APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1993, p. 2204–2209
0099-2240/93/072204-06$02.00/0

Vol. 59, No. 7

Isolation and Identification of Eight Microcystins from Thirteen *Oscillatoria agardhii* Strains and Structure of a New Microcystin

R. LUUKKAINEN,[1] K. SIVONEN,[1]* M. NAMIKOSHI,[2] M. FÄRDIG,[1] K. L. RINEHART,[2] AND S. I. NIEMELÄ[1]

Department of Applied Chemistry and Microbiology, P.O. Box 27, SF-00014 University of Helsinki, Finland,[1] and Department of Chemistry, University of Illinois, Urbana, Illinois 61801[2]

Received 8 March 1993/Accepted 29 April 1993

Microcystins (cyclic heptapeptide hepatotoxins), isolated from 13 freshwater *Oscillatoria agardhii* strains from eight different Finnish lakes by high-performance liquid chromatography, were characterized by amino acid analysis, fast atom bombardment mass spectrometry (FABMS), and tandem FABMS (FABMS/collisionary-induced dissociation/MS). All strains produced two to five different microcystins. In total, eight different compounds, of which five were known microcystins, were isolated. The known compounds identified were [D-Asp^3]MCYST (microcystin)-LR, [Dha^7]MCYST-LR, [D-Asp^3]MCYST-RR, [Dha^7]MCYST-RR, and [D-Asp^3,Dha^7]MCYST-RR. This is the first time that isolation of these toxins from *Oscillatoria* spp., with the exception of [D-Asp^3]MCYST-RR, has been reported. Three of the strains produced a new microcystin, and the structure was assigned as [D-Asp^3,Mser^7]MCYST-RR. The structures of two new microcystins, produced as minor components by one *Oscillatoria* strain, could not be determined because of the small amounts isolated from the cells. Four strains produced [Dha^7]MCYST-RR as the main toxin, but [D-Asp^3]MCYST-RR was clearly the most abundant and most frequently occurring toxin among these isolates of *O. agardhii*.

Mass occurrences of cyanobacteria, so-called water blooms, commonly occur in eutrophic fresh and brackish waters. Many of these water blooms are toxic, causing poisonings of animals and a health risk to human beings (1, 2, 6, 7, 9, 33, 34, 42, 44).

Two main types of cyanobacterial toxins, peptide hepatotoxins and alkaloid neurotoxins, have been found (6, 7, 9). Hepatotoxic blooms are found worldwide (6, 7, 9). Cyclic heptapeptide hepatotoxins, named microcystins, have been previously isolated and characterized from freshwater coccoid *Microcystis* (3, 4, 6, 18, 25, 28, 41, 46) and filamentous *Anabaena* (15, 18, 26, 27, 39, 43), *Nostoc* (24, 37, 40) and *Oscillatoria* (18, 23) cyanobacteria. *Nodularia spumigena* exists only in brackish waters, and it has been previously found to produce a hepatotoxin, nodularin, which is a cyclic pentapeptide closely related to the microcystins (35, 38).

The general structure of the microcystins is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (3, 8, 35). The two acidic amino acids, D-MeAsp and D-Glu, are connected by an isolinkage.

About 40 variants of microcystins have been reported to date (25–28, 37–41, 43). The L amino acid X has been most commonly found to be leucine (L), arginine (R), or tyrosine (Y) (8), but alanine (A) (25), homotyrosine (Hty) (15, 26), phenylalanine (F) (25), homophenylalanine (Hph) (26), methionine *S*-oxide [M(O)] (25), and tryptophan (W) (25) variants of microcystins have also been detected. Correspondingly, Z is arginine (R) or alanine (A) (8), but it might be also aminoisobutyric acid (Aba) (13), homoarginine (Har) (24, 37, 40), or methionine *S*-oxide [M(O)] (4). Common variations also include demethylation of D-MeAsp (i.e., D-Asp) and/or Mdha (i.e., dehydroalanine [Dha]) (8, 15, 16, 18, 23, 25, 39, 41). L-Serine has been found in place of Mdha (26, 27), and D-serine has been found in lieu of D-Ala (40). Mdha has been replaced occasionally by *N*-methylserine (25, 40). α-Monoester variants of D-Glu have been reported previously (25, 43). The unusual amino acid Adda seems to be important for the toxicity of these compounds (10, 14). An acetoxyl group instead of the methoxyl group at the C-9 position of Adda (ADMAdda) (24, 37, 40) and a free hydroxyl group at the same position (DMAdda) (25, 40) have been previously reported to retain the hepatotoxicity, but variants which are stereoisomers at the Δ^6 (double bond at C-6) of the Adda unit are nontoxic (14).

Microcystins and nodularin have recently been reported to be inhibitors of protein phosphatases 1 and 2A (21, 22, 48) and potential tumor promoters (11, 29).

The first report of the hepatotoxicity of *Oscillatoria agardhii* dates from 1981 in Norway (31). To date, two toxins ([D-Asp^3]MCYST-RR and [D-Asp^3,Dha^7]MCYST-LR) have been characterized from a Norwegian *O. agardhii* strain (23) and from two *O. agardhii* bloom samples (6, 18).

We have studied the variation of microcystin structures among different species and strains of cyanobacteria (37, 39, 40). In the present study, we isolated and characterized eight different microcystins from 13 Finnish *O. agardhii* strains and determined the structure of one new toxin.

MATERIALS AND METHODS

Organisms. The 13 *O. agardhii* strains used in this study were isolated from water blooms collected from eight different lakes in Finland. Some of the strains came from the same lake but were collected at different times (Table 1). The strains originated from bloom samples which were either hepatotoxic, neurotoxic, or nontoxic as determined by the

* Corresponding author.

TABLE 1. *O. agardhii* strains used in this study

O. agardhii strain (pigment)	Isolation date (mo/day/yr or yr)	Lake from which strain was isolated	Toxicity of bloom[a]	Amt (g) of cells for extraction
18 (red)	8/22/85	Långsjön	+, H	1.0
49	10/23/85	Valkjärvi	−	12.0
97	7/29/86	Maarianallas	+, H	1.0
195	7/7/87	Haukkajärvi	+, N	21.0
209	8/4/87	Haukkajärvi	+, N	0.3
212	8/11/87	Köyliönjärvi	+, H	0.7
213	8/11/87	Haukkajärvi	+, N	1.0
214	8/11/87	Östra Kyrksundet	+, N	19.0
223	8/26/87	Kotojärvi	+, H	1.0
226	9/8/87	Haukkajärvi	+, H	0.3
CYA 126	1984	Långsjön		1.0
CYA 127	1984	Vesijärvi		1.0
CYA 128 (red)	1984	Vesijärvi		1.0

[a] +, toxic; −, nontoxic; H, hepatotoxic; N, neurotoxic.

intraperitoneal mouse bioassay (42). Three strains (CYA 126, CYA 127, and CYA 128) were gifts from Olav M. Skulberg (Norwegian Institute for Water Research, Oslo, Norway) (30). All strains were isolated and cultured in Z8 medium at ~22°C with continuous illumination of about 50 microeinsteins m^{-2} s^{-1} as detailed earlier (42). Cells were harvested after 12 to 14 days of incubation and lyophilized prior to toxicity testing and toxin isolation.

Isolation of toxins. Toxins were extracted twice (after 3 h and overnight) from lyophilized *Oscillatoria* cells (amounts of cells used are shown in Table 1) with *n*-butanol–methanol–water (1:4:15) (vol/vol) (strains 49, 195, and 214) or water (the rest of the strains). Supernatants were combined, organic solvents were evaporated under air flow (strains 49, 195, and 214), and toxins were applied to a preconditioned C_{18} silica gel column or cartridges (Bond Elut; Analytichem, Harbor City, Calif.) depending on the original amount of cells used in extraction. The toxins were eluted from the column (or cartridges) with 80 to 100% methanol and then evaporated to dryness. The toxic fractions were then separated by high-performance liquid chromatography (HPLC) with either a Varian Vista model 5560 solvent delivery system with a Varian model 220 UV detector or a Waters Delta Prep 3000 solvent delivery system with a Waters model 484 UV detector plus a semipreparative C_{18} silica gel column (19 by 150 mm, μBondapak; Waters Associates, Milford, Mass.). The mobile phase, acetonitrile–10 mM ammonium acetate (15:85 for strain 49, 18:82 for strains 195 and 214, and 26:74 for the rest of the strains), was used at a flow rate of 4 ml/min, and the toxins were detected by UV at 238 nm (absorption maximum of the microcystins). The toxic fractions of strains 49, 195, and 214 were further purified with the same HPLC system but first by using a linear gradient of 15 to 35% acetonitrile (25 or 35 min) at a flow rate of 3 ml/min and then under isocratic conditions with 25 or 35% acetonitrile with 17 mM phosphoric acid (pH 3 to 3.5) at a flow rate of 4 ml/min. The final purification step for the fractions obtained from all of the strains was accomplished by HPLC or thin-layer chromatography (TLC). A Beckman model 114M solvent delivery module equipped with a Beckman model 153 UV detector (254 nm) was used. Isocratic reversed-phase conditions were used with a Nucleosil 7 C_{18} column (10 by 250 mm; Cobert Associates, St. Louis, Mo.) with methanol–0.7% sodium sulfate (60:40) or acetonitrile–0.1% ammonium acetate (27:73) as the mobile phase at 2 ml/min. Few fractions were purified by semipreparative TLC with precoated silica gel plates (Kieselgel 60 F_{254}, 0.25 mm thick; EM Science, Gibbstown, N.J.) and running conditions as detailed below. The purity of the toxins was monitored, and toxins were identified in the isolated fractions by their UV spectra detected with a Hewlett-Packard 1090 M HPLC system and a Hewlett-Packard photodiode array detector. An internal-surface reverse-phase column (4.6 by 150 mm, Regis Pinkerton; Regis Chemical Co., Morton Grove, Ill.) and a mobile phase of CH_3CN–100 mM phosphate buffer (pH 6.8, 15:85) with a flow rate of 1 ml/min were used. The purified toxins were desalted and stored at −20°C prior to amino acid analysis and fast atom bombardment (FAB) mass spectrometry (MS).

HPLC analysis of toxins. Dried cells (50 mg) were extracted with 5% acetic acid (50 ml) by being sonicated for 30 min in a bath sonicator. The cells were filtered with a glass fiber filter (GF/C; Whatman International, Ltd., Maidstone, England) and washed with another 50 ml of extraction solution. Toxins were adsorbed to a preconditioned C_{18} cartridge (3 ml; Analytichem), and the cartridge was washed first with water (6 ml) and then with 20% methanol (6 ml). Toxins were eluted with methanol (6 ml), evaporated to dryness, dissolved in 10% methanol (500 μl), and filtered with an HPLC syringe filter (Acrodisc LC13 PVDF; pore size, 0.22 μm; Gelman Sciences, Inc., Ann Arbor, Mich.) prior to HPLC analysis. HPLC was run on a Hewlett-Packard 1090 M model solvent delivery system equipped with a Hewlett-Packard UV/Vis diode array detector (200 to 600 nm). The column used was a reversed-phase silica gel column (μBondapak C_{18}, 3.9 by 150 mm; Waters Associates), and conditions were as follows: mobile phase, acetonitrile–10 mM ammonium acetate (26:74); flow rate, 1.0 ml/min; and detection at 238 nm with a 4-nm bandwidth. The amounts of toxins were determined by using purified [D-Asp^3]MCYST-RR as a standard and extrapolating peak areas to a standard curve. The quantitation of each sample was made in duplicate.

TLC. TLC was performed on precoated silica gel plates (Kieselgel 60 F_{254}; thickness, 0.25 mm). Adsorbed spots were detected by UV light at 254 nm and by spraying phosphomolybdic acid (10% in ethanol) and by heating. The following solvent mixtures were used: chloroform-methanol-water, 26:15:3; ethyl acetate–2-propanol–water, 8:4:3; ethyl acetate–2-propanol–water, 4:3:2; and 1-butanol–acetic acid–water, 4:1:1.

MS. Mass spectra were run on either a ZAB-SE or a 70-SE4F MS operating in the FAB mode with xenon atoms (8 keV) and a matrix of dithiothreitol-dithioerythritol ("magic bullet") (47). Tandem mass spectra (B/E scan) in the FAB mode were obtained on a four-sector tandem MS (70-SE4F) with helium as a collision gas, and conditions were as follows: resolution of the first and second MS, both 1,000; accelerating potential, 8 keV; collision energy, 4 keV; attenuation, 90%. High-resolution FABMS operated at 10,000 resolution (10% valley). Approximately 5 to 10 μg of each sample was applied as a methanol solution.

Acid hydrolysis of toxins and derivatization of the hydrolysate. Each toxin (50 to 60 μg) was hydrolyzed with 6 N hydrochloric acid (100 μl; Pierce, Rockford, Ill.) at 110°C for 21 h or at 140°C for 40 min. The dried hydrolysate was dissolved in 200 μl of methanol containing 15% hydrogen chloride, and the solution was heated at 110°C for 20 min. The mixture was evaporated to dryness by a gentle stream of nitrogen (N_2), trifluoroacetic anhydride (100 μl) and methylene chloride (100 μl) were added, and the mixture was

TABLE 2. Microcystins isolated from *O. agardhii* strains and their relative abundance

Toxin	Result by FABMS (M + H) [*m/z*]	% of total toxins in strain:												Assignment
		18	49	97	195	209	212	213	214	223	226	CYA 126	CYA 127	CYA 128	
A	981		1		11	2	8	4			4	19			[D-Asp^3]MCYST-LR
B	981	1		1		2	4	4		1	1			1	[Dha^7]MCYST-LR
C	1,010		1					5							[D-Asp^3,Dha^7]MCYST-RR
D	1,024		78	99	88	96	88	87	97		95	81	10	1	[D-Asp^3]MCYST-RR
E	1,024	99	19							99			90	98	[Dha^7]MCYST-RR
F	1,042		1		1				1						[D-Asp^3,Mser^7]MCYST-RR
G	1,044								1						Unknown
H	1,058								1						Unknown

heated at 150°C for 10 min and evaporated by N_2. The residue was dissolved in methylene chloride (25 μl), and 1 μl of each sample was injected for gas chromatography. Authentic amino acids were derivatized in a similar manner.

Gas chromatography. Capillary gas chromatography was carried out on a Varian 3700 gas chromatograph by using a Chirasil Val III column (0.32 mm by 25 m; Alltech Associates, Deerfield, Ill.) (12) and helium as a carrier gas (flow rate, 37 ml/min; split ratio, 20:1). The program rate for the analysis of amino acid derivatives was 90°C (2 min) to 190°C at 8°C/min. The other conditions were as follows: injector temperature, 210°C; detector temperature, 220°C; makeup gas, helium (20 ml/min).

Toxicity testing. Toxicity of the cells and fractions after the first HPLC purification step was tested by mouse bioassay. Aqueous cell suspensions and fractions from HPLC were injected intraperitoneally into mice (20- to 25-g female NIH mice at the University of Helsinki). Mice were observed for 4 h. The death of a mouse within 1 to 3 h and pooling of blood in the liver were taken as signs of poisoning typical of hepatotoxic microcystins.

RESULTS

***O. agardhii* strains.** All *Oscillatoria* strains isolated from Finnish lakes were classified as *O. agardhii* (Gomont 1982) (45). Two of the strains were red variants of *Oscillatoria*, and the rest of the strains were green pigmented. Strain 49 had slightly wider filaments than the rest of the strains.

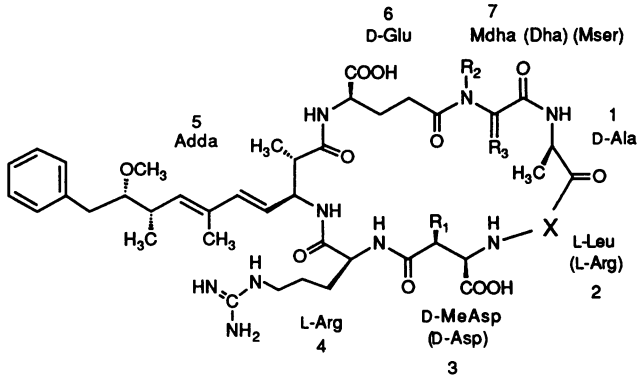
All of these *O. agardhii* strains were hepatotoxic by mouse bioassay. Typical signs of poisoning and autopsy findings were recorded; death occurring within 1 to 3 h and dark, blood-engorged livers weighing about 10% of the body weight were observed.

Two to five microcystins were isolated from each *Oscillatoria* strain (Table 2). Typically, one toxin was clearly the main toxin produced (80 to 99% of the total toxins in each strain). [D-Asp^3]MCYST-RR was the most frequently occurring toxin. In four strains, two of which were red pigmented, the principal toxin was [Dha^7]MCYST-RR. The amounts of total toxins among the different strains varied from 1.0 to 3.7 mg of toxins per g of lyophilized cells. Eight different compounds were isolated from these *O. agardhii* strains. Five of these compounds were known, but three of them were found to be new microcystins (Table 2).

Structure assignment of known microcystins. The microcystins (coded A to H) isolated from 13 different *O. agardhii* strains are listed in Table 2, and the structures of compounds A to F are shown in Fig. 1. The microcystins were first analyzed by FABMS. Toxins A and B showed the same molecular ion peak at *m/z* 981 and were identified by TLC with authentic samples of [D-Asp^3]MCYST-LR and [Dha^7]MCYST-LR, respectively, isolated from *Anabaena* spp. (39). Toxin C (molecular weight, 1,009) was identical to [D-Asp^3,Dha^7]MCYST-RR isolated from *Anabaena* spp. (39). The same molecular ion peak at *m/z* 1,024 was detected for toxins D and E, suggesting demethyl variants of MCYST-RR. The structures of toxins D and E were assigned by direct comparison by TLC with the authentic samples [D-Asp^3]MCYST-RR and [Dha^7]MCYST-RR, respectively, which were isolated from *Anabaena* spp. (39).

Compounds F, G, and H were not identical to known microcystins. Only the structure for toxin F was assigned, since the amounts of toxins G and H were sufficient only for assigning their molecular formulas and amino acid components, as listed in Table 3.

Structure of toxin F. Toxin F showed a molecular ion peak at *m/z* 1,042 by FABMS. The molecular formula of toxin F, $C_{48}H_{75}N_{13}O_{13}$, was deduced from the high-resolution FABMS data (Table 3). Although the molecular weight and



	R_1	R_2	R_3	X
A	H	CH_3	CH_2	L-Leu
B	CH_3	H	CH_2	L-Leu
C	H	H	CH_2	L-Arg
D	H	CH_3	CH_2	L-Arg
E	CH_3	H	CH_2	L-Arg
F	H	CH_3	H, CH_2OH	L-Arg

FIG. 1. Structures of microcystins (designated A to F) isolated from *O. agardhii* strains.

TABLE 3. HRFABMS and amino acid analysis data for toxins F to H obtained from *O. agardhii* strains[a]

Toxin	Result by HRFABMS (M + H) [m/z]	Ion formula	Δ (mDa)[b]	Amino acid components
F	1042.5721	$C_{48}H_{76}N_{13}O_{13}$	−3.5	D-Ala, L-Arg, D-Asp, L-Arg, D-Glu
G	1044.5515	$C_{47}H_{74}N_{13}O_{14}$	−3.7	D-Ala, L-Arg, D-Asp, L-Arg, D-Glu
H	1058.5650	$C_{48}H_{76}N_{13}O_{14}$	−1.5	D-Ala, L-Arg, D-Asp, L-Arg, D-Glu

[a] HRFABMS, high-resolution FABMS.
[b] Difference (in millidaltons) for the calculated value for each composition.

formula of toxin F were identical to those of [L-Ser^7]MCYST-RR, which was isolated from *Anabaena* strain 202 A1 (27), TLC and HPLC showed different R_f values and retention times. The amino acid analysis of toxin F revealed D-Asp together with D-Ala, two L-Arg, and D-Glu, which suggested that the structure of toxin F is [D-Asp^3,Mser^7]MCYST-RR.

The molecular ion $(M + H)^+$ of toxin F obtained by FAB was subjected to FABMS/collisionary-induced dissociation/MS to give the product ions to be used for structure assignments of microcystins (24, 25). The fragment ion peaks at *m/z* 135 [base peak, $PhCH_2CH(OCH_3)$] and 906 (monoisotopic molecular weight [M] − 135) revealed the presence of the Adda unit (25). The sequence of Adda-Glu-Mser-Ala was determined from the fragment ion peaks at *m/z* 393 ($C_{11}H_{14}O$-Glu-Mser), 231 (Glu-Mser plus H), and 173 (Mser-Ala plus H), and that of Asp-Arg was determined from the peak at *m/z* 272 (Asp-Arg plus H). Thus, the structure of toxin F was assigned as [D-Asp^3,Mser^7]MCYST-RR as shown in Fig. 1.

DISCUSSION

Thirteen hepatotoxic *O. agardhii* strains isolated from eight different lakes in Finland produced two to five toxins each. Our study is the first to show that *O. agardhii* frequently contains more than one microcystin. Previous studies have found [D-Asp^3]MCYST-RR in an *O. agardhii* strain (23) and in an *O. agardhii* bloom sample (6, 18) plus [D-Asp^3,Dha^7]MCYST-LR in an *O. agardhii* bloom sample from Norway (6, 18).

The main toxins found in this study were [D-Asp^3]MCYST-RR and [Dha^7]MCYST-RR. The latter was the dominant toxin in two red-pigmented *Oscillatoria* strains (strains 18 and CYA 128) and in two green-pigmented strains (strains 223 and CYA 127). The green-pigmented strain CYA 127 was isolated from the same lake (Lake Vesijärvi) as the red-pigmented strain CYA 128. The red-pigmented strain 18 and the green-pigmented isolate CYA 126 both originated from Lake Långsjön, but their main toxins were different. Four separate isolates from Lake Haukkajärvi differed slightly only in the amounts or presence or absence of some minor toxins. All *Oscillatoria* strains produced only one major toxin at a time, in contrast to the strains of *Anabaena* spp. (39) or *Microcystis* spp. (46), which usually produced two to four main toxins simultaneously.

The toxins in *Oscillatoria* strains varied less structurally than the toxins in *Anabaena* spp. (39) and *Microcystis* spp. (unpublished results) obtained from the same geographical area. For example, 17 different microcystins were identified from seven strains of *Anabaena* spp. (39), compared with 8 microcystins from *Oscillatoria* spp. (this study). Among *Anabaena* and *Microcystis* isolates, MCYST-LR and MCYST-RR were detected, but their demethylated variants were also common (39; also unpublished results). The *O. agardhii* strains produced only demethyl variants. The same demethyl microcystins, i.e., [D-Asp^3]MCYST-LR and -RR, [Dha^7]MCYST-LR and -RR, and [D-Asp^3,Dha^7]MCYST-RR, have now been characterized as common toxins from different isolates of *Anabaena* spp. (39), *Oscillatoria* spp. (this study), and *Microcystis* spp. (unpublished results) from Finland. One *Nostoc* strain and two *Anabaena* strains (26, 40; also unpublished results) from the same area were shown to produce individual varieties of microcystins.

The new microcystin, [D-Asp^3,Mser^7]MCYST-RR, was identified from three different isolates as a minor compound. Previously, variants of microcystins in which *N*-methylserine substitutes for Mdha have been found in a bloom containing several *Microcystis* spp. ([Mser^7]MCYST-LR) (25) and in *Nostoc* sp. strain 152 ([ADMAdda^5,Mser^7]MCYST-LR) (40), and L-serine variants in two *Anabaena* spp., strain 202 ([L-Ser^7]MCYST-LR and [L-Ser^7]MCYST-RR) (27) and strain 66 ([L-Ser^7]MCYST-HtyR) (26) have been found. In this study, a microcystin containing methylserine was found only when large amounts of cells were extracted. According to Pearce and Rinehart (32), serine is the biosynthetic precursor of dehydroalanine, which indicates that the new minor compound identified in this study might be an intermediate product of the main toxin, [D-Asp^3]MCYST-RR, produced by all three strains. However, very little is known of the biogenesis of these compounds at present.

Mass occurrences of toxic *Oscillatoria* spp. are known at least in Scandinavian countries (2, 31, 42; also this study), Italy (5), and The Netherlands (19). *O. agardhii* strains do not usually form scums on the water surface, since they prefer lower light intensities for growth, but they might occur in masses deeper in the water column (20). Protein phosphatase inhibition and tumor promotion make these toxins hazardous to human populations that depend on the water for drinking. Since mass occurrences of *Oscillatoria* spp. may be found close to the water intake line level, they seem to be among the most troublesome species in this respect. Conventional water treatment procedures (slow sand filtration and chlorination) do not remove the toxins, whereas activated carbon and ozonization have been previously found to be effective (17). The toxin production of some of the strains has been studied earlier (36). The main toxin seemed to remain the same, regardless of the different environmental conditions used in this study, but the toxin concentrations in the cells varied at different temperatures, light intensities, and nitrate nitrogen concentrations. We have also isolated 15 *O. agardhii* strains which were not toxic by mouse bioassay (42), and genetic differences between these toxic and nontoxic isolates remain to be studied.

In summary, eight different microcystins were isolated from 13 *O. agardhii* strains isolated from Finnish lakes. [D-Asp^3]MCYST-RR was the main toxin found in most of the isolates, but some isolates contained [Dha^7]MCYST-RR as the main toxin. Five toxins were previously known microcystins, and this was the first time these toxins, except for [D-Asp^3]MCYST-RR, were isolated from *Oscillatoria* spp. Three microcystins were new, and one of these was isolated in amounts adequate to allow structure assignment. The new microcystin was an Mser variant of MCYST-RR and possibly a biosynthetic precursor of the principal toxin found. Determining the biogenesis of these compounds will require future research.

ACKNOWLEDGMENTS

The research at the University of Helsinki was supported by grants from the Academy of Finland, the Maj and Tor Nessling Foundation, and the University of Helsinki. The research at the University of Illinois was supported by grants from the National Institute of Allergy and Infectious Diseases (AI 04769) and the National Institute of General Medical Sciences (GM 27029) to K.L.R.

We thank Olav M. Skulberg for supplying the CYA strains, Riitta Saastamoinen for technical assistance in culturing the cyanobacteria, Furong Sun for mass spectra, and Jarkko Rapala for critically reading the manuscript.

REFERENCES

1. **Beasley, V. R., A. M. Dahlem, W. O. Cook, W. M. Valentine, R. A. Lowell, S. B. Hooser, K.-I. Harada, M. Suzuki, and W. W. Carmichael.** 1989. Diagnostic and clinically important aspects of cyanobacterial (blue-green algae) toxicoses. J. Vet. Diagn. Invest. **1**:359–365.
2. **Berg, K., O. M. Skulberg, R. Skulberg, B. Underdal, and T. Willén.** 1986. Observations of toxic blue-green algae (cyanobacteria) in some Scandinavian lakes. Acta Vet. Scand. **27**:440–452.
3. **Botes, D. P., A. A. Tuinman, P. L. Wessels, C. C. Viljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith, and S. J. Hammond.** 1984. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. J. Chem. Soc. Perkin Trans. I **1984**:2311–2318.
4. **Botes, D. P., P. L. Wessels, H. Kruger, M. T. C. Runnegar, S. Santikarn, R. J. Smith, J. C. J. Barna, and D. H. Williams.** 1985. Structural studies on cyanoginosins-LR, -YR, -YA, and -YM, peptide toxins from *Microcystis aeruginosa*. J. Chem. Soc. Perkin Trans. I **1985**:2747–2748.
5. **Bruno, M. P., M. B. Cucci, E. Pierdominici, P. Sestili, A. Ioppolo, N. Sechi, and L. Volterra.** 1992. Microcystin-like toxins in different freshwater species of *Oscillatoria*. Toxicon **30**:1307–1311.
6. **Carmichael, W. W.** 1989. Freshwater cyanobacteria (blue-green algae) toxins, p. 3–16. *In* C. L. Ownby and G. V. Odell (ed.), Natural toxins: characterization, pharmacology and therapeutics. Pergamon Press, Oxford.
7. **Carmichael, W. W.** 1992. Cyanobacteria secondary metabolites–cyanotoxins. J. Appl. Bacteriol. **72**:445–459.
8. **Carmichael, W. W., V. R. Beasley, D. L. Bunner, J. N. Eloff, I. Falconer, P. Gorham, K.-I. Harada, T. Krishnamurthy, M.-J. Yu, R. E. Moore, K. Rinehart, M. Runnegar, O. M. Skulberg, and M. Watanabe.** 1988. Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon **26**:971–973.
9. **Codd, G. A., and G. K. Poon.** 1988. Cyanobacterial toxins. Proc. Phytochem. Soc. Eur. **28**:283–296.
10. **Dahlem, A. M.** 1989. Ph.D. thesis. University of Illinois, Urbana-Champaign.
11. **Falconer, I. R., and T. H. Buckley.** 1989. Tumour promotion by *Microcystis* sp., a blue-green alga occurring in water supplies. Med. J. Aust. **150**:351.
12. **Frank, H., G. J. Nicholson, and E. Bayer.** 1977. Rapid gas chromatographic separation of amino acid enantiomers with a novel chiral stationary phase. J. Chromatogr. Sci. **15**:174–176.
13. **Gathercole, P. R., and P. G. Thiel.** 1987. Liquid chromatographic determination of the cyanoginosins, toxins produced by the cyanobacterium *Microcystis aeruginosa*. J. Chromatogr. **408**:435–440.
14. **Harada, K.-I., K. Matsuura, M. Suzuki, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley, and W. W. Carmichael.** 1990. Isolation and characterization of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). Toxicon **28**:55–64.
15. **Harada, K.-I., K. Ogawa, Y. Kimura, H. Murata, M. Suzuki, P. M. Thorn, W. R. Evans, and W. W. Carmichael.** 1991. Microcystins from *Anabaena flos-aquae* NRC 525-17. Chem. Res. Toxicol. **4**:535–540.
16. **Harada, K.-L., K. Ogawa, K. Matsuura, H. Nagai, H. Murata, M. Suzuki, Y. Itezono, N. Nakayama, M. Shirai, and M. Nakano.** 1991. Isolation of two toxic heptapeptide microcystins from an axenic strain of *Microcystis aeruginosa*, K-139. Toxicon **29**:479–489.
17. **Keijola, A.-M., K. Himberg, A.-L. Esala, K. Sivonen, and L. Hiisvirta.** 1988. Removal of cyanobacterial toxins in water treatment processes. Laboratory and pilot-scale experiments. Toxicity Assessment **3**:643–656.
18. **Krishnamurthy, T., L. Szafraniec, D. F. Hunt, J. Shabanowitz, J. R. Yates, C. R. Hauer, W. W. Carmichael, O. Skulberg, G. A. Codd, and S. Missler.** 1989. Structural characterization of toxic cyclic peptides from blue-green algae by tandem mass spectrometry. Proc. Natl. Acad. Sci. USA **86**:770–774.
19. **Leeuwangh, P., F. I. Kappers, M. Dekker, and W. Koerselman.** 1983. Toxicity of cyanobacteria in Dutch lakes and reservoirs. Aquat. Toxicol. **4**:63–72.
20. **Lindholm, T., J. E. Eriksson, and J. A. O. Meriluoto.** 1989. Toxic cyanobacteria and water quality problems—examples from a eutrophic lake on Åland, southwest Finland. Water Res. **23**:481–486.
21. **MacKintosh, C., K. A. Beattie, S. Klumpp, P. Cohen, and G. A. Codd.** 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.
22. **Matsushima, R., S. Yoshizawa, M. F. Watanabe, K.-I. Harada, M. Furusawa, W. W. Carmichael, and H. Fujiki.** 1990. *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin and fibroblasts. Biochem. Biophys. Res. Commun. **171**:867–874.
23. **Meriluoto, J. A. O., A. Sandström, J. E. Eriksson, G. Remaud, A. Grey Craig, and J. Chattopadhyaya.** 1989. Structure and toxicity of a peptide hepatotoxin from the cyanobacterium *Oscillatoria agardhii*. Toxicon **27**:1021–1034.
24. **Namikoshi, M., K. L. Rinehart, R. Sakai, K. Sivonen, and W. W. Carmichael.** 1991. Structures of three new cyclic hepatotoxins produced by the cyanobacterium (blue-green alga) *Nostoc* sp. strain 152. J. Org. Chem. **55**:6135–6139.
25. **Namikoshi, M., K. L. Rinehart, R. Sakai, R. R. Stotts, A. M. Dahlem, V. R. Beasley, W. W. Carmichael, and W. R. Evans.** 1992. Identification of 12 hepatotoxins from a Homer lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. J. Org. Chem. **57**:866–872.
26. **Namikoshi, M., K. Sivonen, W. R. Evans, W. W. Carmichael, L. Rouhiainen, R. Luukkainen, and K. L. Rinehart.** 1992. Structures of three new homotyrosine-containing microcystins and a new homophenylalanine variant from *Anabaena* sp. strain 66. Chem. Res. Toxicol. **5**:661–666.
27. **Namikoshi, M., K. Sivonen, W. R. Evans, W. W. Carmichael, F. Sun, L. Rouhiainen, R. Luukkainen, and K. L. Rinehart.** 1992. Two new L-serine variants of microcystins-LR and -RR from *Anabaena* sp. strain 202 A1 and 202 A2. Toxicon **30**:1457–1464.
28. **Namikoshi, M., K. Sivonen, W. R. Evans, F. Sun, W. W. Carmichael, and K. L. Rinehart.** 1992. Isolation and structures of microcystins from a cyanobacterial water bloom (Finland). Toxicon **30**:1473–1479.
29. **Nishiwaki-Matsushima, R., T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W. W. Carmichael, and H. Fujiki.** 1992. Liver tumor promotion by the cyanobacterial

cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. **118:**420–424.

30. **Norwegian Institute for Water Research.** 1985. Culture collection of algae at Norwegian Institute for Water Research, catalog of strains. Norwegian Institute for Water Research, Oslo.
31. **Østensvik, Ø., O. M. Skulberg, and N. E. Søli.** 1981. Toxicity studies with blue-green algae from Norwegian inland waters, p. 315–324. *In* W. W. Carmichael (ed.), The water environment: algal toxins and health. Plenum Press, New York.
32. **Pearce, C. J., and K. L. Rinehart, Jr.** 1979. Berninamycin biosynthesis. 1. Origin of dehydroalanine residues. J. Am. Chem. Soc. **101:**5069–5070.
33. **Pearson, M. J.** 1990. Toxic blue-green algae. Report of the National Rivers Authority, Water Quality Series no. 2. Stanley L. Hunt, Peterborough, United Kingdom.
34. **Repavich, W. M., W. C. Sonzogni, J. H. Standridge, R. E. Wedepohl, and L. F. Meisner.** 1990. Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity. Water Res. **24:**225–231.
35. **Rinehart, K. L., K.-I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley, A. M. Dahlem, and W. W. Carmichael.** 1988. Nodularin, microcystin, and the configuration of Adda. J. Am. Chem. Soc. **110:**8557–8558.
36. **Sivonen, K.** 1990. Effects of light, temperature, nitrogen, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl. Environ. Microbiol. **56:**2658–2666.
37. **Sivonen, K., W. W. Carmichael, M. Namikoshi, K. L. Rinehart, A. M. Dahlem, and S. I. Niemelä.** 1991. Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. Appl. Environ. Microbiol. **56:**2650–2657.
38. **Sivonen, K., K. Kononen, W. W. Carmichael, A. M. Dahlem, K. L. Rinehart, J. Kiviranta, and S. I. Niemelä.** 1989. Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and structure of the toxin. Appl. Environ. Microbiol. **55:**1990–1995.
39. **Sivonen, K., M. Namikoshi, W. R. Evans, W. W. Carmichael, F. Sun, L. Rouhiainen, R. Luukkainen, and K. L. Rinehart.** 1992. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. Appl. Environ. Microbiol. **58:**2495–2500.
40. **Sivonen, K., M. Namikoshi, W. R. Evans, M. Färdig, W. W. Carmichael, and K. L. Rinehart.** 1992. Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. Chem. Res. Toxicol. **5:**464–469.
41. **Sivonen, K., M. Namikoshi, W. R. Evans, B. V. Gromov, W. W. Carmichael, and K. L. Rinehart.** 1992. Isolation and structures of five microcystins from a Russian *Microcystis aeruginosa* strain CALU 972. Toxicon **30:**1481–1485.
42. **Sivonen, K., S. I. Niemelä, R. M. Niemi, L. Lepistö, T. H. Luoma, and L. A. Räsänen.** 1990. Toxic cyanobacteria (blue-green algae) in Finnish fresh and coastal waters. Hydrobiologia **190:**267–275.
43. **Sivonen, K., O. M. Skulberg, M. Namikoshi, W. R. Evans, W. W. Carmichael, and K. L. Rinehart.** 1992. Two methyl ester derivatives of microcystins, cyclic heptapeptide hepatotoxins, isolated from *Anabaena flos-aquae* CYA 83/1. Toxicon **30:**1465–1471.
44. **Skulberg, O. M., G. A. Codd, and W. W. Carmichael.** 1984. Toxic blue-green algal blooms in Europe: a growing problem. Ambio **13:**244–247.
45. **Tikkanen, T.** 1986. Kasviplanktonopas (Phytoplankton guide), p. 278. Forssan Kirjapaino Oy, Helsinki, Finland.
46. **Watanabe, M. F., S. Oishi, K.-I. Harada, K. Matsuura, H. Kawai, and M. Suzuki.** 1988. Toxins contained in *Microcystis* species of cyanobacteria (blue-green algae). Toxicon **26:**1017–1025.
47. **Witten, J. L., M. H. Schaffer, M. O'Shea, J. C. Cook, M. E. Hemling, and K. L. Rinehart, Jr.** 1984. Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Commun. **124:**350–358.
48. **Yoshizawa, S., R. Matsushima, M. F. Watanabe, K.-I. Harada, A. Ichihara, W. W. Carmichael, and H. Fujiki.** 1990. Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. **116:**609–614.