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Nonribosomal peptide synthetase genes occur in most cyanobacterial genera as evidenced by their distribution in axenic strains of the PCC

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Abstract Previous studies largely carried out with environmental samples or axenic and non-axenic cultures suggested that cyanobacteria may be a rich source of hitherto unexplored bioactive compounds. This has been confirmed in the present study by a screening of 146 axenic strains from the Pasteur Culture Collection (PCC) of cyanobacteria. Use of degenerate PCR primers, designed on the basis of conserved sequence motifs in the aminoacyl-adenylation domain of peptide synthetases, revealed the presence of the corresponding genes in the majority (75.3%) of the strains examined. Among unicellular cyanobacteria, only *Chamaesiphon* sp. strain PCC 6605, two strains of *Gloeocapsa* and most *Microcystis* isolates (22 out of 24) contained these genes; no amplicons were detected for any members of the genera *Cyanothece*, *Gloeobacter* and *Gloeothece* and the genetically diverse representatives of *Synechococcus* and *Synechocystis*. By contrast, eight out of ten pleurocapsalean members, 16 out of 25 oscillatorian strains, and all but two of the 63 filamentous heterocystous cyanobacteria tested gave positive amplification results. This information will be highly valuable for further exploring the corresponding cyanobacterial peptides and for elucidating the bioactivity of such non-ribosomally synthesized molecules.

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Introduction

Cyanobacteria have a wide-ranging impact on natural ecosystems and may have both beneficial and harmful effects on human health and activities. Some are also of economic importance (Patterson 1996). Many of these oxygenic phototrophs are important primary producers of combined nitrogen and, for some members, the nutritional value has been shown to be high (Dillon et al. 1996). Although terrestrial representatives are common, the more prominent habitats of cyanobacteria are limnic and marine environments. Depending on the genera or species, they may flourish in rivers and freshwater reservoirs, in brackish to hypersaline habitats, in cold or hot springs, and in environments where eukaryotic phototrophs do not exist (Castenholz and Waterbury 1989). The diversity of cyanobacteria is also reflected by the multitude of structural and functional aspects of their cell morphology and by variations in their metabolic strategies (Carr and Whitton 1983; Castenholz and Waterbury 1989**;** Rippka 1988). Molecular approaches to the systematics of cyanobacteria have been fruitful for elucidating phylogenetic relationships (Rudi et al. 1997; Turner 1997; Willmotte 1994).

In addition to their ecological importance, cyanobacteria are also recognized as a potentially rich, but not yet extensively examined, source of pharmacologically and structurally interesting secondary metabolites (Falch et al. 1995; Namikoshi and Rinehart 1996). A multitude of different cyanobacterial peptides, such as microcystins, cyanopeptolins, microviridins, microcolins and cryptophycins, has been described (Koehn et al. 1992; Moore 1996; Namikoshi and Rinehart 1996; Smith et al. 1994). Such peptides (including depsipeptides) may exhibit potent calcium antagonistic, angiotensin-converting enzyme inhibitory and cytotoxic effects and may also act as inhibitors of tumor

growth (Falch et al. 1995; Moore 1996). However, screening efforts have been limited to a number of selected representatives, many of which were studied in natural material or impure cultures. Apart from *Lyngbya*, *Schizothrix* and *Scytomema* species, members of the bloom-forming taxa *Microcystis aeruginosa*, *Anabaena flos-aquae* and *Planktothrix agardhii* (ex *Oscillatoria agardhii*) have been extensively investigated.

Molecular approaches have been successful in identifying the biosynthetic genes involved in the synthesis of microcystin in *Microcystis aeruginosa* (Dittmann et al. 1997; Nishizawa et al. 2000; Tillett et. al. 2000) and of anabaenopeptilide in *Anabaena flos-aquae* (Rouhiainen et al. 2000). Large multienzyme complexes, comprising peptide synthetases, polyketide synthases and a number of addional enzyme activities, have been shown to be responsible for the formation of these compounds. Non-ribosomal peptide synthetases (NRPS) are known to produce a complement of secondary metabolites in bacteria and lower eukaryotes. They have been intensively investigated in relation to the biosynthesis of peptide antibiotics such as gramicidin S in *Bacillus subtilis*, immunosuppressors as represented by cyclosporin in *Tolypocladium niveum*, or toxins such as the HC-toxin of *Cochliobolus carbonum* (Kleinkauf and von Döhren 1996; Marahiel et al. 1997). These multifunctional enzymes are composed of homologous modules, each being responsible for recognition, aminoacyl-adenylation, thioesterification or modification of a specific amino acid substrate, and for the elongation of the growing peptide product. Adenylate-forming domains exhibit a high degree of sequence conservation, permitting the search for peptide synthetase genes with degenerate primers (Turgay and Marahiel 1994).

We have previously reported that both toxic and nontoxic strains of *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes (Meissner et al. 1996). Furthermore, the presence of these biosynthetic genes has been demonstrated for bloom-forming strains such as *Anabaena flos-aquae*, *Oscillatoria agardhii* and *Nodularia spumigena* as well as for a number of *Nostoc* strains (Neilan et al. 1999). Here, we have extended the search for NRPS genes in cyanobacterial genera (Dittmann et al. 1999) and report the results of a screening using degenerate PCR primers for the detection of NRPS amplicons in 146 axenic cyanobacteria of the PCC. These strains are representative of 35 genera and the five sections (I–V), respectively equivalent to the traditional botanical orders *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales* and *Stignomatales*. We show that the majority of the investigated strains contains NRPS genes, although they occur more frequently in filamentous heterocystous members. This knowledge may prove useful for the isolation of the corresponding peptide molecules; moreover, the sequences of such genes, their modules and domains should also be valuable for the design and generation of entirely new bioactive compounds by a genetic combinatorial approach (Mootz and Marahiel 1999).

Materials and methods

Cyanobacterial strains and cultivation

All cyanobacterial strains were obtained from the PCC (Paris, France) of cyanobacteria as axenic isolates. Most were grown in the appropriate growth medium (see Rippka and Herdman 1992) at 25–28 °C under a continuous light regime and in the presence of white light (Osram Universal White) with a photosynthetic photon flux density (PPFD) of 40 µmol quanta m^{-2} s^{-1} (measured with a LICOR LI-185B quantum radiometer/photometer equipped with a LI-190SB quantum sensor). Some strains were cultivated at 23 °C in a light/dark cycle of 14 h/10 h and a PPFD of 10 µmol quanta m^{-2} s^{-1}. The cultures (2–20 ml) were either sent to Humboldt University (Berlin, Germany) in the living state, or were washed three times with sterile distilled water (150 mM NaCl for marine strains) and dispatched as frozen pellets on dry ice. Strains not mentioned in the PCC catalogue (Rippka and Herdman 1992) are new axenic isolates and include seven representatives isolated from French rivers and freshwater reservoirs by one of the authors (L. Via-Ordorika). Their respective strain histories and growth media are described in an update of the PCC Website (http://www.pasteur.fr/recherche/banques/PCC/).

DNA extraction, amplification and cloning

Total genomic DNA was extracted as described by Franche and Damerval (1988).

The primer pair MTF2/MTR2 (Dittmann et al. 1997) was used for amplification as described previously (Neilan et al. 1999). The PCR thermal cycling protocol included an initial denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s at an annealing temperature of 50 °C for 30 s, and at 72 °C for 1 min. Amplification components were as described by the manufacturer (Perkin-Elmer, Roche Molecular Systems, Branchburg, New Jersey, USA) and were incubated in a PTC-200 apparatus (MJ Research, Watertown, Mass., USA).

Aliquots of the PCR products were loaded onto 0.8% agarose gels containing ethidium bromide and separated by electrophoresis. The amplicons were visualized under UV-light (234 nm) on a transilluminator (UniEquip, Martinsried, Germany), and bands representing DNA fragments of about 1 kb were cut out of the gel. DNA was purified using the NucleoSpin Extract kit (Machery-Nagel, Düren, Germany).

Purified DNA fragments were cloned in the pGEM-T-Vector (Promega, Madison, Wis., USA) according to the instructions of the manufacturers.

DNA sequencing

DNA was sequenced on both strands with the Dye Terminator Cycle Sequencing Kit using an Automatic Sequencer (ABI, Weiterstadt, Germany).

Nucleotide sequences were analyzed using programs of the University of Wisconsin Genetics Computer Group (Genetics Computer Group 1994). Translated protein sequences were compared to those in the databases through the National Center for Biotechnology Information using the BLAST program (US National Institutes of Health, Bethesda, Md.).

Results

DNA was isolated from 146 strains representative of all five sections of cyanobacteria: *Chroococcales* (section I), *Pleurocapsales* (section II), *Oscillatoriales* (section III), *Nostocales* (section IV) and *Stigonematales* (section V)

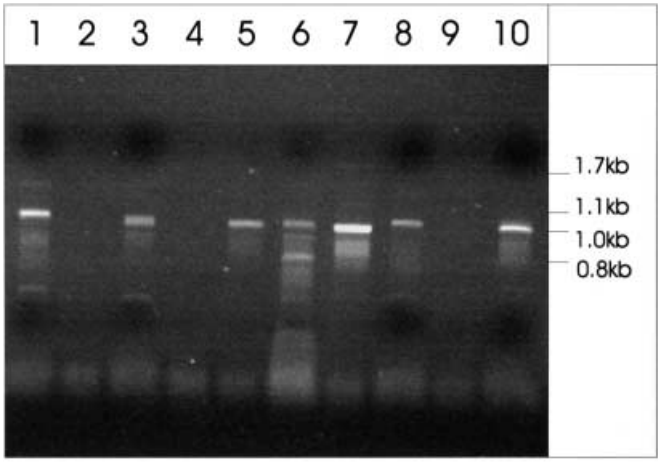

Fig. 1 Ethidium-bromide-stained agarose gel showing the presence or absence of PCR amplification products of nonribosomal peptide synthetase genes in 9 different cyanobacteria. *Chamaesiphon* sp. strain PCC 6605 (*lane 1*), *Synechocystis* sp. strain PCC 6803 (*lane 2*), *Pleurocapsa* sp. strain PCC 7319 (*lane 3*), *Stanieria* sp. strain PCC 7301 (*lane 4*), *Oscillatoria* sp. strain PCC 7515 (*lane 5*), *Nostoc* sp. strain PCC 7120 (*lane 6*), *Chlorogloeopsis* sp. strain PCC 6912 (*lane 7*) and *Fischerella* sp. strain PCC 7414 (*lane 8*). *Lanes 9 and 10* show, respectively, a negative control without a DNA template and the PCR product of *Microcystis* sp. strain PCC 7806, known to contain NRPS genes

(Rippka et al. 1979). As adenylate-forming domains of peptide synthetases contain well-conserved sequence motifs, the degenerate forward and reverse primers (MTF2 and MTR2, respectively), designed by Dittmann et al. (1997) for the detection of such motifs, should result in PCR amplification products in all strains harboring the corresponding genes.

Out of the 146 strains examined, 110 (75.35%) reproducibly yielded PCR products of the expected size (about 1,000 bp) as determined by gel electrophoresis. A representative gel is shown in Fig. 1. In order to confirm that the amplicons resulted from sequence motifs of the adenylate-forming domains of NRPS genes, they were cloned and sequenced for a number of representative strains (*Microcystis* sp. strain PCC 7806, *Planktothrix (ex Oscillatoria agardhii*) sp. strain PCC 7811, *Nostoc* (*Anabaena*) sp. strain PCC 7120, *Tolypothrix* sp. strain PCC7 601/1, *Scytonema* sp. strain PCC 7110, and *Chlorogloeopsis* sp. strain PCC 6912). In all cases, the derived amino acid sequences showed the characteristic core motifs of adenylate-forming domains of peptide synthetases. Sequence data of the strains PCC 7811, 7601/1, 7110 and 6912 have been deposited in the EMBL database (accession no. AJ318785–8). The results demonstrated that the applied PCR method safely amplified the expected gene fragments.

The data summarizing the presence or absence of NRPS genes for all PCC strains examined are shown in Tables 1 and 2. Among representatives of Section I, NRPS genes were found in 52% of the isolates analyzed (25 out of 48), although the majority (88%) of strains harboring these genes were 22 representatives of the genus *Microcystis* (Table 1). The observed frequency of NRPS genes in the latter is in full agreement with the high incidence of hepatotoxin (microcystin) production in representatives of this genus (Dittmann et al. 1999; Neilan et al. 1997; Nishizawa et al. 1999; Otsuka et al 1999). Interestingly, the single strain (PCC 6605) of the genus *Chamaesiphon* and two strains of *Gloeocapsa* examined also showed a NRPS amplicon, although nonribosomally synthesized peptides have never been described for these taxa. NRPS genes were not detected in members of the genera *Cyanothece*, *Gloeobacter*, *Gloeothece*, *Synechococcus* and *Synechocystis*. The lack of detection of such genes in the former four genera may be due to the low number (1–3) of representatives examined, but in the "genera" *Synechococcus* and *Synechocystis*, for which a higher number of isolates (6–8) was analyzed, it seems that NRPS genes are rare or totally absent. It should be mentioned that the latter two "genera" are genetically highly diverse (Herdman et al. 2001; Honda et al. 1999; Waterbury and Rippka 1989) and thus NRPS gene analysis included representatives of the three different GC clusters for *Synechococcus* and of the two GC clusters for *Synechocystis* (compare the PCC strains in Table 1 with the description of the genetic clusters in Herdman et al. 2001; Waterbury and Rippka 1989).

Among members of Section II, NRPS genes were found in 80% of the strains (8 out of 10) examined (Table 1). Although only a single representative of *Cyanocystis* and *Dermocarpella* was analyzed, and sampling of *Pleurocapsa* and *Chroococcidiopsis* was small (four and two strains, respectively), NRPS genes were detected in all. In contrast, such genes were not found in the single representatives of the genera *Stanieria* (ex *Dermocarpa*, see Rippka et al. 2001) and *Xenococcus* investigated.

Among the filamentous, nonheterocystous strains of Section III, NRPS gene amplicons were obtained for 16 of the 25 isolates examined, which amounts to 64% (Table 1). As expected for members of *Planktothrix* (a genus created to accommodate the gas-vesicle-containing species *Oscillatoria agardhii* and *O. rubescens*, Anagnostidis and Komárek 1988), most of which produce microcystins (Dittmann et al. 1997; Moore 1996; Neilan et al. 1999; Rinehart et al. 1994), NRPS genes were found in five of the six isolates investigated. However, the same frequency of NRPS amplicons (83.3%) was also observed for the morphologically and genetically diverse strains (Castenholz et al. 2001; Turner 1997; Wilmotte and Herdman 2001; Wilmotte 1994) still maintained in the "genus" *Oscillatoria* but assigned to several distinct clusters (Rippka and Herdman 1992). Although not known to be toxin producers, NRPS genes were found in strains of cluster 1 (PCC 7515 and PCC 9014), cluster 2 (PCC 7112), cluster 4 (PCC 6412 and PCC 6602), but not in cluster 3 (strain PCC 6304). The marine cosmopolitan species *Microcoleus chthonoplastes* (Garcia-Pichel et al. 1996) represented by strain PCC 7420 and the marine aerobic nitrogen fixer *Symploca atlantica* sp. strain PCC 8002 (Rippka and Herdman 1992) also harbor NRPS genes. In contrast, *Leptolyngbya* sp. strain PCC 6306, formerly a representative of the LPP-B group (Castenholz et al. 2001; Rippka et al. 1979), did not give a PCR product. The same is true for the only represen-

Table 1 Nonribosomal peptide synthetases (NRPS) genes in cyanobacteria of sections I–III as revealed by PCR amplification using primers MTF2 and MTR2 (see Methods). *PCC* Pasteur Culture Collection, + NRPS genes detected by PCR, – NRPS genes not detected

Section	Genus	Strain (PCC number)	NRPS genes	NRPS positive strains/ total number tested
I	*Chamaesiphon*	6605	+	1/1 (100%)
	Cyanothece	7424, 7425, 7418	–	0/3 (0%)
	Gloeobacter	7421	–	0/1 (0%)
	Gloeocapsa	73106, 7428	–	2/4 (50%)
		9352, 9817	+	
	Gloeothece	6501	–	0/1 (0%)
	Microcystis	9624, 9805	–	
		7005, 7806, 7813, 7820, 7941, 9354, 9355, 9432, 9443, 9603, 9622, 9701, 9717, 9804, 9806, 9807, 9808, 9809, 9810, 9811, 9812, 9814	+	22/24 (87.5%)
	Synechococcus	6301, 6307, 6715, 6716, 7002, 7202, 7502; 9630	–	0/8 (0%)
	Synechocystis	6308, 6701, 6803, 6906, 7008, 9632	–	0/6 (0%)
NRPS genes detected in total number of strains of section I				25/48 (52%)
II	*Cyanocystis*	9504	+	1/1 (100%)
	Chroococcidiopsis	6712, 7203	+	2/2 (100%)
	Dermocarpella	7326	+	1/1 (100%)
	Pleurocapsa	7319, 7321, 7327, 7516	+	4/4 (100%)
	Stanieria	7301	–	0/1 (0%)
	Xenococcus	7305	–	0/1 (0%)
NRPS genes detected in total number of strains of section II				8/10 (80%)
III	*Arthrospira*	7345, 8005, 9223	–	1/4 (25%)
		9444	+	
	Leptolyngbya	6306	–	0/1 (0%)
	Lyngbya	8937	–	0/1 (0%)
	Microcoleus	7420	+	1/1 (100%)
	Oscillatoria	6304	–	5/6 (83.3%)
		6412, 6602, 7112, 7515, 9014	+	
	Planktothrix	9214	–	5/6 (83.3%)
		7805; 7811, 7821, 9625, 9637,	+	
	Pseudanabaena	6802, 7429	–	1/3 (33.3%)
		7409	+	
	Spirulina	6313, 9445	+	2/2 (100%)
	Symploca	8002	+	1/1 (100%)
NRPS genes detected in total number of strains of section III				16/25 (64%)

tative of the genus *Lyngbya* examined (strain PCC 8937), though a morphologically similar species, *Lyngbya majuscula*, is known to be a rich source of bioactive peptides (Luesch et al. 2000; Mitchell et al. 2000; Sitachitta et al. 2000). For the helically coiled representatives, it seems possible that NRPS genes may occur more often in *Spirulina* than in *Arthrospira* (Table 1). However, more intensive sampling, particularly of representatives that proved to give negative amplification results, will be required to allow more definitive conclusions about the frequency of NRPS genes in taxa of Section III.

The filamentous heterocystous strains of Sections IV and V investigated were all shown to contain NRPS genes, with the exception of the two *Nostoc* strains PCC 8113 and PCC 9346 (Table 2). Whether the extraordinary frequency of such genes in members of Section IV (96.7 % for a total of 60 strains examined) is also typical of the branching heterocystous cyanobacteria of Section V, remains to be investigated in more representative genera and strains. Our findings are particularly remarkable for the genera *Anabaena* and *Nostoc*, known to be morphologically, ecologically and genetically diverse (Lachance 1981; Rippka et al. 1979; Rippka et al. 2001) and for which a relatively large number (10 and 32, respectively) of strains were examined. In some of the *Anabaena* strains (*A. flos-aquae* PCC 9302, PCC 9332 and PCC 9349), the NRPS genes detected may be involved in microcystin synthesis, since this cyclic peptide commonly occurs in planktonic species of this genus (Neilan et al. 1999; Rinehart et al. 1994) and has been described for strain PCC 9302, which corresponds to the non-axenic isolate NRC 525-17 (Harada et al. 1991; Rinehart et al. 1994). Two representatives of *Nodularia*, strain PCC 9350 (corresponding to the impure isolate AV2 from the Baltic Sea, Martin et al. 1990) and strain PCC 7804, are known to synthesize the cyclic pentapeptide nodularin (Martin et al. 1990; G. Codd, University of Dundee, personal communication), whereas this needs to be examined for strains PCC 9234

Table 2 Nonribosomal peptide synthetases (NRPS) genes in cyanobacteria of sections IV–V as revealed by PCR amplification using primers MTF2 and MTR2 (see Methods). *PCC* Pasteur Culture Collection, + NRPS genes detected by PCR, – NRPS genes not detected

Section	Genus	Strain (PCC number)	NRPS genes	NRPS positive strains/ total number tested
IV	*Anabaena*	6309, 7108, 7122, 73105, 7938, 9302, 9332, 9349, 9404, 9406, 9424	+	11/11 (100%)
	Aphanizomenon	7905	+	1/1 (100%)
	Calothrix	6303, 7103, 9314	+	3/3 (100%)
	Cylindrospermum	7417, 7604, 9238	+	3/3 (100%)
	Microchaete	7126	+	1/1 (100%)
	Nodularia	73104,7804, 9234, 9235, 9236, 9350	+	6/6 (100%)
	Nostoc	8113, 9346	–	29/31 (93.4%)
		6302, 6310, 6314/1, 6705, 6720, 7107, 7119, 7120[a], 73102, 7416, 7422, 7423, 7524/1, 7706, 7803, 7808, 7906, 7936, 7937, 8009/1, 8107/1, 8108, 8111, 8901, 9225, 9230, 9335, 9237/1, 9347	+	
	Scytonema	7110	+	1/1 (100%)
	Tolypothrix	7101, 7504, 7601/1[b]	+	3/3 (100%)
NRPS genes detected in total number of strains of section IV				58/60 (96.7%)
V	*Chlorogloeopsis*	6912	+	1/1 (100%)
	Fischerella	7414	+	1/1 (100%)
	Matteia	9605	+	1/1 (100%)
NRPS genes detected in total number of strains of section V				3/3 (100%)

[a]Strain also known as "*Anabaena*" sp. strain PCC 7120
[b]Strain also known as *Calothrix* sp. or *Fremyella diplosiphon* PCC 7601 (Rippka and Herdman 1992)

and PCC 9235. The detection of a NRPS gene amplicon for *Nodularia* sp. strain PCC 9236 is in full agreement with previous results : this strain is a new subisolate of UTEX 2091 and, based on a common strain history, would have been expected to be identical to the nontoxic *Nodularia* sp. strain PCC 73104, shown to contain NRPS genes (Neilan et al. 1999).

Discussion

It is possible that the 36 strains (24.7%) for which no PCR products were detected may contain NRPS genes with much less conserved aminoacyl adenylation core motifs. If this were the case, the primers used in this study would not have been suitable for PCR amplification. However, we are confident that the lack of NRPS amplicons truly reflects the absence of the corresponding genes in the respective strains for the following reasons. Firstly, we performed an *in silico* analysis of several cyanobacterial NRPS gene sequences available in the database: the *mcy* cluster of *Microcystis* sp. strain PCC 7806 (Tillett et al. 2000), the *apd* cluster of *Anabaena* sp. strain 90 (Rouhiainen et al. 2000) and the *nos* cluster of *Nostoc* sp. strain GSV224 (Genbank accession no. AF204805). Furthermore, several gene clusters encoding peptide synthetases in the genomes of *Anabaena* (*Nostoc*) sp. strain PCC 7120 (http://www.kazusa.or.jp/cyano/) and *Nostoc punctiforme* PCC 73102 (http://spider.jgi-psf.org/JGI_microbial/html/nostoc/nostoc_homepage.html) were analyzed**.** In agreement with our positive amplification results for 110 cyanobacterial strains, as well as with the sequences of such fragments determined in this and an earlier study (Neilan et al. 1999), no striking deviations were identified in the region used for primer design in any of the NRPS core motifs analyzed. Hence, it seems that all cyanobacterial NRPS genes share these sequences and, unless NRPS genes are lacking, they should be detectable by PCR with the primers used for amplification. Secondly, *Synechocystis* sp. strain PCC 6803, which did not yield a NRPS gene product (Table 1), indeed does not contain any peptide synthetase genes as evidenced by *in silico* analysis of its genome (http://www.kazusa.or.jp/cyano/).

Based on the relatively large number of strains analyzed (Table 1), it seems that unicellular cyanobacteria of the "genera" *Synechococcus* and *Synechocystis* may generally lack the ability to produce nonribosomally synthesized peptides, at least by means of a modular peptide synthetase of the type here investigated. However, this needs to be explored in more detail. Although the majority of cyanobacteria examined contain NRPS genes, they may be present or absent in strains of the same genus, or even among isolates of the same species. This has been particularly shown for taxa in which a sufficiently large number of strains was examined and for which close genetic relationships are known, as is the case for *Microcytis*, *Planktothrix* and *Nodularia* (Bolch et al. 1999; Lehtimäki et al. 2000; Neilan et al. 1997; Rudi et al. 1997). Since closely related strains may differ with respect to their content of NRPS genes, it is obvious that the presence or absence of such genes does not reflect distinct phylogenetic lineages, and thus this trait is not a reliable taxonomic marker.

Cyanobacterial peptide synthetase gene clusters involved in the biosynthesis of microcystins and anabaenopep-

tilides, from *Microcystis aeruginosa* (Nishizawa et al. 1999; Tillett et. al. 2000) and *Anabaena flos-aquae* (Rouhiainen et al. 2000), respectively, have been sequenced and characterized. Their analyses has permitted a number of unique features, such as novel condensation and modification domains, to be identified. The high number of strains yielding NRPS gene amplicons discovered in the course of this study demonstrates the great potential of cyanobacteria as a source for secondary peptide metabolites. Given the genetic diversity of the NRPS-positive strains, it is anticipated that sequencing of the entire gene clusters of the corresponding peptide synthetases may reveal additional new or interesting domains that may prove to be of value for the combinatorial synthesis of bioactive compounds by genetic engineering (Mootz and Marahiel 1999).

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