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Phylogenetic and Chemical Diversity of Three Chemotypes of Bloom-Forming *Lyngbya* Species (*Cyanobacteria*: *Oscillatoriales*) from Reefs of Southeastern Florida▽

Koty Sharp,[1]† Karen E. Arthur,[1] Liangcai Gu,[2] Cliff Ross,[3] Genelle Harrison,[1] Sarath P. Gunasekera,[1] Theresa Meickle,[1] Susan Matthew,[4] Hendrik Luesch,[4] Robert W. Thacker,[5] David H. Sherman,[2] and Valerie J. Paul[1]*

Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, Florida 34949[1]; Life Sciences Institute and Departments of Medicinal Chemistry, Chemistry, and Microbiology & Immunology, University of Michigan, 210 Washtenaw Ave., Ann Arbor, Michigan 48109[2]; University of North Florida, 1 UNF Drive, Jacksonville, Florida 32224[3]; Department of Medicinal Chemistry, University of Florida, 1600 SW Archer Road, Gainesville, Florida 32610[4]; and Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-1170[5]

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The cyanobacterial genus *Lyngbya* includes free-living, benthic, filamentous cyanobacteria that form periodic nuisance blooms in lagoons, reefs, and estuaries. *Lyngbya* spp. are prolific producers of biologically active compounds that deter grazers and help blooms persist in the marine environment. Here, our investigations reveal the presence of three distinct *Lyngbya* species on nearshore reefs in Broward County, FL, sampled in 2006 and 2007. With a combination of morphological measurements, molecular biology techniques, and natural products chemistry, we associated these three *Lyngbya* species with three distinct *Lyngbya* chemotypes. One species, identified as *Lyngbya* cf. *confervoides* via morphological measurements and 16S rRNA gene sequencing, produces a diverse array of bioactive peptides and depsipeptides. Our results indicate that the other two *Lyngbya* species produce either microcolins A and B or curacin D and dragonamides C and D. Results from screening for the biosynthetic capacity for curacin production among the three *Lyngbya* chemotypes in this study correlated that capacity with the presence of curacin D. Our work on these bloom-forming *Lyngbya* species emphasizes the significant phylogenetic and chemical diversity of the marine cyanobacteria on southern Florida reefs and identifies some of the genetic components of those differences.

Marine harmful algal blooms are increasing in frequency and severity as a result of eutrophication in the marine environment, changes in global climate patterns, and increased monitoring and use of marine habitats (22, 23, 60). The genus *Lyngbya* consists of filamentous cyanobacteria that cause periodic, but in some cases long-lasting, blooms in shallow (usually <30 m) tropical and subtropical marine and estuarine environments (42, 43). *Lyngbya* species are prolific producers of secondary metabolites, primarily lipopeptides, cyclic peptides, and depsipeptides. To date, over 200 biologically active compounds have been isolated from *Lyngbya* spp. collected around the world (4, 5, 57). Many *Lyngbya*-derived bioactive secondary metabolites confer a competitive advantage to the cyanobacteria by deterring grazers, allowing the cyanobacteria to overgrow other organisms in benthic habitats (45). In addition to rendering *Lyngbya* spp. unpalatable, bioactive peptides in *Lyngbya* spp. may have additional impacts, such as allelopathy against sympatric benthic organisms (3, 27, 46). Many compounds produced by *Lyngbya* spp. are of significant concern to human and animal health and have been implicated in intoxication, dermatitis, and asthma-like symptoms in humans (43), formation of tumors in marine turtles (1), and alterations in turtle blood biochemistry and increased dugong strandings (2). A better understanding of chemical diversity of *Lyngbya* has many implications for ecosystem, animal, and human health.

It is not well known how external, abiotic, environmental factors regulate bioactive compound biosynthesis in cyanobacteria. Taxonomic studies of the *Oscillatoriales*, which includes many chemically rich species in genera such as *Lyngbya*, *Oscillatoria*, and *Phormidium*, have conventionally been based on morphological characteristics, including cell length and width, colony formation, extracellular sheath width, and pigmentation. Unfortunately, these diagnostic features exhibit plasticity in response to environmental parameters such as salinity, turbidity, and nutrient content (44, 54, 59). Molecular approaches to taxonomy, based on nitrogenase (*nifH*) and 16S rRNA gene sequences (18, 24, 25, 59, 63, 64) and random amplified polymorphic DNA analysis (6, 40), have revealed enormous phylogenetic diversity among environmental cyanobacteria not indicated by morphological classifications, suggesting that cyanobacterial taxonomy remains widely unresolved. As a result, it has been a challenge to identify species-specific patterns of bioactive compound production in the *Oscillatoriales*. Genetically distinct strains of the cyanobacteria *Anabaena* and *Aphanizomenon* have been shown to have different bioactive compound compositions (20, 31), but Thacker and Paul (59) demonstrated that variation in 16S rRNA gene sequences often does not

* Corresponding author. Mailing address: Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, FL 34949. Phone: (772) 462-0982. Fax: (772) 461-8154. E-mail: paul@si.edu.

† Present address: Ocean Genome Legacy Foundation, New England Biolabs, 240 County Rd., Ipswich, MA 01938.

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correlate with chemical variability among samples from the genus *Lyngbya* collected in Guam. These data suggest that other mechanisms, such as responses to environmental conditions or faster rates of genetic change in biosynthetic genes, may contribute to the chemical variation observed among *Lyngbya* spp.

Elucidation of biosynthetic pathways encoding *Lyngbya*-derived compounds, including the barbamides, lyngbyatoxin A, curacin A, and jamaicamides (7, 8, 11, 12), illustrates some novel biosynthetic mechanisms for the secondary metabolites in *Lyngbya* spp. Identification of these genes allows the development of cyanobacteria-specific probes for secondary metabolite biosynthesis. Surveys for biosynthetic genes and 16S rRNA gene sequencing across the five taxonomic sections of cyanobacteria have revealed a wide variability of secondary metabolite biosynthesis (13). The majority of the compounds found in *Lyngbya* spp. are synthesized via nonribosomal peptide synthetases (NRPSs) or mixed polyketide synthase-NRPSs (57). The identification of specific natural product biosynthetic genes provides an effective indicator for the presence of a pathway in a target genome. For example, the curacin A pathway includes a series of genes that are unusual for polyketide synthase and NRPS systems. A GNAT-like domain in the chain initiation module was demonstrated to mediate the chain initiation by catalyzing malonyl coenzyme A decarboxylation and *S*-acetyl transfer (19). In the chain termination module, a sulfotransferase has been predicted to mediate decarboxylative chain termination (8). In this report, the genes encoding these two atypical enzymes, as well as the condensation (C) domain from the NRPS module in the middle of the curacin A pathway (8), were selected in order to identify the highly similar curacin D pathway in the genomes of *Lyngbya* collections.

Methods developed for understanding the molecular basis of natural products biosynthesis can be combined with species identification—based on both 16S rRNA gene sequence and morphology—to present a powerful approach to evaluate the genetic potential for microbial production of secondary metabolites. Probing and surveying uncultured environmental strains for the presence of genes for secondary metabolite biosynthesis can reveal mechanisms driving the distribution of bioactive compounds in the environment.

Since the description of the 2002 to 2005 *Lyngbya* blooms (46), *Lyngbya* spp. have continued to be prevalent on the Broward County reefs in the summer and fall months. Here, we describe three dominant chemotypes of *Lyngbya* collected from reefs near Fort Lauderdale, FL, and we assess their diversity based on morphology, 16S rRNA gene sequences, and presence of biologically active compounds. One of these chemotypes contains curacin D, and here we show that the presence of this molecule corresponds to the presence of curacin biosynthetic gene clusters. The curacin biosynthetic genes are absent in the genomes of other *Lyngbya* species collected from the site, indicating a species-specific genetic basis to the chemical variation observed among *Lyngbya* species occurring in southeastern Florida.

MATERIALS AND METHODS

Sample collection and preservation. *Lyngbya* spp. were collected from hard bottom reefs running parallel to the shore near Fort Lauderdale, Broward County, Florida (26°04′N, 80°06′W), an area that has been described in detail by Paul et al. (46). *Lyngbya* samples were collected between August 2006 and July 2007 (Table 1) and preserved for morphological, molecular, and chemical analyses. The chemical diversity of additional collections from 2004 to 2006 was characterized. Collections were made by hand while scuba diving from reefs at depths ranging from 8 to 15 m. Samples were placed in plastic zip-lock bags at depth and brought to the surface, where they were immediately separated from other macroalgae and any remaining hard substrate, rinsed in seawater, and placed in seawater-filled coolers for transportation to the laboratory (3 h). Morphological measurements were made immediately upon return to the laboratory. For each sample, the majority of the biomass was frozen at −20°C for chemical analysis. Voucher specimens were preserved in 5% formalin–seawater, and approximately 250 mg (wet weight) was frozen in RNA*later* (Ambion, Foster City, CA) for DNA analysis. During each collection from August 2006 to July 2007, light measurements were made using an underwater quantum light sensor (LI-COR, Inc., Lincoln, NE).

TABLE 1. *Lyngbya* samples evaluated in this study[a]

Collection date	Species	Cell morphology [mean (SE)]		
		Filament width (μm)	Cell width (μm)	Cell length (μm)
8 June 2007	*L.* cf. *confervoides*	14.3 (0.5)	13.0 (0.3)	4.8 (0.5)
11 July 2007	*L.* cf. *confervoides*	13.1 (0.3)	12.8 (0.3)	5.8 (0.4)
3 August 2006	*Lyngbya* sp. strain A	37.5 (0.8)	31.8 (0.5)	2.4 (0.1)
4 May 2007	*Lyngbya* sp. strain A	38.8 (1.1)	30.3 (1.1)	3.0 (0.3)
8 June 2007	*Lyngbya* sp. strain A	39.3 (0.7)	33.0 (1.2)	2.5 (0.2)
11 July 2007	*Lyngbya* sp. strain A	44.0 (0.8)	36.1 (0.8)	3.8 (0.4)
3 August 2006	*Lyngbya* sp. strain B	43.6 (1.1)	33.8 (0.7)	9.5 (0.7)
20 October 2006	*Lyngbya* sp. strain B	43.8 (0.4)	35.5 (0.5)	10.3 (0.8)
8 June 2007	*Lyngbya* sp. strain B	43.8 (0.9)	34.8 (0.7)	7.4 (0.8)

[a] For each sample collection date, $n = 10$.

Morphological observations. Filament width, cell width, and cell length of *Lyngbya* spp. were measured on a compound light microscope (Zeiss, Germany) with a 40× nonimmersion objective and 10× ocular lens with a calibrated optical micrometer. Ten separate filaments were measured for each sample, and means and standard errors were calculated. Samples were identified based on morphological characteristics according to the methods described by Littler and Littler (30).

Morphological characteristics of *Lyngbya* spp. were compared by multivariate analysis using Primer 6 (Primer-E Ltd., Plymouth, United Kingdom). Descriptive parameters used for comparison were filament width, cell width, and cell length. Data were normalized using a log transformation to ensure that the magnitude of the measurement did not affect the comparative analysis. A Bray-Curtis similarity matrix was established and nonmetric multidimensional scaling was used to produce a two-dimensional ordination of the data (10). Cluster analysis based on morphological characters was performed to determine whether the samples grouped a priori differed from one another based on their multivariate structure. A similarity profile (SIMPROF) test was incorporated to assess the significance of divisions within the cluster analysis (9). Significant differences were determined at a P level of 0.05.

Bioactive compound isolation and analysis. Frozen bulk samples of *Lyngbya* were freeze-dried and extracted in a nonpolar (1:1 ethyl acetate-methanol) followed by a polar (1:1 ethanol-water) solvent scheme. Resulting nonpolar crude extracts were separated using column chromatography followed by reverse-phase high-performance liquid chromatography (HPLC). This study documents the presence of compounds across a range of species from Fort Lauderdale collections. Previous studies detailed the bioassay-guided fractionation and nuclear magnetic resonance (NMR) methods used to isolate and elucidate structures of the compounds of interest (21, 34, 35, 48, 58). For comparisons among chemotypes, an Econosil (Alltech, Deerfield, IL) C_{18} 10-μm column was used with a solvent scheme consisting of methanol-water (80:20) run isocratically for 10 min followed by a linear gradient to 100% methanol over the course of 60 min.

16S rRNA gene sequencing and clone library construction. Cyanobacterial samples fixed in RNA*later* were further separated under the dissection microscope (10×) so that only the dominant filamentous morphotype was apparent in the sample. Approximately 50 mg of the filamentous material was used for nucleic acid purification. Genomic DNA was extracted from each sample using a protocol adapted from that of Preston et al. (50). RNA*later* was removed and replaced by 1 mg/ml lysozyme–TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0), and the samples were incubated at 37°C for 30 min. Proteinase K was added to a final

concentration of 0.5 mg/ml, and the samples were incubated at 55°C for 1 h, until the solution was transparent. To complete lysis, the sample was boiled for 60 s. After lysis, the DNeasy genomic extraction kit (Qiagen) bacterial DNA extraction protocol was used.

PCR with the cyanobacteria-specific forward primer 359F (5′-GGGGAATY TTCCGCAATGGG-3′) (41) and general eubacterial reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) was done under the following profile conditions: initial denaturation (3 min at 95°C); 35 cycles of denaturation (30 s at 95°C), annealing (1 min at 50°C), and elongation (1 min at 72°C); and a final extension step (7 min at 72°C). The resulting PCR fragment for each sample was cloned into a PCR 2.1 vector (Invitrogen), which was transformed into TOP10 cells (Invitrogen). Transformants were selected using Luria-Bertani plates containing 1 μg/ml kanamycin topspread with 50 ng/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. For each sample, inserts were amplified from three white colonies picked from the selective plates with plasmid-specific primers (M13F, 5′-GTAAAACGACGGCCAG-3′; M13R, 5′-CAGGAAACAGCTATGAC-3′ [Invitrogen]). Inserts were sequenced for full 2× sequence coverage, using the ABI BigDye version 3.1 sequencing mix.

For some samples, additional PCRs were required, pairing the cyanobacteria-specific forward primer 106F (5′-CGGACGGGTGAGTAACGCGTGA) with reverse primer 1492R (above). These PCR products were gel purified and cleaned using the Wizard PCR Preps system (Promega) and then were ligated into plasmids using the pGEM-T Easy vector system (Promega). For each sample, plasmids were harvested from at least three white colonies using the QIAprep Spin miniprep kit (Qiagen). Inserts were sequenced using two plasmid-specific primers (T7, 5′-TAATACGACTCACTATAGGG-3′; SP6, 5′-ATTTAG GTGACACTATAGAA-3′) and three cyanobacteria-specific primers (359F, above; 781F, 5′-AAWGGGATTAGATACCCCWGTAGTC-3′; 781R, 5′-GAC TACWGGGGTATCTAATCCCWTT-3′) (41).

For each clone, reads were assembled into contigs in Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI) and CodonCode Aligner 2.0.6 (CodonCode Corp., Dedham, MA). For each sample, contigs from at least three clones were aligned to construct a single inclusive consensus sequence. Individual clone sequences and sample consensus sequences were compared to sequences in the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp) and GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analyses. Consensus sequences were aligned using Clustal W, as implemented in CodonCode Aligner 2.0.6. GenBank BLAST searches identified the sequences most closely related to the consensus sequences for phylogenetic comparisons. Modeltest 3.7 (49) was used to select the best model of DNA substitution, the general time reversible model with an estimated proportion of invariable sites, and an estimated gamma distribution of variable substitution rates among sites (GTR+I+G).

Bayesian phylogenetic analyses were conducted by using MrBayes 3.1.2 (51) to calculate the posterior probabilities of branch nodes under the GTR+I+G model. The Monte Carlo Markov chain length was set at 3.5 million generations with sampling every 100th generation and a burn-in value of 8,750 cycles; the temperature parameter was set at 0.10. Convergence after 1.4 million generations was determined by an average standard deviation of split frequencies of <0.01 and by the values of all potential scale reduction factors equal to 1.00. Maximum likelihood (ML) phylogenetic analyses were performed by implementing the GTR+I+G model in GARLI 0.96 (65); data were resampled with 100 bootstrap replicates. Neighbor-joining (NJ) phylogenetic analyses were performed in MEGA 4.0 (56), using the maximum composite likelihood method with an estimated proportion of invariable sites and an estimated gamma distribution of variable substitution rates among sites; data were resampled using 1,000 bootstrap replicates.

Identification of curacin biosynthetic genes in *Lyngbya* spp. Filaments from the two most similar *Lyngbya* spp. were flash-frozen in liquid nitrogen and homogenized with a Dounce tool in microcentrifuge tubes. Homogenized cell powder was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and extracted three times with phenol-chloroform (1:1). The final aqueous layer was transferred to a new tube, and genomic DNA was precipitated with 95% ethanol, dried in air, and solubilized in TE buffer.

Three representative catalytic domains in the curacin A biosynthetic gene cluster (8) were selected as probes for the presence of curacin D biosynthetic genes in *Lyngbya* genomes: (i) a $GNAT_L$ domain in the CurA loading module; (ii) a C domain in the CurF NRPS module; (iii) an ST domain in the CurM chain termination module (8, 19). Degenerate primers were designed based on the conserved protein sequences in the following domains (restriction sites are underlined): (i) $GNAT_L$ (F), 5′-CATATGATHGTIGGIGCIATHTAY-3′, and (R), 5′-CTCGAGICCRTGDATYTGRTGRAA-3′; (ii) C domain (F), 5′-CATATGATHCARCARGCITAYTGG-3′, and (R), 5′-CTCGAGYTCRTTIACYT GIGGRTG-3′; (iii) ST (F), 5′-CATATGTTYAAYACIATGAARGAR-3′, and (R), 5′-CTCGAGRTAIGGRTTYTCYTC ICC-3′. PCR products were visualized by gel electrophoresis, purified from agarose gels by using a Wizard SV gel cleanup kit (Promega), and inserted into the pGEM-T Easy vector (Promega) for propagation. Insertions for PCR products of the $GNAT_L$, C domain, and ST genes were sequenced and compared to corresponding regions in the curacin A biosynthetic gene cluster (8).

Nucleotide sequence accession numbers. Consensus sequences were deposited in GenBank under accession numbers FJ602745 to FJ602753.

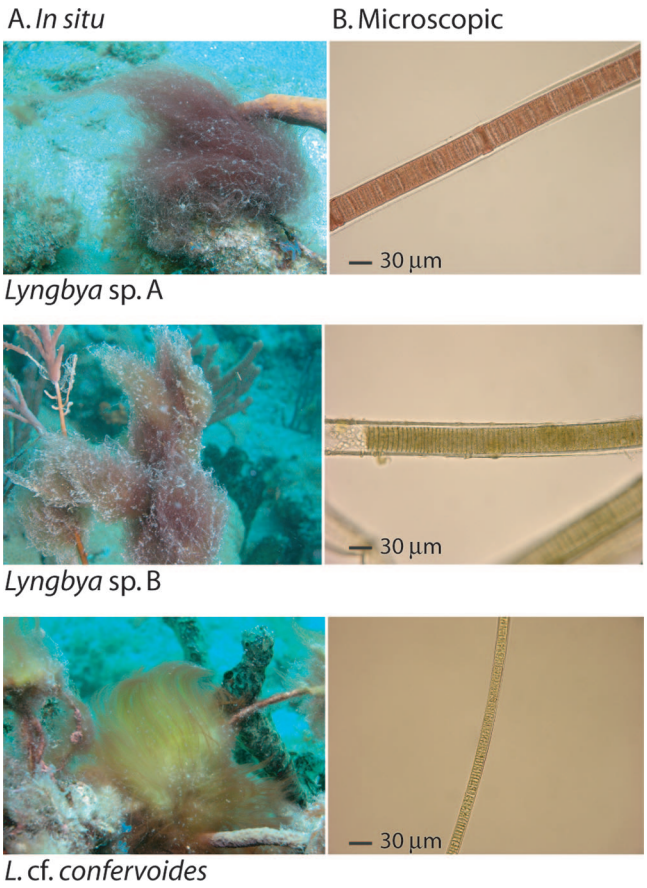

FIG. 1. *Lyngbya* spp. collected in Fort Lauderdale, FL. Each species, as identified based on morphological features, is shown in situ at the collection site (A) and in dissecting light micrographs (B).

RESULTS

Morphological variation. *Lyngbya* spp. were observed on Broward County hard bottom reefs during the spring and summer months of 2004 to 2007. Colonies of *Lyngbya* spp. appeared as dark red, brown, or black tufts ranging from 5 to 20 cm in length and grew attached to gorgonians, macroalgae, and hard or soft bottom substrates (Fig. 1). All samples were collected from reefs between 8 and 15 m in depth at light levels ranging from 20 to 30% of surface irradiation. Initially, based on field observations, the samples were identified as *Lyngbya* cf. *confervoides* (i.e., a *Lyngbya* sp. that looks like *Lyngbya confervoides*), which resembled fine, silky hair underwater, and what appeared to be two color morphs of *Lyngbya polychroa*, both of which were darker and coarser than the *L.* cf. *confervoides*. Underwater, the two *L. polychroa* color morphs appeared different from one another in both color and growth

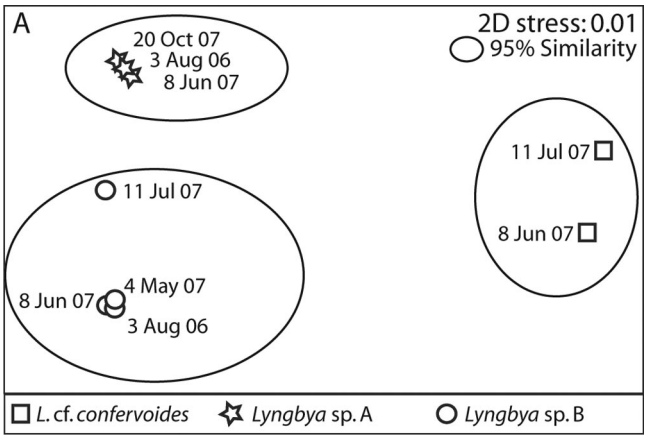

FIG. 2. Multivariate analysis of morphological characteristics of *Lyngbya* samples collected from Fort Lauderdale, FL. A multidimensional scaling plot of normalized cell measurements (filament width, cell width, and cell length) for each sample collected is shown.

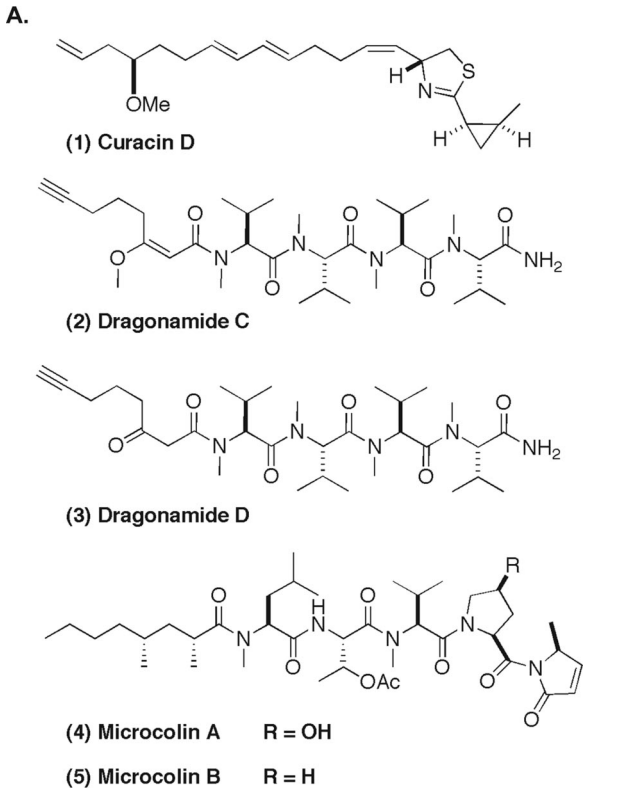

FIG. 3. Bioactive compounds from *Lyngbya* species in this study. (A) *Lyngbya* sp. strain B contains curacin D and dragonamides C and D. *Lyngbya* sp. strain A contains microcolins A and B. (B) *L.* cf. *confervoides* contains many compounds, including lyngbyastatins 4, 5, and 6, pompanopeptins A and B, and largamides A to H.

form. *Lyngbya* sp. strain A (formerly *Lyngbya polychroa* [red]) colonies appeared as long, loosely associated hair-like filaments, unlike *Lyngbya* sp. strain B (previously identified as *L. polychroa* [brown]), which appeared as clumps (Fig. 1).

Microscopic measurements demonstrated that *Lyngbya* sp. strain A and *Lyngbya* sp. strain B were similar in cell width. However, *Lyngbya* sp. strain B filaments had longer cells, appeared dark brown as opposed to red, and were often covered in epiphytic diatoms (Fig. 1). *Lyngbya* cf. *confervoides* had the narrowest filaments of the three chemotypes, with a smaller cell width-to-length ratio than either of the other two *Lyngbya* samples (Table 1; Fig. 1). Multivariate analysis based on the morphological characteristics placed the *Lyngbya* collections into three significantly different groups (SIMPROF, $P < 0.05$) (Fig. 2) with the *Lyngbya* sp. strain A and *Lyngbya* sp. strain B more similar to one another than to *L.* cf. *confervoides*.

Chemotypes of different *Lyngbya* collections. Several bioactive metabolites were isolated and identified from the three *Lyngbya* species (Fig. 3). The major products in *Lyngbya* sp. strain B were identified as curacin D and dragonamides C and D (21). Microcolins A and B were present in *Lyngbya* sp. strain A (Fig. 3A). The *L.* cf. *confervoides* collected in this study does not contain either curacins or microcolins; however, a diverse array of secondary metabolites has been isolated and identified from this species, including the lyngbyastatins 4 to 6 (35, 58), pompanopeptins A and B (34), largamides A to C (as revised by Matthew et al. [33]), and largamides D to H (48) (Fig. 3B).

Consistent differences in chemical composition between *Lyngbya* sp. A and B were observed upon HPLC (Fig. 4). All four collections of *Lyngbya* sp. strain A contained microcolins A and B, but they contained neither curacin D nor dragonamides. *Lyngbya* sp. strain B did not contain microcolins A and B, but two of the three samples (3 August 2006 and 20 October 2006) had curacin D and dragonamides. There was insufficient *Lyngbya* sp. strain B available from the 8 June 2007 collection to determine the chemical composition of this sample.

Identification of samples with 16S rRNA gene sequence. A 1,000-bp fragment from the 16S rRNA gene was sequenced for identification of the cyanobacterial chemotypes. At least three clones per sample of each chemotype were sequenced to ensure the identification of the dominant ribotype in the sample. All recovered sequences matched most closely to a previously described species from the family *Oscillatoriales* (Fig. 5). The two samples identified as *L.* cf. *confervoides* were very closely related to each other (<0.4% pairwise sequence divergence) and to a previously described *L.* cf. *confervoides* strain collected from the same location in Florida (46). Three samples of the curacin D-producing chemotypes collected in this study, denoted as *Lyngbya* sp. strain B, fell into a group of cyanobacteria previously identified as *Lyngbya majuscula*, including *L. majuscula* 3L, originally isolated as a curacin A producer (17). In contrast, sequences from four samples of the *Lyngbya* sp. A strains were more closely related to cyanobacteria identified as *Oscillatoria* sp. strain PAB-21, a strain shown to produce the antimalarial venturamides (29), and *Oscillatoria nigroviridis* 3LOSC.

For each sample but one, sequences of the recovered clones showed less than 1% pairwise sequence divergence across the 1,000-bp fragment of the 16S rRNA gene. In one sample, *Lyngbya* sp. strain A from 11 July 2007, four of the eight cloned sequences matched sequences from the other *Lyngbya* sp. strain A samples with less than 1% pairwise sequence divergence, while the remaining sequences did not match closely to either *Lyngbya* sp. strain A or *Lyngbya* sp. strain B and were instead more similar to sequences from uncultured environmental bacteria.

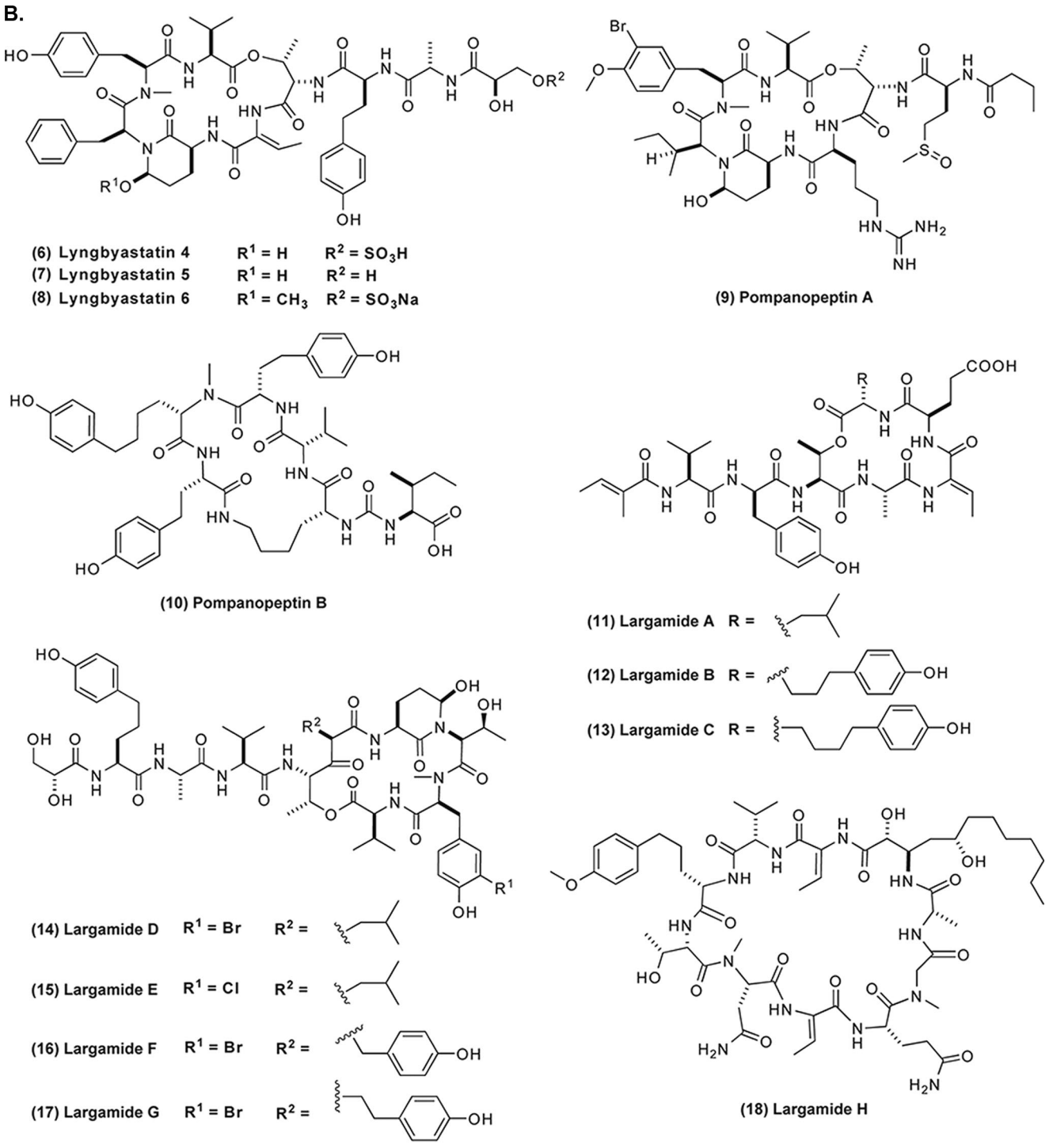

FIG. 3—*Continued.*

Identification of curacin biosynthetic genes in *L. polychroa*. Genomic DNA from each of seven *Lyngbya* sp. strain A and B samples were surveyed by PCR for the presence of genes in the curacin D biosynthetic gene cluster. Three pairs of degenerate primers were designed based on the conserved sequences in three essential catalytic domains in the curacin A pathway (8), including CurA $GNAT_L$ (GCN5-related acetyltransferase-like decarboxylase/*S*-acetyltransferase) (19), CurF C (condensation) domain, and CurM ST (sulfotransferase), which are responsible for the chain initiation, thiazoline ring formation, and decarboxylative chain termination of curacin A, respectively. Each of the *Lyngbya* sp. strain B samples yielded PCR products with expected sizes for all three primer pairs (Fig. 6). The resulting products were sequenced and compared with the

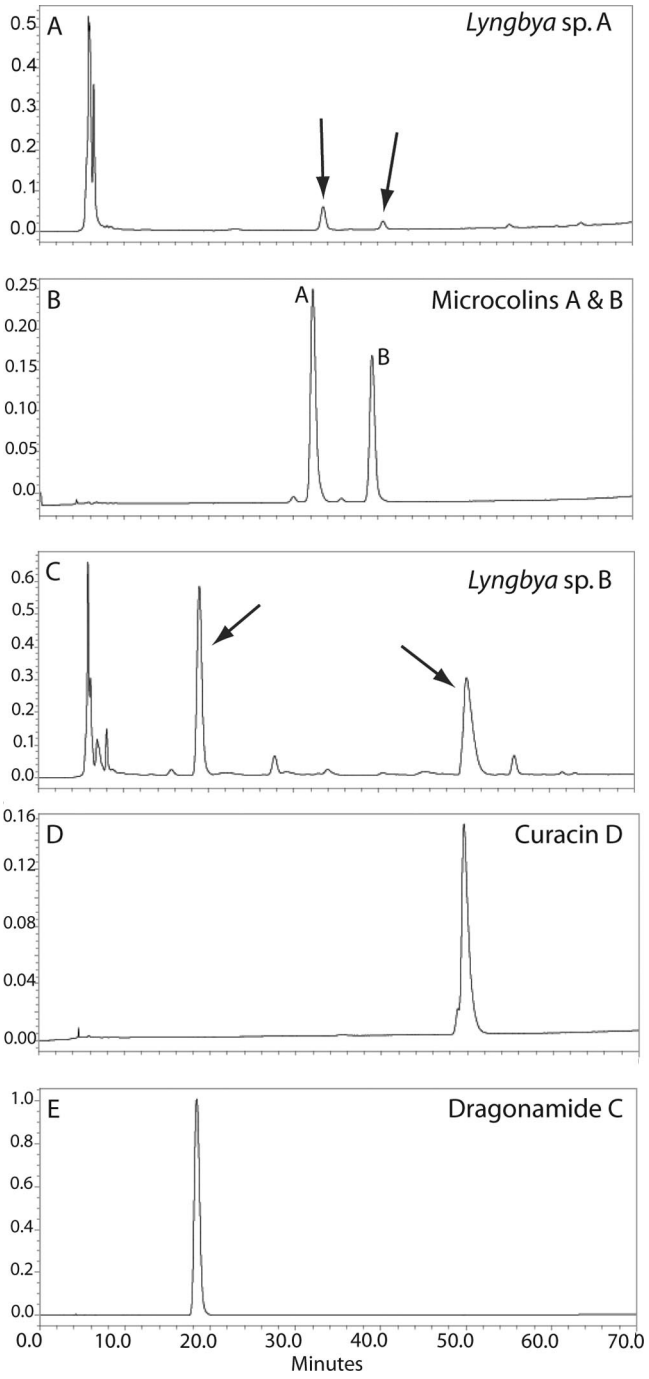

FIG. 4. Chemical differences between *Lyngbya* sp. strain A and *Lyngbya* sp. strain B. (A) HPLC chromatograms of a crude extract from *Lyngbya* sp. strain A, showing the presence of microcolins A and B (arrows). (B) Pure microcolins A and B. (C) Crude extract from *Lyngbya* sp. strain B, showing the presence of curacin D and dragonamide C. (D) Pure curacin D. (E) Pure dragonamide C.

regions in curacin A biosynthetic genes. The encoded amino acid sequences in $GNAT_L$ and ST domains are identical to those of the curacin A pathway, and only one amino acid difference was found for the sequences in the C domains, indicating that the *Lyngbya* sp. strain B samples contain curacin biosynthetic genes. The CurA $GNAT_L$ and CurM ST gene fragments were not detected in any of the *Lyngbya* sp. strain A samples with these primers (Fig. 6). The primers targeting the CurM ST domain did amplify DNA from one of the *Lyngbya* sp. strain A samples, but the other two gene fragments were not detected in that sample.

DISCUSSION

In this study, we characterized nine collections of *Lyngbya* from one site by analyzing their morphologies, 16S rRNA gene sequences, and secondary metabolite compositions. Classification based on cell width, cell length, and sheath width showed that the Fort Lauderdale *Lyngbya* collections in this study fall into three groups, identified in this study as *L*. cf. *confervoides*, *Lyngbya* sp. strain A, and *Lyngbya* sp. strain B. Our analyses of 16S rRNA gene sequences are congruent with these morphological groupings and indicate that the three groups are genetically distinct from each other, with more than 8% pairwise sequence divergence among them. These results stress that *Lyngbya* species can be especially difficult to differentiate based on morphology alone. Cryptic species, or groups of species whose morphological similarities mask significant genetic variation among them, have been identified in other genera of cyanobacteria. For example, Casamatta et al. (6) demonstrated that the freshwater cyanobacterium *Phormidium retzii* represents several cryptic species and suggested that cryptic speciation is likely to be common among cyanobacteria. Our results with the *Lyngbya* sp. strain A collection from 11 July 2007 further emphasize the importance of using cloning and DNA sequencing techniques to identify the source of chemistry in a complex, environmental sample. In this sample, only four of the eight recovered clones matched the sequences from the other *Lyngbya* sp. strain A collections. The cyanobacterial mat for this sample likely contained other bacteria and cyanobacteria in a multispecies assemblage. A combination of DNA sequencing and microscopic analysis is essential for the accurate identification and detection of species.

Lyngbya species and other marine cyanobacteria are prolific producers of diverse bioactive compounds with significant pharmaceutical applications, but some of the compounds can have detrimental impacts on benthic ecosystems. Each of the three morphologically and genetically distinct groups of *Lyngbya* samples consistently contained a distinct secondary metabolite profile. *Lyngbya* sp. strain A samples contained cytotoxic metabolites, primarily the microcolins A and B, which were previously found in *Lyngbya* samples collected in Venezuela (26). 16S rRNA gene sequences indicated that the *Lyngbya* sp. strain B samples are almost identical (>99.9% sequence identity) to the curacin A- to C-producing *L. majuscula* strains from Curaçao (17, 62), and they contain curacin D, a highly cytotoxic metabolite previously isolated from *L. majuscula* collected in the U.S. Virgin Islands (32). In addition, *Lyngbya* sp. strain B contains dragonamides C and D (21), which are closely related to dragonamides A and B found in a Panamanian strain of *L. majuscula* (36).

Microcolins A and B, first isolated from a *Lyngbya majuscula* strain collected in Venezuela, are lipopeptides that are of clinical interest for their potent inhibition of the mammalian murine mixed lymphocyte response and murine P-388 leukemia

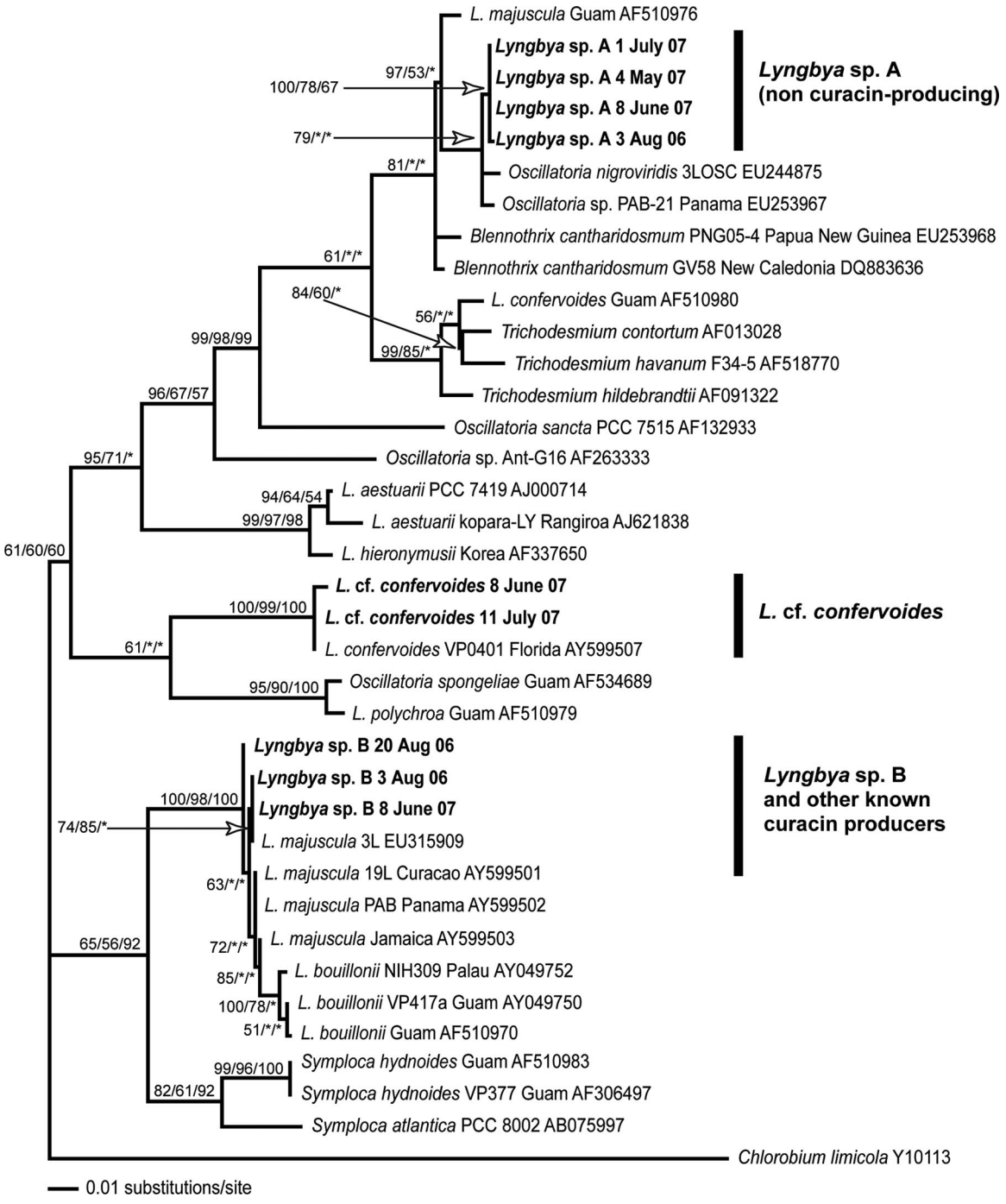

FIG. 5. Phylogram of 16S rRNA gene sequences from three chemotypes of bloom-forming *Lyngbya* spp. collected from Fort Lauderdale, FL. Labels on terminal nodes indicate the chemotype and date of sample collection; comparison sequences are identified according to their GenBank accession numbers. Bars on the right indicate the curacin-producing *Lyngbya* sp. strain B samples, the non-curacin-producing *Lyngbya* sp. strain A specimens, and *L*. cf. *confervoides*. Tree topology was constructed using Bayesian inference. Percentage Bayesian posterior probability (PP), ML bootstrap, and NJ bootstrap support values are shown at each node (PP/ML/NJ); values less than 50 are indicated by asterisks. Bar, 0.01 substitutions per site.

(26). In the marine environment, natural concentrations of microcolin B act as a feeding deterrent to *Stylocheilus longicauda*, a specialist predator of *L. majuscula* (38). The curacins are a group of mixed polyketide nonribosomal-peptide compounds with cytotoxic activity against several mammalian cancer cell lines (17, 32, 61, 62). Like many of the bioactive compounds identified from *Lyngbya* spp., the curacins and microcolins have been studied for their pharmaceutical and biotechnological applications, but very little is known about their ecological impacts on marine environments. Although

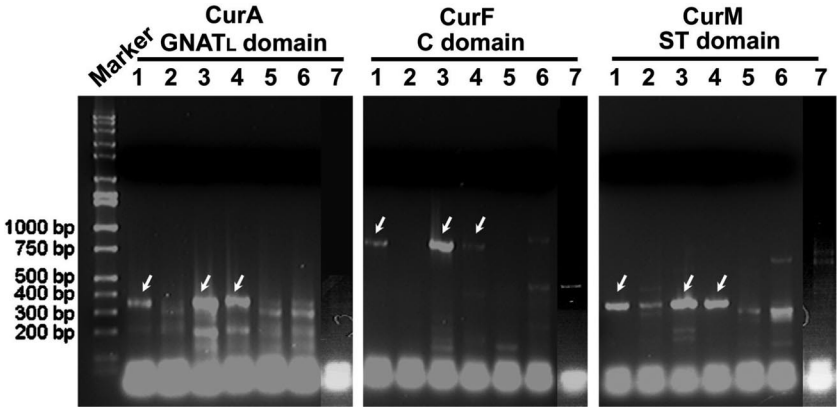

FIG. 6. Gel electrophoresis of PCR amplification products for detection of three different gene fragments in the curacin D biosynthetic cluster. CurA $GNAT_L$, primer pair for GCN5-related acetyltransferase-like decarboxylase/*S*-acetyltransferase; CurF C, primer pair for the condensation domain and CurM; ST, primer pair for sulfotransferase. Lanes 1 to 7 correspond to *Lyngbya* samples as follows: 1, 3 August 2006 (*Lyngbya* sp. strain B); 2, 3 August 2006 (*Lyngbya* sp. strain A); 3, 20 October 2006 (*Lyngbya* sp. strain B); 4, 8 June 2007 (*Lyngbya* sp. strain B); 5, 8 June 2007 (*Lyngbya* sp. strain A); 6, 11 July 2007 (*Lyngbya* sp. strain A); 7, 4 May 2007 (*Lyngbya* sp. strain A).

ecological activities of the dragonamides and curacins have not yet been demonstrated, it is likely that the compounds have an impact on sympatric species, including grazers, potentially altering the landscape of the reef habitat.

Although it was very similar in 16S rRNA gene sequence to *Lyngbya bouillonii* from Guam (accession number AF510970), *L. majuscula* from Jamaica (accession number AY599503), and *L. majuscula* from Panama (accession number AY599502), the curacin-producing *Lyngbya* sp. strain B in this study was morphologically distinct from these other strains, including the curacin-producing strain from Curaçao. It had thinner filaments, a longer cell length, a larger ratio of cell length to width, and thicker sheath than described for the Curaçao strain (15). None of these strains fit the morphology typical of *L. majuscula* with short cells 2 to 4 μm in length (16, 30). Sequence analysis of *Lyngbya* sp. strain A suggests that it is most closely related to *L. majuscula* from Guam (AF510976) and *Oscillatoria nigroviridis* (EU244875). Trichome width and cell length measurements of *Lyngbya* sp. strain A filaments were similar to those of *L.* cf. *confervoides* from Guam (AF510980) (59), but interestingly, the *Lyngbya* sp. strain A samples from this study have thinner trichomes than *L. majuscula* from Guam but have similar cell lengths (15).

Some of the compounds identified in *L.* cf. *confervoides* are protease inhibitors, which are widespread among cyanobacteria and are commonly regarded as digestion inhibitors due to their ability to inhibit trypsin and/or other digestive enzymes (3). Similar structures are found in taxonomically diverse cyanobacteria present in both freshwater and marine habitats, suggesting either an ancient biosynthetic origin for this class of compounds or horizontal gene transfer. For example, lyngbyastatins 4 to 6, pompanopeptin A, and largamides D to G are closely related to cyanopeptolins, planktopeptins, oscillapeptins, and scyptolin A, all of which are serine protease inhibitors from freshwater cyanobacteria (reviewed in reference 28). Pompanopeptin B is an analogue of the anabaenopeptins (37), the second prevalent class of cyanobacterial cyclopeptides from freshwater sources. Protease inhibitors in *Microcystis* spp. are often cosynthesized with microcystins and may enhance microcystin activity (39) or induce expression of the microcystin (*mcy*) gene cluster (52). Recently, it has been demonstrated that protease inhibitors have a significant ecological impact, controlling cyanobacterial population density and blooms by triggering viral lysis of cyanobacteria (52, 53).

Molecular detection of biosynthetic genes, commonly used to screen for the hepatotoxic microcystins and nodularins in freshwater cyanobacteria (reviewed in reference 47), is a powerful approach for documenting and tracking the potential for toxin production in the environment. Here, we present the first application for screening benthic marine cyanobacteria for secondary metabolite biosynthesis. Molecular surveys with PCR primers targeting curacin biosynthetic genes show that each of the *Lyngbya* sp. strain B samples collected from Fort Lauderdale reefs possesses homologues to major genes of the curacin biosynthetic cluster. HPLC and NMR showed that two out of the three *Lyngbya* sp. strain B samples produce curacin D. Although there was an insufficient amount available for chemical analysis of the third sample (8 June 2007), the presence of the curacin biosynthetic cluster in that sample indicates the capacity to produce curacin D. Other collections from the same site, which were previously thought to be a different color morph of the same species, possess neither the curacins nor a full suite of curacin biosynthetic genes, demonstrating that at least some of the chemical diversity in *Lyngbya* in the Fort Lauderdale reefs has a genomic basis and is not simply the product of shifting biosynthetic gene expression over time or in response to varying environmental conditions. This is consistent with previous findings on polychlorinated peptide production by the symbiotic cyanobacterium *Oscillatoria spongeliae* in the sponge *Dysidea herbacea*. Biosynthetic genes for the bioactive peptides are absent from *O. spongeliae* genomes in the populations of *D. herbacea* that do not possess polychlorinated peptides (15).

Several morphological species (including *L. majuscula* and *L.* cf. *confervoides*) are represented in multiple, genetically distinct clades in our molecular phylogeny, illustrating a common difficulty in studies that combine morphological and molecular phylogenetic approaches to cyanobacterial taxonomy.

This pattern could reflect a combination of high morphological plasticity and relatively high conservation of 16S rRNA gene sequences or the reverse, low morphological plasticity combined with high rRNA gene sequence variability. Other molecular markers can be used to characterize closely related cyanobacteria and increase fine-scale phylogenetic resolution, including the 16S-23S rRNA internal transcribed spacer region (14, 55). The use of such markers is recommended for future identification of cyanobacteria from environmental samples. Proper characterization of cyanobacteria, combining morphology and sequence analysis for identification, is critical for improving our understanding of the global patterns of natural product biosynthesis in the marine environment and will further clarify the relationship between biodiversity and chemical diversity within the *Oscillatoriales*.

In previous studies, *Lyngbya* spp. have been compared from several sites, spanning free-living and symbiotic strains from a wide range of locations (46, 59). Classification based on morphological characteristics, 16S rRNA gene sequence, and secondary metabolite traits has demonstrated a tremendous level of morphological plasticity and chemical diversity within the species *L. majuscula*, suggesting that a combination of environmental factors and genomic differences controls the production of bioactive compounds in *Lyngbya* (59). In this study, however, we characterized diversity among *Lyngbya* samples from a single site. Variations in morphological traits, 16S rRNA gene sequences, and bioactive compound profiles among the *Lyngbya* samples were congruent, suggesting a stronger genetic influence on compound production and a weaker environmental impact on biosynthetic gene expression. Additional studies on the environmental factors that may drive changes in *Lyngbya* community composition and secondary metabolite production are clearly needed to understand the mechanisms that ultimately control the distribution and diversity of cyanotoxins on coral reefs.

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