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Anatoxin-a Synthetase Gene Cluster of the Cyanobacterium *Anabaena* sp. Strain 37 and Molecular Methods To Detect Potential Producers▽†

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Cyanobacterial mass occurrences are common in fresh and brackish waters. They pose a threat to water users due to toxins frequently produced by the cyanobacterial species present. Anatoxin-a and homoanatoxin-a are neurotoxins synthesized by various cyanobacteria, e.g., *Anabaena*, *Oscillatoria*, and *Aphanizomenon*. The biosynthesis of these toxins and the genes involved in anatoxin production were recently described for *Oscillatoria* sp. strain PCC 6506 (A. Méjean et al., J. Am. Chem. Soc. 131:7512-7513, 2009). In this study, we identified the anatoxin synthetase gene cluster (*anaA* to *anaG* and *orf1*; 29 kb) in *Anabaena* sp. strain 37. The gene (81.6% to 89.2%) and amino acid (78.8% to 86.9%) sequences were highly similar to those of *Oscillatoria* sp. PCC 6506, while the organization of the genes differed. Molecular detection methods for potential anatoxin-a and homoanatoxin-a producers of the genera *Anabaena*, *Aphanizomenon*, and *Oscillatoria* were developed by designing primers to recognize the *anaC* gene. *Anabaena* and *Oscillatoria anaC* genes were specifically identified in several cyanobacterial strains by PCR. Restriction fragment length polymorphism (RFLP) analysis of the *anaC* amplicons enabled simultaneous identification of three producer genera: *Anabaena*, *Oscillatoria*, and *Aphanizomenon*. The molecular methods developed in this study revealed the presence of both *Anabaena* and *Oscillatoria* as potential anatoxin producers in Finnish fresh waters and the Baltic Sea; they could be applied for surveys of these neurotoxin producers in other aquatic environments.

Cyanobacteria frequently form mass occurrences (blooms) worldwide. Blooms hinder the use of water for drinking as well as for recreation due to the high risk of exposure to cyanobacterial toxins. Although neurotoxic blooms are less common than hepatotoxic blooms, they are widespread in some countries, especially in North America, Europe, and Australia (48), and have been connected to several incidents of animal poisoning (34, 46).

Cyanobacteria produce a variety of toxins, including the neurotoxic anatoxin-a and its methylene homologue homoanatoxin-a (3, 48). Their toxic effect is due to disruption of the normal signal transmission between neurons and muscles, which can lead to death by respiratory arrest (3, 9). Anatoxin-a was first identified in the 1970s from an *Anabaena flos-aquae* strain isolated from Burton Lake, Canada (14). Since then, these neurotoxins have been detected in various cyanobacteria; among the strains isolated, *Anabaena* and *Oscillatoria* are the most common genera (34).

Not all cyanobacterial strains produce toxins. However, the toxin-producing strains cannot be distinguished from the nontoxin-producing strains by traditional light microscopy, commonly used to monitor water bodies. An alternative for the differentiation of potentially toxic strains from nontoxic strains is to use molecular methods to detect the presence of toxin biosynthetic genes (20, 36, 45). Such methods are already available and could be used for the detection and identification of potential microcystin and nodularin producers present in environmental samples, e.g., blooms (37, 45). The elucidation of the biosynthetic gene clusters for cylindrospermopsin (29, 32, 52) and saxitoxins (24, 33) has enabled the development of molecular detection methods for the producers of these toxins (1, 39).

Recently, biosynthetic genes responsible for anatoxin-a production were reported in a benthic *Oscillatoria* strain, PCC 6506 (7, 30, 31). Subsequently, methods for the detection of the *anaF* genes of the anatoxin-producing *Oscillatoria* (7), *Phormidium* (54), and *Aphanizomenon* (5) strains were designed. Our aim was to identify the anatoxin-a synthetase (*ana*) gene cluster in the genus *Anabaena* by sequencing the entire cluster in *Anabaena* sp. strain 37, a planktonic strain originally isolated from a cyanobacterial bloom in Lake Sääskjärvi that caused cattle deaths in the summer of 1985 (47). Comparison of the *ana* gene clusters of *Anabaena* sp. strain 37 and *Oscillatoria* sp. strain PCC 6506 and the *anaC* gene sequences from additional anatoxin-a-producing strains of the genera *Anabaena*, *Aphanizomenon*, and *Oscillatoria* enabled us to design the primers needed for molecular detection methods. The primers were applied in PCR to detect these three producer genera at the same time (general primers) or to identify either *Anabaena* or *Oscillatoria anaC* gene variants (genus-specific primers). In

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TABLE 1. Primers used in this study to amplify regions of the anatoxin-a synthetase gene (*anaC*)

Primer pair (annealing temp [°C])	Gene	Amplicon length (bp)	Position[a]	Orientation	Primer sequence (5′–3′)	Application
anxC (52)	*anaC*	813	5211	F	TGAGGGAACAAGTGAGTT	Sequencing
			6023	R	ATCATCTCCGATCCCAATCC	
anxgen (52)	*anaC*	861	5247	F	ATGGTCAGAGGTTTTACAAG	General PCR, sequencing
			6107	R	CGACTCTTAATCATGCGATC	
anaC-gen (58)	*anaC*	366	5588	F	TCTGGTATTCAGTCCCCTCTAT	General PCR, sequencing, RFLP
			5953	R	CCCAATAGCCTGTCATCAA	
anaC-anab (60)	*Anabaena anaC*	263	5802	F	GCCCGATATTGAAACAAGT	Genus-specific PCR
			6064	R	CACCCTCTGGAGATTGTTTA	
anaC-osc (60)	*Oscillatoria anaC*	216	5604	F	CTCTATTCTCACAAGTTTGGTCT	Genus-specific PCR
			5819	R	GTTAGTTCAATATCAAGTGGTGGA	

[a] Numbered according to the *Oscillatoria* sp. PCC 6506 anatoxin-a and homoanatoxin-a biosynthetic gene cluster sequence (GenBank accession number FJ477836).

addition, restriction fragment length polymorphism (RFLP) analysis of the general PCR products allowed simultaneous identification of the three producer genera studied. The methods were applied to cyanobacterial strains as well as to environmental DNA samples so as to establish molecular tools for the detection of these potential anatoxin producers.

MATERIALS AND METHODS

Cyanobacterial strains and growth conditions. The cyanobacterial strain *Anabaena* sp. 37 was isolated from Lake Sääskjärvi, Finland, in 1985 (47) and was made axenic (42). All heterocystous strains were grown in Z8 medium (26) without nitrate, while oscillatorian strains were grown in Z8 or BG11 medium (41) (see Table S1 in the supplemental material) at approximately 23°C with continuous illumination of 5 μmol photons s^{-1} m^{-2}. All PCC strains are maintained at the Pasteur Culture Collection of Cyanobacteria, Institut Pasteur; the other strains are maintained at the Helsinki University Cyanobacteria Culture Collection.

DNA extraction. The DNAs of *Anabaena* sp. 37 and *Oscillatoria* sp. strain 193 were isolated according to a method described previously (17). DNAs of other *Anabaena* strains and *Aphanizomenon* strains were extracted with the E.Z.N.A. SP plant DNA kit (Omega Bio-Tek) according to the manufacturer's instructions. Before extraction, the harvested cells (approximately 150 mg) were disrupted with a FastPrep FP120 bead beater (Savant Instruments Inc.) for 30 s at a speed of 5 m s^{-1}. Cells of 10 *Oscillatoria* PCC strains (see Table S1 in the supplemental material) were harvested from 40-ml cultures and were immediately frozen in liquid nitrogen prior to being lyophilized. DNA was extracted from the lyophilized pellets by using NucleoBond AXG columns (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions for bacterial genomic DNA. Environmental DNAs from eight samples collected from Finnish fresh waters and the Baltic Sea in 2004 and 2005 (see Table S1) were extracted in the laboratory of the Finnish Environment Institute with the FastDNA kit (Qbiogene, Inc.). The DNA of the Baltic Sea sample collected in 2009 (see Table S1) was extracted by bead beating and the cetyltrimethylammonium bromide (CTAB) method as described by Koskenniemi et al. (25). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

Sequencing of the anatoxin-a synthetase gene cluster. The ongoing *Anabaena* sp. 37 genome sequencing project was started by use of the 454 method at the Institute for Biomedical Technologies (National Research Council, Milan, Italy). A single-stranded DNA (ssDNA) library and a paired-end library were prepared according to the Roche-454 library preparation manual. Shotgun and paired-end sequencing were performed using the Titanium version of the Roche-454 GS FLX system. From the sequences produced, an initial set of open reading frames (ORF) for anatoxin-a synthetase genes was predicted by Glimmer, version 3.02 (13) (www.cbcb.umd.edu/software/glimmer/), on the basis of similarity with the corresponding genes of *Oscillatoria* sp. PCC 6506 (30). The Needle program of the EMBOSS package (the European Molecular Biology Open Software Suite) (40) was used for comparison of *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506 gene clusters with default parameters. Sequence similarity searches in databases were carried out with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (2). The InterProScan Sequence Search (www.ebi.ac.uk/Tools/InterProScan) and the PKS/NRPS (polyketide synthase/nonribosomal peptide synthetase) Analysis Web-site (http://nrps.igs.umaryland.edu/nrps) were used to predict the protein functions and identify the domain structures.

Sequencing of the *anaC* gene. For sequencing, the *anaC* gene regions of nine cyanobacterial strains (*Anabaena* sp. strains 54 and 86; *Oscillatoria* sp. strains 193, PCC 6407, PCC 9029, PCC 9240, PCC 10601, and PCC 10608; and *Aphanizomenon* sp. strain 3) and the Baltic Sea Helsinki (BSH) sample were amplified with the primer pair anxgen (861 bp) or anxC (813 bp; only for *Anabaena* sp. 54) (Table 1). In addition, a 366-bp *anaC* region of *Oscillatoria* sp. strain PCC 10111 was amplified with anaC-gen primers (Table 1). Amplicons either were sequenced directly with gene-specific primers or were cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. Inserts were sequenced with vector (M13 and T7)- or gene-specific primers on an ABI 310 sequencer (Applied Biosystems). Sequences were checked manually with Chromas, version 2.24 (Technelysium Pty Ltd.) and were combined in contigs with the BioEdit (version 7.0.9.0) sequence alignment editor (19). Sequences were analyzed with Blastn (NCBI) against *ana* sequences of *Anabaena* sp. 37 (GenBank accession number JF803645) and *Oscillatoria* sp. PCC 6506 (accession number FJ477836).

PCR. For the detection of the *anaC* gene in cyanobacterial strains and environmental samples, the general primer pairs, anxgen and anaC-gen (Table 1), were used. These primers were designed on the basis of the *anaC* gene region conserved between *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506 in order to enable the amplification of several producer genera. For the identification of potential anatoxin-producing *Anabaena* and *Oscillatoria* strains, the primers amplifying only *Anabaena anaC* (anaC-anab) or *Oscillatoria anaC* (anaC-osc) (Table 1) were designed based on the alignment of the *anaC* sequences of *Anabaena* sp. 37 (this study) and *Oscillatoria* sp. PCC 6506 (GenBank accession number FJ477836) and the cloned 860-bp *anaC* sequences produced in this study (see above). The limits of detection for the anaC-gen, anaC-anab, and anaC-osc primer pairs were determined by amplifying a 10-fold dilution series (25 ng to 25 fg) of genomic DNA of *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506. To calculate the corresponding genome copy numbers, genome sizes of 5.6 Mb and 6.7 Mb (31) were used for *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506, respectively.

The PCR mixtures included 1× DyNAzyme PCR buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM primers, and 0.5 U DyNAzyme II polymerase (Finnzymes) in a total volume of 20 μl. As a template, 20 to 25 ng of cyanobacterial strain DNA or 10 to 50 ng of environmental sample DNA was used. The PCR program was 94°C for 2 min; 25 to 35 cycles of 94°C for 30 s, 50 to 60°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. The annealing temperature for each primer pair is specified in Table 1. An annealing temperature of 50°C was used in the amplification of environmental DNAs. For cloning, reaction mixtures were cycled 25 times, whereas for specificity and restriction fragment length polymorphism (RFLP) experiments, 30 or 35 cycles were used for strains or environmental samples, respectively. The success of amplification was assessed by running the PCRs in 1.5% agarose gels stained with ethidium bromide.

RFLP of *anaC* amplicons. The enzymes for RFLP analysis were chosen on the basis of *in silico* analysis of the 366-bp *anaC* sequences of *Anabaena*, *Aphanizomenon*, and *Oscillatoria* obtained in this study and the corresponding locus of *Oscillatoria* sp. PCC 6506 (GenBank accession no. FJ477836). The aim was to find enzymes that cut the *anaC* amplicons of *Anabaena*, *Aphanizomenon*, and *Oscillatoria* differentially, enabling identification of the potential producer on the

TABLE 2. Comparison of *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506[a] anatoxin-a synthetase genes (*ana*) and corresponding amino acid sequences

Gene	Length of gene (bp)/protein (aa)		Identity (%)[b]		Predicted protein function[d]
	Anabaena 37	PCC 6506	Gene	Amino acid	
orf1	723/241	717/239	87.6	84.4	Cyclase[c]
anaA	750/250	753/251	89.2	84.1	Type II thioesterase
anaB	1,143/381	1,143/381	85.2	85.6	Proline dehydrogenase
anaC	1,596/532	1,614/538	81.6	78.8	Proline adenylation
anaD	273/91	261/87	79.0	79.6	Acyl carrier
anaE	6,438/2,146	6,438/2,146	86.4	84.9	Modular type I PKS: KS, AT, DH, ER, KR, ACP domains
anaF	5,619/1,873	5,595/1,865	86.4	85.5	Modular type I PKS: KS, AT, DH, KR, ACP domains
anaG	4,896/1,632	4,899/1,633	88.0	86.9	Modular type I PKS: KS, AT, CM, ACP domains

[a] GenBank accession number FJ477836. See reference 30.
[b] Determined by use of the Needle program of the EMBOSS package with default parameters.
[c] A FASTA comparison (35) (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi) showed 26.3% identity and 52.6% similarity to the cyclase domain of *Stigmatella aurantiaca* StiJ (16).
[d] PKS, polyketide synthase; KS, ketosynthase; AT, acyltransferase; DH, dehydrogenase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein; CM, C-methyltransferase.

basis of the fragment lengths. Preliminary selection was made with the REPK program (Restriction Enzyme Picker Online, version 1.3) (12) to exclude all the type IIA enzymes that either did not cut the *anaC* amplicon sequences at all (163 of 191 enzymes) or had the same restriction site for all strains or for strains of different genera (24 of 28 enzymes). Since REPK determines only the terminal fragment lengths, the fragmentation patterns (number of restriction sites, lengths of all fragments) of potentially suitable enzymes ($n = 4$) were further inspected with NEBcutter, version 2.0 (53), in order to identify enzymes with a distinct fragmentation pattern for each anatoxin-a producer genus.

For RFLP analysis, the 366-bp *anaC* amplicons (amplified with anaC-gen [Table 1]) from four parallel PCRs were combined and purified with an Amicon Ultra-0.5 kit (Millipore). The length of the purified amplicon was checked by an agarose gel run (1.5% agarose in 0.5× Tris-acetate-EDTA [TAE]), and the concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). In single-strain reactions, 200 ng of the purified PCR product was digested with 1 μl HhaI or HinfI FastDigest enzyme (Fermentas) in 0.67× FastDigest Green Buffer (Fermentas) in a total volume of 30 μl. In digestions of cyanobacterial strain mixtures, the total amount of amplified DNA was 200 ng for two-, 300 ng for three-, and 400 ng for four-strain mixtures. For environmental sample PCRs, 200 to 600 ng was used in the RFLP analysis. Reaction mixtures were incubated at 37°C for 15 min and were inactivated at 65°C for 20 min. The restriction fragments were separated in ethidium bromide-stained 3% MetaPhor (FMC BioProducts) or 3% TopVision (Fermentas) agarose gels in 1× Tris-borate-EDTA (TBE) buffer. Gels were documented with a Kodak DC290 camera and the Kodak 1D imaging program, version 3.5.0. The sizes of fragments were estimated by comparison to fragments of the size marker (O'GeneRuler low-range DNA ladder; Fermentas).

Toxin analysis. Anatoxins were extracted from cyanobacterial strains by mixing 1,000 μl of the late-logarithmic-phase culture with 2 μl 50% formic acid (final concentration, 0.1% formic acid). Cells were disrupted with glass beads in a FastPrep FP120 bead beater (Savant Instruments Inc.) for 10 s at 6.5 m s^{-1}. After centrifugation for 5 min at 20,000 × *g*, the supernatant was diluted 1:10 with acetonitrile and was analyzed by high-performance liquid chromatography (HPLC) coupled with a diode array detector (Agilent 1100) and mass spectrophotometer (MS; Agilent XCT Plus ion trap). The injection volume was 10 μl. The samples were separated with a Cogent Diamond Hydride column (particle size, 4 μm; pore size, 100 Å; length, 150 mm; inner diameter, 2.0 mm; MicroSolv Technology Corporation, Eatontown, NJ). For liquid chromatography (LC), the mobile phase consisted of 1% formic acid ammonium salt (solvent A) and acetonitrile (solvent B). The linear gradient was as follows: 90% solvent B at 0 min and 60% solvent B at 20 min. A flow rate of 0.15 ml min^{-1} was used with a column temperature of 30°C. Electrospray ionization was performed in positive-ion mode. The nebulizer gas (N_2) pressure was 30 lb/in^2, and the drying gas flow rate and temperature were 9 liters min^{-1} and 350°C, respectively. The capillary voltage was 1,300 V, and the trap drive value was 30. Mass spectra were recorded at a scan range of 40 to 500 *m/z*. Tandem MS (MS^2) spectra were recorded in an auto-MS mode with the following parameters: 2 precursor ions, an isolation width of 4 *m/z*, and a fragmentation amplitude value of 0.65 V. Commercial anatoxin-a (Enzo Life Sciences International, Inc.) was used as a standard. The identification of anatoxin-a (166 *m/z*) and homoanatoxin-a (180 *m/z*) in the samples was based on the masses of the precursor ions, the retention time (18.6 min for anatoxin-a and 16.7 min for homoanatoxin-a), and the MS^2 fragmentation pattern.

The presence of anatoxin-a, homoanatoxin-a, and their degradation products in environmental samples was analyzed in the laboratory of the Finnish Environment Institute by a fluorimetric liquid chromatographic method as described by James et al. (21).

Nucleotide sequence accession numbers. The 29.5-kb sequence of *Anabaena* sp. 37 determined in this study, including the *ana* gene cluster, has been submitted to GenBank under accession number JF803645. The sequences of the *anaC* gene (366 to 897 bp) determined here have been submitted to GenBank under accession numbers JF803646 to JF803657.

RESULTS

Anatoxin-a synthetase gene cluster of *Anabaena* sp. 37. The anatoxin-a biosynthesis genes (*ana*) of *Anabaena* sp. 37 (GenBank accession number JF803645) were identified on the basis of comparison to the *ana* genes of *Oscillatoria* sp. PCC 6506 (GenBank accession number FJ477836) (30). The *ana* genes and proteins of *Anabaena* sp. 37 were very similar, with ≥81.6% and 78.8% identity, respectively, to those of *Oscillatoria* sp. PCC 6506 (Table 2). Accordingly, the protein functions predicted and the functional domains identified in the polyketide synthases (Table 2) were the same as those of *Oscillatoria* sp. PCC 6506. The main difference between the two *ana* gene clusters was seen in the organization of the genes (Fig. 1). In *Anabaena* sp. 37, the biosynthetic genes were found in two clusters spanning approximately 29 kb. The *anaB* to *anaG* genes formed one cluster of 20.3 kb, while *anaA* and the putative cyclase gene, *orf1* (1.7 kb), were separated from the main cluster by a 6.9-kb section of DNA and were transcribed in the opposite direction (Fig. 1). The biosynthetic genes were putatively organized in four or five operons. The −10 (Pribnow box) and −35 sequences were recognized upstream of the gene regions *anaB* to *anaD*, *anaEF*, *orf1*, and *anaA*, but not before *anaG*. Although RNA polymerase recognition sequences were not identified, a long gap (288 bp) (Fig. 1) between *anaF* and *anaG* suggested that *anaG* forms its own operon. The 6.9-kb insert contained genes coding for four hypothetical proteins, an N-acetyltransferase, a Rieske domain-containing protein, an oxidoreductase, and a putative multidrug exporter, which

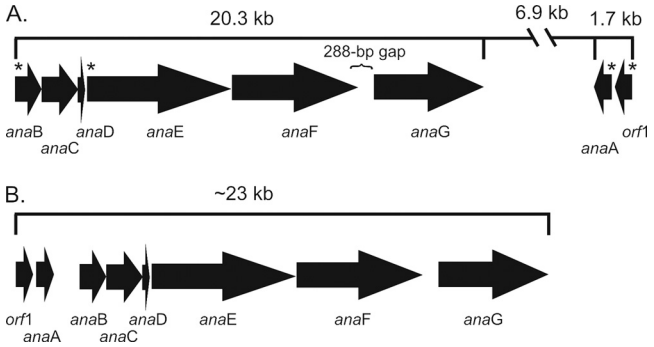

FIG. 1. Anatoxin-a biosynthetic gene (*ana*) clusters of *Anabaena* sp. strain 37 and *Oscillatoria* sp. strain PCC 6506. (A) *Anabaena* sp. strain 37. Asterisks indicate genes upstream of which RNA polymerase recognition sequences were identified. (B) *Oscillatoria* sp. strain PCC 6506. Adapted from reference 30.

were absent from the vicinity of the anatoxin-a synthetase gene cluster of *Oscillatoria* sp. PCC 6506.

Detection of the *anaC* genes in cyanobacterial strains. Molecular methods for the detection and identification of potential anatoxin producers were designed on the basis of the *anaC* gene. The *anaC* sequences of *Anabaena* strains 54 and 86 were either identical or highly similar (99%) to the corresponding *anaC* region of *Anabaena* sp. 37. The sequences of *Oscillatoria* strains were divided into two main groups: the *anaC* sequences (861 bp) of strains PCC 6407, PCC 9029, and PCC 10608 were completely identical (group OscI), while the *anaC* sequences (772 to 861 bp) of strains 193, PCC 9240, and PCC 10601 had 92% identity (group OscII), to the *anaC* sequence of *Oscillatoria* sp. PCC 6506. The shorter *anaC* sequence (366 bp) obtained from *Oscillatoria* sp. PCC 10111 had 91% identity to group OscI and 95% identity to group OscII sequences. The *anaC* sequence of *Aphanizomenon* sp. 3 showed 88% identity to both *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506 sequences.

In PCR using the general primer pairs anxgen and anaC-gen, the *anaC* sequences were detected in 14/17 and 15/17 cyanobacterial strains previously identified as anatoxin producers but not in the hepatotoxic *Anabaena* sp. strain 90 (Table 3). With the genus-specific primer pair anaC-anab or anaC-osc (Table 1), the *anaC* gene was amplified from anatoxin-a- or homoanatoxin-a-producing *Anabaena* or *Oscillatoria* strains, respectively, while no amplification from *Aphanizomenon* sp. 3 or *Anabaena* sp. 90 DNA was detected (Table 3). The limit of detection with the genus-specific primers was 250 fg of genomic DNA of *Anabaena* sp. 37 or *Oscillatoria* sp. PCC 6506, corresponding to 40 or 34 genome copies, respectively. For the anaC-gen primer pair, the limit of detection was 250 pg of DNA, corresponding to 40×10^3 or 34×10^3 genome copies.

Of the cyanobacterial strains in which *anaC* was detected, *Anabaena* sp. strains 14, 37, 54, and 130, as well as *Oscillatoria* sp. 193, produced anatoxin-a according to LC-MS results (Table 3; see also Fig. S2 and S3 in the supplemental material). The *anaC* gene was also present in *Anabaena* sp. strain 86 and *Aphanizomenon* sp. 3, in which toxin production was no longer detectable. All the *Oscillatoria* PCC strains studied were previously identified as homoanatoxin-a and/or anatoxin-a producers (4, 7). In this study, homoanatoxin-a production was confirmed for strains PCC 6506, PCC 9029, and PCC 10111 but not for strains PCC 10608 and PCC 10702 (Table 3; see also Fig. S2 and S3). However, *anaC* was detected in all *Oscillatoria* strains except for strain PCC 10702 (Table 3).

The RFLP method was developed for simultaneous detection (amplification with anaC-gen primers [Table 1]) and identification of different anatoxin producer genera present. Identification is based on the RFLP patterns produced after amplicons are digested with HhaI or HinfI. When the cyanobacterial strains were subjected to RFLP analysis, the fragmentation patterns observed were similar to those predicted by *in silico* analysis of the *anaC* sequences (Table 4; Fig. 2). In addition, strains *Anabaena* sp. 130, *Oscillatoria* sp. PCC 6412, and *Oscillatoria* sp. PCC 9107, the *anaC* gene of which was not sequenced prior to RFLP analysis, produced fragmentation patterns identical to those of groups Ana, OscI, and OscII, respectively (Table 4). Due to the failure of amplification with anaC-gen primers, the method was not applied to *Oscillatoria* sp. PCC 10608. In accordance with the sequencing results, the *Oscillatoria* strains produced differing fragmentation patterns when digested with HhaI (groups OscI and OscII [Table 4]). *Oscillatoria* strain PCC 10111 was digested with HinfI similarly to other *Oscillatoria* strains, but not at all with HhaI. Sequencing of the anaC-gen amplicon (GenBank accession number JF803654) of this strain revealed a silent nucleotide substitution that abolished the only HhaI recognition site (G<u>C</u>GC → G<u>T</u>GC). All the characteristic fragments could also be recognized when the anaC-gen amplicons of several strains were

TABLE 3. Anatoxin-a and homoanatoxin-a production as determined by LC-MS and detection of the *anaC* gene by PCR in the cyanobacterial strains studied

Strain	Toxin[a]	PCR[b]			
		General primers		Genus-specific primers	
		anxgen	anaC-gen	anaC-anab	anaC-osc
Anabaena sp.					
14	ana	+	+	+	−
37	ana	+	+	+	−
54	ana	+	+	+	−
86	—[c]	+	+	+	−
130	ana	+	+	+	−
Oscillatoria sp.					
193	ana	+	+	−	+
PCC 6407	NA[c]	+	+	−	+
PCC 6412	NA[c]	+	+	−	+
PCC 6506	hana	+	+	−	+
PCC 9029	hana	+	+	−	+
PCC 9107	NA[c]	−	+	−	+
PCC 9240	NA[c]	+	+	−	+
PCC 10111	hana	−	+	−	+
PCC 10601	NA[c]	+	+	−	+
PCC 10608	—[c]	+[d]	−	−	−
PCC 10702	—[c]	−	−	−	−
Aphanizomenon sp. 3	—[c]	+	+	−	−
Anabaena sp. 90	mc	−	−	−	−

[a] ana, anatoxin-a; hana, homoanatoxin-a; —, no toxin production detected; NA, not analyzed; mc, microcystin-producing strain (49).

[b] Primer pair anaC-anab was specific for *Anabaena* strains, and primer pair anaC-osc was specific for *Oscillatoria* strains. +, amplification product detected by PCR; −, no amplification by PCR.

[c] Anatoxin-a or homoanatoxin-a was detected previously by Sivonen et al. (47), Aráoz et al. (4), or Cadel-Six et al. (7).

[d] The annealing temperature was 50°C instead of 52°C.

TABLE 4. Predicted and observed fragmentation patterns of the amplified *anaC* (366-bp) gene region upon digestion with the HhaI or HinfI restriction enzyme

Group	Predicted fragment length(s) (bp)[a]		Strain(s) in which predicted fragments were observed[b]
	HhaI	HinfI	
Ana	337, 29	205, 161	*Anabaena* sp. 14, 37, 54, 86, 130
OscI	261, 76, 29	234, 132	*Oscillatoria* sp. PCC 6407, PCC 6412, PCC 6506, PCC 9029
OscII	261, 105	234, 132	*Oscillatoria* sp. 193, PCC 9107, PCC 9240, PCC 10601
OscIIb	366	234, 132	*Oscillatoria* sp. PCC 10111
Aph	124, 213, 29	366	*Aphanizomenon* sp. 3

[a] Predicted by *in silico* digestion of the *anaC* sequences. The order in which the fragments are presented corresponds to the order of restriction cut sites, starting from the 5′ ends of the amplicons.

[b] Fragment sizes were estimated by comparison to the size marker.

mixed before digestion (see Fig. S1 in the supplemental material), suggesting that the RFLP method could be used to study environmental samples, where the population of potential anatoxin producers could be composed of a mixture of strains.

Identification of potential anatoxin producers in environmental samples. The molecular methods developed were also tested with nine environmental DNA samples from Finnish fresh waters or the Baltic Sea (see Table S1 in the supplemental material). In eight of the samples, anatoxin-a, homoanatoxin-a, or their degradation products were detected (Table 5). The neurotoxin content of the Baltic Sea Helsinki sample could not be determined, since it was originally collected for a study on the presence of microcystins (unpublished results), and there was no sample left for anatoxin-a analysis. The *anaC* gene was detected in all the samples by PCR with the anaC-gen primers (Table 5). However, the amplification products were often weak, even when the annealing temperature was lowered to 50°C. This could be due to inhibiting substances present in the DNA extracts, as indicated by suboptimal A_{260}/A_{280} and A_{260}/A_{230} ratios. With the anxgen primers, which amplify a longer region, the presence of the *anaC* gene was confirmed in two samples: the Lake Kymijärvi and Baltic Sea Helsinki samples (Table 5). Genus-specific primers identified *Anabaena* as the potential producer in five samples and *Oscillatoria* in six samples. Both genera were detected in three of the samples: the River Ruskonjoki, Lake Ranuanjärvi, and Baltic Sea Helsinki samples (Table 5).

RFLP analysis could be performed with three environmental samples that showed strong enough amplification products: the Lake Pyhäjärvi, Lake Kymijärvi, and Baltic Sea Helsinki samples (Table 5; Fig. 2). The fragmentation patterns of the Lake Pyhäjärvi and Lake Kymijärvi samples were similar to those of *Oscillatoria* strains belonging to the OscI and OscII groups, respectively (Fig. 2). However, in the Lake Pyhäjärvi sample, the presence of *Anabaena anaC* instead of *Oscillatoria anaC* was indicated by PCR (Table 5). In this sample, all the PCR amplicons and RFLP fragments detected were faint, and it is possible that some amplification products did not reach the level of detection at all. In the Baltic Sea Helsinki sample, both *Anabaena* and *Oscillatoria* OscII fragmentation patterns were clearly recognized (Fig. 2), in accordance with the PCR results (Table 5). The presence of both producers was also verified by cloning of the amplified *anaC* region. Of the 16 clones checked, 15 clones were identified as *Oscillatoria anaC* and one as *Anabaena anaC* by genus-specific PCR. In addition, the sequences of two clones showed high similarity (99 to 100%) either to the *anaC* sequence of *Oscillatoria* sp. PCC 9240 (clone cBSH.1; GenBank accession number JF803656) or *Anabaena* sp. 37 (clone cBSH.2; GenBank accession number JF803657). Accordingly, fragmentation patterns similar to that of *Oscillatoria* group OscII (cBSH.1) or *Anabaena* (cBSH.2) were detected in the RFLP analysis (Fig. 2).

DISCUSSION

We identified anatoxin-a synthetase (*ana*) genes required for anatoxin-a production in the genomic sequence of *Anabaena* sp. 37. The genes resembled those identified in *Oscillatoria* sp. strain PCC 6506 (30); the *ana* gene content was the same, with high sequence identity. In addition, the protein functions and

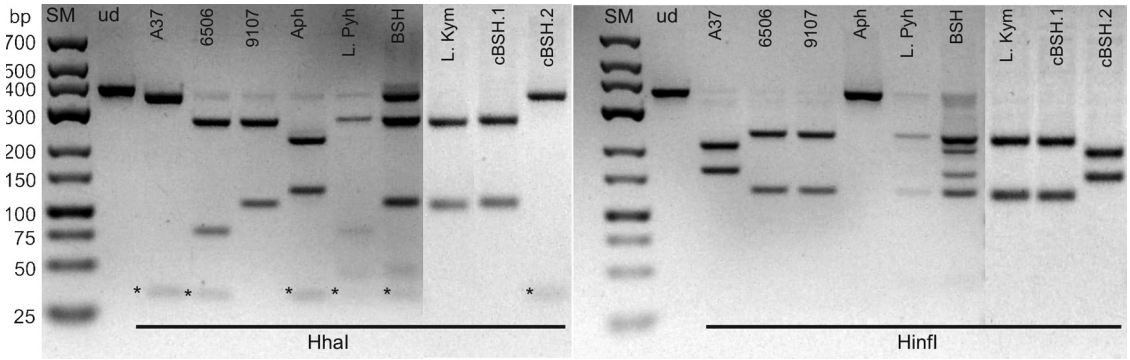

FIG. 2. RFLP analysis of *anaC* amplicons (366 bp) of cyanobacterial strains and environmental samples with the HhaI and HinfI restriction enzymes. The amplicons are indicated above the lanes according to the strain/sample used for amplification: *Anabaena* sp. 37 (A37), *Oscillatoria* sp. PCC 6506 (6506), *Oscillatoria* sp. PCC 9107 (9107), and *Aphanizomenon* sp. 3 (Aph). Lake Pyhäjärvi (L. Pyh), Lake Kymijärvi (L. Kym), and Baltic Sea Helsinki (BSH) samples were amplified directly from environmental DNA, while cBSH.1 and cBSH.2 indicate the cloned *anaC* fragments of the BSH sample. ud, digestion reactions without the enzyme; SM, size marker. Asterisks indicate the 29-bp fragment detected in HhaI digestion. The panel for each enzyme is assembled from two separate gel images.

TABLE 5. Detection of the *anaC* gene in environmental samples by PCR and RFLP analysis

Sample[a]	Toxin[b]	PCR[c]				RFLP pattern[d]
		Genus-specific primers		General primers		
		anaC-anab	anaC-osc	anxgen	anaC-gen	
Lake Pyhäjärvi	epohana	(+)	−	−	(+)	OscI
Lake Kymijärvi	hana	−	+	+	+	OscII
Lake Kirkkojärvi	dhana	−	+	−	(+)	NA
Lake Kyrösjärvi	ana	−	+	−	(+)	NA
Lake Ranuanjärvi	dhana, ana	+	+	−	(+)	NA
River Ruskonjoki	ana	(+)	(+)	−	(+)	NA
Baltic Sea, Halikko	epohana	(+)	−	−	(+)	NA
Baltic Sea, Luvia	dhana	−	−	−	(+)	NA
Baltic Sea, Helsinki	NA	+	+	+	+	Ana, OscII

[a] All environmental samples were taken from locations in Finland.
[b] ana, anatoxin-a; hana, homoanatoxin-a; epohana, epoxyhomoanatoxin-a; dhana, dihydroanatoxin-a; NA, not analyzed.
[c] +, amplification product detected; (+), weak amplification; −, no amplification.
[d] OscI, HhaI RFLP pattern identical to that of *Oscillatoria* sp. PCC 6506; OscII, HhaI RFLP pattern identical to that of *Oscillatoria* sp. PCC 9240; Ana, RFLP pattern identical to that of *Anabaena* strains (see Table 4 for details).

domains predicted agreed with those of *Oscillatoria* sp. PCC 6506 proteins, further confirming the identification of the sequences as anatoxin-a synthetase genes. The most prominent difference between the gene clusters was the organization of the genes. In *Anabaena* sp. 37, *orf1* and *anaA* are located on the opposite strand downstream from the other biosynthetic genes (*anaB* to *anaG*), while in *Oscillatoria* sp. PCC 6506, *orf1* and *anaA* lie upstream and are transcribed in the same direction as the other genes. Similar differences in gene order have been recognized in biosynthetic gene clusters of other cyanobacterial toxins: microcystins (37), cylindrospermopsin (29, 52), and saxitoxins (33). The DNA (81.6 to 89.2%) and encoded amino acid (78.8 to 86.9%) sequence similarities between the *Anabaena* sp. 37 and *Oscillatoria* sp. PCC 6506 clusters were at the same level as those between the biosynthetic gene clusters of different microcystin (43) and saxitoxin producers (33).

The molecular methods developed in this study to detect potential anatoxin-a or homoanatoxin-a producers were based on recognition of the *anaC* gene, which encodes AnaC protein, thought to be responsible for the first step in anatoxin-a synthesis, proline adenylation (30). Sequencing of the 860-bp *anaC* gene region revealed unexpectedly that *Oscillatoria* strains were divided into two groups—one with sequences identical to that of *Oscillatoria* sp. PCC 6506 and the other with approximately 92% sequence identity to *Oscillatoria* sp. PCC 6506—while variation between *Anabaena* strains was almost nonexistent. Sequence variation, even when it does not affect the protein function, challenges the design and use of molecular detection methods, which rely on the tight connection between a certain sequence variant and the identity of the producer organism. In our study, the genus-specific PCR assays with both *Anabaena anaC*- and *Oscillatoria anaC*-specific primers performed highly specifically and detected the target gene only in *Anabaena* or *Oscillatoria* strains, respectively, except for *Oscillatoria* strains PCC 10608 and PCC 10702. These strains were previously reported to produce homoanatoxin-a (8), but toxin production could not be detected in this study. The lack of amplification with any or most of the *anaC*-targeted or *anaF*-targeted (data not shown) primers suggests that the strains have undergone extensive genetic rearrangements that have caused deletion of some or all of the biosynthetic genes. Deletions and insertions in the microcystin (10, 11, 15, 44) and cylindrospermopsin (39) biosynthetic gene clusters have been determined to cause a lack of toxin production. Smaller changes, e.g., point mutations in gene or regulatory regions (10, 22, 27), have been suspected to cause a lack of toxin production in cases where all the biosynthetic genes were shown to be intact. This could be the case with *Anabaena* sp. strain 86 and *Aphanizomenon* sp. strain 3, which had both the *anaC* and *anaF* (data not shown) genes but produced no anatoxin-a according to LC-MS results. These strains have been kept in culture continuously since 1986 and were shown to produce anatoxin-a at that time (38, 47). It is hypothesized that at some point during the 25 years of culture, they underwent spontaneous mutations inactivating the gene clusters. However, a more detailed investigation of the gene clusters or their remnants is needed in order to elucidate what has happened in the *ana* gene clusters of these strains.

The molecular methods developed were also applied to environmental DNA samples. The *anaC* gene was detected in all nine samples. In addition, the potential anatoxin-a producer was identified as *Anabaena* or *Oscillatoria* in eight samples. Surprisingly, the presence of potentially neurotoxic *Anabaena* and *Oscillatoria* was also indicated in the coastal Baltic Sea samples, where neurotoxic cyanobacteria have not been reported previously. In Finnish lakes, anatoxin-a-producing *Anabaena* strains have been detected frequently and have caused cattle deaths (28, 47, 50). In addition, anatoxin-a-producing *Anabaena* strains have commonly been encountered in fresh waters in the United States and Canada (6, 14, 23, 51). While the first anatoxin-a-producing *Oscillatoria* strain (strain 193) was isolated in Lake Hormajärvi, Finland, in 1986 (47), anatoxin-a- and homoanatoxin-a-producing benthic *Oscillatoria* and *Phormidium* strains have been encountered more frequently in Western Europe, e.g., in France, where they have caused dog deaths (8, 18). The results of this study suggest that anatoxin-a-producing *Oscillatoria* strains could be more widespread than previously suspected, in Finland as well.

In this study, we identified and sequenced the anatoxin-a synthetase gene cluster of a planktonic cyanobacterial strain, *Anabaena* sp. 37. The genes were closely related to anatoxin-a

synthetase genes previously recognized in *Oscillatoria* sp. PCC 6506, although the organization of the gene clusters differed. The *Anabaena* gene cluster sequence contributes to knowledge of the biosynthesis of this important neurotoxin and opens possibilities for future research avenues, e.g., to reveal the evolutionary history of anatoxin-a production. This sequence and the additional sequences of the *anaC* genes of several strains of the cyanobacteria *Anabaena*, *Oscillatoria*, and *Aphanizomenon* were used to design methods for the detection (PCR with general primers) and identification (PCR with genus-specific primers, RFLP) of the *anaC* gene, and thus of potential anatoxin-a and homoanatoxin-a producers, in environmental samples. The molecular tools developed here can be used to monitor the potential anatoxin producers of the genera *Anabaena*, *Aphanizomenon*, and *Oscillatoria* in ecosystems and can help to rapidly estimate the risks of their occurrence for water users.

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