Cyclic heptapeptide microcystin biosynthesis requires the glutamate racemase gene

Tomoyasu Nishizawa,^{1,3} Munehiko Asayama¹ and Makoto Shirai^{1,2}

Author for correspondence: Makoto Shirai. Tel: +81 298 88 8652. Fax: +81 298 88 8653.
e-mail: shirai@ipc.ibaraki.ac.jp

^{1,2} Laboratory of Molecular Genetics, School of Agriculture¹, and Gene Research Center², Ibaraki University, Ami, Ibaraki 300-0393, Japan

³ The United Graduate School, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan

It was demonstrated previously that the operon consisting of the non-ribosomal peptide synthetase (NRPS) gene coupled with the polyketide synthase (PKS) gene involved in cyclic heptapeptide microcystin synthesis includes two different D-amino acid synthetase genes, an epimerization domain at the 3' end of module 2, and the racemase gene *mcyF*. To determine the role of *mcyF* in microcystin synthesis, gene-disruption and complementation analyses were carried out. Insertional mutagenesis in the *mcyF* gene, generated by homologous recombination, abolished only microcystin synthesis, but did not influence cell growth. Furthermore, *McyF* supported D-Glu-independent growth of a strain of *Escherichia coli* defective in D-Glu synthesis. It is concluded that *mcyF* is the glutamic acid racemase gene involved in the synthesis of D-Glu residues in the microcystin molecule. This is the first report of the racemase in prokaryotic NRPS.

Keywords: amino acid racemase, cyanobacteria, *Microcystis*, peptide synthetase gene, microcystin biosynthesis

INTRODUCTION

The cyanobacteria (blue-green algae) are Gram-negative and are exceptionally large photosynthetic prokaryotes. These bacteria proliferate in eutrophic marine and freshwater habitats, resulting in the formation of water blooms. Toxic cyanobacterial water blooms are found worldwide in eutrophic lakes, ponds and dams (Carmichael, 1994; Watanabe *et al.*, 1996). The major cause of toxicity is hepatotoxic microcystin, and the toxicity of microcystins is due to the inhibition of protein phosphatases 1 and 2A (MacKintosh *et al.*, 1990). Microcystins cause a variety of human illnesses and death in indigenous and domestic animals. Recently, the deaths of more than 50 haemodialysis patients in Caruaru, Brazil, were attributed to exposure to microcystin in the dialysis water (Jochimsen *et al.*, 1998). 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, Mdha is N-methyldehydroalanine, D-MeAsp is D-erythro-β-methylaspartic acid, and Adda is 3-amino-9-methoxy-10-

phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid (Fig. 1). More than 60 structural variants of microcystins have been found (Sivonen & Jones, 1999).

Peptides including microcystins that may contain non-protein amino acids are synthesized non-ribosomally by a large multifunctional enzyme, utilizing a thio-template mechanism called non-ribosomal peptide synthetase (NRPS) (Stachelhaus & Marahiel, 1995; Marahiel, 1997). We have cloned the microcystin synthetase genes (*mcyA*, -B, -C, -D, -E, -F and -G) encoding seven NRPS modules and polyketide synthases (PKSs) for synthesis of the Adda molecule (Nishizawa *et al.*, 2000). There are three NRPSs (*McyA*, *McyB* and *McyC*), two NRPS-PKS hybrids (*McyE* and *McyG*) and one PKS (*McyD*). Furthermore, in addition to the epimerase gene of module 2, a racemase gene for D-amino acid synthesis was also observed. The microcystin molecule has three D-amino acids: D-Ala, D-MeAsp and D-Glu. The same D-amino acids incorporated in non-ribosomally synthesized peptides by known prokaryotic NRPSs are synthesized by epimerization domains. Only one epimerization domain was found at the carboxy-terminal end of the thiolation domain in module 2 of *McyA*. Since this module is responsible for activation and incorporation of Ala, this epimerase domain must be for D-Ala synthesis.

Many D-amino acid racemase genes for peptidoglycan

Abbreviations: Adda, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid; MCYST, microcystin; Mdha, N-methyldehydroalanine; D-MeAsp, D-erythro-β-methylaspartic acid; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; UDP-MurNAc-L-Ala-D-Glu, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate.

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

synthesis have been reported in eubacteria (Liu *et al.*, 1997; Malathi *et al.*, 1999; Pucci *et al.*, 1995). However, no racemase genes have been reported in prokaryotic NRPS. The alanine racemase genes responsible for synthesis of cyclosporin A and HC-toxin were identified in fungal NRPS (Cheng & Walton, 2000; Hoffmann *et al.*, 1994). In this study, we showed that the *mcyF* gene encodes the glutamate racemase involved in microcystin synthesis.

METHODS

Bacterial strains and growth conditions. *Microcystis aeruginosa* K-139 (which produces 7-desmethyl-MCYST-LR and 3,7-didesmethyl-MCYST-LR) and *Microcystis* sp. S-70 (which produces MCYST-LR, -RR and -YR), both of which were isolated from Lake Kasumigaura, Ibaraki (Harada *et al.*, 1991; Sakamoto *et al.*, 1993), were used in this study. *Microcystis* strains were grown under continuous illumination (2000 lx) at 30 °C in CB medium (Shirai *et al.*, 1991). *Escherichia coli* DH5 α MCR (Cosmo Bio.) and *E. coli* JM109 (ToYoBo) were used as hosts for recombinant plasmids and were grown at 37 °C in 2 $\times$ TY broth and 2 $\times$ TY agarose. *E. coli* strain WM335 (*leu pro trp his arg thyA met lac gal rpsL hsdM hsdR murl gltS*) was used for a complementation test (Lugtenberg *et al.*, 1973). Antibiotics were used for recombinants, which were grown at final concentrations of 75 μ g ampicillin ml⁻¹ and 25 μ g chloramphenicol ml⁻¹.

DNA manipulation, Southern blotting and sequencing. Total DNA of *Microcystis* cells was isolated by the method described previously (Sakamoto *et al.*, 1993), and DNA manipulations were performed as described by Sambrook *et al.* (1989). Digested cyanobacterial DNA was separated in 0.8% agarose gels and transferred onto Hybond-NX membranes. DNA fragments (as probes) were labelled using an ECL random prime labelling kit (Amersham Pharmacia Biotech) as described previously (Nishizawa *et al.*, 1999). Genomic Southern blotting was performed according to the manufacturer's instructions. The nucleotide sequence was determined by dideoxy chain termination, using an Applied Biosystems automated sequencer (model 373S) (Nishizawa *et al.*, 1999).

Construction of a gene-disruption plasmid, and integrative conjugation of *Microcystis*. The plasmids for gene disruption

of *mcyF* by homologous recombination were constructed as follows. The 2.5 kb *NcoI*-*EcoRI* fragment containing *mcyF* was subcloned from pMCQ3 (Nishizawa *et al.*, 2000) by blunt-end ligation into the *SmaI*-*BamHI* site of pUC119 (ToYoBo), generating pCHI621. The 1.2 kb *XbaI* fragment containing the Cm^R gene cassette from pR107XH (Nishizawa *et al.*, 1999) and the 1.8 kb *BamHI* fragment containing the *mob* gene from pSUP5011 (Simon, 1984) were inserted by blunt-end ligation into the *BamHI* site (in *mcyF*) and the *HindIII* site (in the multi-cloning site) of pCHI621, respectively, generating pJXS10. The plasmids for gene disruption were introduced into *Microcystis* cells (by conjugation) from *E. coli* S17-1, and chloramphenicol-resistant (8 μ g chloramphenicol ml⁻¹) conjugants were selected as described previously (Nishizawa *et al.*, 1999).

HPLC analysis of microcystins. Microcystins were extracted from the dried cells with 5% aqueous acetic acid, purified using BondElute ODS cartridges (Varian) and analysed by HPLC as described previously (Nishizawa *et al.*, 1999).

Bacterial complementation test. *E. coli* WM335, which requires D-glutamic acid for growth, was grown in Luria-Bertani (LB) medium supplemented with 50 μ g D-glutamic acid ml⁻¹ and 20 μ g thymine ml⁻¹. The racemase expression vector pQE-McyF was constructed as follows (see Fig. 4a). The entire *mcyF* gene was amplified by PCR with primers 5'-McyF/*SphI* (5'-CT CGCATGCAG ACA AAA CTA CCG-3') and 3'-McyF/*HindIII* (5'-CTC AAGCTT TTT GGG TTT GAA GGC-3') (restriction sites are underlined and modified sequences are shown in italics). PCR was carried out on chromosomal *M. aeruginosa* K-139 DNA with *Taq* DNA polymerase (Nippon Gene). PCR products were purified, digested with *SphI* and *HindIII*, and cloned into the *SphI* and *HindIII* sites of the pQE70 vector (Qiagen). Both the 5'- and the 3'-cloning sites were confirmed by sequencing. The primers (and sequences) used were as follows: QE/F2 primer, 5'-TTG CTT TGT GAG CGG ATA AC-3'; QE/R1 primer, 5'-CAT TAC TGG ATC TAT CAA CAG G-3'. *HindIII* digestion of pQE70 resulted in removal of the His-tag sequence and appended the amino acid sequence SLIS at the C-terminus of the recombinant protein.

Expression of McyF. *E. coli* WM335 cells transformed with pQE-McyF were inoculated into LB medium supplemented with ampicillin (75 μ g ml⁻¹) and thymine (20 μ g ml⁻¹) and were grown at 37 °C with shaking for over 20 h. Expression

was analysed by SDS-PAGE and staining with Coomassie brilliant blue according to the method of Laemmli (1970).

Computer analysis of DNA. The DNA sequences were assembled and analysed using GENETYX-MAC software (Software Development).

RESULTS AND DISCUSSION

Characterization of the *mcyF* gene of *M. aeruginosa*

Previously, we characterized the microcystin synthetase gene cluster and found the racemase gene, *mcyF*, in this cluster (Nishizawa *et al.*, 2000). The *mcyF* gene is located 35 bp downstream of the PKS–NRPS hybrid gene *mcyE*, which contains the NRPS module at its 3' end. The *mcyF* gene is 753 bp in length, encoding a 251-residue polypeptide with a predicted molecular mass of 27990 Da. The putative Shine–Dalgarno sequence (AGGAGA) was found before the putative initiation codon. The NRPS module present at the 3' end of the *mcyE* gene is presumed to be involved in activation and incorporation of glutamic acid. Moore *et al.* (1991) demonstrated (by ^{13}C -NMR analysis) that D-Glu in the microcystin molecule is derived from L-Glu, and that D-MeAsp does not arise from rearrangement of Glu. In the database search, McyF showed 30.4% identity with respect to the aspartate racemase of *Desulfurococcus* (Nishizawa *et al.*, 2000).

D-Glutamic acid is fairly widespread in eubacteria, as D-amino acids constitute part of the fundamental tetrapeptide chain of murein in the cell wall (as common components of peptidoglycans) (Fotheringham *et al.*, 1998; Liu *et al.*, 1997; Malathi *et al.*, 1999; Pucci *et al.*, 1995). D-Glutamate in a free form is incorporated into the common peptidoglycan precursor UDP-N-acetyl-muramoyl-L-alanyl-D-glutamate (UDP-MurNAc-L-Ala-D-Glu), through the action of UDP-MurNAc-L-Ala-D-Glu synthetase (EC 6.3.2.9). In bacteria, enzymes are involved in the biosynthesis of D-glutamic acid through

two different pathways: D-glutamate racemase and D-amino acid aminotransferase (Malathi *et al.*, 1999). Cysteine residues at the active sites of motif 1 [(V/I)x(P/A)CNTA(H/T)] and motif 2 [(I/V)(A/L)GCTH(E/H)x(S/P)], which are thought to form the catalytic centres of these cofactor-independent racemases (Yohda *et al.*, 1996), were also conserved in McyF (Nishizawa *et al.*, 2000).

Disruption of the *mcyF* gene

To determine whether the cloned racemase gene, *mcyF*, in the *mcy* operon is required for microcystin biosynthesis, gene disruption of *mcyF* in *M. aeruginosa* K-139 and *Microcystis* sp. S-70 was carried out. However, no disruptants were isolated for strain K-139. The restriction barrier of strain K-139 may interfere with recombination (Takahashi *et al.*, 1996). Genomic Southern hybridization demonstrated the presence of *mcyF* in *Microcystis* sp. S-70, which produces MCYST-LR, -RR and -YR (Nishizawa *et al.*, 2000). Therefore, insertional mutagenesis of *mcyF* in strain S-70 was performed, and the chloramphenicol-resistant conjugant *Microcystis* sp. S-70JX1 was isolated.

To demonstrate integration of the Cm^R cassette into the *mcyF* gene on the chromosome, genomic Southern hybridization was carried out. Total DNA from mutant S-70JX1 was digested with either *Hinc*II or *Hind*III and then probed with the 0.75 kb *Bgl*II fragment containing a part of the racemase gene (Fig. 2a). A 2.9 kb signal and a 5.0 kb signal, obtained with *Hinc*II and *Hind*III, respectively, were detected in the control (*Microcystis* sp. S-70). A 0.8 kb signal and a 6.2 kb signal, however, were detected in mutant JX1 (Fig. 2b). In the case of *Hind*III digestion, the position of this signal coincided with that of a signal obtained with the probe of the Cm^R cassette fragment (data not shown). Furthermore, to confirm *mcyF* gene disruption and gene organization in

Fig. 2. Southern blot analysis of the *mcyF*-disrupted conjugant. (a) Schematic representation of insertional inactivation of the *mcyF* gene. The *mcyF* probe (black bar) is shown. *Hc*, *Hinc*II; *H*, *Hind*III. (b) Genomic Southern hybridization analysis. Chromosomal DNA of *Microcystis* sp. S-70 was digested with either *Hinc*II or *Hind*III. Lanes: 1, wild-type with *Hinc*II; 2, wild-type with *Hind*III; 3, JX1 mutant with *Hinc*II; 4, JX1 mutant with *Hind*III. The sizes of the signals are indicated in kb on both sides.

Fig. 3. HPLC of methanol extracts of *Microcystis* sp. S-70 cells. Microcystins were extracted from dried cells of the wild-type (a) and the mutant JX1 (b). Peaks: RR, MCYST-RR; YR, MCYST-YR; LR, MCYST-LR. Column, Cosmosil 5C18ARII (150 × 4.6 mm i.d.); mobile phase, CH₃CN/0.01 M trifluoroacetic acid (30:70, v/v); flow rate, 1.0 ml min⁻¹; detection, 238 nm.

strain S-70JX1, the 6.2 kb *Hind*III fragment, including *mcyE*–*mcyF*–*mcyG* (Fig. 2b), was cloned from this strain. Sequence analysis showed that the gene organization in strain S-70 was the same as that in strain K-139, and nucleotide sequence identity between both strains was 95.5 %.

To investigate the role of *mcyF*, we used HPLC to analyse microcystin production. The extracts from the control (*Microcystis* sp. S-70) showed three peaks for MCYST-LR, -RR and -YR (Fig. 3a). In contrast, the S-70JX1 mutant lacked all of these microcystin peaks (Fig. 3b). Moreover, the *mcyF* mutants showed normal growth and development on agarose plates and in liquid culture, indicating that McyF has no essential house-keeping function. These observations revealed that the *mcyF* gene encoding racemase is specifically involved in microcystin biosynthesis.

Genomic Southern hybridization using a *mcyF* probe revealed the absence of *mcyF* in microcystin-non-producing *Microcystis* strains and in *Synechocystis* PCC 6803 (Nishizawa *et al.*, 2000). Two racemase genes, D-glutamate racemase and D-alanine racemase, are found in microcystin-non-producing *Synechocystis* PCC 6803 in the database of the Kazusa Institute, Chiba, Japan. The *mcyF*-disruptant exhibited significant growth similar to that of the control strain. Therefore, the *mcyF* gene is required only for microcystin synthesis, but not for fundamental growth.

Fig. 4. Overexpression of the recombinant McyF protein for complementation of an *E. coli* glutamate racemase mutant. (a) Recombinant plasmid pQE-McyF. T5, promoter; stop, stop codon. (b) SDS-PAGE analysis of McyF expressed from pQE-McyF in *E. coli* WM335. A Coomassie-stained gel of the total cell extracts is shown. Lanes: 1, pQE70; 2, pQE-McyF; MM, molecular mass markers (kDa). Arrowhead, position of the 28 kDa expressed McyF protein.

Complementation of a bacterial mutant deficient in D-Glu synthesis

To confirm that the *mcyF* gene encodes the glutamate racemase, the McyF racemase was expressed in D-glutamic-acid-requiring *E. coli* WM335, using the T5 promoter transcription–translation system of pQE vectors (Fig. 4a). *E. coli* WM335 transformed with pQE-McyF expressed a protein of about 28 kDa (Fig. 4b), the molecular mass of which coincided well with the value predicted for McyF on the basis of its nucleotide sequence. The pQE-McyF-transformed *E. coli* grew exponentially and survived on agarose plates and in LB liquid medium (data not shown) without D-glutamic acid as well as in those supplemented with 50 µg D-glutamic acid ml⁻¹. However, *E. coli* WM335 carrying the expression vector pQE70 or pCHI621 showed no

Fig. 5. Phylogenetic tree representation of the apparent evolutionary distances of amino acid racemase proteins from several bacteria and fungi. The tree was constructed by the neighbour-joining method. The scale for the tree indicates inferred evolutionary distances. *Anabaena* PCC 7120_Ala, AAF33755; *Aquifex aeolicus*_Ala, O67687; *Aquifex aeolicus*_Glu, O66662; *Aquifex pyrophilus*_Glu, P56868; *Bacillus sphaericus*_Glu, P52972; *Bacillus stearothermophilus*_Ala, P10724; *Bacillus subtilis*_Ala, P94494; *Bacillus subtilis*_Asp, Z99118; *Bacillus subtilis*_Glu, P94556; *Borrelia burgdorferi*_Ala, O51182; *Cochliobolus carbonum*_Ala, AF169478; *Corynebacterium glutamicum*_Glu, AB020624; *Desulfurococcus* SY_Asp, D84067; *Escherichia coli*_Ala, AAC43147; *Escherichia coli*_Glu, AE000471; *Haemophilus influenzae*_Ala, P45257; *Haemophilus influenzae*_Glu, P52973; *Helicobacter pylori*_Ala, O25595; *Helicobacter pylori*_Glu, P56068; *Lactobacillus brevis*_Glu, P48797; *Lactobacillus fermentum*_Glu, Q03469; *Lactobacillus plantarum*_Ala, O08445; *Listeria monocytogenes*_Ala, O85045; *Microcystis aeruginosa* K-139, AB032549; *Mycobacterium leprae*_Glu, P46705; *Mycobacterium tuberculosis*_Ala, Q50705; *Mycobacterium tuberculosis*_Glu, Q10626; *Pediococcus pentosaceus*_Glu, Q08783; *Pyrococcus horikoshii*_Asp, AP00003; *Rickettsia prowazekii*_Ala, Q9ZE52; *Salmonella typhimurium*_Ala, P06655; *Staphylococcus aureus*_Ala, Q9ZAH5; *Staphylococcus haemolyticus*_Glu, P52974; *Streptococcus thermophilus*_Asp, X61301; *Streptomyces coelicolor*_Ala, O86786; *Synechocystis* sp. PCC 6803_Ala, S75775; *Synechocystis* sp. PCC 6803_Glu, P73737; *Thermococcus* sp._Asp, BAA35091.

growth on agarose plates without D-glutamic acid, and a rapid decrease in OD_{660} was observed shortly after inoculation into liquid medium without D-glutamic acid (data not shown). These observations showed that the *mcvF* gene was capable of complementation of the D-glutamic-acid-requiring phenotype in *E. coli*. Gene disruption and complementation analysis demonstrated that D-Glu in the cyclic heptapeptide microcystin is synthesized from the primary metabolite L-Glu by a glutamate racemase (McyF). These results indicated that McyF is involved in D-glutamic acid synthesis. Moreover, this is the first report showing the occurrence

of a glutamate racemase gene in prokaryotic non-ribosomal peptide synthesis.

Phylogenetic analysis of the D-amino acid racemase

The microcystin molecule is composed of the following three D-amino acid residues: D-Ala, D-MeAsp and D-Glu (Fig. 1). The microcystin synthetase was shown previously to contain one epimerization domain in McyA and one racemase, McyF (Nishizawa *et al.*, 2000). In NRPS, D-amino acid synthesis is generally catalysed by an epimerase, which is encoded by an epimerization

domain at the carboxy-terminal end of the thiolation domain (Stein *et al.*, 1995). Our results indicated that a glutamic acid racemase, McyF, is responsible for the production of D-Glu.

On the other hand, a sequence similarity search of D-amino acid racemases from bacteria and fungi, which are available in the GenBank, EMBL and DDBJ databases, was carried out using the program in GENETYX-MAC (see Methods). Our results revealed that McyF has 22.4–30.1 % sequence similarity to aspartate racemase, 16.9–23.7 % similarity to glutamate racemase, and 15.1–21.2 % similarity to alanine racemase. The phylogenetic relationships between these racemase genes were analysed by the neighbour-joining method, using GENETYX-MAC, and the resulting dendrogram is shown in Fig. 5. These racemase genes were tightly clustered, except for the glutamate racemase of *Haemophilus influenzae*. D-Amino acid racemases can be roughly divided into two groups, namely the Ala type and the Glu type. Aspartate racemases belong to the glutamate racemase cluster. Two cysteine residues, which are thought to form the catalytic centre, were highly conserved among the aspartate and glutamate racemases, as well as among their surrounding amino acid sequences (Nishizawa *et al.*, 2000).

The only racemase previously reported to be involved in non-ribosomal peptide synthesis was the alanine racemase in fungi (Cheng & Walton, 2000; Hoffmann *et al.*, 1994). The involvement of specific racemases in prokaryotic non-ribosomal peptide synthesis has not been reported. Their racemase genes are unrelated, at the primary amino acid level, to any known bacterial alanine racemases (Cheng & Walton, 2000; Hoffmann *et al.*, 1994). This analysis indicated that McyF belongs to the glutamic acid racemase group. Microcystin synthetase, including both types of D-amino acid synthesis enzymes, epimerase and racemase, is a unique NRPS. Interestingly, McyF is grouped with aspartate racemases in the phylogenetic tree (Fig. 5). Since the microcystin molecule includes D-MeAsp, it is possible that McyF is responsible for D-MeAsp synthesis together with D-Glu synthesis. However, this possibility has been excluded by the work of Moore *et al.* (1991), who concluded from the [1,2-¹³C]acetate labelling pattern that MeAsp is synthesized from acetyl-CoA and pyruvic acid.

ACKNOWLEDGEMENTS

We thank Drs K.-I. Harada and K. Fujii for their help with HPLC analysis. We are grateful to Drs W. Messer and M. Wachi for gifts of the strains used in this study.

REFERENCES

- Carmichael, W. W. (1994). The toxins of cyanobacteria. *Sci Am* **270**, 64–72.
- Cheng, Y. & Walton, J. D. (2000). A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. *J Biol Chem* **275**, 4906–4911.
- Fotheringham, I. G., Bledig, S. A. & Taylor, P. P. (1998). Characterization of the genes encoding D-amino acid transaminase and glutamate racemase, two D-glutamate biosynthetic enzymes of *Bacillus sphaericus* ATCC 10208. *J Bacteriol* **180**, 4319–4323.
- Harada, K., Ogawa, K., Matsuura, K. & 7 other authors (1991). Isolation of two toxic heptapeptide microcystins from an axenic strain of *Microcystis aeruginosa* K-139. *Toxicon* **29**, 479–489.
- Hoffmann, K., Scheider-Scherzer, E., Kleinkauf, H. & Zocher, R. (1994). Purification and characterization of eucaryotic alanine racemase acting as key enzyme in cyclosporin biosynthesis. *J Biol Chem* **269**, 12710–12714.
- Jochimsen, E. M., Carmichael, W. W., An, J. S. & 9 other authors (1998). Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N Engl J Med* **338**, 873–878.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liu, L., Yoshimura, T., Endo, K., Esaki, N. & Soda, K. (1997). Cloning and expression of the glutamate racemase gene of *Bacillus pumilus*. *J Biochem* **121**, 1156–1161.
- Lugtenberg, E. J. J., Wijsman, H. J. W. & van Zaane, D. (1973). Properties of a D-glutamic acid-requiring mutant of *Escherichia coli*. *J Bacteriol* **114**, 499–506.
- MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. & Codd, G. A. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett* **264**, 187–192.
- Malathi, K. C., Wachi, M. & Nagai, K. (1999). Isolation of the *murl* gene from *Brevibacterium lactofermentum* ATCC 13869 encoding D-glutamate racemase. *FEMS Microbiol Lett* **175**, 193–196.
- Marahiel, M. A. (1997). Protein templates for the biosynthesis of peptide antibiotics. *Chem Biol* **4**, 561–567.
- Moore, R. E., Chen, J. L., Moore, B. S. & Patterson, G. M. L. (1991). Biosynthesis of Microcystin-LR. Origin of the carbons in the Adda and Masp units. *J Am Chem Soc* **113**, 5083–5084.
- Nishizawa, T., Asayama, M., Fujii, K., Harada, K.-I. & Shirai, M. (1999). Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. *J Biochem* **126**, 520–529.
- Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K.-I., Ochi, K. & Shirai, M. (2000). Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. *J Biochem* **127**, 779–789.
- Pucci, M. J., Thanassi, J. A., Ho, H., Falk, P. J. & Dougherty, T. J. (1995). *Staphylococcus haemolyticus* contains two D-glutamic acid biosynthetic activities, a glutamate racemase and a D-amino acid transaminase. *J Bacteriol* **177**, 336–342.
- Sakamoto, T., Shirai, M., Asayama, M., Aida, T., Sato, A., Tanaka, K., Takahashi, H. & Nakano, M. (1993). Characteristics of DNA and multiple *rpoD* homologs of *Microcystis* (*Synechocystis*) strains. *Int J Syst Bacteriol* **43**, 844–847.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shirai, M., Ohtake, A., Sano, T. & 8 other authors (1991). Toxicity and toxins of natural blooms and isolated *Microcystis* spp. (Cyanobacteria) and improved procedure for purification of cultures. *Appl Environ Microbiol* **57**, 1241–1245.
- Simon, R. (1984). High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn5-Mob transposon. *Mol Gen Genet* **196**, 413–420.
- Sivonen, K. & Jones, G. (1999). Cyanobacterial toxins. In *Toxic Cyanobacteria in Water: a Guide to their Public Health Conse-*

quences, *Monitoring and Management*, pp. 41–111. Edited by I. Chorus & J. Bertram. London: E. & F. N. Spon.

Stachelhaus, T. & Marahiel, M. A. (1995). Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEMS Microbiol Lett* **125**, 3–14.

Stein, T., Kluge, B., Vater, J., Franke, P., Otto, A. & Wittmann-Liebold, B. (1995). Gramicidin S synthetase 1 (phenylalanine racemase), a prototype of amino acid racemases containing the cofactor 4'-phosphopantetheine. *Biochemistry* **34**, 4633–4642.

Takahashi, I., Hayano, D., Asayama, M., Futagami, M., Watahiki, M. & Shirai, M. (1996). Restriction barrier composed of an extracellular nuclease and restriction endonuclease in the uni-

cellular cyanobacterium *Microcystis* sp. *FEMS Microbiol Lett* **145**, 107–111.

Watanabe, M. F., Harada, K., Carmichael, W. W. & Fujiki, H. (editors) (1996). *Toxic Microcystis*. Boca Raton, FL: CRC Press.

Yohda, M., Endo, I., Abe, Y., Ohta, T., Iida, T., Maruyama, T. & Kagawa, Y. (1996). Gene for aspartate racemase from the sulfur-dependent hyperthermophilic archaeum, *Desulfurococcus* strain SY. *J Biol Chem* **271**, 22017–22021.

Received 3 October 2000; revised 6 December 2000; accepted 29 January 2001.