC contains four C-, A- and T-domains. An *N*-methyltransferase (MT) domain, which corresponds to 421 amino acids, was found in the fourth module of McnC. McnE has one module, containing C-, A-, T- and thioesterase (TE) domains. A TE-domain is located at the C-terminal end of McnE and its function is most likely to release the linear intermediate of a micropeptin from the NRPS complex and to catalyse its cyclization.

A potential Shine–Dalgarno (SD) sequence (AAAT G) was recognized 10 bp upstream of the ATG codon of *mcnA*. The *mcnB* gene is separated by 3 bp from *mcnA*'s stop codon and overlaps with the 5′-end of *mcnC* over a length of 2 bp at its 3′-end. However, putative SD sequences were not found upstream of the start codons of *mcnB* and *mcnC*. The ATG start codon of *mcnE* was located 264 bp downstream of the 3′-end of *mcnC*, and a potential SD sequence (AAAG GG) was found 3 bp upstream of it. The 2067-bp *mcnF*, which is located 432 bp upstream of the 5′-end of *mcnA*, and transcribed in the opposite direction to *mcnA*, encodes a 78,375-Da protein with high homology to ABC transporters, NosG (accession number, AF204805; identity, 65%), NcpC (AY167420; 68%) and McyH (AB032549; 63%), suggesting that ABC transporters involve in peptides export (*35*). A potential SD sequence (AAATGC) was observed 3 bp upstream of the start codon of *mcnF*.

The genes coding for the putative cyanopeptolin synthetase in the genus *Microcystis* has been sequenced. A comparison of the *mcn* gene clusters was carried out among four *Microcystis* strains; *Microcystis* cf. *wesenbergii* N-C 172/5 and *M. aeruginosa* K-139, NIES-843 and PCC 7806 (Fig. 2). Basically, the organization and order (*mcnA*, *mcnB*, *mcnC* and *mcnE*) were conserved among the genus. However, there were some differences between the *mcn* gene clusters in *Micrcosytis* strains: (i) homology with McnA was lower among strains K-139, N-C 172/5, NIES-843 and PCC 7806 (Table I); (ii) NIES-843 contained a transposase gene (gene ID, MAE60050) and a gene coding for an unknown protein (MAE60040) between *mcnF* and *mcnA* and (iii) N-C 172/5 retained the *mcnD* gene encoding chlorination.

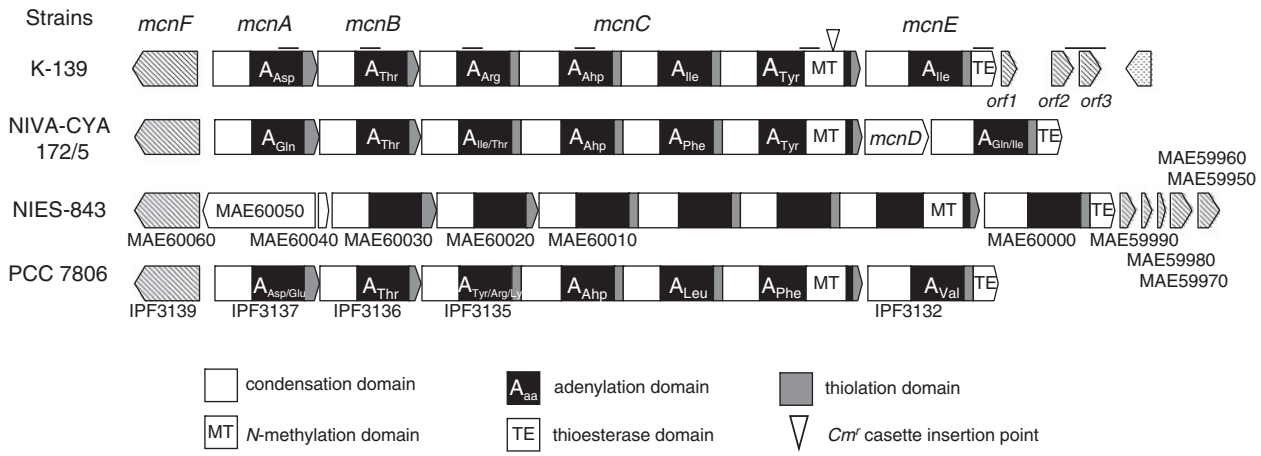

Fig. 2 Organization of the gene clusters for micropeptin. *mcnA-mcnE*, NRPSs; *orf1*, protein of unknown function; *orf2*, δ(2,4)-sterol C-methyltransferase; *orf3*, 3-oxoacyl-[acyl-carrier protein] reductase; *mcnD*, halogenase; *mcnF*, ABC transporter ATP-binding protein. The domains encoded by *mcnA*, *mcnB*, *mcnC* and *mcnE* are indicated by boxed gene modules as follows: the white box is the condensation domain; A_{aa} is the adenylation domain with the predicted amino acid that is activated noted as a subscript; the cross-hatched box is the thiolation domain; MT is the *N*-methylation domain; TE is the thioesterase domain. A white triangle indicates the insertion point of the Cm cassette. Bars indicate the probe for Southern hybridization (Table II). The sequence ID is given under the gene cluster in *M. aeruginosa* NIES-843 (accession number, AP009552) and PCC 7806 (contig C312) (AM778942).

Downstream from *mcnE*, we found three ORFs, *orf1*, *orf2* and *orf3*, transcribed in the same direction from the micropeptin K139 synthetase gene cluster (Fig. 2). The putative initiation codon of *orf1* is located 223 bp downstream of the (TGA) stop codon of the *mcnE* gene. The polypeptide (18,792 Da) encoded by *orf1* had no known function in the Genbank database. The putative start codon of *orf2* is located 1,012 bp downstream of *orf1*'s stop codon TGA and 7 bp downstream of a potential ribosomal binding site (AAGTG AG). This Orf, which calculated size of 30,267 Da, shows homology (62, 34 and 33% identity, respectively) to a putative methyltransferase, ApdE, of *Anabaena* sp. strain 90, and delta-(*2*,*4*)-sterol-C-methyltransferases of *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803. Additionally, the Orf2 possessed the specific conserved motif [ILDVGCGLG] of a *S*-adenosylmethionine-dependent methyltransferase. *orf3* is located 158 bp downstream of the TAA stop codon of *orf2* and encodes a 25,615-Da protein with homology (79, 26 and 27% identity, respectively) to a putative acyl-carrier protein, ApdF, in strain 90, and the 3-oxoacyl-[acyl-carrier protein] (ACP) reductase of PCC7120 and PCC6803. A putative SD sequence (GG AGA) is located 7 bp upstream of the initiation codon of *orf3*. NIES-843 also possessed MAE59990, MAE59960 and MAE59950 downstream of *mcnE* (MAE60000) and these ORFs showed high similarity to Orf1, Orf2 and Orf3 of strain K-139, respectively (Table I). In the CyanoBase (Kazusa DNA Research Institute), MAE59970 and MAE59980 were registered as hypothetical proteins (Fig. 2).

Selectivity-conferring code of NRPS A-domain

Based on the known non-ribosomal consensus code (*36*, *37*), the signature sequences of the substrate-binding pocket derived in the order of the McnA, McnB, McnC and McnE A-domain modules are shown in Table I. In the case of strain K-139, NRPS signature codes from A-domains of McnA were identified as the signature sequence for Asp (estimated identity, 89%). In the case of McnB, the Thr signature code was shared in all strains. The signature of McnC1 was similar to the Arg signature code (50%). The signatures of McnC3 and McnE were most similar to the signature of Ile (78%), and the signature of McnC4 was most similar to the signature of Tyr (44%). The signature of McnC2 corresponds to none of the precedents in the database, but the signature of McnC2 in the genus *Microcystis* was consistent with that of ApdB2 for anabaenopeptilide biosynthesis in *Anabaena* sp. strain 90. The putative Ahp activating McnC2's A-domain showed 78% identity to ApdB2's A-domain.

Screening of the mcn *genes in* Microcystis *strains*

To confirm which strains retain the *mcn* gene cluster, the presence of *mcnA*, *mcnB*, *mcnC*, *mcnE*, *orf2* and *orf3* in laboratory strains of *Microcystis* was examined by genomic Southern hybridization using the gene-specific probes. Due to detection of the *mcn* gene cluster in *Microcystis*, we mainly selected microcystin biosynthesis (*mcy*) gene cluster-possessing strains as a source. Chromosomal DNA was isolated from the 13 strains of *Microcystis* listed in Table II, and digested with HindIII. Our results indicated that almost all the *Microcystis* strains isolated possessed both the *mcn* and microcystin biosynthetic (*mcy*) gene clusters. Notably, in the microcystin synthetase gene-negative strains *M. aeruginosa* B-19 and NIES-87, probes detected no hybridization signal for the *mcn* gene. This result indicates that B-19 and NIES-87 did not retain any vestige of the *mcn* gene cluster in the genome. On the other hand, *M. aeruginosa* S-77, NIES-89, NIES-90, NIES-298 and *M. viridis* NIES-103 gave no signal hybridizing to the *mcnA* probe. This observation suggested the *mcnA* genes have little or no similarity to $mcnA_{K\text{-}139}$.

Table I. Deduced functions and domain organization of the ORFs in *M. aeruginosa* K-139 and comparison of the NRPS of *mcn* genes among the genus *Microcystis*.

Gene	Amino acids	Proposed function	Protein	Percentage identity of amino acids[a]			Strain	Signature Sequence[b]	Putative activated Substrates[c]
				N-C 172/5	NIES-843	PCC 7806			
mcnF	689	ABC transporter	McnF	99 (689)	99 (689)	98 (689)			
mcnA	1112	NRPS	McnA	74 (1098)	80 (1099)	94 (1112)	K-139	DLTKIGHVGK	Asp
							N-C 172/5	DAWQTGLIDK	Gln
							NIES-843	DTKCLGVVDK	n.d.
							PCC 7806	DLTKIGHVGK	Asp/Gln
mcnB	1105	NRPS	McnB	98 (1105)	98 (1106)	95 (1109)	K-139	DFWNIGMVHK	Thr
							N-C 172/5	DFWNIGMVHK	Thr
							NIES-843	DFWNIGMVHK	n.d.
							PCC 7806	DFWNIGMVHK	Thr
mcnC	4732	NRPSs	McnC	89 (4721)	94 (4725)	92 (4713)			
			McnC1				K-139	DAEGVGAIAK	Arg
							N-C 172/5	DAFFLGVTFK	Ile
							NIES-843	DAESVGGIAK	n.d.
							PCC 7806	DAEGFGAIAK	Tyr/Arg/Lys
			McnC2				K-139	DVENAGVVTK	Ahp
							N-C 172/5	DVENAGVVTK	Ahp
							NIES-843	DVENAGVVTK	n.d.
							PCC 7806	DVENAGVVTK	Ahp
			McnC3				K-139	DAFFLGVTFK	Ile
							N-C 172/5	DAWTIAAVCK	Phe
							NIES-843	DAWTIGASVK	n.d.
							PCC 7806	DAWTIGASVK	Leu
			McnC4				K-139	DASTIAGVCK	Tyr
							N-C 172/5	DASTIAGVCK	Tyr
							NIES-843	DASTIAGVCK	n.d.
							PCC 7806	DAWTIAGVCK	Phe
mcnE	1386	NRPS	McnE	97 (1418)	97 (1419)	88 (1372)	K-139	DAFFLGVTFK	Ile
							N-C 172/5	DAQDLGVVDK	Gln
							NIES-843	DAFFLGVTSK	n.d.
							PCC 7806	DAFFLGGTTK	Val
orf1	177	Unknown			98 (159)				
orf2	262	δ-(2,4)-sterol-C-methyltransferases			98 (262)				
orf3	244	3-oxoacyl-[acyl-carrier protein] reductase			96 (244)				

[a]Parentheses indicate the number of amino acids.
[b]Ten variable amino acids of the signature sequence indicated based on the known non-ribosomal consensus code (36, 37).
[c]Substrate as predicted from the depsipeptide compound. n.d. not decided. [K-139 (25); NIVA-CYA 172-5 (7); PCC 7806 (1, 4, 24)].

Table II. Summary of the results of genomic Southern hybridization of *Microcystis* DNA with specific probes.

Probes	*mcnA* Module 1	*mcnB* Module 2	*mcnC* Module 3	*mcnC* Module 4	*mcnC* Module 6	*mcnE* Module 7	*orf2-3*	*mcy*[a]	Depsipeptide Production[b]	Reference
Strain										
Microcystis aeruginosa										
B-19	−	−	−	−	−	−	−	−	n.d.	30
B-35	+	+	+	+	+	+	+	+	n.d.	29
B-47	+	+	+	+	+	n.t.	+	+	n.d.	29
K-81	+	+	+	+	+	+	+	+	n.d.	27
K-139	+	+	+	+	+	+	+	+	micropeptin K139	28
S-77	−	+	+	+	+	+	+	+	n.d.	30
NIES-87	n.t.	−	−	−	−	n.t.	−	−	n.d.	NIES collection
NIES-89	−	+	+	+	+	+	+	+	n.d.	NIES collection
NIES-90	−	+	n.t.	+	n.t.	+	+	+	micropeptin 90	NIES collection
NIES-298	−	+	+	+	+	+	+	+	n.d.	NIES collection
Microcystis viridis										
S-70	+	+	+	+	+	+	+	+	micropeption S70-A/-B	30
NIES-102	+	+	+	+	+	+	+	+	n.d.	NIES collection
NIES-103	−	+	+	+	+	+	+	+	micropeptin NIES103	NIES collection

Probes indicate in Fig. 1 and were as follows: Module 1, 606-bp HincII fragment containing A7 and A8 of the *mcnA* AT-domain; Module 2, 555-bp HindIII fragment containing A3 of the *mcnB* A-domain; Module 3, 944-bp StyI-EcoRI fragment containing A4, A5 and A6 of the *mcnC1* A-domain; Module 4, 944-bp *Sty*I-EcoRI fragment containing A6, A7 and A8 of the *mcnC2* A-domain; Module 6, 720-bp A*se*I fragment containing the MT-domain of the *mcnC4* A-domain; Module 7, 582-bp HincII fragment containing the thioestrease region of *mcnE*; *orf2-3*, 1,700-bp BamHI-HindIII fragment containing the *orf2* and *orf3* genes; −, no signal; +, signal; n.t, not tested.
[a]*mcy*-possessing and non-possessing strains are indicated by plus and minus signs, respectively. Reference to Nishizawa *et al.* (*18*).
[b]n.d. not determined.

Substrate specificity of McnB

To examine the substrate specificity of McnB, the AT-domain in McnB, whose putative activated substrates were conserved in the depsipeptides of *Microcystis* strains producing cyanopeptolins (Table I), was over-expressed as His_6-tagged fusion proteins. The recombinant $McnB_{K\text{-}139}$ protein was expressed as soluble form and had a molecular weight consistent with the calculated mass of ~75 kDa. After affinity purification, its purity was >98%. The substrate specificity of the purified AT-domains was determined by an ATP-PPi exchange reaction. In this assay, no comparable alternative activation was identified. The $McnB_{K\text{-}139}$ protein exclusively activated L-Thr (Fig. 3). This result corresponds well to the amino acid order in the constituent of micropeptin K139 as well as with predictions considering the organization of the gene.

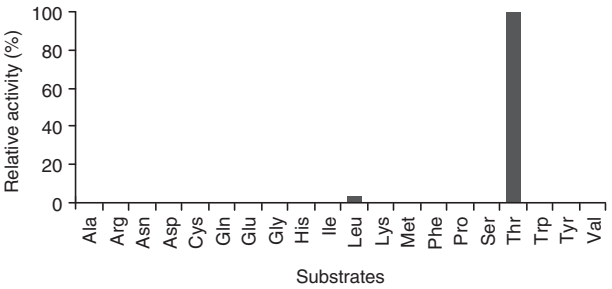

Fig. 3 Substrate specificity of the recombinant McnB enzyme as measured by amino acid-dependent ATP-PPi exchange assays. Values are relative with the highest activity defined as 100%. Data are expressed as means for duplicate independent experiments.

Construction and analysis of a mcnC *knockout mutant*

To obtain genetic evidence of the involvement of *mcn* in micropeptin production, the knockout of the *mcn* gene was performed. Although we tried to disrupt the *mcn* gene in K-139 by homologous recombination (*13*), the $mcn_{K\text{-}139}$ gene-knockout mutant was not obtained. It seemed that a strong host-restriction system of K-139 strain interfered with the frequency of homologous recombination (*38*). Previous gene-disruption experiments suggested that *M. viridis* S-70 has a comparatively weak restriction barrier (*14*, *39*). According to genomic Southern hybridization profiling, we revealed that *M. viridis* S-70 possess the *mcn* gene cluster in its genome (Table II). In related to productivity of depsipeptide, Harada *et al.* (*25*) have reported that S-70 produces heptadepsipeptide, micropeptins S70-A/-B. The structure of micropeptins S70-A/-B, octanoic acid (OA)-Glu–Thr–[Arg/*N*-methyl Lys (MeLys)]–Ahp–Leu–*N*-methyl Tyr (MeTyr)–Val, was close to that of micropeptin K139, hexanoic acid (HA)-Asp–Thr–Arg–Ahp–Ile–*N*-MeTyr–Ile (Fig. 1). These observations suggested a putative $mcn_{S\text{-}70}$ gene cluster similar to the gene assembly of K-139. Therefore, S-70 was utilized for the *mcn* gene disruption experiment in this study.

The knockout of the MT-domain region in *mcnC* was successfully achieved, and chloramphenicol-resistant conjugants, *M. viridis* S-70/405B-1 and -2, were isolated. To confirm that gene replacement had occurred through double crossover at the intended locus, genomic Southern hybridization was carried out. Total DNA from the S-70/405B mutants was digested with HindIII and probed with the 720-bp fragment containing a part of the A- and

MT-domain of *mcnC*/Module 6 derived from K-139 (Fig. 4A). A 2.3-kb band was detected in the wild-type S-70, while only a 3.5-kb band was detected in the two mutants (Fig. 4B). This 3.5-kb signal corresponds to one expected from the correct gene replacement by double crossover, indicating the 405B mutants to be *mcnC* knockout strains.

To determine the productivity of peptide compounds in knockout strains, high-performance liquid chromatography (HPLC) analysis was performed. HPLC of an extraction of the knockout mutant 405B-1 showed the disappearance of two peaks corresponding to micropeptins S70-A and S70-B, revealing that the *mcn* gene cluster is required for the biosynthesis of the two variants in S-70 (Fig. 5), whereas microcystin production in S-70 (*14*) was not affected at all in this mutant (data not shown).

Nucleotide sequences of the mcnC-E *region*

The halogenase genes *mcnD* are located upstream of the C-domain of *mcnE* in N-C 172/5 (Fig. 2). In the case of the *mcn* gene cluster of K-139, a possible halogenase gene has not been found: there is no additional ORF encoding a halogenase in front of McnE that has a TE-domain. To investigate the presence of a halogenase gene in seven *mcn*-possessing strains of *Microcystis*, B-35, B-47, S-70, NIES-89, NIES-90, NIES-102 and NIES-103 (Table II), PCR amplification of the region between *mcnC* containing a T-domain and *mcnE* containing a C-domain was carried out. Sequencing of the 0.9-kb amplicons from B-47, NIES-89, NIES-90, S-70, NIES-102 and NIES-103 revealed 94.9–96.8% identity to the region between *mcnC* and *mcnE* from K-139. In the case of B-35, a 2.6-kb PCR fragment was amplified and sequenced (accession number, AB481216). One ORF (designated *mcnD*) was found in the sequence and showed homology to two halogenase genes, *apdC* of *Anabaena* sp. strain 90 and *mcnD* of N-C 172/5 (nucleotide sequence, 86.4 and 97.9% identity), respectively. A detailed comparison of the *mcnC* and *mcnE* genes of B-35 and K-139 was performed. The sequence between *mcnC* and *mcnE* was divided into four (33, 36, 101, and 92 bp) fragments in K-139 compared with the *mcnC-E* of B-35 (Fig. 6A). This result suggests that three deletion events occurred between *mcnC* and *mcnE* of K-139.

A BLAST-search of the database showed that the deduced $McnD_{B-35}$ (625 amino acids) contained the conserved motifs [GxGxxG] and [WxWxIP] in $FADH_2$-dependent halogenases (*40*). Comparisons of the *mcnD* sequences in *Microcystis* sp. PCC 7005, PCC 7941, PCC 9808, PCC 9812 and PCC 9905 against the *mcnC-D*$_{B-35}$ region showed 85.9–94.3% similarity. Although two 107-bp imperfect direct repeats in the *mcnC-mcnE* region of PCC 7005, PCC 7941, PCC

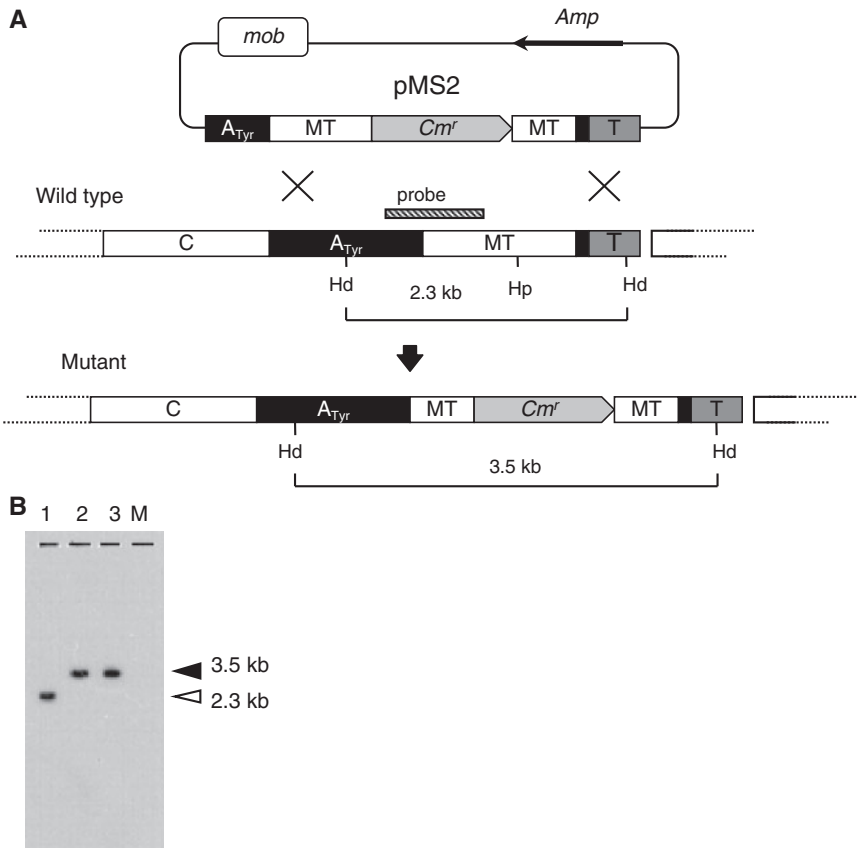

Fig. 4 Inactivation of the micropeptin biosynthetic gene by homologous recombination. (A) Schematic representation of the disruption of the *mcnC* gene. C, condensation domain; A, adenylation domain; MT, *N*-methylation domain; T, thiolation domain. (B) Southern hybridization analysis of mutant *M. viridis* S-70 using the *mcnC* gene probe. Lane 1, *M. viridis* S-70 wild-type; lanes 2 and 3, *mcnC* mutant 405B-1 and 405B-2; M, marker.

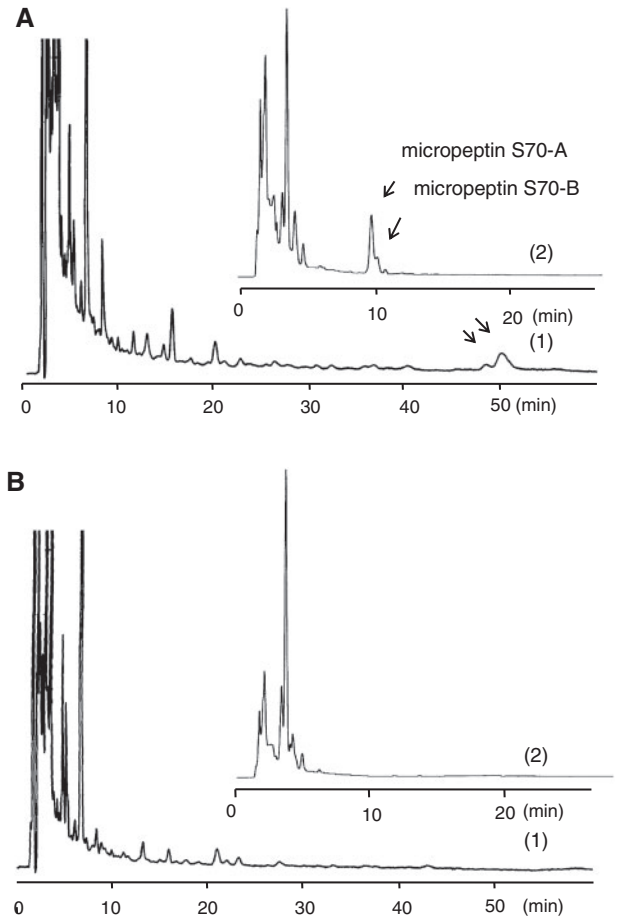

Fig. 5 HPLC of the methanol extract monitored for the detection of micropeptin. (A) Wild-type cell. (B) Mutant 405B-1 cell of *M. viridis* S-70. The following mobile phases were used at a flow rate of 1.0 ml/min: (1) acetonitrile : 0.01 M triethanolamine (aq.) = 30 : 70; (2) acetonitrile : 0.02 M sodium sulphate = 60 : 40.

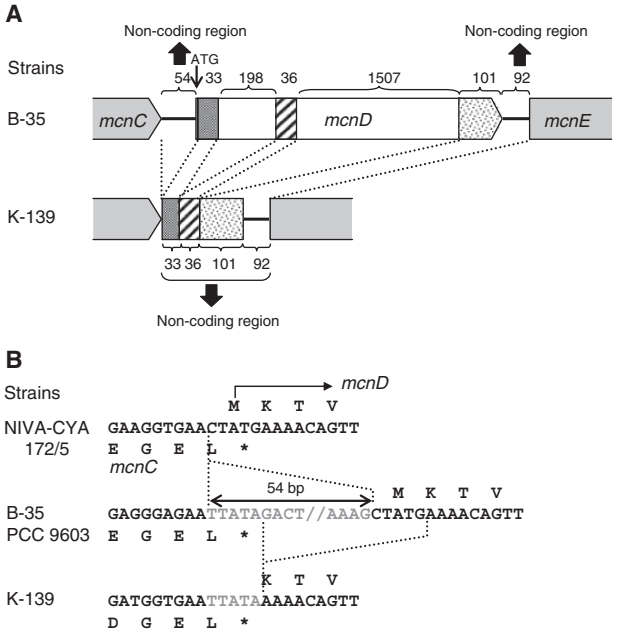

Fig. 6 Comparison analysis of downstream of *mcnC*. Comparison of the genomic sequence for the junction region between *mcnC, mcnD* and *mcnE* (A) and a comparison of the gene arrangement downstream of *mcnC* (B). Numbers indicate base pairs.

9808, PCC 9812 and PCC 9905 were identified (*41*), a conspicuous direct repeat sequence was not observed in the *mcnC*-$D_{B\text{-}35}$ region. The $mcnD_{B\text{-}35}$ gene is located 54 bp downstream of the stop codon (TAG) of the putative $mcnC_{B\text{-}35}$ (Fig. 6B). Except for the non-coding region, $mcnCDE_{B\text{-}35}$, corresponded to *mcnCDE* of N-C 172/5. A detailed analysis showed that the 54-bp non-coding region between *mcnC* and *mcnD* of B-35 had low similarity (48.8%) to the 96-bp non-coding region between *mcnC* and *mcnE* of PCC 9807 (AM773673).

Discussion

A specific region (33.7 kb) of *M. aeruginosa* K-139's chromosome was cloned and sequenced, revealing the existence of a *mcn* gene cluster remarkably similar to the gene responsible for anabaenopeptilide biosynthesis (*apd*) in *Anabaena* sp. strain 90 (*21*) and the putative gene for cyanopeptolin biosynthesis (*mcn*) of *M. wesenbergii* N-C 172/5 (*7*). According to a biochemical analysis in this study, the truncated McnB A-domain strongly activated a Thr residue (Fig. 3), whose residue is highly conserved in the cyanopeptolin-class depsipeptides (*1–4*, *7*, *25*, *42–44*).

As an alternative, the gene knockout experiments for the inactivation of MT-domain of *mcnC* demonstrated that the *mcn* gene cluster is responsible for micropeptin production using micropeptins-producing *M. viridis* S-70. Moreover, we clarified the recombination events responsible for the deletion of the halogenase gene from an analysis of the *mcn* gene cluster.

In this study, we proposed recombination events of the *mcnD* gene (Fig. 6). Cadel-Six *et al.* (*41*) investigated the presence or absence of the halogenase gene (*mcnD*) in 28 *Microcystis* laboratory strains by PCR amplification and nucleotide sequencing. They suggested a horizontal transfer of the *mcnD* gene because of the lower GC content of *mcnD* compared with that of genomic DNA and the direct repeats sequence between *mcnC* and *mcnE*. As a result of a detailed comparison of nucleotide sequence, we revealed that a deletion of three regions occurred in the truncated *mcnD* gene of *Microcystis*. We speculate that the N-C 172/5 type and the K-139 type appeared as a result of individual deletion events at different sites. In addition, PCC 9603 has one of 54-bp and one of 477-bp fragment upstream and downstream of *mcnD*, respectively (*41*), but the 477-bp insert was not retained between downstream of $mcnD_{B\text{-}35}$ and the initiation codon of $mcnE_{B\text{-}35}$. In particular, because the 5′-end of the [TTATA] sequence in the 54-bp insert was also observed in the truncated *mcnD* gene of K-139 (Fig. 6B), it seems rational that K-139 originates appeared from B-35. These results indicate that the *mcnD* gene originates from $mcnD_{B\text{-}35}$ and that further recombination occurred in the intact halogenase gene. Additionally, we assume that variation in the

truncated *mcnD* gene region occurred more frequently due to the relaxed selection pressures on the truncated type as a result of the loss of function, indicating that *mcnD* is not directly responsible for cyanopeptolin/micropeptin biosynthesis.

The cyanopeptolin-class depsipeptides contain Ahp as a structural element. Considering the substrate of the Ahp-activating A-domain, Rouhiainen *et al.* (*21*) have proposed a pathway leading to the formation of Ahp as a derivative of glutamate (Gln/Glu) involved in ApdF (a putative 3-oxoacyl ACP reductase). Tooming-Klunderud *et al.* (*7*) reported that Ahp-activating A-domains could clearly be divided into Gln- and Glu-activating A-domains according to phylogenetic analysis of the cyanobacterial NRPS A-domains. In this study, Orf3 and MAE59950 corresponding to ApdF were found in downstream of the *mcnE* gene and MAE60000 in K-139 and NIES-843, respectively (Fig. 2), indicating that the pathway to Ahp may exist in the genus *Microcystis* as well. However, further amino acid alignment analysis based on the Genbank and DDBJ database revealed that Orf3 (3-oxoacyl ACP reductase) was similar to a putative ketoacyl reductase (P16544), which belongs to the short-chain dehydrogenases/reductase family, involved in the biosynthesis of actinorhodin. We surmise that Orf3 is involved in the formation of the fatty acid moiety rather than the formation of Ahp. Further biochemical work is necessary to elucidate the pathway leading to the formation of Ahp.

A common feature of many cyanopeptolin/micropeptins is the moiety located at the N-terminal. In micropeptin K139, this moiety was composed of L-Asp and a HA fatty acid side chain. In the case of depsipeptide cyanopeptolin-984, which is produced by N-C 172/5, the first amino acid residue possessed acylated-Gln (*7*). The nucleotide sequence of the A-domain of $mcnA_{\text{K-139}}$ showed similarity to the A-domain of $mcnA_{\text{N-C 172/5}}$ with 63% identity, $mcnA_{\text{NIES-843}}$ (73%), and $mcnA_{\text{PCC 7806}}$ (96%), respectively. However, authentic NRPS C-domain of the initiating module was highly conserved the C-domain of *mcnA* (N-C 172/5, 91% identity; NIES-843, 96%; PCC 7806, 95%) against the C-domain of $mcnA_{\text{K-139}}$. Southern hybridization showed *mcnA*-undetectable strains S-77, NISE-89, NIES-90, NIES-298 and NIES-103 (Table II). NIES-90 produced a hexapeptide, micropeptin 90, containing glyceric acid 3-*O*-sulphate (D-Ga sulphate) due to an amino acid bound to the side chain (*42*, *43*). NIES-103 produces the octapeptide micropeptin NIES103 containing a HA bound to a Gly residue (*44*). Unfortunately, no depsipeptide was identified in strains S-77, NIES-89 and NIES-298. Considering these circumstances, we suppose that the absence of a hybridization signal in *mcnA*-undetectable strains shows a lower level of conservation or a lack of the *mcnA* gene. Further study of the NRPS gene cluster related to peptide biosynthesis in the genus *Microcystis* is needed to understand the diversity and propagation of NRPs in cyanobacteria.

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Conflict of interest
None declared.

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