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Genetic Analysis of the Peptide Synthetase Genes for a Cyclic Heptapeptide Microcystin in *Microcystis* spp.[1]

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Peptide-synthetase-encoding DNA fragments were isolated by a PCR-based approach from the chromosome of *Microcystis aeruginosa* K-139, which produces cyclic heptapeptides, 7-desmethylmicrocystin-LR and 3,7-didesmethylmicrocystin-LR. Three open reading frames (*mcyA*, *mcyB*, *mcyC*) encoding microcystin synthetases were identified in the gene cluster. Sequence analysis indicated that McyA (315 kDa) consists of two modules with an *N*-methylation domain attached to the first and an epimerization domain attached to the second; McyB (242 kDa) has two modules, and McyC (147 kDa) contains one module with a putative C-terminal thioesterase domain. Conserved amino acid sequence motifs for ATP binding, ATP hydrolysis, adenylate formation, and 4′-phosphopantetheine attachment were identified by sequence comparison with authentic peptide synthetase. Insertion mutations in *mcyA*, generated by homologous recombination, abolished the production of both microcystins in *M. aeruginosa* K-139. Primer extension analysis demonstrated light-dependent *mcy* expression. Southern hybridization and partial DNA sequencing analyses of six microcystin-producing and two non-producing *Microcystis* strains suggested that the microcystin-producing strains contain the *mcy* gene and the non-producing strains can be divided into two groups, those possessing no *mcy* genes and those with *mcy* genes.

Key words: cyanobacteria, microcystin, *Microcystis*, multifunctional enzyme complex, peptide synthetase gene.

Toxic cyanobacterial (blue-green algal) waterblooms are found worldwide in eutrophic lakes, ponds, and dams (*1, 2*). The deaths of animals after drinking freshwater containing toxic cyanobacteria have been reported. Strains of several cyanobacterial genera, such as *Microcystis, Anabaena, Oscillatoria*, and *Nostoc*, produce cyclic peptide hepatotoxin microcystins, and a number of microcystin-producing strains produce multiple microcystins. *Microcystis* species are some of the most common waterbloom-forming species of cyanobacteria. The toxic effects of microcystins are due to the inhibition of protein phosphatases 1 and 2A (*3*). Microcystins cause cytokeratin hyperphosphorylation which leads to a disruption of cytoskeletal components and cell deformation, followed by disruption of the liver architecture (*1*). Moreover, microcystins have been reported to act as tumor promoters in human liver (*2, 4*). In 1996, liver failure and death of patients after exposure to microcystins occurred at a hemodialysis center in Brazil (*5*). Microcystins produced by cyanobacteria have become a serious environmental problem. Cyanobacteria, including microcystin-producing strains, produce a large number of peptide compounds, *e.g.* micropeptins, cyanopeptolins, microviridin, with varying bioactivities (*6*). However, very little is known about the peptide synthetase genes in cyanobacteria.

The general structure of microcystins (MCYST-XZ) is cyclo (-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) in which X and Z are variable L amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Adda is 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid, and Mdha is *N*-methyldehydro-alanine (Fig. 1) (*2*). More than 50 structural variants of microcystins have been found. Since microcystins contain unusual residues that are not present in proteins, in addition to the known proteogenic amino acids, microcystin is thought to be produced non-ribosomally by a large multifunctional enzyme complex utilizing a thio-template mechanism (*7-12*). In this mechanism, multifunctional enzymes, called peptide synthetases, activate the amino acid constituents as amino-acyladenylate at the expense of ATP and thioesterify them to the thiol moiety of an enzyme-attached cofactor, 4′-phosphopantetheine. One module harbors these catalytic activities to incorporate single amino acid residues into the peptide (*8, 9*). Sequence alignment of peptide synthetase revealed the domains that are the functional building units, and each domain shows highly conserved core regions of 6 to 20

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Abbreviations: MCYST, microcystin; D-MeAsp, D-*erythro*-β-methylaspartic acid; Adda, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid; Mdha, *N*-methyldehydro-alanine; bp, base pair; PCR, polymerase chain reaction; Cm, chloramphenicol.

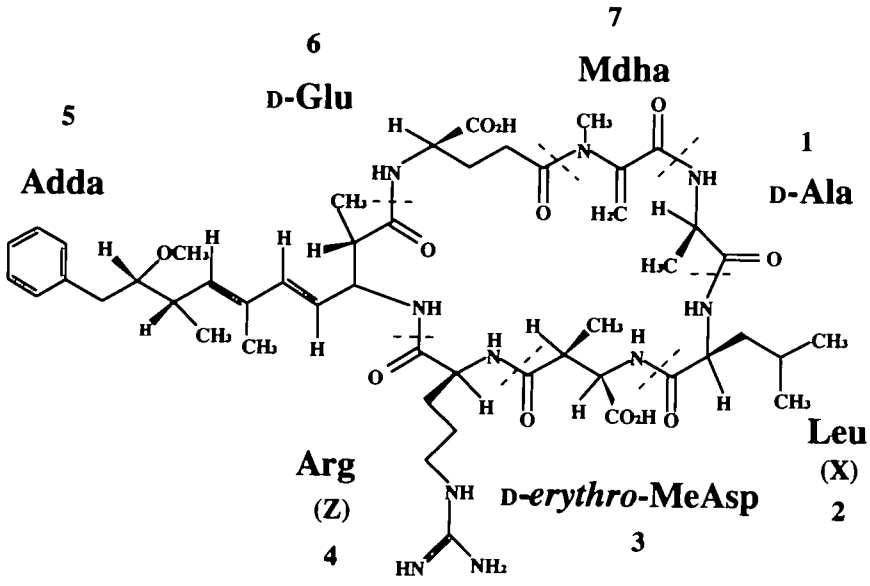


Fig. 1. **General structure of microcystin.** The general structure of microcystin (MCYST-XZ) is cyclo (-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), in which X and Z are variable L amino acids, D-*erythro*-MeAsp is D-*erythro*-β-methylaspartic acid, Adda is 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid, and Mdha is *N*-methyldehydroalanine. This figure shows microcystin-LR (MCYST-LR).

amino acid residues that are essential for amino acid activation, thioester formation, and condensation (*8-10, 13*). Furthermore, additional domains carry out modification of the activated amino acid, such as epimerization and *N*-methylation. In addition to these domains, a putative thioesterase domain that might be involved in product release and/or cyclization of the peptide chain, was found at the C-terminal end (*7, 14*). The arrangement of these domains determines the number and order of the amino acid constituents of the peptide product.

Recently, Börner and co-workers isolated and sequenced the putative peptide synthetase gene from microcystin-producing *M. aeruginosa* (*15-17*). Furthermore, they performed gene disruption experiments to confirm that the cloned gene was responsible for microcystin production (*15*). However, the entire gene structure involved in microcystin synthesis has not been identified. Environmental conditions, *e.g.* light intensity, temperature, and culture age, influence toxin production (*2*). We reported that *M. aeruginosa* retains light-responsive and rhythmic gene expression (*18, 19*) and possesses multiple *rpoD* homologs encoding a principal sigma factor (*18, 20, 21*). Furthermore, several alternative sigma factors have been isolated from cyanobacteria and characterized (*22*). These sigma factors are probably expressed differentially under various environmental conditions. However, the regulation of microcystin synthetase gene expression has not been determined.

M. aeruginosa K-139, which produces 7-desmethylmicrocystin-LR (7-desmethyl-MCYST-LR) and 3,7-didesmethylmicrocystin-LR (3,7-didesmethyl-MCYST-LR), was isolated from Lake Kasumigaura (*23*). To isolate the microcystin synthetase genes, we synthesized oligonucleotides corresponding to conserved amino acid sequences (core 1 and core 2) of the adenylate-forming domains (*24*) as primers for PCR with *M. aeruginosa* K-139 cDNAs. Using amplified PCR fragments, the peptide synthesis genes corresponding to microcystin synthetase were isolated and analyzed.

EXPERIMENTAL PROCEDURES

Cyanobacterial Strains, Culture Conditions, and Plasmids—The following *Microcystis* strains were used; *Microcystis aeruginosa* K-139, which produces 7-desmethyl-MCYST-LR and 3,7-didesmethyl-MCYST-LR; *M. aeruginosa* S-77, *M. aeruginosa* B-35, and *Microcystis* sp. S-70 (formerly identified as *M. viridis*, but the characteristic cell arrangement was lost and identification is necessary), which produce MCYST-LR, -RR, and -YR; *M. aeruginosa* M-20, which produces MCYST-LR, -RR, -YR, and 3-desmethyl-MCYST-LR; and microcystin-non-producing strains *M. aeruginosa* K-81 and B-19, isolated from Lake Kasumigaura, Ibaraki (*23, 25*). *Microcystis* strains were grown in CB medium under continuous illumination with fluorescent (cool white) light at 30°C and 2,000 lux (*25*). *Escherichia coli* DH5αMCR (Cosmo Bio., Tokyo) was used as a host for recombinant plasmids and grown at 37°C in 2× TY broth, 2×TY agar, or LB broth. *E. coli* S17-1 (*26*) was used for conjugation and grown at 37°C for 16 h in LB broth. Antibiotics were added as necessary at the following final concentrations: ampicillin 75 μg/ml and neomycin 30 μg/ml. Lorist 6 (Nippon Gene, Toyama) was used to construct the cosmid library, pUC118/119 was used for cloning, and pSUP5011 (*26*) was used for conjugation.

DNA Manipulation—Total *Microcystis* strain DNA was isolated from cells grown to the late logarithmic phase by the previously described procedure (*27*). DNA manipulations were performed as described previously (*28*).

Southern Hybridization—Digested cyanobacterial DNA was separated in 0.8 or 0.3% agarose gels and transferred to Hybond-N or Hybond-NX membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (*27*). DNA fragments for use as probes were labeled using the ECL random prime labeling system (Amersham Pharmacia Biotech); Southern hybridization was performed according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)—To amplify the adenylate-forming domain regions of the peptide synthetase genes, core 1 primer [TTI AA(A/G) GC(A/G)

GG(C/T) GGI GCI TAT GTG CCG AT(C/T) GA(C/T) CC] and core 2R primer [CC TTT TGG CTT ICC TGT IGT ICC (A/G)GA IGT (G/A)TA IAT] derived from conserved core 1 and core 2 sequences (*24*) were synthesized and used as forward and reverse primers, respectively. The reactions were performed in a final volume of 25 μl containing 5 ng of total DNA from *Microcystis* cells, 20 pmol of each primer, 0.2 mM dNTPs, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 1.4U Taq DNA polymerase (Nippon Gene). The reactions were performed in a MiniCycler (MJ Research, Watertown, MA) for 30 cycles after preincubation at 95°C for 2 min, with denaturation at 95°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 65°C for 3 min. In the case of amplification of the microcystin synthetase gene from *Microcystis* strains, the reaction was performed under the following conditions: 1 min at 96°C, then 25 cycles of 96°C (30 s), 56°C (30 s) and 72°C (1 min), and 3 min at 72°C.

Construction of M. aeruginosa Genomic DNA Library and Cloning—Partially *Hin*dIII-digested *M. aeruginosa* K-139 total DNA (ab. 40 kb in size) was ligated to cosmid Lorist6 DNA digested with *Hin*dIII. *In vitro* packaging using Gigapack II Gold Packaging Extract (Stratagene, La Jolla, CA) was performed according to the manufacturer's instructions. The packaged DNA was transfected into *E. coli* strain DH5αMCR. The genomic library was screened using probes derived from cloned PCR-amplified fragments. For subcloning, plasmid pUC119/118 was used as the vector with *E. coli* strain DH5αMCR as the host.

Sequencing—Dideoxy chain termination using an Applied Biosystems Automated Sequencer (model 373S, Foster, CA) was used to determine the nucleotide sequences of double-stranded template DNA fragments derived from deletion clones generated using Exonuclease III and Mung Bean Nuclease.

Integrative Conjugation of Microcystis—A plasmid that causes gene disruption by double homologous recombination events that lead to the insertion of a chloramphenicol (Cm) resistance cassette into the peptide synthetase gene was introduced into *Microcystis* cells by conjugation with *E. coli* cells. Fifty milliliter aliquots of log-plase *Microcystis* culture and overnight culture, of *E. coli* S17-1 containing conjugative plasmids were centrifuged, washed with MilliQ water, and resuspended in 0.25 ml of MilliQ water. Aliquots of cell suspensions (50 μl) were mixed in 1.5-ml sample tubes or on Millipore membrane filters (HAWP 025 00, Bedford, MA) placed on CB agarose plates (*25*) without antibiotics and incubated at 30°C for 20-24 h in the dark. Cell mixtures or membrane filters were transferred to 50 ml of CB medium and incubated at 30°C under continuous illumination at 2,000 lux. After 2 days, chloramphenicol (8 μg/ml)-resistant cells were selected.

HPLC Analysis of Microcystin—Microcystins were extracted from dried cells with 5% aqueous acetic acid, purified on Bond Elute ODS cartridges (Varian, CA) and analyzed by HPLC (*2, 29*) under the following conditions: System, Shimadzu LC-9A pump (Kyoto) and Waters 991J photodiode array detector (Milford, MA); column, Cosmosil 5C18ARII (150×4.6 mm I.D., Nacalai Tesque, Kyoto); mobile phase, CH_3CN:0.01 M trifluoroacetic acid=30:70 (v/v); flow rate, 1.0 ml/min; detection, UV 200-300 nm by photodiode array detection.

Primer Extension Analysis—Total RNA was isolated from *Microcystis* cells using hot phenol and subjected to high-resolution primer extension analysis using the primers NSZW1 (5′-CTTGAAGTTGCCGAATTTGG-3′, 20 mer) and NSZW2 (5′-GAACTACAGGAAACCCGAC-3′, 19 mer), as described previously (*20*).

Computer Analysis of DNA and Protein Sequences—The DNA sequences were assembled and analyzed using GENETYX-MAC from Software Development (Tokyo, Japan).

Nucleotide Sequence Accession Number—The nucleotide sequences in this report have been submitted to DDBJ under the following accession numbers: AB019578 (*mcy* from *M. aeruginosa* K-139), AB019708 (*M. aeruginosa* K-81 peptide synthetase gene), AB019709 (*M. aeruginosa* B-35 peptide synthetase gene), AB019710 (*M. aeruginosa* B-47 peptide synthetase gene), AB019711 (*M. aeruginosa* S-77 peptide synthetase gene), and AB019712 (*Microcystis* sp. S-70 peptide synthetase gene).

RESULTS

Amplification of Adenylation Domain Sequences of Peptide Synthetase—To clone microcystin synthetase genes from *M. aeruginosa* K-139, we amplified the internal gene fragment corresponding to the adenylation domain by PCR using two oligonucleotides designed to encode conserved motifs, core 1 and core 2, of peptide synthetase (*24*). An amplified band of approximately the expected length (260 bp) was detected. DNA from this band was cloned into the *Sma*I site of pUC119, and 45 clones were isolated. The nucleotide sequences of these clones were determined; six different clones were obtained and designated as the TN series. Nucleotide identity among these six clones was 27%, and each clone was approximately 260 bp in length, suggesting that these six PCR products were derived from the adenylation domains of peptide synthetase genes of *M. aeruginosa* K-139. To investigate the strain-specific distributions of these putative adenylation domains, genomic Southern hybridization analysis of toxic and non-toxic strains using the cloned DNA fragments as probes was carried out and the results are summarized in Table I. All six fragments hybridized with both *Hin*dIII-digested and *Xba*I-digested total DNA of toxic *M. aeruginosa* K-139 and B-47, whereas no hybridization signals were observed with the DNA digests from non-toxic *M. aeruginosa* B-19. In the case of non-toxic *M. aeruginosa* K-81, all fragments except TN29 generated signals. TN9 and TN18 hybridized with DNAs from all toxic strains. The results of Southern analysis suggested that TN9 and TN18 may be parts of the adenylation domains of microcystin synthetase genes and hybridize with different modules of the peptide synthetase genes. Therefore, both of these PCR products were used to identify corresponding sequences in a genomic library of *M. aeruginosa* K-139.

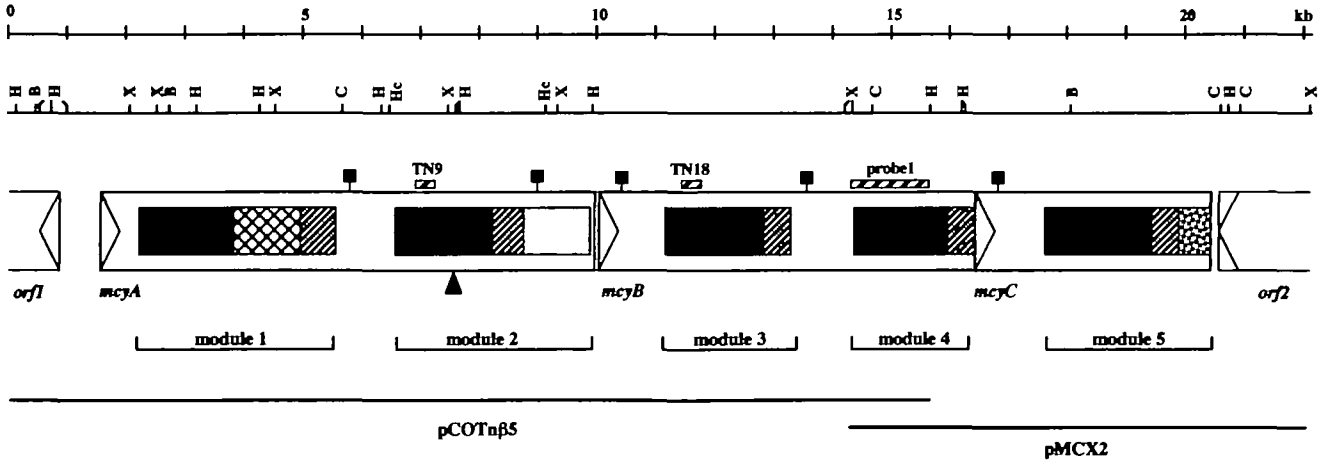
Cloning and Sequencing—ECL-labeled DNA fragments of TN9 and TN18 were used to screen approximately 1,000 colonies of the cosmid library of *M. aeruginosa* K-139. One colony, including the recombinant plasmid pCOTnβ5, hybridized with both probes and was studied further (Fig. 2). The nucleotide sequence of the junction region between the inserted fragment and cosmid vector showed that part of the 3′-end of the peptide synthetase gene was missing in pCOTnβ5 (data not shown). To obtain the rest of the

peptide synthetase gene, *Xba*I-digested total strain K-139 DNA was shotgun cloned into the *Xba*I site of pUC119. Using a 1.42 kb *Xba*I-*Hin*dIII fragment (probe1) from the 3′-end region of the insert of pCOTnβ5 as a probe, recombinant pMCX2 containing a 7.8 kb *Xba*I fragment was isolated (Fig. 2). Genomic Southern hybridization analysis using the cloned DNA fragments as probes revealed that the deletion in the large DNA fragment had not occurred during the cloning experiments. The nucleotide sequence of a 22,125 bp DNA fragment was determined on both strands. The overall G+C content of this clone was 40%, similar to that of *M. aeruginosa* K-139 genomic DNA (*27*). Analysis of the obtained nucleotide sequence revealed the presence of three open reading frames (ORFs) transcribed in the same direction, which were designated *mcyA*, *mcyB*, and *mcyC* (microcystin synthetase genes). The first ORF, *mcyA*, starting at putative position 1,670 bp (ATG) and ending at position 10,031 (TAA), was 8,361 bp in length, encoding a polypeptide of 2,787 amino acids (aa) with a predicted molecular mass of 315,268 Da (Fig. 2 and Fig. 6C). A putative Shine-Dalgarno sequence was found 12 nucleotides upstream from the start codon. The second ORF, *mcyB*, was located 12 bp downstream of the two tandem TAA stop codons of *mcyA*, starting with a putative ATG codon at position 10,049 bp and ending with a TGA codon at position 16,427. A possible Shine-Dalgarno sequence was located 9 bp upstream of the ATG codon. This ORF (6,378 bp) encodes a putative protein of 2,126 aa with a predicted molecular mass of 242,240 Da. The third ORF (*mcyC*) had a possible ATG start codon at position 16,426, overlapping the *mcyB* TGA stop codon and ending with a TAA stop codon at position 20,296 (Fig. 2). This ORF

TABLE I. **Summary of genomic Southern hybridization of *Microcystis* DNA with PCR products as probes.**

Strains	Signals with PCR products (TN)						Microcystin
	8	9	10	12	18	29	
M. aeruginosa							
K-81	+	++	++	+	++	−	non
K-139	++	++	++	++	++	+	[Dha⁷]MCYST-LR, [D-Asp³,Dha⁷]MCYST-LR
B-19	−	−	−	−	−	−	non
B-35	NT	++	NT	NT	++	NT	MCYST-LR, RR, YR
B-47	++	++	+	++	++	+	[Dha⁷]MCYST-LR, [D-Asp³,Dha⁷]MCYST-LR
M-20	−	++	−	−	+	−	MCYST-LR, RR, YR, [D-Asp³]MCYST-LR
S-77	−	++	NT	NT	++	NT	MCYST-LR, RR, YR
Microcystis sp.							
S-70	−	++	++	++	++	−	MCYST-LR, RR, YR

−, no signal; +, weak signal; ++, intense signal; NT, not test; [Dha⁷]MCYST-LR, 7-desmethylmicrocystin-LR; [D-Asp³,Dha⁷]MCYST-LR, 3,7-didesmethylmicrocystin-LR; [D-Asp³]MCYST-LR, 3-desmethylmicrocystin-LR; MCYST, microcystin. Genomic Southern hybridization proceeded at 60°C.



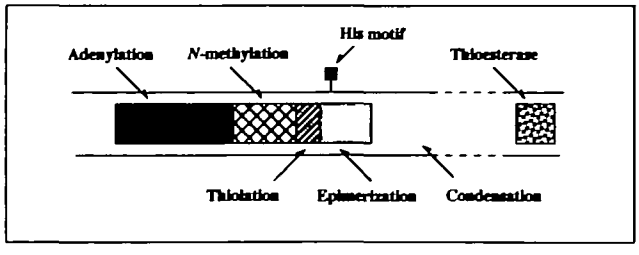


Fig. 2. **Organization and physical map of the *mcy* operon.** The domain organizations of the microcystin synthetase deduced from *mcyA*, *mcyB*, and *mcyC* are shown. The structure of a typical module including the domains and His motif conserved in the peptide synthetase are illustrated in the box. Restriction map and number of modules are shown above and below the diagram, respectively. The lower part of the figure shows the location of cosmid pCOTnβ5 and plasmid pMCX2. Positions corresponding to PCR products TN9 and TN18 are indicated. Probe 1 was used to clone pMCX2. The closed triangle shows the insertion site of the Cm cassette for gene disruption. Putative *orf1* and *orf2* read in the opposite direction. Abbreviations: H, *Hin*dIII; B, *Bam*HI; X, *Xba*I; C, *Cla*I; Hc, *Hin*cII.

(3,870 bp) encodes a putative protein of 1,290 aa with a predicted molecular mass of 147,605 Da, but no putative ribosome binding site was found. We detected two other putative ORFs, *orf1* and *orf2*, 743 bp upstream of *mcyA* and 92 bp downstream of *mcyC*, respectively, both in the opposite orientation from *mcyA, B*, and *C* (Fig. 2).

Homology Analysis of the Peptide Synthetase Genes—Non-ribosomal peptide synthetases have a unique modular structure in which each module is responsible for the activation and incorporation of a single amino acid (*8, 9*). Each module can be subdivided into several specific domains, *i.e.* the adenylation domain, thioester-binding domain, and condensation domain. In some cases, an additional modification domain, *e.g.* for epimerization or *N*-methylation, has also been found. Each domain contains a highly conserved sequence motif (*8-10, 13*). Sequence homology analysis of the ORFs revealed that *mcyA* consists of two modules (units) of amino acid activation including an *N*-methylation domain and an epimerization domain for the incorporation of two amino acids; the second (*mcyB*)

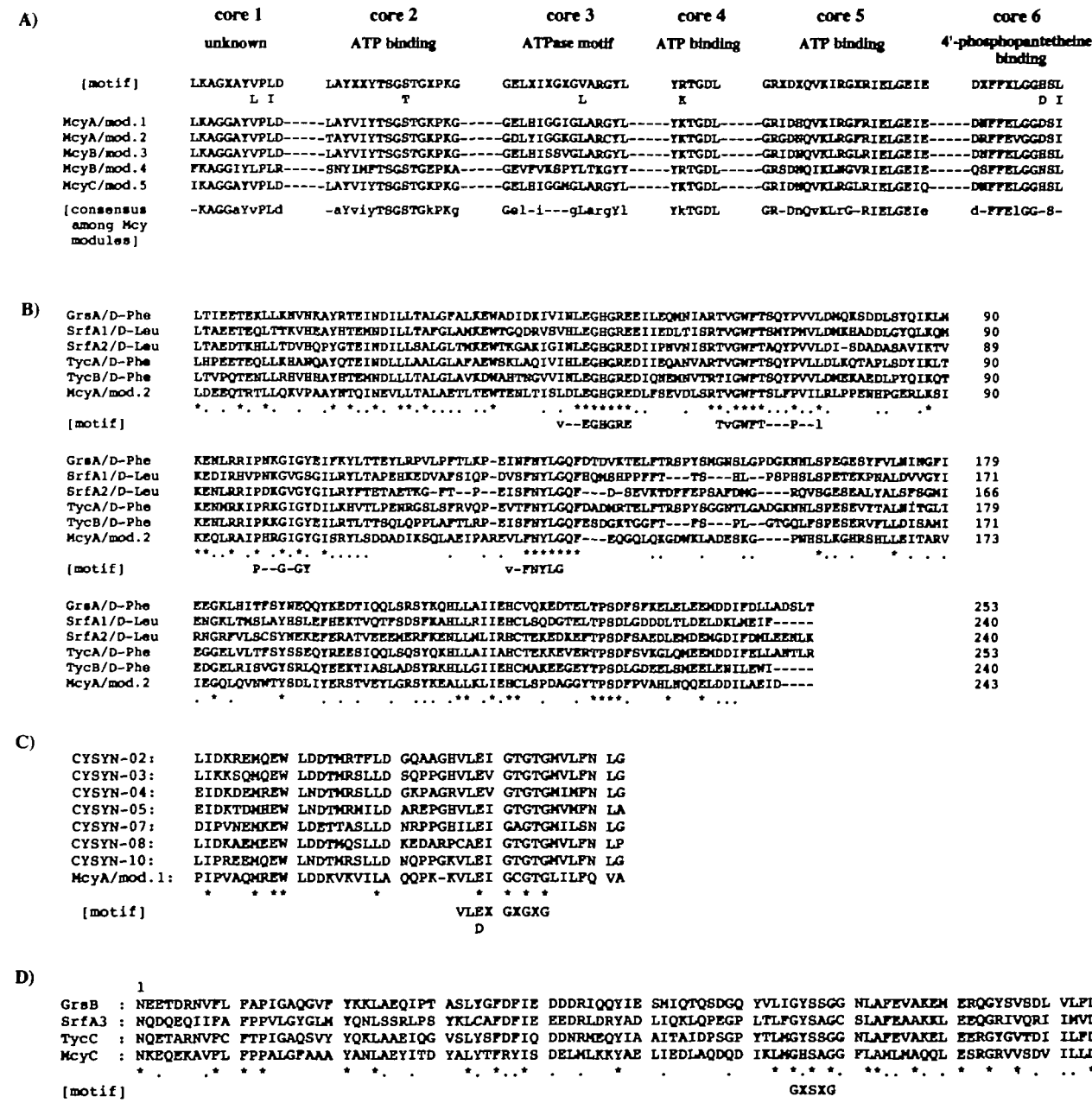


Fig. 3. **Alignment of conserved motifs among peptide synthetases.** (A) The alignment of core sequences of Mcy is shown (*8, 9*). Putative function and consensus motif sequences of core 1-core 6 are shown in the upper part and consensus sequences among modules of Mcy are shown in the lower part. (B) Comparison of the epimerization domains. (C) Comparison of the *N*-methylation domains. (D) Comparison of the thioesterase domains. Identical residues are indicated by asterisks, similar residues by dots. Sequence motifs for the epimerization (*30*), *N*-methylation (*33*) and thioesterase domains (*10*) are shown under the sequence alignment. GrsA and GrsB, gramicidin S synthetases (X15577, X61658); SrfA, surfactin synthetase (X70356); Tyc, tyrocidine synthetase (AF004835); CYSYN, cyclosporin synthetase (Z28383); Mcy, microcystin synthetases (AB019578).

and third (*mcyC*) ORFs contain two modules and one module, respectively (Fig. 2). Adenylation domains comprise about 500 amino acids while thiolation domains comprise about 90 amino acids. These domains in the five modules in *Microcystis* peptide synthetase show a high degree of amino acid sequence identity with each other (32-59% in the adenylation domain, 30-49% in the thiolation domain). Furthermore, the minimal modules of *Microcystis* peptide synthetase without module 4 exhibited a higher degree of sequence identity (31-52%) than those of other peptide synthetases such as gramicidin S (*grsA*, X15577; *grsB*, X61658), surfactin (*srfA1, srfA2, srfA3*, X70356), and tyrocidine (*tycA, tycB, tycC*, AF004835) synthetases. Module 4 shows 22 to 39% identity to the same module of other peptide synthetases. Sequence alignment of the conserved motifs, core 1-core 6 (*9, 10*), is shown in Fig. 3A. The function of core 1 [LKAGXAY(V/L)P(L/I)D] is unknown, while core 2 [LAYXXYTSG(S/T)TGXPKG], core 3 [GELXIXGXG(V/L)ARGYL], core 4 [Y(R/K) TGDL], and core 5 [GRXDXQVKIRGXRIEKGEIE] are believed to be involved in ATP binding and hydrolysis, and core 6 [DXFFXXLGG(H/D)S(L/I)] is present in the thioester-forming domain (*8, 9, 30, 31*). The sequences of these core motifs are conserved in each domain of the five modules of Mcy. An additional epimerization domain (400 aa) was observed at the carboxy-terminal end of the thiolation domain of McyA (Fig. 2). Highly conserved sequence motifs were observed in the D-Phe domains of GrsA, TycA, and TycB, and the D-Leu domains of SrfA1 and SrfA2 (Fig. 3B) (*9, 13, 32*). Four conserved sequences in the epimerization motif were observed in McyA, indicating that the second module of McyA is responsible for the recognition, activation, and epimerization of an amino acid residue. A His motif [HHXXXDG], which is called the spacer motif, is conserved in condensation (elongation) and epimerization domains (*9, 13, 33*). In Mcy, a His motif was found between the two amino acid-activating domains, in the epimerization domain and at the N-termini of McyB and McyC (Fig. 2). The N-terminus of the first module lacks a His motif. The condensation domains of Mcy show a low degree of amino acid identity with each other. However, the condensation domains in modules 3 and 5, which represent the N-terminus (480 aa) of McyB and McyC, are relatively similar (24.5%). Also, an additional *N*-methylation domain of about 420 amino acids was found inserted between core 5 and core 6 in module 1 of McyA (Fig. 2). A glycine-rich sequence [VL(E/D)XGXGXG], which is a common sequence motif in *S*-adenosylmethionine (SAM)-binding sites (*34, 35*), is conserved in the *N*-methylation domain of McyA (Fig. 3C). A thioesterase active site (TAS)-like domain, which might be involved in the release and/or cyclization of peptide chains (*10, 36*), was found only at the carboxy-terminal end of McyC and contains the motif GXSXG (*5, 9, 37*) (Figs. 2 and 3D). On the other hand, Orf1 and Orf2 show high degrees of similarity to polyketide synthetases PksE, PssD, PksC, and PksD of *Mycobacterium*, and a hypothetical protein (Sll0471) of *Synechocystis* PCC 6803. These observations indicate that the three ORFs, *mcyA*, *mcyB*, *mcyC*, encode peptide synthetase subunits making up the five amino acid activating domains. Recently, Dittman *et al.* cloned a 4,149 bp DNA locus of a microcystin-producing *M. aeruginosa* strain PCC7806, and demonstrated microcystin synthetase genes, *mcyA* and *mcyB*, in this locus by insertional mutagenesis (*15*). Homology analysis indicated that McyA and McyB from strain PCC7806 correspond to the C-terminal region of McyA and the N-terminal region of McyB from *M. aeruginosa* K-139, respectively, and that Mcy from strain K-139 show an extremely high degree of predicted amino acid sequence identity (97%) to McyA and McyB from strain PCC7806. These observations indicate that *mcy* encodes a microcystin synthetase. Furthermore, it can be speculated that the *mcy* genes are organized in an operon structure.

Disruption of the mcyA Gene—To determine whether the cloned peptide synthetase gene *mcy* is required for microcystin biosynthesis, gene disruption of *mcyA* in the *M. aeruginosa* K-139 genome was performed by homologous recombination. First, we performed transformation using double-strand plasmids, but no transformants were isolated. The restriction barrier of *M. aeruginosa* K-139 maybe interfere with transformation (*38*). Therefore, we used a conjugation procedure. The 2,698 *Hin*cII fragment containing the amino acid activation and epimerization domains of *mcyA* (Fig. 3) was inserted by blunt-end ligation into the *Sma*I site of pUC119, generating pMOT1. To obtain effective expression of the Cm resistance gene in *Microcystis* cells, the original promoter of the Cm resistance gene was replaced by the P1 and P2 promoters of

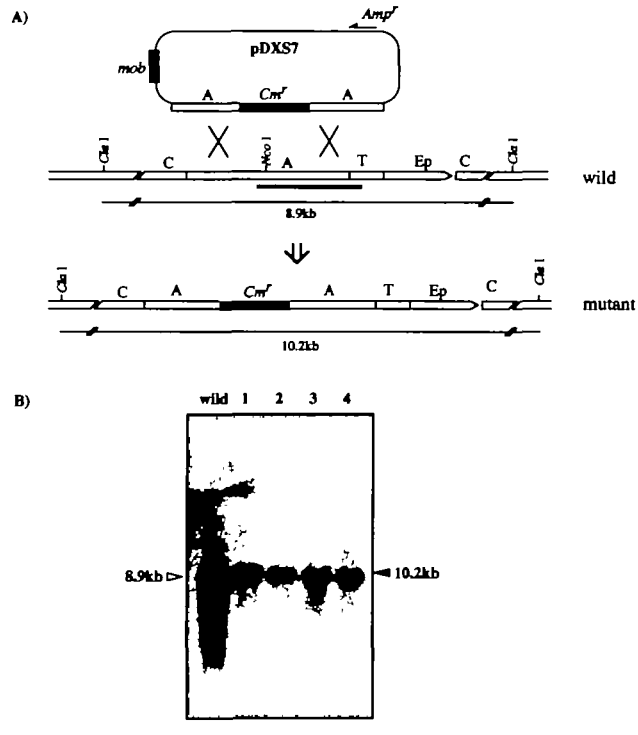


Fig. 4. **Disruption of the *mcy* gene by homologous recombination.** (A) Schematic representation of the insertional inactivation of the *mcyA* gene. Plasmid pDXS7 is a suicide gene disruption plasmid. *Cla*I digestion fragments hybridized with a *mcyA* probe (bold bar) are shown with their sizes. Locations of the corresponding domains are indicated as C, condensation domain; A, adenylation domain; T, thiolation domain; Ep, epimerization domain; Cm, Cm cassette. (B) Genomic Southern hybridization analysis of wild-type and mutant *M. aeruginosa* K-139. Lanes 1-4 are DX1, 2, 3, and 4 mutants, respectively. Sizes of the detected signals are indicated on both sides.

rpoD1 from *M. aeruginosa* K-81 (*20*). Transcription from the P2 promoter is strongly expressed in *Microcystis* cells grown under light and dark conditions. A 550 bp *Sac*I-*Nco*I fragment containing the *rpoD1* promoter region from pKXC-R (*20*) was blunt-end ligated into the *Sma*I and *Hin*dIII sites of the promoter probe vector pKK232-8 including a promoter-less Cm resistance gene (Amersham Pharmacia Biotech). After deletion of parts of the promoter region, modification, and subcloning, pR107XH was obtained. pR107XH consists of the Cm resistance gene with *rpoD1* promoter 1 and 2 and pUC118 with additional multicloning sites. The Cm gene cassette (ab. 1.2 kb) could be easily isolated from pR107XH by digestion with *Sac*I, *Sma*I, *Hin*dIII, *Bam*HI, or *Xba*I. The *Sma*I fragment including the Cm gene cassette was blunt-end ligated into the *Nco*I site of the cloned *mcyA* fragment of pMOT1, generating pMOT-CM. Then, a 1.8 kb *Bam*HI fragment containing the *mob* gene from pSUP5011 (*26*) was inserted by blunt-end ligation into the *Sal*I site in the multicloning region of pMOT-CM, generating pDXS7 (Fig. 4A). After transfer from *E. coli* S17-1 containing pDXS7, four independent mutants, *M. aeruginosa* K-139 DX1, 2, 3, and 4, were isolated. To confirm integration of the Cm cassette into the *mcyA* gene on the chromosome, genomic Southern hybridization was carried out. Total DNAs from four mutants were digested with *Cla*I and probed with the 1.8 kb *Xba*I fragment containing the 3′-end of *mcyA* (Fig. 4A) or the 0.7 kb *Eco*RI-*Xba*I Cm cassette fragment. Since pDXS7 cannot replicate in *M. aeruginosa*, only conjugants that acquire the resistance gene on their chromosomes by homologous recombination would be obtained. Only a 10.2 kb signal was detected in all mutants with the *mcyA* probe (Fig. 4, A and B), and the position of this signal coincides with that of the signal obtained with the Cm probe (data not shown). Southern hybridization analysis revealed that four mutants were double-crossover recombinants and the Cm cassette was integrated into the expected region of *mcyA* (Fig. 4A). These results were confirmed by other hybridization experiments using *Xba*I-digested DNA (data not shown). To investigate the influence of *mcyA* on microcystin biosynthesis, we analyzed the microcystin production of these four mutants by HPLC. Methanol extracts of cells were applied to an ODS silica gel column as described in "EXPERIMENTAL PROCEDURES". The extracts from the *M. aeruginosa* K-139 wild-type strain showed two peaks for microcystin, a major peak for 7-desmethyl-MCYST-LR and a minor peak for 3,7-didesmethyl-MCYST-LR (Fig. 5A). In all four mutants, neither microcystin peak was present (Fig. 5B). This is strong evidence that the *mcyA* gene is specifically involved in microcystin biosynthesis.

Analysis of Other Microcystis Strains—Southern hybridization analysis of *Microcystis* strains using two PCR amplified fragments, TN9 from *mcyA* and TN18 from

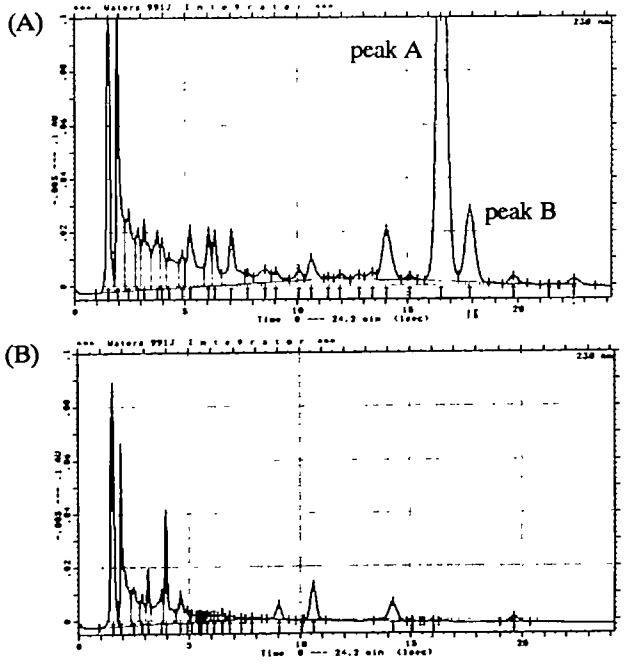


Fig. 5. **HPLC of the methanol extracts from *M. aeruginosa* K-139 cells.** Microcystins were extracted from dried cells of wild-type (A) and mutant DX1 (B). Peak A, 7-desmethyl-MCYST-LR; peak B, 3,7-didesmethyl-MCYST-LR; column, Cosmosil 5C18ARII (150× 4.6 mm I.D.); mobile phase, CH_3CN:0.01 M trifluoroacetic acid = 30:70 (v/v); flow rate, 1.0 ml/min; detection, 238 nm.

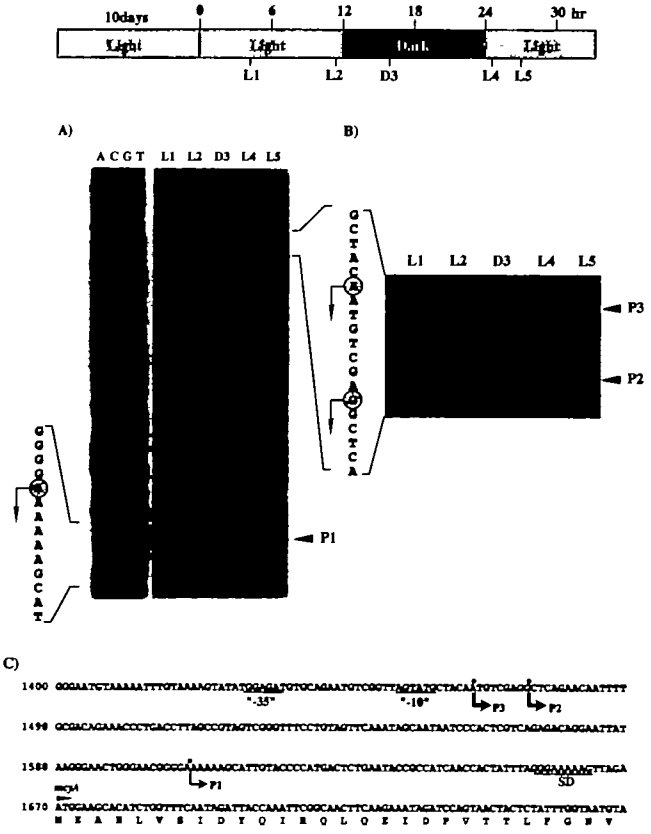


Fig. 6. **5′-ends of the *mcy* transcripts.** Cells were grown under continuous illumination for 10 days and then transferred to a 12/12 h light/dark cycle. Total RNA was extracted from the cells at L1, 4 h/light; L2, 11 h/light; D3, 4 h/dark; L4, 1 h/light; and L5, 4 h/light, and analyzed by primer NSZW1 (5′-CTTGAAGTTGCCGAATTTGG-3′) (panel A) and NSZW2 (5′-GAACTACAGGAAACCCGAC-3′) (panel B). The positions of the 5′-end of the *mcy* transcripts are indicated on the side and the 5′-end is circled. Nucleotide and deduced amino acid sequence in 5′-end region of *mcyA* is shown in panel C. Putative promoter and SD sequence are indicated under the nucleotide sequence. P1, P2, and P3 with arrows are 5′-ends of the transcripts.

mcyB (Table I), indicated that microcystin-producing strains *M. aeruginosa* B-35, B-47, S-77, M-20, and *Microcystis* sp. S-70 retain the *mcy* gene encoding a microcystin synthetase. Furthermore, a DNA fragment derived from *orf1* was hybridized to the genomic DNAs of the microcystin-producing strains (data not shown). However, both PCR fragments and an *orf1* probe DNA hybridized with genomic DNA from non-producing *M. aeruginosa* K-81, but not with genomic DNA from non-producing *M. aeruginosa* B-19. To confirm the conservation of *mcy* gene sequences among these strains, the region containing no conserved motifs was analyzed. The 693 bp DNA fragment including the 3′-end of *mcyB* and 5′-end of *mcyC* was amplified by PCR using each genomic DNA as a template and sequenced directly. A forward primer (5′-CAGTCTG-AAGGCGATGCG-3′) corresponding to positions 16297-16314 and a reverse primer (5′-CTGTAGGGTAAGAGGG-GGT-3′) corresponding to positions 16971-16989 in the sequence were used. A band of the approximate expected length was amplified from all strains tested including non-producing *M. aeruginosa* K-81. The amplified regions showed strong identity (nucleotide: 98%, amino acid: 96%) to each other and overlapping was observed in all clones (data not shown).

5′-End Mapping of the mcy Transcript—M. aeruginosa K-139 cells were grown under continuous illumination for 10 days, then transferred to a 12/12 h light/dark cycle. Total RNA was extracted from the cells at various times as indicated in Fig. 6. Primer extension analysis revealed three bands whose start points were designated as P1, P2, and P3 from each RNA preparation under both light and dark conditions (Fig. 6, A and B). Three transcripts were expressed weakly in the dark, suggesting that the expression of *mcy* is light-dependent. The transcription levels from P2 and P3 were considerably higher than that from P1. A potential *E. coli* consensus promoter sequence (−35: TGGAGA-(16 bp)-- −10:TAGTAT) was found just upstream of P3, but no consensus promoter sequences were observed upstream of P1 (Fig. 6C).

DISCUSSION

Recently, a multiple carrier thiotemplate model was proposed (*7, 8, 10, 36*). According to this model, each amino acid of a peptide molecule is activated as an amino acyladenylate and linked to the enzyme as a thioester of a phosphopantetheinyl group; then elongation occurs by transfer of the activated carboxyl to the amino group of the next amino acid. We cloned and sequenced a ~22 kb DNA locus of microcystin-producing *M. aeruginosa*. Homology analysis revealed that this DNA locus contains peptide synthetase genes encoding five modules. Each module of *mcy* includes the minimal module containing at least adenylation and thiolation domains. Furthermore, modules 1 and 2 include an *N*-methylation domain and an epimerization domain, respectively. Disruption of this peptide synthetase gene abolishes both 7-desmethyl-MCYST-LR and 3,7-didesmethyl-MCYST-LR production, indicating that the *mcy* gene is responsible for microcystin synthetase activity. Recently, Börner and co-workers isolated a peptide synthetase gene (*mapep1*, 2,982 bp) from toxic *M. aeruginosa* HUB 5-2-4 that specifically hybridizes to DNA from toxic strains of *M. aeruginosa* (*16, 17*). Using two oligonucleotides designed according to the sequence of *mapep1*, they cloned and sequenced a 4,149 bp DNA locus of a microcystin-producing *M. aeruginosa* strain, PCC7806, and insertional mutagenesis revealed microcystin-synthetase genes, *mcyA* and *mcyB*, on this locus (*15*). Homology analysis indicated that *mcyA* and *mcyB* in strain PCC7806 correspond to the 3′-end of *mcyA* and 5′-end of *mcyB* in *M. aeruginosa* K-139, respectively (Fig. 2), and the nucleotide and putative amino acid sequence identities between PCC7806 and K-139 are 98 and 97%, respectively. The sequences of the overlapping region between *mcyB* and *mcyC* in strain K-139 are highly conserved among *M. aeruginosa* K-81, B-35, B-47, S-77, and *Microcystis* sp. S-70. This region does not contain any conserved motifs. These observations suggest that the amino acid sequences of microcystin synthetase are highly conserved among microcystin-produing *Microcystis*. On the other hand, Mapep1 from *M. aeruginosa* HUB 5-2-4 shows 82% identity to McyB of strain K-139 (module 3). If the *mapep1* gene is involved in microcystin synthesis, this gene product may activate a different amino acid from constituents of MCYST-LR.

Our results indicate that microcystin-non-producing strains can be divided into two groups. One is an *M. aeruginosa* B-19 type group, which possess no *mcy* genes; the other is an *M. aeruginosa* K-81 type group, which possess the *mcy* genes. No microcystins were detected in strain K-81 cells, and the LD_{50} of this strain was >1,000 mg of dry cells per kg of mouse-weight (*23*). The reason why strain K-81 does not synthesis microcystins is unclear. Microcystin synthetase genes might be mutated or lacking in strain K-81 resulting in the observed phenotype.

Generally, peptide synthetases contain modules for activating the amino acids in a peptide molecule. Since microcystin is a cyclic peptide comprising seven amino acids (Fig. 1), microcystin synthetase genes have to include at least seven modules for amino acid activation. The bacterial peptide synthetase genes *grs, srfA*, and *tyc* are organized into an operon (*8, 39*). Putative *orf1* and *orf2* in the opposite orientation were found in this study (Fig. 2). However, neither of these *orf* genes show any homology to peptide synthetase genes reported to date. We analyzed two other peptide synthetase genes which hybridize with TN8, TN10, and TN12 (Table I), from *M. aeruginosa* K-139. Sequence analysis and gene disruption revealed that these genes are not responsible for microcystin synthesis. Since TN29 does not hybridize with the genomic DNAs of microcystin-producing strains, *M. aeruginosa* M-20 and *Microcystis* sp. S-70, this gene may be not responsible for microcystin synthesis. It is known that microcystin-producing strains produce other peptides, *e.g.*, cyanopeptolin (*15*). In addition to microcystin, another peptide was isolated from *M. aeruginosa* K-139 and the structural analysis of this peptide is currently under way. Interestingly, the *orf1* gene shows a high degree of similarity to the polyketide synthetase genes. Moore *et al.* reported that Adda in the microcystin molecule is biosynthesized from the carbon skeleton of phenylalanine and four molecules of acetate (*37*). To examine the participation of this polyketide synthetase homolog in microcystin synthesis, further sequencing and gene disruption experiments are currently in progress.

The His motif, which is generally found in condensation

(elongation or inter-) and epimerization domains (*9, 13, 33*), was observed upstream of all internal adenylation domains and in the epimerization domain. However, this motif was not found at the N-terminus of McyA. Generally, the subunit activating the first amino acid of the peptide lacks a His motif, when the peptide synthetase is composed of several subunits (*33*). On the other hand, a putative thioesterase domain, which might be involved in product release and/or cyclization of the peptide chain, is located only at the C-terminal end of the last module that is responsible for incorporation of the last amino acid (*8, 39*). A thioesterase-like domain was observed at the C-terminal end of module 5 of McyC (Fig. 2). We have identified five modules for the activation and incorporation of five amino acid residues, but have not yet found the other two modules. The operon organization of microcystin synthetase in *Microcystis* sp. may be unique.

The modules are aligned in a sequence that is colinear with the sequence of the peptide product (*8*). According to this theory, Mcy can be assumed to activate and cause condensation of five amino acids, Mdha, D-Ala, L-Leu, β-MeAsp, and L-Arg. Microcystin includes two methylated amino acids, Mdha and β-MeAsp. The *N*-methylation domain is located in the peptide synthetase gene (*34*), but as yet no β-methylation domain has been reported in any module. Furthermore, the amino acids on either side of β-MeAsp are in the L-form. Therefore, the first module, including the *N*-methylation domain of McyA, is thought to be responsible for domain of Mdha activation. Modules 2 including an epimerization domain, modules 3, 4, and 5, are presumed to be responsible for the activation domains of D-Ala, L-Leu, β-MeAsp, and L-Arg, respectively. Cosmina *et al.* compared 13 different amino acid binding domains in peptide synthetases from *Bacillus* strains and observed high degrees of similarity between domains that bind the same amino acid (*40*). To determine the substrate specificity, the Mcy amino acid activating domains were compared with similar domains of other peptide synthetases, Grs, SrA, and Tyc (*36, 39-41*). However, it was difficult to infer the adenylation domain involved in specific amino acid recognition from sequence comparison.

The disruption of *mcyA* indicated that *mcy* is responsible for the synthesis of 7-desmethyl-MCYST-LR and 3,7-didesmethyl-MCYST-LR. The question has been raised as to whether this gene is responsible for the synthesis of two kinds of microcystin. Dittmann *et al.* reported that the disruption of chromosomal *mcyA* resulted in MCYST-LR and 3-desmethyl MCYST-LR. Furthermore, they observed the disappearance of MCYST-RR and MCYST-YR as a minor component (*15*). Our results indicated that there are no other *mcy* homologs on the K-139 chromosome. These observations indicate that the *mcy* gene is responsible for the production of two microcystins. To clarify the role of each module in amino acid recognition and modification, biochemical characterization is required.

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