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LACUSTRINE *NOSTOC* (NOSTOCALES) AND ASSOCIATED MICROBIOME GENERATE A NEW TYPE OF MODERN CLOTTED MICROBIALITE[1]

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Microbialites are mineral formations formed by microbial communities that are often dominated by cyanobacteria. Carbonate microbialites, known from Proterozoic times through the present, are recognized for sequestering globally significant amounts of inorganic carbon. Recent ecological work has focused on microbial communities dominated by cyanobacteria that produce microbial mats and laminate microbialites (stromatolites). However, the taxonomic composition and functions of microbial communities that generate distinctive clotted microbialites (thrombolites) are less well understood. Here, microscopy and deep shotgun sequencing were used to characterize the microbiome (microbial taxa and their genomes) associated with a single cyanobacterial host linked by 16S sequences to *Nostoc commune* Vaucher ex Bornet & Flahault, which dominates abundant littoral clotted microbialites in shallow, subpolar, freshwater Laguna Larga in southern Chile. Microscopy and energy-dispersive X-ray spectroscopy suggested the hypothesis that adherent hollow carbonate spheres typical of the clotted microbialite begin development on the rigid curved outer surfaces of the *Nostoc* balls. A surface biofilm included >50 nonoxygenic bacterial genera (taxa other than *Nostoc*) that indicate diverse ecological functions. The Laguna Larga *Nostoc* microbiome included the sulfate reducers *Desulfomicrobium* and *Sulfospirillum* and genes encoding all known proteins specific to sulfate reduction, a process known to facilitate carbonate deposition by increasing pH. Sequences indicating presence of nostocalean and other types of nifH, nostocalean sulfide:ferredoxin oxidoreductase (indicating anoxygenic photosynthesis), and biosynthetic pathways for the secondary products scytonemin, mycosporine, and microviridin toxin were identified. These results allow comparisons with microbiota and microbiomes of other algae and illuminate biogeochemical roles of ancient microbialites.

Key index words: **microbialite; microbiome; *Nostoc*; sulfate reduction; X-ray spectroscopy**

Abbreviations: **EDS, energy-dispersive X-ray spectroscopy; ML, maximum likelihood; ORF, open reading frame**

The involvement of prokaryotic and eukaryotic algae in the formation of diverse types of sedimentary carbonates is important to the sequestration of inorganic carbon for very long periods of time (Graham et al. 2009). Cyanobacteria are thought to have generated the earliest algal carbonate deposits in the form of layered stromatolites, clotted thrombolites, and other formations collectively known as microbially induced sedimentary structures or microbialites (Burne and Moore 1987). Most micro-

bialite formations are largely composed of carbonates, although other mineral types occur (Riding 2011).

Cyanobacterial photosynthesis plays a role in microbialite formation by increasing ambient pH (Garcia-Pichel et al. 2004), thereby fostering carbonate precipitation, and extracellular polymeric substances produced by cyanobacteria and associated bacteria are thought to aid carbonate formation by binding Ca^{2+} (Van Lith et al. 2003, Braissant et al. 2007). Sulfate reducing bacteria may also be crucial to the formation of microbialites, because sulfate reduction fosters carbonate formation by increasing local pH and carbonate alkalinity (Dupraz and Visscher 2005, Baumgartner et al. 2006). The microbiomes, metagenomes, and other features of diverse modern cyanobacteria-dominated mats and microbialites are being studied as model systems expected to yield insights into the biotic interactions and formation of modern and ancient microbialites (Laval et al. 2000, Arp et al. 2001, Sheehan and Harris 2004, Breitbart et al. 2009, Couradeau et al. 2011, Harris et al. 2012, Oliver and Rowland 2002). For example, 16S amplicon sequencing of microbial mats in hypersaline salterns at Guerrero Negro (Mexico) revealed very high bacterial diversity (752 species identifiable at the 97% level, in 42 phyla, 15 of those new to science). In this model system the filamentous, nonheterocytous (nonheterocystous) genus *Microcoleus* (= *Coleofasciculus*; Siegesmund et al. 2008) was the dominant cyanobacterial component (Ley et al. 2006), as is the case for most marine mats (Green and Jahnke 2010).

Some modern freshwater lakes display large microbialite formations that are likewise regarded as model systems for decoding the fossil record. 16S clone library analysis revealed that mats whose cyanobacterial components were mostly *Pleurocapsa*-like coccoid forms promote carbonate precipitation to form giant cone-shaped microbialites in Lake Van, Turkey (Kempe et al. 1991, López-Garcia et al. 2005). *Calothrix,* a heterocytous cyanobacterial genus, and unidentified nonheterocytous filamentous cyanobacteria are associated with the formation of structurally similar microbialites in freshwater Pavilion Lake, British Columbia, Canada (Schulze-Makuch et al. 2013).

Although layered stromatolites extend back to the Archaean, clotted microbialites become abundant only later in the Neoproterozoic (Riding 2011), therefore representing a later-evolved microbialite type. Clotted microbialites also occur in more recent deposits known as "disaster forms," which are regarded as microbial responses to major extinction events (Mata and Bottjer 2012). Two previous SSU rDNA analyses of clotted microbialites (Airo 2010, Myshrall et al. 2010) indicated that nostocaleans are important in the formation of modern clotted microbialites, so the relatively late divergence of heterocytous cyanobacterial lineages (Schirrmeister

et al. 2013) might explain the later appearance of clotted microbialites in the fossil record. Modern clotted microbialites are known from marine and freshwater settings, and understanding their microbial ecology would inform our understanding of modern biogeochemical function as well as the fossil record.

Previous SSU rDNA analyses of clotted microbialites (Airo 2010, Myshrall et al. 2010) indicated the presence of diverse cyanobacterial components. Although vertically oriented, tapered filaments of heterocytous cyanobacteria identified as *Dichothrix* dominate button-size clotted microbialites in shallow marine systems (Highbourne Cay, Bahamas), 15 additional diverse cyanobacterial OTUs also occur (as do nonoxygenic Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Spirochaetes, and Verrucomicrobia; Myshrall et al. 2010). In a study of freshwater clotted microbialites, hemispherical colonies of vertically oriented, tapered, false-branching filaments having basal heterocytes, identified as the cyanobacterial genus *Rivularia*, were reported as the prominent components of clotted microbialite communities in Lago Sarmiento in southern Chile, although more than 60 additional cyanobacterial species were detected by SSU rDNA (in addition to Proteobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia; Airo 2010). The complexity of such cyanobacterial communities challenges efforts to understand how particular cyanobacterial species might influence microbialite structure and functional interactions between the oxygenic photosynthesizers and associated microbiota. While eukaryotic algae are known to provide diverse communities of epibiontic microbes with substratum, oxygen, and organic exudates (Amin et al. 2012, Zulkifly et al. 2012) and to receive growth-enhancing vitamins from bacterial associates (Croft et al. 2005), interactions among bacterial epibionts and individual cyanobacterial host species are poorly understood.

Here, we report the results of correlative microscopy, energy-dispersive X-ray spectros-copy (EDS), and shotgun pyrosequencing analyses of a simpler microbialite system in which a single cyanobacterial species, a conspicuous ball-forming *Nostoc* species linked by 16S sequence analysis to *N. commune* Vaucher ex Bornet & Flahault, hosts a community of >50 epibiontic bacterial genera having diverse physiological phenotypes. This newly discovered system reveals the first microbiome associated with a single, free-living cyanobacterial species. Microbiomes are inferred from metagenomic data that allow not only the identification of microbial taxa, but also analysis of their genomes, as has been accomplished for the endobiont *Prochloron* (Donia et al. 2011). The simplicity of the new *Nostoc* system facilitates inference of host-epibiont interactions, information that can be used to understand the physiological properties

and biogeochemical significance of ancient carbonates having similar architecture (e.g., Tang et al. 2013). The results also expand knowledge of algal microbiota, allowing comparisons that reveal common and distinctive features.

MATERIALS AND METHODS

Clotted microbialites textured by macroscopic cavities and whose surfaces featured numerous, conspicuous dark-brown ball-forming cyanobacteria were collected from the littoral zone of shallow, subpolar, freshwater Laguna Larga in southern Chile (51°01.544′ S, 72°52.556′ W). Laguna Larga is 207 m above sea level near Lago Sarmiento in Torres del Paine National Park and is considered oligotrophic (chl a <1.0 μg · L^{-1}; De los Rios and Soto 2009). Lakes in this area occur in a semi-arid steppe region over sandstones, siltstones, and conglomerates of the Cerro Toro Formation; the main source of water is precipitation from westerly winds (Solari et al. 2010). Laguna Larga is a small (<0.1 km^2) lake, whose pH was 9.62 at the time of sampling, and displays high conductivity (3,448 μS · cm^{-1}) arising from wind-induced evaporation (De los Rios and Soto 2009).

Sample handling and transport. A near-surface submerged microbialite ~5 cm by 3 cm, bearing brown *Nostoc* balls at the upper surface, was nudged into a transparent, sterile Nasco Whirl-Pack (Fort Atkinson, WI, USA) along with lake water sufficient to cover the rock before the bag was closed. During transport within Chile, the sample was regularly exposed to indirect natural light and not exposed to desiccation or temperatures below 10°C or above 27°C. Mid-way during transit, a second sterile Whirl-Pack was opened over the top of the sample bag, then the sample bag was opened to allow gas exchange for 24 h before being closed, still within the covering Whirl-Pack.

Microscopy and imaging. Cyanobacterial material was examined with the use of an Olympus BX-60 epifluorescence microscope equipped with UV filter set BP 36-370 DM400 BA420. For SEM, replicate *Nostoc* balls were briefly fixed with 2% glutaraldehyde in distilled water (to avoid adding ions that might influence EDS), followed by dehydration in an ethanol series. At the University of Wisconsin-Milwaukee Electron Microscopy Laboratory, cyanobacterial specimens were critical point dried, iridium-coated, and surfaces examined using a Hitachi S-4800 Ultra High Resolution Cold Cathode Field Emission SEM operated at 5 kV (Hitachi, Tokyo, Japan). Elemental mapping of Ca, Mg, and Si at *Nostoc* ball surfaces was accomplished by EDS at the same facility.

Mineral evaluation. Concentrated HCl was dripped onto a representative microbialite rock surface, using a glass pipette, to test for presence of carbonates. Carbonates are indicated by the appearance of a foaming reaction.

DNA extraction and shotgun pyrosequencing. DNA was extracted for shotgun pyrosequencing in the United States on the 11th day after collection using the FastDNA SPIN Kit for Soil (MPBio, Santa Ana, CA, USA), modified by adding 100 μL of lysozyme (100 mg · mL^{-1}) and sonicating for 10 min during the lysis step. Pyrosequencing is sequencing by synthesis; polymerase extension of a primed template releases inorganic phosphate that is used in formation of ATP that powers light emission by luciferin. Single nucleotides are added in each cycle. Fragmentation, library preparation (during which adaptors are added to fragments), immobilization of the library onto capture beads, emulsification of the library, amplification, loading of beads onto plate wells, and chemiluminescent sequencing (flow order TACG) were accomplished by UW-Madison Biotechnology Center personnel using Roche FLX+ chemistry (Roche, Madison, WI, USA). Average length of shotgun pyrosequencing reads was 562 bp, median length was 617 bp, and modal length was 700 bp; only reads greater than 200 bp were used in bioinformatic analyses.

Sequence processing and analysis. Demultiplexed sff files were used as input into SeqMan NGen 4 (DNASTAR, Madison, WI USA), and a metagenomic de novo assembly was performed using default parameters, except that unassembled reads and short contigs were saved. Output files were converted to FASTA format (when required) and combined into one multiFASTA file using seqret from the EMBOSS package (Rice et al. 2000). Both unassembled and assembled data were run through the RAMMCAP workflow through CAMERA2 (Sun et al. 2011). 16S sequences identified within the unassembled data by the RAMMCAP workflow were then run through the rRNA Taxonomy Binning workflow against the RDP (Ribosomal Database Project) Database, and also through CAMERA2. Bacterial classifications were typically accomplished by ascertaining sequence similarities at the 0.50 or 0.80 level, the latter representing the higher standard and the one used for inferring generic composition in this analysis. Outputs were analyzed within Microsoft Excel and MEGAN (Huson et al. 2011). The assembled data were used to create BLAST (NCBI) nucleotide databases against which query sequences representing genes of interest could be BLASTed locally. Sequences identified through the local BLAST were then BLASTed against the NCBI nr database to more accurately estimate their true identity. Contigs were annotated in SeqBuilder (DNASTAR) with rRNAs, tRNAs, and open reading frames (ORFs) identified by RAMMCAP. Using BLASTX, contigs were then compared against the NCBI nr database sequentially along their length to identify matches to known proteins. These matches were annotated onto the contigs, and the match information combined with ORF annotation was used to identify indels leading to frameshifts and false ORF annotation. Putative ORFs that BLASTed to a single gene of interest were aligned using the ClustalW algorithm in MEGA5 (Tamura et al. 2011) and the alignment was edited by hand to reflect known conserved residues. The alignment in turn was used in MEGA5 to create a maximum likelihood (ML) tree with 100 bootstraps. The entirety of the assembled data was compared locally against the NCBI nr database using BLASTX, and the output was used for a KEGG analysis in MEGAN for identification of metabolic pathways present in the metagenome.

To classify the host organism, the Laguna Larga microbialite 16S pyrosequencing reads classifiable to cyanobacteria were compared to reference sequences from public databases and then phylogenetically analyzed using ML, under the GTR + I + gamma model of sequence evolution (selected with ModelTest 3.7, Posada and Crandall 1998). Sequences known to be involved in both anaerobic and aerobic pathways for vitamin B$_{12}$ biosynthesis, nostocalean genes encoding the enzyme sulfide:ferredoxin oxidoreductase, the scytonemin biosynthetic pathway of *N. punctiforme* ATCC 29133 (Soule et al. 2007), cyanobacterial mycosporine biosynthetic pathways (Balskus and Walsh 2010, Kehr et al. 2011), and cyanobacterial toxin biosynthesis (Ziemert et al. 2010, Kehr et al. 2011, Dittmann et al. 2012) were used to search the Laguna Larga *Nostoc* metagenome for related sequences.

Sequence archiving. Reads classifiable to 16S have been deposited in the NCBI SRA (short read archive) http://www.ncbi.nlm.nih.gov/sra. The entire Laguna Larga *Nostoc* metagenome has been deposited with the CAMERA (Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis) data repository (https://portal.camera.calit2.net/gridsphere/gridsphere.)

RESULTS

The Laguna Larga microbialites displayed a foaming reaction when treated with concentrated HCl, demonstrating that the minerals were primarily composed of carbonate. Microbialite structure was distinctive in having a texture of adherent hollow carbonate spheres of diameters similar to those of associated *Nostoc* balls (Fig. 1). The internal diameter of the six largest spherical clot cavities visible from the external surface of one microbialite rock averaged 4.8 mm. The mean diameter of five cyanobacterial balls from the sample used for microbiome and microscopic analyses was 4.3 mm; the mean diameter of 10 of the larger balls shown in Figure 1 was 3.4 mm.

Light microscopy examination of the ball-forming cyanobacterium revealed unbranched nontapering filaments having numerous intercalary heterocytes (heterocysts), embedded in a rigid mucilaginous matrix. This morphology was consistent with the genus *Nostoc*, an identification confirmed by analysis of 16S sequences occurring within the microbiome. Of numerous 16S reads that were >200 bp in length and identified as cyanobacteria at the 0.8 confidence level, all but one clustered with *N. commune* (Fig. 2). No cyanobacterial morphotypes other than the host *Nostoc* were observed. The Laguna Larga microbialite microbiome included a *wspA* gene that encodes a 36 kDa water stress protein and sequences that in ML analysis grouped with the nitrogen fixation indicator gene *nifH* of other nostocaleans (*Anabaena*; Figure S1 in the Supporting Information). Diverse additional *nifH*-like sequences were also found in the metagenome (Figure S1).

Within the *Nostoc* balls collected at the Laguna Larga site, cyanobacterial filaments were loosely

Fig. 1. Carbonate clotted microbialites at Laguna Larga. Darkly pigmented, ball-forming cyanobacteria typically occur on surfaces of submerged microbialites.

assembled, but at the periphery, filaments were tightly packed and associated with nonfluorescent brown pigment (Fig. 3, A and B). Numerous loose heterocytes were observed. Inspection of the ball surface in UV excitation (Fig. 3C) revealed red fluorescence indicating chlorophyll autofluorescence of close-packed near-surface *Nostoc* filaments, and a conspicuous blue-fluorescent surface biofilm, corresponding with the occurrence of irregularly shaped carbonate crystals (Fig. 3, D–F). Clusters of longer, thinner crystals were present less commonly (Fig. 3, G and H). EDS revealed that crystals formed on cyanobacterial ball surfaces were rich in Ca and Mg, but Si mapped only to the occasional diatom (Fig. 3, I–K). Diverse bacterial morphotypes were revealed by SEM examination of the *Nostoc* surface (Fig. 4).

Within the Laguna Larga *Nostoc* microbiome, >50 genera of nonoxygenic bacteria could be identified by database sequence comparisons (Fig. 5). Ecologically significant functional features inferred for the Laguna Larga microbialite from the presence of classifiable 16S bacterial sequences included diazotrophy, anoxygenic photosynthesis, methanotrophy, phosphate accumulation, metal detoxification, and predation (see Table S1 in the Supporting Information). Many reads were found of 16S sequence indicating the sulfate-reducers *Desulfomicrobium* (Deltaproteobacteria) and *Sulfurospirillum* (Epsilonproteobacteria). The Laguna Larga *Nostoc* microbiome also included the following genes specific to the sulfate reduction pathway: CysJ (K00380), CysI (K00381), Sir (K00392), and CysH (K00390).

The majority of genes associated with anaerobic and aerobic vitamin B_{12} biosynthetic pathways were found in the metagenome; these included the key *cbiA* (anaerobic pathway) and *cobB* (aerobic pathway) genes that encode cobyrinic acid a,c-diamide synthase (Table S2 in the Supporting Information). Sequences similar to nostocalean genes encoding the anoxygenic photosynthesis-related enzyme sulfide:ferredoxin oxidoreductase were observed (Fig. S2 in the Supporting Information). The metagenome also contained sequences relatable to genes associated with cyanobacterial biosynthetic pathways for scytonemin, mycosporines, and microviridin (Table S3 in the Supporting Information).

16S analyses revealed only two eukaryotic chloroplast sequences, and then only at the 0.5 confidence level. Occasional diatoms (see Fig. 3I), other photosynthetic stramenopiles, flagellate haptophyte cells, and nonflagellate colonial green algae were observed via microscopy, as were at least one ciliate and a rotifer.

DISCUSSION

The results reported here indicate that a ball-forming *Nostoc* (linked by 16S sequence to *N. commune*) and its epibiontic community of anoxygenic
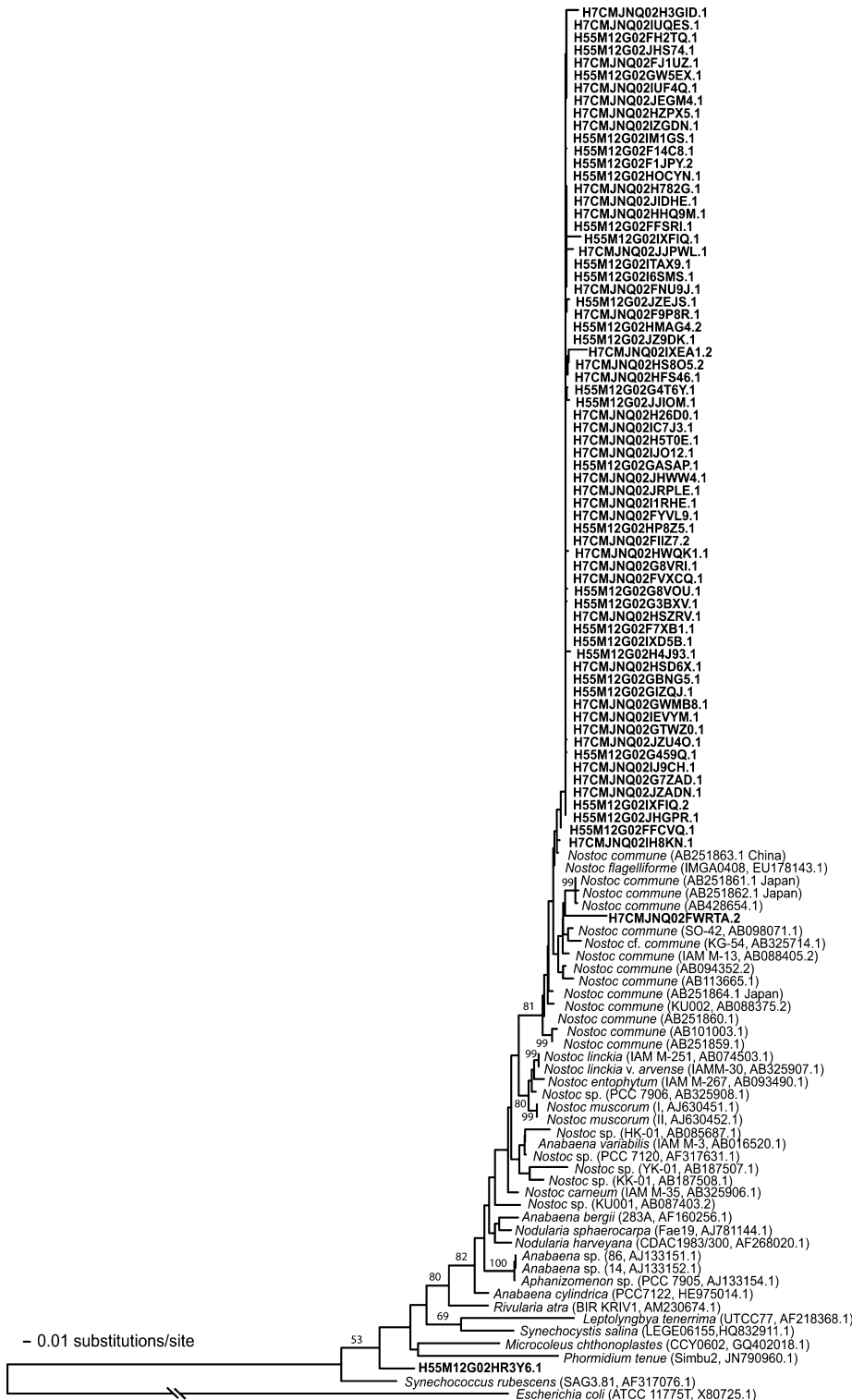
Fig. 2. Classification of host as *Nostoc commune* based on comparison of all microbiome 16S sequences clustering with cyanobacteria (boldface) to database sequences. Shown is one of 434601 best maximum likelihood trees of score lnL = −10,056.00, determined under GTR + I + gamma model of sequence evolution, rooted with *Escherichia coli* sequence. Taxon labels of published cyanobacterial data indicate corresponding cyanobacterial strains and GenBank accession numbers. Bootstrap (100 replicates) values over 50% indicated on nodes. Scale bar indicates expected number of substitutions per site.

bacteria together manufacture a new modern type of microbialite having structural similarities to certain ancient biogenic clotted carbonates. The Laguna Larga microbialite is distinctive among modern microbialites by association with a single cyanobacterial species. In contrast, other modern microbialites, including other studied clotted microbialites, display mixtures of cyanobacterial species and are not dominated by *Nostoc*. The simplicity of the *Nostoc* microbialite system fosters the inference of algal-microbial interactions, which are more difficult to disentangle in other modern microbialites characterized by multiple cyanobacterial species. The microbial community associated with
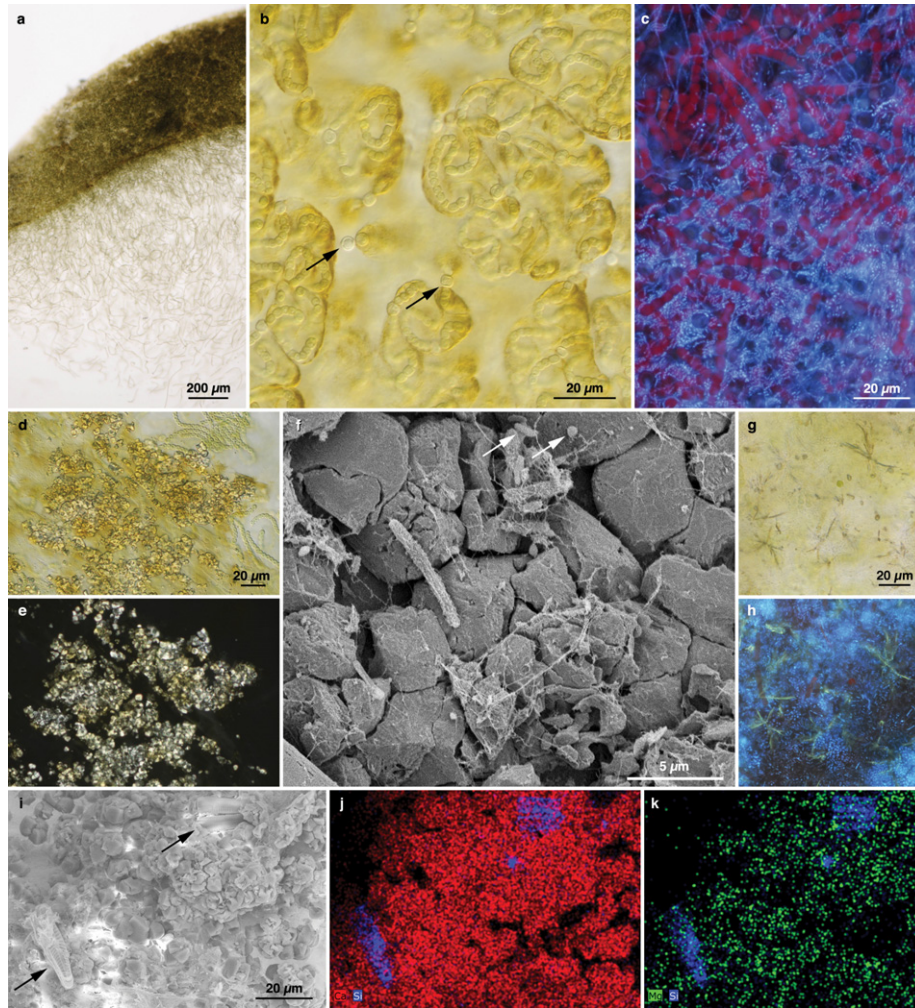
Fig. 3. Structure of Laguna Larga *Nostoc* ball and surface carbonates. (A) Low-magnification brightfield light microscopy (LM) view of small excised portion with firm, pigmented peripheral mucilage toward top; filaments more tightly packed in periphery of ball. (B) Differential Interference Contrast view looking through surface of mucilaginous matrix, showing tightly packed and contorted outermost *Nostoc* filaments. Intercalary heterocytes are evident (arrows). (C) Fluorescence of *Nostoc* ball surface in UV excitation, showing red chlorophyll autofluorescence of cyanobacterial filaments within mucilage and blue fluorescence of calcite deposited between and onto surfaces of bacterial cells. (D) LM view of carbonate crystals on surface; *Nostoc* filaments at upper right corner. (E) Crossed polarizers show that surficial crystals are birefringent. (F) Scanning electron microscopy (SEM) view of irregularly shaped carbonate crystals and bacterial cells (arrows). (G) Bright-field view of longer, thinner carbonate crystals on surface of *Nostoc* ball. (H) Fluorescence view of longer, thinner carbonate crystals. (I) Reference backscatter SEM showing diatom frustules (arrows) amid carbonate crystals. (J) Energy-dispersive X-ray spectroscopy (EDS) image of field shown in I, revealing Ca distribution in red, Si in blue. (K) EDS image of field shown in I, revealing Mg distribution shown in green, Si in blue.

Laguna Larga *N. commune* includes >50 bacterial genera and genes indicating diverse functional phenotypes that extend algal biogeochemical impacts in surprising ways. The metagenome contains sequences indicating that the community is able to synthesize vitamin B_{12} and that the *Nostoc* host has the capacity for anoxygenic photosynthesis and the biosynthesis of scytonemin, mycosporines, and microviridin.

Host features. Based on 16S data, presence of *nifH* sequence characteristic of Nostocales but not other cyanobacterial types, and a *wspA* sequence known to occur only in *N. commune* and one other *Nostoc* species (Arima et al. 2012), we conclude that Laguna Larga microbialite cyanobacterial populations were dominated by a single *Nostoc* species closely related to *N. commune*. Although *N. commune* is considered to be a terrestrial species (Řeháková et al. 2007, Arima et al. 2012), the Laguna Larga *Nostoc* was observed only on submerged carbonates. The brown pigmentation typical of Laguna Larga *Nostoc* likely represents the indole-alkaloid ultraviolet A (UVA) sunscreen scytonemin, which is widely produced by cyanobacteria in response to UVA exposure (Gao and Garcia-Pichel 2011). This hypothesis is supported by presence in the metagenome of genes associated with the scytonemin biosynthetic pathway in the *Nostoc* metagenome. Production of scytone-
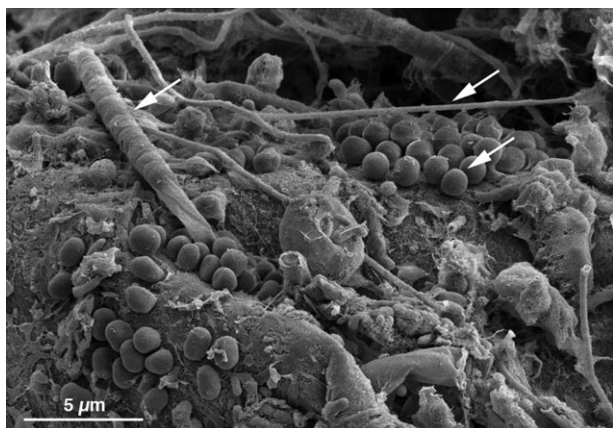
Fig. 4. Representative scanning electron microscopy of *Nostoc* ball surface showing diverse bacterial morphotypes (arrows).

min is consistent with relatively high latitude, littoral habitat.

Similar dimensions of microbialite clot cavities and *Nostoc* balls suggest the hypothesis that deposition of carbonates onto surfaces of the ball-forming cyanobacteria is responsible for clot architecture. This hypothesis is supported by our observation of fluorescent material associated with bacteria on *Nostoc* ball surfaces having fluorescence features similar to calcite deposited on the outer surfaces of other bacteria (Yoshida et al. 2010) and evidence that diverse bacterial types precipitate carbonate on their surfaces in alkaline conditions (Zamarreño et al. 2009). Further evidence that carbonates are precipitated on the surfaces of cyanobacterial balls is our observation on *Nostoc* surfaces of patches of mineral crystals having LM and SEM features similar to those known for calcite produced in stromatolites (Spadafora et al. 2010) and cave environments (Banks et al. 2010), as well as the bright (birefringent) appearance of ball surfaces when examined with crossed polarizers. In Laguna Larga, HCO_3^- has been determined to occur at nearly 3,000 mg $\cdot$ L^{-1}, Ca^{2+} 20.6 mg $\cdot$ L^{-1}, and Mg 470 mg $\cdot$ L^{-1} (De los Rios and Soto 2009). Together, these observations support the hypothesis that surfaces of *Nostoc* balls serve as templates for the structurally distinctive Laguna Larga clotted microbialites and suggest the likelihood that epibiontic bacteria aid carbonate precipitation. The occurrence of Mesoproterozoic clotted microbialites displaying similar clot sizes (Tang et al. 2013), suggests that ancient carbonate formations might likewise have formed on the surfaces of ball-forming nostocaleans.

Laguna Larga Nostoc *microbiota.* Consistent with Laguna Larga sulfate levels of 295 mg $\cdot$ L^{-1} (De los Rios and Soto 2009), presence in the modern *Nostoc* microbiome of *Sulfospirillum* (Sikorski et al. 2010) and another sulfate reducer, *Desulfomicrobium,* were inferred from 16S sequence. These data, together with evidence for the presence in the Laguna Larga

Nostoc microbiome of all known gene sequences associated with sulfate reduction, strongly suggest the occurrence of sulfate reduction, although expression analyses would be helpful in confirming this function. Sulfate reducing bacteria facilitate carbonate deposition by reducing sulfate ions thereby increasing alkalinity, consuming organic acids, and producing copious amounts of negatively charged exopolymeric substances that bind calcium and other metals (Braissant et al. 2007). The activities of sulfate reducing bacteria are considered important in interpreting the early record of biogenic carbonate rocks and also understanding modern global sulfur cycling.

Alphaproteobacterial nitrogen-fixing genera inferred to occur in the microbiome included *Rhizobium* and *Azospirillum* and possibly *Devosia,* based on the observation that *D. neptuniae* is an N-fixing symbiont in root nodules of the aquatic legume *Neptunia natans* (Rivas et al. 2002). ML analysis of *nifH*-like sequences in the Laguna Larga *Nostoc* microbiome revealed sequences that grouped with *Rhizobium leguminosarum* with a high level of support. Possible betaproteobacterial diazotrophs whose presence was inferred include *Ideonella* (Noar and Buckley 2009) and *Shinella* (Lin et al. 2008). Microbiome sequences clustering with known betaproteobacterial, epsilonproteobacterial, and gammaproteobacterial *nifH* genes were observed. These alphaproteobacterial, betaproteobacterial, epsilonproteobacterial, gammaproteobacterial, and cyanobacterial sequences include the majority of known *nifH* sequences and encode conventional FeMo nitrogenases (Gaby and Buckley 2011). We did not observe sequence evidence for alternative FeV and FeFe nitrogenases, but we did observe sequences related to *nifH* genes typical of anaerobes (Gaby and Buckley 2011) and of *Clostridium acidurici* 9a. Several microbiome sequences grouped with the nitrogen reductase genes of deltaproteobacterium *Desulfovibrio vulgaris,* which is interesting because *Desulfovibrio* is a sulfate reducer and several species are known nitrogen fixers. These observations suggest that the Laguna Larga microbiota may include taxa that likewise reduce both sulfate and N$_2$.

The inference that nitrogen-fixing anoxygenic bacteria associate with a nitrogen-fixing host (*Nostoc*) might seem surprising. Lacking anthropogenic input, Laguna Larga nitrate concentration has been determined to be 362 μg N $\cdot$ L^{-1}, ammonium 8.6 μg N $\cdot$ L^{-1}, and total N 38,400 μg N $\cdot$ L^{-1} (De los Rios and Soto 2009). Putative N-fixers were also inferred to occur in the microbiota of freshwater *Cladophora glomerata* sampled from a hypereutrophic lake that supports high populations of planktonic N-fixing cyanobacteria (Zulkifly et al. 2012), another remarkable location for epibiontic anoxygenic N-fixers.

The finding of sequence evidence for presence of the nitrogen-fixer *Azospirillum* (Alphaproteobacteria,
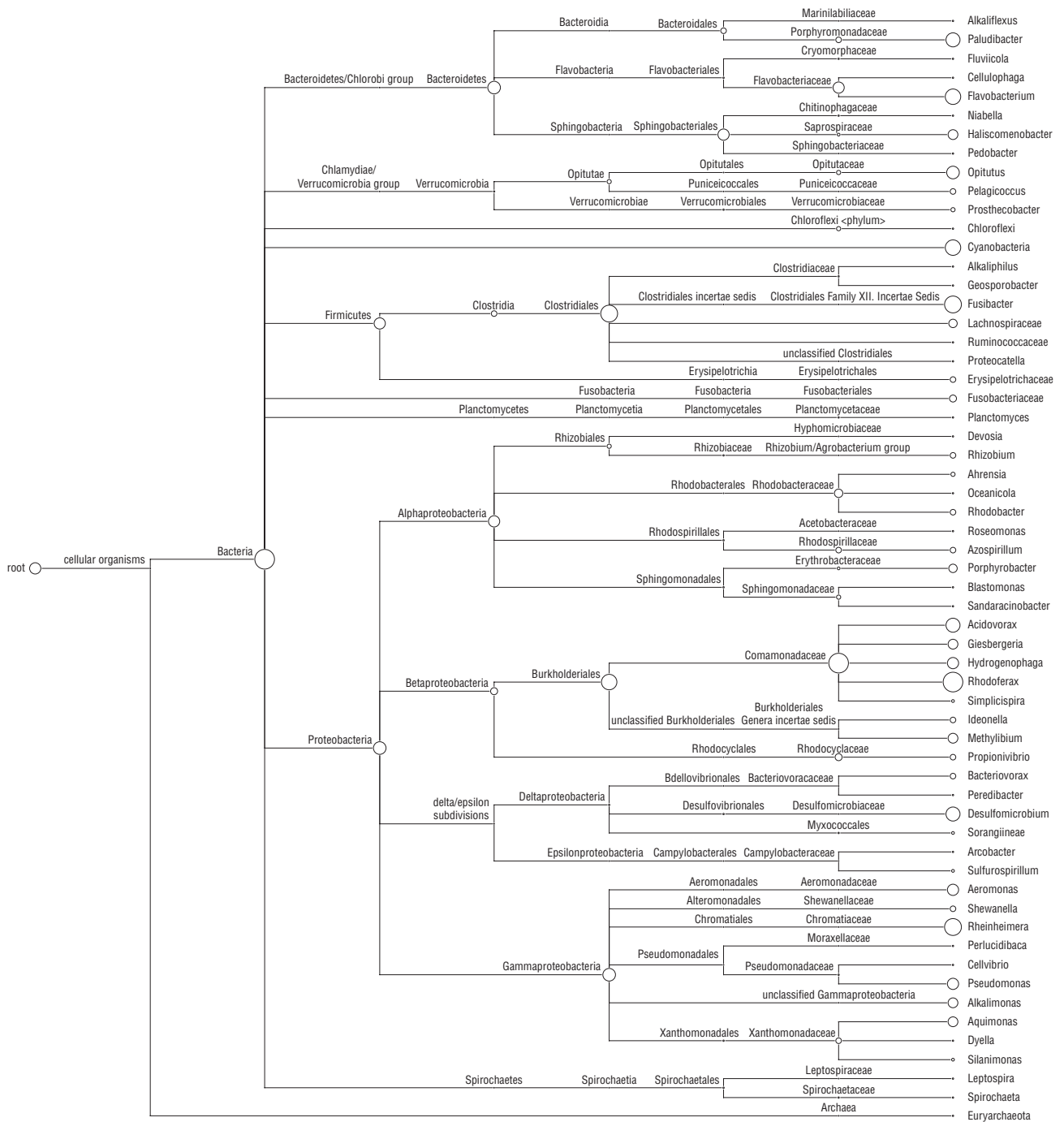
FIG. 5. Maximum likelihood phylogeny of bacterial taxa in Laguna Larga *Nostoc* microbiome that could be identified to genus at 0.80 confidence level with existing database resources. Diameters of circles represent relative numbers of reads.

Rhodospirillaceae) in the Laguna Larga *Nostoc* microbiome was notable because some authorities consider that modern *Azospirillum* occurs primarily with the roots of vascular land plants and that the evolutionary origin of this genus was tied to that of vascular plants (Wisniewski-Dyé et al. 2011). Since grasses are known to host *Azospirillum*, it is possible that *Azospirillum* washes into Laguna Larga from upland grassland (steppe) vegetation. However, the high

littoral wave action typical for this lake (De los Rios and Soto 2009) constantly agitates and washes *Nostoc* surfaces, in the process likely removing casual microbial associates. *Azospirillum* is known to possess pili that foster attachment to surfaces (Wisniewski-Dyé et al. 2011).

Several anoxygenic photosynthesizing members of Alphaproteobacteria were also inferred to occur in the *Nostoc* microbiome: *Rhodobacter* and *Porphyrobacter*

(which were also components of the microbiota of freshwater *Cladophora glomerata*; Zulkifly et al. 2012) as well as *Sandaracinobacter*. Many reads of photosynthetic *Rhodoferax* (Betaproteobacteria) were also observed. Association with littoral *Nostoc* keeps these photosynthetic taxa within the illuminated zone, and they likely contribute organic production to the community. Inferred presence of *Methylibium* (Betaproteobacteria) suggests methanotrophy, supported by oxygen exuded by the *Nostoc* host, as has been hypothesized for other freshwater algal microbiotas that include methanotrophs (Zulkifly et al. 2012). Many other obligate aerobes occur that would likewise benefit from *Nostoc* oxygen production.

Other ecologically significant functions inferred for the Laguna Larga microbialite microbiomes include metal reduction and detoxification, performed by *Shewanella* (Gammaproteobacteria), which is known to occur in marine environments, sometimes as an epibiont (Fredrickson et al. 2008), and phosphate accumulation suggested by presence of *Giesbergeria* (Betaproteobacteria; Grabovich et al. 2006). In the microbiota of the periphytic freshwater green alga *Cladophora*, *Gemmatimonas* may play a similar P-accumulation role (Zulkifly et al. 2012). The microbiome of Laguna Larga microbialites also included *Bacteriovorax* and *Peredibacter*, obligate predators on Gram-negative bacteria that are likely to influence community composition and function (Davidov and Jurkevitch 2004). Related *Bdellovibrio* was inferred to occur in the microbiome of the freshwater periphytic green alga *Cladophora glomerata* (Zulkifly et al. 2012). Together, these findings suggest that freshwater algal epibiontic microbial communities having high bacterial densities may generally include bacteria that function as predators.

Consistent with the high-alkalinity freshwater habitat, the Laguna Larga *Nostoc* microbiota contained species typical of both freshwater and marine habitats (see Table S1). Consistent with the high southern latitude location, the Laguna Larga *Nostoc* microbiota also included psychrophiles such as *Proteocatella*, which was first described from penguin guano in Chilean Patagonia (Pikuta et al. 2009).

Comparison of the Laguna Larga Nostoc *microbiome with microbial associates of other cyanobacteria.* Metagenomic data have been reported for *Prochloron* in a tunicate symbiosis (Donia et al. 2011), but unlike Laguna Larga *Nostoc*, which does not seem to include other cyanobacteria, *Prochloron* generally occurs with a variety of other cyanobacterial taxa, so it is more difficult to infer relationships between particular bacterial genera and cyanobacteria in that system. 16S-based studies of bacterial communities associated with free-living *Microcystis* have been done (Dziallas and Grossart 2011, Parveen et al. 2013) but those studies did not generate metagenomic data. Of the bacterial genera suggested to be associated with *Microcystis*, only *Rhizobium*, *Pseudomonas*, and *Flavobacterium* were also inferred to be present in the Laguna Larga *Nostoc* microbiome, and the Laguna Larga *Nostoc* microbiota did not include *Sphingomonas*, a common *Microcystis* associate (Dziallas and Grossart 2011). These limited data suggest absence of a common cyanobacterial microbiota, but additional studies of cyanobacterial microbiomes would be needed to test this hypothesis.

Laguna Larga Nostoc *metagenomic features.* Our observation that sequences related to the majority of genes associated with anaerobic and aerobic vitamin B_{12} biosynthetic pathways occur in the Laguna Larga *Nostoc* metagenome suggests that vitamin B_{12} can be produced by the microbiota, fostering microbialite growth. In previous studies (Bertrand et al. 2011), *cbiA* (anaerobic pathway) and *cobB* (aerobic pathway) that encode cobyrinic acid a,c-diamide synthase (both found in the *Nostoc* microbiome) have been used as amplification targets in studies focused on marine B_{12} producing bacteria. Even so, we did not identify particular bacterial genera known to possess complete vitamin B_{12} biosynthetic pathways.

The capacity for biosynthesis of the sunscreen molecule scytonemin is indicated not only by the dark color of the Laguna Larga *Nostoc*, but also by presence of metagenomic sequences closely related to genes known to be involved in scytonemin biosynthesis in *N. punctiforme* (Soule et al. 2007). Presence in the Laguna Larga *Nostoc* metagenome of sequences closely related to genes associated with biosynthesis of mycosporines (Balskus and Walsh 2010, Kehr et al. 2011) indicates additional capacity to resist UV damage.

Our observation in the Laguna Larga *Nostoc* metagenome of sequences similar to genes in the biosynthetic pathway for microviridin (Ziemert et al. 2010, Kehr et al. 2011, Dittmann et al. 2012) suggests that this cyanobacterium may be capable of cyanotoxin production. Microviridins are peptides having a cage structure, and most forms inhibit serine-type proteases; one isoform has been shown to inhibit molting in *Daphnia*, thereby leading to death (Rohrlack et al. 2004). We sought but did not find metagenomic evidence for biosynthesis of microcystin, nodularin, cylindrospermopsin, anatoxin-a, saxitoxin, lyngbyatoxin, anabaenopeptilide, aeruginosin, anabaenopeptin, barbamide, jamaicamide, curacin A, hectochlorin, nostopeptolide, nostocyclopeptide, cryptophycin, patellamide, or lantipeptide. Future expression studies would be helpful in determining the extent to which this *Nostoc* produces cyanotoxins and may be important in monitoring toxin occurrence in the natural habitat.

The diverse biochemical and ecological functionalities we inferred for the Laguna Larga *Nostoc* microbiome suggest that, today and in the geological past, cyanobacterial microbialites play more extensive ecological roles than inorganic carbon sequestration alone. Comparisons among freshwater algal microbiota (e.g., the data reported here and in Zulkifly et al. 2012) reveal both common and

distinctive features of ecological significance. In view of modern concerns regarding excess atmospheric CO_2 and methane, and recent interest in methods for sequestering CO_2 into carbonates and using methanotrophs to generate liquid fuels from methane, the microorganisms and genes described here may have useful biotechnological applications.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Laguna Larga *Nostoc* metagenomic *nifh*-like sequences indicated by dark green dots, in ML analysis together with database reference sequences indicated by other colored dots. The arrows point to metagenomic sequences clustering with known nostoclean *nifH* genes (Contig

2139.10, Metagene H7CMJNQ02GWNM5.1, and Contig 996.3).

Figure S2. ML analysis of Laguna Larga *Nostoc* metagenomic sequences (indicated by green dots) in relation to known cyanobacterial sulfide:ferredoxin oxidoreductase genes.

Table S1. Epibiontic bacterial genera inferred to occur on *Nostoc commune* in Laguna Larga and typical ecological function or habitat.

Table S2. Laguna Larga *Nostoc* metagenomic sequences related to genes encoding enzymes in anaerobic and aerobic vitamin B_{12} biosynthesis pathways.

Table S3. Sequences associated with biosynthesis of secondary metabolites that were sought in the Laguna Larga *Nostoc* metagenome. Colors indicate sequences found in the same contig. A needle alignment is provided for microviridin, the only cyanotoxin for which sequence evidence was found.