Identification of a Polyketide Synthase Coding Sequence Specific for Anatoxin-a-Producing *Oscillatoria* Cyanobacteria^{∇†}

Sabrina Cadel-Six,¹ Isabelle Iteman,¹ Caroline Peyraud-Thomas,¹ Stéphane Mann,²
Olivier Ploux,² and Annick Méjean^{1,2,3*}

Unité des Cyanobactéries (URA CNRS 2172), Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris Cedex 15, France¹;
Laboratoire de Biochimie des Microorganismes: Enzymologie, Métabolisme et Antibiotiques (UMR CNRS 7223),
ENSCP, 11 Rue Pierre et Marie Curie, 75231 Paris Cedex 05, France²; and Université Paris Diderot-Paris 7,
75013 Paris, France³

Received 29 October 2008/Accepted 12 May 2009

We report the identification of a sequence from the genome of *Oscillatoria* sp. strain PCC 6506 coding for a polyketide synthase. Using 50 axenic cyanobacteria, we found this sequence only in the genomes of *Oscillatoria* strains producing anatoxin-a or homoanatoxin-a, indicating its likely involvement in the biosynthesis of these toxins.

Anatoxin-a and homoanatoxin-a (Fig. 1) are potent neurotoxins produced by cyanobacteria (3, 20, 21). Cases of animal death, after the animals ingested water contaminated by cyanobacteria producing these alkaloids, are regularly reported in different places around the world (2, 6–8, 20, 22). Therefore, the release of such cyanobacterial toxins into water bodies and water supplies is a major public health and environmental issue (4).

To find a reliable genetic marker for the detection of anatoxin-a- and homoanatoxin-a-producing cyanobacteria, we searched for a gene involved in the biosynthesis of these toxins. Based on the biosynthetic route proposed by Hemscheidt et al. (9), we suspected that a polyketide synthase (PKS) was involved in the elongation of the glutamic semialdehyde starter. We chose *Oscillatoria* sp. strain PCC 6506 for this work because it produces anatoxin-a and homoanatoxin-a, it is a fast-growing cyanobacterium under standard conditions, and it has lost its benthic character after multiple subcultures since its isolation (R. Rippka and A. Méjean, unpublished observations). To amplify PKS genes from the genome of PCC 6506, we used the degenerate primers DKF and DKR, designed by Moffitt and Neilan (13). The PCC 6506 strain was cultivated (1, 10, 16) and its genomic DNA was isolated (12) by using previously described protocols. The PCR amplification, achieved under conditions described previously (2, 13), afforded a diffuse band corresponding to about 650 bp, as judged by gel electrophoresis (17), and the product was purified and cloned into the pGEM-T vector (Promega). After the transformation of *Escherichia coli* JM109, the selection of positive clones, the purification of the plasmids, and sequencing by the GATC Company (Konstanz, Germany), we obtained three distinct

DNA sequences, designated ks1-630, ks2-682, and ks3-716 (corresponding to fragments ks1, ks2, and ks3, of 630, 682, and 716 bp, respectively). Using Southern blotting experiments with digoxigenin-labeled probes (17), we obtained a larger ks1 fragment: ks1-3711. Using the adaptor-mediated gene walking technology (18) with the APAGene BT 501 kit (BIO S&T, Canada), we obtained a longer ks2 fragment: ks2-1680. A BLAST analysis of the ks2 sequence against the GenBank database revealed three sequences (accession no. AY768507, AY768508, and AY210784) almost identical to ks2-1680. Interestingly, the AY210784 sequence was from *Anabaena flos-aquae* NRC 44-1, a cyanobacterium producing anatoxin-a (3, 14), while the AY768507 and AY768508 sequences, which were identical, were from PCC 6506.

The putative functions of the products translated from partial coding DNA sequences (CDS) in the fragments ks1-3711, ks2-1680, and ks3-676 (Table 1) were inferred from bioinformatic analyses (using NCBI BLAST and the websites <http://www.tigr.org/jravel/nrps> and <http://www.nii.res.in/nrps-pks.html> for predicting PKS domains). As expected, each CDS coded for a ketosynthase (KS) domain of PKS. Furthermore, an acyltransferase (AT) domain, a classical feature in PKS (5), was found downstream of the KS domain in the product of the ks2-1680 CDS. Comparison of the sequence translated from ks2-1680 with PKS sequences involved in the biosynthesis of cyanobacterial secondary metabolites, such as curacin, jamai-camid, and microcystin, showed a degree of sequence identity higher than 50% (see Fig. S1 in the supplemental material), suggesting that the ks2 gene product is likely a PKS (Table 1). Fragment ks3-676 coded for a KS domain homologous (89% identical) to CyrB, a PKS involved in the biosynthesis of cylindrospermopsin (11). The fragment ks1-3711 encoded, beside the KS domain, a partial pyridoxal phosphate-dependent decarboxylase and a methyltransferase. It is very likely that other PKS genes are present in the genome of PCC 6506. However, the degenerate primers did not anneal to these sequences, preventing their isolation by our strategy.

The genomes of 49 other axenic cyanobacteria belonging to several genera, obtained from the Pasteur Culture Col-

* Corresponding author. Mailing address: Laboratoire de Biochimie des Microorganismes: Enzymologie, Métabolisme et Antibiotiques (UMR CNRS 7573), ENSCP, 11 Rue Pierre et Marie Curie, 75231 Paris Cedex 05, France. Phone and fax: 33 1 44 27 67 01. E-mail: annick-mejean@enscp.fr.

† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 15 May 2009.

FIG. 1. Structures of anatoxin-a and homoanatoxin-a.

lection of Cyanobacteria (PCC) (10), were searched for the presence of the PKS-encoding DNA sequences identified in the genome of strain PCC 6506 (Table 2; see also Table S1 in the supplemental material). We designed specific primers (see Table S2 in the supplemental material) to amplify a 400-bp sequence within fragment ks1, ks2, or ks3. The PCR was run using purified genomic DNA (19) or cell lysate (2) under standard conditions (17). When a PCR product was obtained, it appeared, in each case, as a clean single band of the predicted size upon agarose gel electrophoresis. Therefore, it is reasonable to postulate that the presence of a PCR product indicated the presence of the corresponding gene in the genome of the strain of interest and that the absence of a PCR product indicated the absence of the corresponding gene.

In parallel, we tested the abilities of each axenic strain to produce anatoxin-a and homoanatoxin-a (Table 2). The toxin contents of the cellular extracts were determined as described previously (2). Ten strains among the 50 strains studies produced anatoxin-a and homoanatoxin-a, and these 10 strains gave a ks2 PCR product (Table 2), whereas the 40 nonproducers gave no ks2 PCR product. In contrast, no such correlation for ks1 and ks3 PCR products could be observed. The 10 ks2 amplicons were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by the GATC Company to confirm their identities or similarities. The alignment (see Fig. S2 in the supplemental material) of the 10 DNA sequences showed that they were of exactly the same length (412 bp), with high degrees of identity (94 to 100%). The corresponding translated sequences also showed high levels of identity (see Fig. S2 in the supplemental material). Thus, we are confident that the 10 ks2 amplicons correspond to the same partial CDS. Interestingly, the *A. flos-aquae* NRC 44-1 strain, which harbors a homologous ks2 sequence (the sequence translated from the gene with accession no. AY210784 and that translated from ks2-1680 shared 96% identity), produces anatoxin-a (3, 14). Although these facts are only indirect evidence, we suggest that ks2-1680 is part of the gene cluster responsible for the biosynthesis of anatoxin-a and homoanatoxin-a. Insertional gene inactivation was not attempted at this stage of the work because, so far, it has not been possible to perform such genetic experimentation on PCC 6506.

We have provided the first tools toward a simple and reliable PCR-based methodology (using primers CGCAAATCGATG CTCACCTTA and CCACTGGCTCCATCTTGATT) to detect, in field samples, the presence of anatoxin-a- and homoanatoxin-a-producing *Oscillatoria* cyanobacteria that represent a risk for animal and human health (15).

Nucleotide sequence accession numbers. The three new DNA sequences determined in this study were deposited in the GenBank database with the following accession numbers: ks1-

TABLE 1. Putative functions of the CDS obtained from *Oscillatoria* sp. strain PCC 6506

DNA sequence designation	Size (bp)	Position (bp) of partial CDS	Accession no. and corresponding protein (organism) for Psi-BLAST similarity match	% Identity	Description of putative product
ks1-3711	3,711	1-1392	EAV71836.1 (<i>Clostridium cellulolyticum</i> H10)	58	Pyridoxal phosphate-dependent decarboxylase
		1398-2204	ACC81856.1 (<i>Nostoc punctiforme</i> PCC 73102)	42	Methyltransferase
		2254-3711	ACC81837.1 (<i>Nostoc punctiforme</i> PCC 73102)	73	KS domain of PKS
ks2-1680	1,680	1-1680	AAT70104.1; Cnr1 (<i>Lyngbya majuscula</i>)	60	KS and AT domains of PKS
ks3-676	676	1-676	ABX60161.1; CnrB (<i>Cylindrospermopsis raciborskii</i> AWT205)	89	KS domain of PKS

TABLE 2. Correlation between the production of anatoxin-a and homoanatoxin-a and the amplification of DNA sequences coding for PKS in PCC strains

PCC strain	Presence of anatoxin-a and homoanatoxin-a ^a	Presence of PCR product ^b for:		
		ks1	ks2	ks3
<i>Anabaena flos-aquae</i> strains				
PCC 9302	—	+	—	+
PCC 9332	—	+	—	—
PCC 9349	—	+	—	—
<i>Aphanizomenon flos-aquae</i> strain				
PCC 7905	—	+	—	+
<i>Geitlerinema</i> strain				
PCC 10602	—	—	—	—
<i>Lyngbya</i> strains				
PCC 7419 ^c	— ^d	+	—	—
PCC 8103 ^c	— ^d	+	—	—
PCC 8937 ^c	— ^d	+	—	—
PCC 8992 ^c	— ^d	+	—	—
<i>Microcystis</i> strain				
PCC 7806	—	—	—	—
<i>Nostoc</i> strains				
PCC 7120	—	—	—	—
PCC 73102	—	—	—	—
<i>Oscillatoria</i> strains				
PCC 6304	—	—	—	—
PCC 6407	+	+	+	+
PCC 6412	+	+	+	+
PCC 6506	+	+	+	+
PCC 6602	—	—	—	—
PCC 7112	—	+	—	—
PCC 7515	—	—	—	—
PCC 7816	—	—	—	—
PCC 7817 ^c	— ^d	—	—	+
PCC 7823 ^c	— ^d	—	—	—
PCC 7907 ^c	— ^d	—	—	+
PCC 7926	—	—	—	—
PCC 7928 ^c	— ^d	—	—	—
PCC 7929	—	—	—	—
PCC 7930	—	+	—	+
PCC 8704	—	—	—	+
PCC 8808	—	—	—	—
PCC 8914	—	—	—	+
PCC 8919	—	+	—	—
PCC 8922	—	—	—	—
PCC 8923	—	—	—	—
PCC 8935	—	+	—	+
PCC 8946	—	—	—	—
PCC 8963	—	+	—	—
PCC 8973	—	—	—	—
PCC 8989	—	—	—	—
PCC 9029	+	+	+	+
PCC 9107	+	+	+	+
PCC 9240	+	—	+	—
PCC 9609	—	+	—	—
PCC 10016	—	+	—	—
PCC 10111	+	+	+	+
PCC 10601	+	—	+	—
PCC 10608	+	—	+	—
PCC 10702	+	+	+	+
<i>Planktothrix</i> strains				
PCC 7805 ^c	— ^d	+	—	—
PCC 7811 ^c	— ^d	+	—	—
<i>Synechocystis</i> strain				
PCC 6803	—	—	—	—

^a Presence (+) or absence (—) of anatoxin-a and homoanatoxin-a in the cellular extract as analyzed by gas chromatography-mass spectrometry as described previously (2).

^b Presence (+) or absence (—) of a single PCR amplicon obtained using primers specific for the ks1, ks2, or ks3 sequence.

^c Cell lysate instead of purified genomic DNA was used for the PCR amplifications.

^d Data are from Aráoz et al. (1).

3711, FJ418584; ks2-1680, FJ418585; and ks3-676 (equivalent to ks3-716 but excluding the sequence of the degenerate primers), FJ418586.

This work was supported by the Institut Pasteur, the Ecole Nationale Supérieure de Chimie de Paris, and the Centre National de la Recherche Scientifique (URA 2172 and UMR 7573) and by a Multiorganismes grant from Aventis Pharma (Groupe Sanofi-Aventis) and Bayer Pharma. C. Peyraud-Thomas was the recipient of a fellowship from Aventis Pharma (Groupe Sanofi-Aventis) and Bayer Pharma (Multiorganismes grant). S. Cadel-Six was the recipient of a Ph.D. fellowship funded by the Agence Française de Sécurité Sanitaire de l'Environnement et du Travail through an Environnement et Santé grant.

We thank R. Rippka for providing the axenic PCC strains and for valuable advice concerning the cyanobacterial cultures. N. Tandeau de Marsac is thanked for her constant support and for valuable scientific discussions during the course of this work. C. Monard and A. Coquin are both warmly thanked for their technical contributions at the early stage of this work.

REFERENCES

- Aráoz, R., H. O. Nghiêm, R. Rippka, N. Palibroda, N. Tandeau de Marsac, and M. Herdman. 2005. Neurotoxins in axenic oscillatorian cyanobacteria: coexistence of anatoxin-a and homoanatoxin-a determined by ligand-binding assay and GC/MS. *Microbiology* **151**:1263–1273.
- Cadel-Six, S., C. Peyraud-Thomas, L. Briant, N. Tandeau de Marsac, R. Rippka, and A. Méjean. 2007. Different genotypes of anatoxin-producing cyanobacteria coexist in the Tarn River, France. *Appl. Environ. Microbiol.* **73**:7605–7614.
- Carmichael, W. W., D. F. Biggs, and M. A. Peterson. 1979. Pharmacology of anatoxin-a, produced by the freshwater cyanophyte *Anabaena flos-aquae* NRC-44-1. *Toxicon* **17**:229–236.
- Chorus, I., and J. Bartram (ed.). 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. Spon Press, London, United Kingdom.
- Donadio, S., P. Monciardini, and M. Sosio. 2007. Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Nat. Prod. Rep.* **24**:1073–1109.
- Edwards, C., K. Beattie, C. Scrimgeour, and G. Codd. 1992. Identification of anatoxin-a in benthic cyanobacteria (blue-green algae) and in associated dog poisoning at Loch Insh, Scotland. *Toxicon* **30**:1165–1175.
- Gugger, M., S. Lenoir, C. Berger, A. Ledreux, J. C. Druart, J. F. Humbert, C. Guette, and C. Bernard. 2005. First report in a river in France of the benthic cyanobacterium *Phormidium favosum* producing anatoxin-a associated with dog neurotoxicosis. *Toxicon* **45**:919–928.
- Gunn, G. J., A. G. Rafferty, G. C. Rafferty, N. Cockburn, C. Edwards, K. A. Beattie, and G. A. Codd. 1992. Fatal canine neurotoxicosis attributed to blue-green algae (cyanobacteria). *Vet. Rec.* **130**:301–302.
- Hemscheidt, T., J. Rapala, K. Sivonen, and O. M. Skulberg. 1995. Biosynthesis of anatoxin-a in *Anabaena flos-aquae* and homoanatoxin-a in *Oscillatoria formosa*. *J. Chem. Soc. Chem. Commun.* **13**:1361–1362.
- Herdman, M., I. Itean, and R. Rippka. 2005. Catalogue of cyanobacterial strains, 2nd ed. Institut Pasteur, Paris, France.
- Mihali, T. K., R. Kellmann, J. Muenchhoff, K. D. Barrow, and B. A. Neilan. 2008. Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. *Appl. Environ. Microbiol.* **74**:716–722.
- Mlouka, A., K. Comte, A. M. Castets, C. Bouchier, and N. Tandeau de Marsac. 2004. The gas vesicle gene cluster from *Microcystis aeruginosa* and DNA rearrangements that lead to loss of cell buoyancy. *J. Bacteriol.* **186**:2355–2365.
- Moffitt, M. C., and B. A. Neilan. 2001. On the presence of peptide synthetase and polyketide synthase genes in the cyanobacterial genus *Nodularia*. *FEMS Microbiol. Lett.* **196**:207–214.
- Moffitt, M. C., and B. A. Neilan. 2003. Evolutionary affiliations within the superfamily of ketosynthases reflect complex pathway associations. *J. Mol. Evol.* **56**:446–457.
- Pearson, L. A., and B. A. Neilan. 2008. The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. *Curr. Opin. Biotechnol.* **19**:281–288.
- Rippka, R. 1988. Isolation and purification of cyanobacteria. *Methods Enzymol.* **167**:3–27.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Siebert, P. D., A. Chenchik, D. E. Kellogg, K. A. Lukyanov, and S. A. Lukyanov. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* **23**:1087–1088.

19. **Sivonen, K., K. Himberg, R. Luukkainen, S. I. Niemelä, G. K. Poon, and G. A. Codd.** 1989. Preliminary characterization of neurotoxic cyanobacteria blooms and strains from Finland. *Toxic. Assess.* **4**:339–352.
20. **van Apeldoorn, M. E., H. P. van Egmond, G. J. Speijers, and G. J. Bakker.** 2007. Toxins of cyanobacteria. *Mol. Nutr. Food Res.* **51**:7–60.
21. **Wonnacott, S., and T. Gallagher.** 2006. The chemistry and pharmacology of anatoxin-a and related homotropans with respect to nicotinic acetylcholine receptors. *Mar. Drugs* **4**:228–254.
22. **Wood, S. A., A. I. Selwood, A. Rueckert, P. T. Holland, J. R. Milne, K. F. Smith, B. Smits, L. F. Watts, and C. S. Cary.** 2007. First report of homo-anatoxin-a and associated dog neurotoxicosis in New Zealand. *Toxicon* **50**: 292–301.