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Phylogenetic Analysis of Frequent Recombinant Region in Micropeptin Biosynthetic Gene Cluster among the Genus *Microcystis*

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Abstract

The gene cluster for the biosynthetic of a nonribosomal peptide, cyanopeptolins and micropeptin (MCN), was identified in *Microcystis* strains and halogenated MCN-producing *Microcystis* were found to possess the halogenase gene, *mcnD*, between nonribosomal peptide synthetase genes, *mcnC* and *mcnE*. A comparative sequence analysis of the *mcn* gene cluster between halogenated and non-halogenated MCN-producing strains revealed mosaic sequence traces from *mcnD* in the non-coding region between *mcnC* and *mcnE* in the latter strains. A phylogenetic analysis based on a 170-bp non-coding region including the *mcnD* traces suggests that the recombination events occurred in a particular region of the *Microcystis*' *mcn* gene. This study provides novel insight into the ecological patterning of widespread *Microcystis* species.

Keywords

Cyanobacteria, *Microcystis*, Micropeptin Biosynthetic Gene, Phylogenetic Analysis

Eutrophic and hypertrophic freshwater bodies worldwide are habitable environments for cyanobacteria [1]. As the unicellular cyanobacterium, *Microcystis* can be collected comparatively easily from lakes and ponds. It has been proposed as a model for studying the ecological patterning in free-living bacteria [2]. Ecological patterning

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may affect the concept of species through historical and/or contemporary environmental processes such as local selection, indicating that there are such ecologically distinct populations based on the Stable Ecotype Model [3] [4]. To investigate the concept of bacterial ecological species, the bacterial ribosomal RNA gene region is utilized [5]. However, it is difficult to differentiate distinct ecotypes of *Microcystis* according to the internal transcribed spacer sequence of 16S-23S rRNA [2].

Whole genome sequences of 12 genera of cyanobacteria have been decoded to date. The entire and draft genome sequences of *Microcystis aeruginosa* strains NIES-843 [6] and PCC 7806 [7], respectively, have been established. Notably, 11.8% of the genome of NIES-843 was occupied by mobile elements: insertion sequences and miniature inverted-repeat transposable elements [6]. This indicates that rearrangements of the genome, including the deletion and mutation of genes, have frequently occurred in *Microcystis*. Moreover, the genus is known to produce natural products *via* secondary metabolism. The gene for a cyclic heptapeptide microcystin (*mcy*) whose production involves nonribosomal peptide synthetase (NRPS) and polyketide synthase was identified in *M. aeruginosa* K-139 [8] [9]. A highly conserved *mcy* gene cluster was confirmed among the *Microcystis* [10]-[12]. Moreover, there were hotspots for the insertion and deletion of a fragment between a non-coding region of the ORF gene and the area adjacent to the cyclic heptapeptide microcystin and micropeptin gene (*mcn*) clusters among the genus [10] [11] [13].

We cloned and sequenced the genes *mcnABCE*, responsible for producing the heptadepsipeptide micropeptin belonging to the cyanopeptolin class, in *M. aeruginosa* K-139 and demonstrated that NRPS is involved in micropeptin production by means of *mcn* gene-knockout [13]. In addition, according to a comparative analysis of the *mcn* genes, we revealed the loss of a recombination event in relation to a halogenase gene, designated *mcnD*, within the *mcn* gene cluster as well as proposed a clear evolutional history for the absence of a transcriptional region in *mcnD*. Therefore, we concluded that the *mcnD* gene does not contribute to the production of non-halogenated MCNs [13]. Regarding NRPS-related halogenases, Cadel-Six *et al*. [14] proposed that the ancestral *Microcystis* had a *mcn* gene cluster containing a halogenase that was subsequently lost. Strain PCC 9812, which was isolated in Lake Mendota, United States, produces non-halogenated-MCN, but has an intact *mcnD* [14]. On the other hand, non-halogenated-MCN producing *Microcystis* strains, K-139, NIES-102, NIES-103, and S-70, which were isolated in Lake Kasumigaura, Japan, and NIES-90, which was isolated from Lake Kawaguchi, Japan, were also identified (**Table 1**). Although we revealed that strain B-35, which strain possesses an intact *mcnD*, retained the *mcnA*, *mcnB*, *mcnC*, and *mcnE* genes according to genomic Southern hybridization analysis [13], we have not identified the MCN compound in strain B-35 to date (T. Nishizawa and M. Shirai, unpublished data). To identify the transcription of the *mcn* gene, a reverse transcribed-PCR analysis was carried out. For the reverse transcribed-PCR analysis, cells in a logarithmic growth phase were harvested, total RNA was isolated from *M. aeruginosa* B-35 and K-139 using hot phenol, and cDNA was prepared as described previously [8]. The reverse transcribed reaction was performed as described [15]. The primers sets for the *mcnA* and *mcnD* genes were F-mcnA-RT (5'-CGCCCAAAAATGTCACC-3') and R-mcnA (5'-AAGGGGAAATCTTGGGC-3') based on the $mcnA_{K\text{-}139}$ gene (accession number, AB481215) and F-hal-RT (5'-GGCGAATCAATCTTTACATCG-3') and R-hal-RT (5'-TCACTTACCAATTGCCTC-3') based on the $mcnD_{B\text{-}35}$ gene (AB481216), respectively. PCR (30 µL) was performed under the following condition; 3 min at 95˚C, then 25 cycles of 95˚C (30 s), 58˚C (30 s), and 72˚C (45 s), and 72˚C (2 min). In this study, no transcription of *mcnA* and *mcnD* in strain B-35 was found though the transcription of a NRPS-related microcystin biosynthetic gene was observed (data not shown). From these results and our previous investigation of genomic Southern hybridization [13], it was suggested that the *mcn* gene cluster of strain B-35 may be pseudogenes. Therefore, strain B-35 is the first *Microcystis* strain which does not produce MCN although it has the *mcn* gene cluster including the halogenase gene.

In this study, a detailed comparison of the *mcn*-related halogenase gene of *Microcystis* species was carried out. According to a comparative analysis of strains K-139 and B-35 based on *mcnC* and *mcnE*, we propose that a loss of three DNA fragments occurred through recombination in a mosaic-like manner and then a nontranslated region containing a part of *mcnD* arose in strain K-139 [13]. To examine the deletion of the intact *mcnD* gene among the genus *Microcystis*, we selected 22 sequences of *mcnC-mcnE* from the MCN-producing *Microcystis* strains (**Table 1**). The acceleration of the rate of nucleotide substitutions in the *mcnC-mcnE* gene region among the genus *Microcystis* was calculated based on a phylogenetic analysis. Nucleotide sequences of the *mcnC-E* region were aligned using Clustal W [27]. The bootstrap test was performed on 500 replicates. A neighbor-joining (NJ) tree was constructed [28] and evolutionary distances were computed using the Tajima's test in MEGA 4

Table 1. Summary of cyanopeptolin-producing *Microcystis* and *Anabaena* strains.

Strain	Geographic origin	Peptide	Sequence of *mcnC-mcnE*, accession number	Reference
M. aeruginosa				
B-35	Japan[a]	no	AB481216	This study
B-47	Japan[a]	n.d.[c]	AB481217	[13]
K-139	Japan[a]	micropeptin K139	AB481215	[16]
NIES-89	Japan[b]	n.d.	AB481218	[14]
NIES-90	Japan[b]	micropeptin 90	AB481219	[17]
NIES-298	Japan[a]	n.d.	AM773670	[14]
PCC 7005	United States	cyanopeptolin 954[d]	AM773675	[18]
PCC 7806	Netherlands	cyanopeptolin A cyanopeptolin B cyanopeptolin 963A	AM773668	[19] [19] [20]
PCC 7813	Scotland	aeruginopeptin 228A	AM773665	[21]
PCC 7941	Canada	cyanopeptolin 1040A[d] cyanopeptolin 1006A	AM773676	[14] [22]
PCC 9603	Sweden	cyanopeptolin 989[d]	AM773677	[14]
PCC 9622	France	cyanopeptolin S	AM773669	[23]
PCC 9807	South Africa	cyanopeptolin 1020 cyanopeptolin 1005	AM773673	[22] [14]
PCC 9808	Australia	cyanopeptolin 1040B cyanopeptolin 1040C[d]	AM773674	[14] [14]
PCC 9809	United States	aeruginopeptin 228-A aeruginopeptin 228-B	AM773671	[21] [21]
PCC 9812	United States	cyanopeptolin 1020	AM773679	[22]
PCC 9905	United States	cyanopeptolin 954[d]	AM773678	[18]
PCC 10025	France	cyanopeptolin	AM773672	[14]
M. viridis				
NIES-102	Japan[a]	cyanopeptolin	AB481220	[14]
NIES-103	Japan[a]	micropeptin NIES103	AB481221	[24]
S-70	Japan[a]	micropeptin S70-A micropeptin S70-B	AB481222	[16] [16]
M. wesenbergii				
NIVA-CYA 172/5	Denmark	cyanopeptolin 984[d]	DQ075244	[25]
Anabaena sp.				
Strain 90	Finland	anabaenopeptilide 90-A anabaenopeptilide 90-B[d]	AJ269505	[26] [26]

[a]Isolated in Lake Kasumigaura. [b]Isolated in Lake Kawaguchi. [c]n.d. not determined. [d]Chlorinated cyanopeptolin.

program [29]. When a phylogenetic analysis of the 92-bp sequence (downstream non-coding region) using 21 strains excluding strain NIVA-CYA 172/5 [14] was conducted with a non-coding region between downstream of *apdC* involved in halogenation of the depsipeptide anabaenopeptilide and upstream of *adpD* in the heterocyst *Anabaena* sp. strain 90 [26] as an outgroup, these 92-bp sequences formed one group (data not shown). Then, a

phylogenetic analysis was performed based on the non-coding region of 170-bp, which is mosaic sequence traces, between *mcnC* and *mcnE* except for the 92-bp fragment in strain K-139 [13]. In this phylogenetic analysis, the *apdC* gene was used as an outgroup. The phylogenetic relationship shown in **Figure 1(a)** clearly divided into two clades, mosaic sequence traces (clade I) and the intact *mcnD* gene (clade II). Interestingly, strain K-139 placed outside of clade I (**Figure 1(a)**). Except for strain K-139, the relative rate of substitution was examined at a number of sites (170-bp) using the shortest branch of strain PCC 9622 in clade I and the longest branch of strain B-35, which has a 54-bp insertion sequence upstream of *mcnD* [13], in clade II. The number of nucleotide substitutions per site was 0.112:0.018 (6-folds, $\chi^2 = 11.64$, $P < 0.001$). When strain K-139 was used instead of strain PCC 9622, the number was 0.064:0.035 ($\chi^2 = 1.47$, $P = 0.23$). Otherwise, excluding the outgroup of *adpC*, we estimate the average evolutionary divergence of the 170-bp and 92-bp sequence pairs to be 0.097 and 0.045, respectively. These investigations indicate an acceleration of the rate of nucleotide substitutions in clade I. Moreover, the rate increased in the truncated *mcnD* gene region rather than the non-coding region (92-bp) between *mcnD* and *mcnE*.

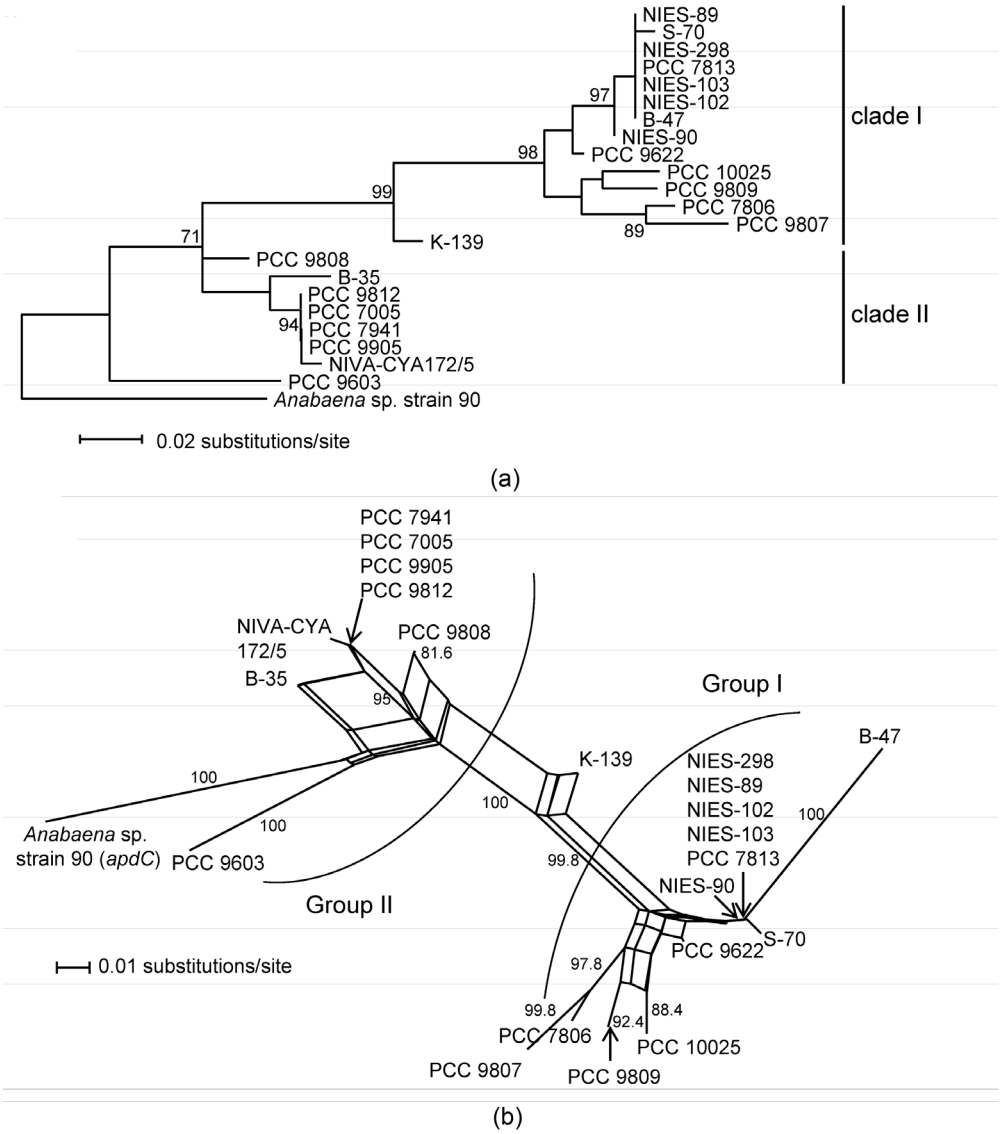

Figure 1. Phylogenetic relationship determined using the neighbor-joining method (a) and a splits decomposition analysis (b) of the *mcnC-E* region of *Microcystis* strains. Only bootstrap values exceeding 70% are shown. There were a total of 170 positions in the final dataset.

To determine the genetic relationship of strain K-139 to both clades, a splits decomposition analysis was carried out using the 170-bp sequence. Recombination was investigated by a split decomposition analysis using SplitsTree Version 4 [30] with default settings (uncorrected P method) and 500 bootstrap replicates. As shown in **Figure 1(b)**, the 170-bp non-coding region of strain K-139 placed in between the two groups. Phylogenetic analyses revealed that the truncated *mcnD* gene of strain K-139 is a unique sequence, indicating the strain to have a unique non-halogenated-MCN genotype. In addition, lower divergence of the 170-bp non-coding region was found within the non-halogenated-MCN genotypes isolated in Lake Kasumigaura and Lake Kawaguchi, Japan except strain B-47 in group I (**Figure 1(b)**). Our results indicate that the region between *mcnC* and *mcnE* is the evolutionary hotspot among non-halogenated MCN-producing *Microcystis*.

Conclusively, the diversified sequences of the intact and truncated *mcnD* gene may imply that halogenated MCN- and non-halogenated MCN-producing *Microcystis* occur individually. In the present study, the existence of a truncated *mcnD*-possessing *Microcystis* peculiar to Lake Kasumigaura was showed and it is assumed that truncated *mcnD* such as strain K-139 is purged. Our results show new insight into the ecological patterning of widespread *Microcystis* species. Further isolation of *mcy* and *mcn*-possessing *Microcystis* strains is needed to elucidate the ecological species of *Microcystis* that have adapted genetically to local ecosystems.

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