Research Paper

Molecular Diversity of Cyanobacteria Inhabiting Coniform Structures and Surrounding Mat in a Yellowstone Hot Spring

EVAN LAU, CODY Z. NASH, DETLEV R. VOGLER, and K.W. CULLINGS

ABSTRACT

Lithified coniform structures are common within cyanobacterial mats in Yellowstone National Park hot springs. It is unknown whether these structures and the mats from which they develop are inhabited by the same cyanobacterial populations. Denaturing gradient gel electrophoresis and sequencing and phylogenetic analysis of 16S rDNA was used to determine whether (1) three different morphological types of lithified coniform structures are inhabited by different cyanobacterial species, (2) these species are partitioned along a vertical gradient of these structures, and (3) lithified and non-lithified sections of mat are inhabited by different cyanobacterial species. Our results, based on multiple samplings, indicate that the cyanobacterial community compositions in the three lithified morphological types were identical and lacked any vertical differentiation. However, lithified and non-lithified portions of the same mat were inhabited by distinct and different populations of cyanobacteria. Cyanobacteria inhabiting lithified structures included at least one undefined Oscillatorialean taxon, which may represent the dominant cyanobacteria genus in lithified coniform stromatolites, Phormidium, three Synechococcus-like species, and two unknown cyanobacterial taxa. In contrast, the surrounding mats contained four closely related Synechococcus-like species. Our results indicate that the distribution of lithified coniform stromatolites may be dependent on the presence of one or more microorganisms, which are phylogenetically different from those inhabiting surrounding non-lithified mats. Key Words: Lithified coniform structures—Cyanobacteria—Hot spring stromatolites. Astrobiology 5, 83–92.

INTRODUCTION

Modern stromatolites are organosedimentary structures, composed of dense, laminated, benthic, and/or precipitating microbial communities that consist principally of cyanobacteria (e.g., Awramik, 1992; Stal, 1995). They may also be analogous to ancient fossil stromatolites that possibly date back to 3.5 billion years (e.g., Walter, 1983; Schopf and Packer, 1987; Hofmann et al., 1999). Fossil stromatolites are among the most tangible indications of life on early Earth.
and are presumed to have formed as a result of biologically mediated lithification (precipitation) of multiple layers of microbial mats (Schopf and Walter, 1983; Walter, 1994; Hofmann et al., 1999). Among early (presumably biogenic) stromatolitic fossils are coniform *Conophyton* (e.g., Vaslov, 1970; Hofmann et al., 1999), with distinctive conical and pseudocolumnar structures. Believed to have become extinct near the end of the Precambrian, extant structures resembling *Conophyton* have been recorded in the hot springs of Yellowstone National Park and the North Island of New Zealand, as well as in ice-covered lakes of Antarctica (Walter et al., 1976; Love et al., 1983; Wharton, 1994; Jones et al., 2001, 2002; Konhauser et al., 2001). The study of cyanobacterial communities inhabiting modern stromatolites that resemble *Conophyton* may shed light on the interplay between the microbial diversity of ancient lithifying microorganisms and the environment.

Many pH-neutral hot springs of Yellowstone National Park contain lithified coniform stromatolites that bear remarkable resemblance to formation and morphogenesis of these extant coniform stromatolites have been attributed, in part, to filamentous cyanobacteria dominated by the genus *Phormidium* (Walter et al., 1976; Cady and Farmer, 1996; Farmer et al., 1997). Earlier microscopic observations and isolation from several hot springs in Yellowstone National Park indicated that flat-topped columns may be dominated by one *Phormidium* species (*Phormidium truncatum var. thermalis*), while cones appeared to be dominated by another (*Phormidium tenue var. granuliferum*). In addition, other genera of cyanobacteria have been inconsistently observed in these structures, including *Synechococcus*, *Plectonema*, and *Isocystis* (Walter et al., 1976).

The non-lithified, higher temperature regions of hot spring cyanobacterial mats appear to be dominated by *Synechococcus* (see e.g., Ward et al., 1990, 1994; Weller et al., 1991; Ferris et al., 1996, 1997; Ramsing et al., 2000), and the lower temperature lithifying regions of hot spring mats appear to be dominated by *Phormidium* (Jones et al., 2002). While the former has been relatively well characterized, the same cannot be said of the latter. For example, it is not known whether any correlation exists between stromatolite morphology and the diversity of their component cyanobacterial community. Stromatolite formation in hot springs may be influenced by aspects of cyanobacterial physiology such as motility, temperature sensitivity, and orientation to light (Walter et al., 1976; Walter, 1983). In addition, stromatolite morphology may be determined by light availability and water flow rates, which vary with the depth of the submerged structures (Cady and Farmer, 1996; Jones et al., 2002). These differences can be attributed to cyanobacterial species forming "clumps" of filaments, from which stromatolitic morphological structures are believed to originate (Walter et al., 1976; Walter, 1983). Together these suggest that the distribution of coniform stromatolite is likely to be dependent on cyanobacterial species diversity and that different cyanobacterial species may stratify along a vertical gradient of the morphological structures.

Our study focused on an approximately 0.5 m² portion of the microbial mat in the outflow of Black Sand Pool of Yellowstone National Park. The two major siliceous and stromatolite-forming morphological types were (1) flat-topped columns and (2) columnar cones with and without ridges, prevalent in thermal outflows (described in Walter et al., 1976) (see Fig. 1). Recent molecular studies have shown that cyanobacterial structural and morphological characters are insufficient to distinguish between taxonomically similar taxa and species from the non-heterocystous filamentous Oscillatoriales (as defined by Anagnostidis and Komárek, 1988). These taxa, which include the taxonomic *Lyngbya-Phormidium-Plectonema* (LPP) group B (as designated by Rippka et al., 1979) and their close relatives, appear to be polyphyletic and cannot be distinguished by structural criteria alone (Wilmutte and Golubic, 1991; Castenholz, 1992; Wilmutte, 1994; Garcia-Pichel et al., 1998; Honda et al., 1999; Ishida et al., 2001; Turner et al., 1999; Jones et al., 2001; Wilmutte and Herdman, 2001). In addition, rapid silification of coniform stromatolites tends to destroy the morphological and structural characters of *Phormidium* filaments, which are crucial for taxonomic identification (Jones et al., 2002). Hence, molecular techniques based on polymerase chain reaction (PCR) amplification of cyanobacterial 16S rDNA, which are more reliable for identification of cyanobacteria in the absence of informative morphological characters, were used in this study.

In our study, primers specific for cyanobacterial 16S rDNA were used in nested PCR followed by denaturing gradient gel electrophoresis (DGGE) and phylogenetic analysis, to test null hypotheses regarding lithifying systems in Yellowstone hot springs. Three hypotheses, based in part on the observations of Walter et al. (1976) and Cady and Farmer (1996), were tested:
1. There are no differences between the cyanobacterial communities inhabiting lithified flat-topped columns and cones (ridged and columnar).
2. There are no differences in the vertical distribution of cyanobacterial communities within each lithified morphological structure.
3. There are no differences between the cyanobacterial communities inhabiting lithified morphological structures and the immediate surrounding non-lithified mat.

MATERIALS AND METHODS

Sample collection and DNA extraction

Black Sand Pool is located in the Punch Bowl Spring Group in the original Black Sand Basin of Yellowstone National Park. Multiple samples of the morphological structures from one of the outflows of Black Sand Pool (Fig. 1) were collected in October 2000. From Black Sand Pool, nine of each morphological structure types (for a total of 27 samples) were collected in sterile 1.5-ml polypropylene tubes, transported from the field in dry ice, and stored in a –80°C freezer. These samples were from (1) lithified flat-topped columns (3–4 cm long), (2) lithified columnar cones (1–2 cm long), and (3) lithified ridged cones (1–2 cm). These were later divided into “top,” “middle,” and “bottom” sections (for a total of 81 sections). Each section measured approximately 1.2 cm long and weighed between 0.3 and 0.5 g.

In June 2001, nine samples (three sets of three) of the non-lithified mat surface, which were within 30 cm of the morphological structures (square plugs measuring approximately 1 cm² and 2–3 mm thick), were collected in sterile 25-ml tubes. These plugs weighed approximately 0.2–0.4 g. Water depth (measured from bottom of morpho-
logical structures) ranged from approximately 5 to 20 cm, depending on water eruptions. Water temperatures measured from collection sites were between 58°C and 60°C. DNA was extracted using the FastPrep® (For Soil) bead beater method (Qbiogene, Inc., Carlsbad, CA). Each section of the morphological structure or mat plug was placed in MultiMix 2 Tissue Matrix tubes with 0.375 ml of sodium phosphate (120 mM) and 190 ml of sodium dodecyl sulfate solution (10% sodium dodecyl sulfate, 0.1 M sodium chloride, 0.5 M Tris/HCl at pH 8.0) (T. Norris, personal communication; adapted from More et al., 1994) and processed in the FastPrep at a speed of 6.5 for 45 s.

PCR, DGGE, and sequencing of partial 16S rDNA

PCR assays were conducted in PTC-100 (MJ Research, Watertown, MA) thermocyclers. We could not consistently PCR-amplify cyanobacterial 16S rDNA directly from environmental DNA extracts using the cyanobacteria-specific primers. Instead, PCR amplification using universal primers specific for the 16S rDNA of domain Bacteria, followed by band excision and extraction of amplified products from agarose gel, and a second, nested PCR using cyanobacterial 16S rDNA-specific primers was adopted. This method consistently produced PCR products from the environmental DNA extracts. Universal primers were GM5F (Muyzer et al., 1995) and 806r (Wilson et al., 1990; Relman et al., 1992). Reaction mixtures contained 1 X PCR buffer, 2.5 mM MgCl2, 200 mM final concentration of each deoxynucleotide triphosphate, a 400 mM final concentration of each primer, and 2.5 U of Taq polymerase (reaction mix from PCR kit, Roche Diagnostics, Indianapolis, IN) in a final volume of 50 ml. PCR (35 cycles) conditions were as follows: initial denaturation at 94°C for 1 min 25 s, with 13 cycles of denaturation for 35 s at 95°C, primer annealing for 55 s at 55°C, and extension for 45 s at 72°C, with another 13 cycles with extension time extended to 1 min, followed by an additional nine cycles with a 3-min extension, and a final 10-min extension at 72°C. Amplified DNA was electrophoresed in 1.5% NuSieve® GTG® agarose (Cambrex BioProducts, East Rutherford, NJ), and bands were excised and extracted (in TE buffer at 65°C for 15 min) for a second, nested PCR using primers specific for cyanobacterial 16S rDNA, CYA39F (Nübel et al., 1997), with a 40-base 5’ GC clamp incorporated, and CYA781R (Nübel et al., 1997). The cyanobacteria-specific PCR primers used in this study have been successfully employed to amplify cyanobacterial 16S rDNA from natural environments (see, e.g., Nübel et al., 1999, 2000; Garcia-Pichel et al., 2001; MacGregor et al., 2001; Abed and Garcia-Pichel, 2002; Abed et al., 2002; Grötzschel et al., 2002; Redfield et al., 2002), as well as numerous cultures of Phormidium-like organisms previously isolated from various Yelowstone hot springs (L. Jahnke, personal communication). PCR assays were carried out in 30 cycles under the same conditions mentioned above. PCR products were electrophoresed on a DGGE apparatus (Bio-Rad Laboratories, Hercules, CA) under 40–70% denaturing conditions for PCR products from lithified morphological structures only and under 20–80% denaturing conditions when PCR products from both lithified morphological structures and non-lithified mats were analyzed. DGGE gels were stained in ethidium bromide, and discernible bands from the community profiles of morphological structures and non-lithified mats were excised, reamplified, and sequenced in both directions via an ABI377 (ABI Perkin-Elmer, Foster City, CA) using rhodamine chemistry in the Baysdorfer Laboratory at California State University at Hayward, CA. Sequence alignment

Between 360 and 380 nucleotides of the 16S rDNA (with primer sequence omitted) were determined for bands R1A, R2A, R3A, R4A, R5A, and R6A from the lithified morphological structure community and M1A, M2A, M3A, and M4A from the non-lithified surrounding mat community. BLAST (Altschul et al., 1990) and Seqmatch (Cole et al., 2003) searches were conducted individually on the sequences. Calculations of nucleotide similarities were based on comparison of the entire DGGE cutout sequence (with primer sequences omitted) with sequences from GenBank via BLAST and PAUP version 4.0b10 (Swofford, 2002). Partial 16S rDNA sequences were syntactically superimposed on the stem-loop higher-order structure of the mature 16S rRNA molecule of a cyanobacteria (Weller et al., 1992) from positions (relative) 379–782 of Escherichia coli and checked by the CHECK CHIMERA program [at RDP-II (Cole et al., 2003)] to ensure they were
not chimeras. The 16S rDNA gene sequences of close relatives (up to 85% similarity based on BLAST search) of these sequences, as well as other representative cyanobacterial sequences, including members of the genera Phormidium, Lyngbya, Anabaena, Nostoc, Oscillatoria, and Trichodesmium, and E. coli (the out group), were downloaded from GenBank (Benson et al., 2003) and RDP-II (Cole et al., 2003) for alignment. All sequences were aligned and adjusted in Sequencer version 4.1 (Gene Codes Corp., Ann Arbor, MI).

Phylogenetic analysis

Phylogenetic trees were constructed using PAUP version 4.0b10 (Swofford, 2002) using neighbor joining and maximum parsimony. Of the 395 characters, 158 were parsimony informative. A heuristic search was performed in maximum parsimony phylogeny using tree tree bi-section reconnection. Starting trees were obtained by stepwise addition of sequences, with 100 random addition replicates, and multiple parsimonious trees were combined as a strict consensus tree. Bootstrapping (500 pseudoreplicates) was used to determine the significance of the clades under maximum parsimony. For neighbor-joining measures, a tree using systematically taxon-order-dependent break ties was used. The tree was inferred with 1,000 bootstrap pseudoreplicates. Both trees were rooted with E. coli as the out group.

Nucleotide sequence accession numbers

The nucleotide sequences for the partial 16S rDNA gene of bands R1A, R2A, R3A, R4A, R5A, R6A, M1A, M2A, M3A, and M4A were deposited in the GenBank (Benson et al., 2003) database and assigned the accession numbers AY354460–AY354469.

RESULTS

The DGGE band patterns (under 20–70% denaturing conditions) describing the cyanobacterial community composition in the top, middle, and bottom of all lithified flat-topped columns, columnar cones, and ridged cones collected in multiple replicates (81 DGGE profiles in all) were entirely identical. This indicated that there were no differences among cyanobacterial communities inhabiting the three types of lithified coniform structures. There were also no differences in the vertical distribution of cyanobacterial community composition in the structures, based on the profiles of top, middle, and bottom sections analyzed. A representation of the DGGE profile of the cyanobacterial community is shown in Fig. 2, with the six defined bands R1A–R6A indicated.

In contrast, DGGE analysis indicated a consistent difference in cyanobacterial community composition between lithified morphological structures and the immediately surrounding non-lithified mat (Fig. 3). Cyanobacterial community profiles of mats 1–6 (represented by the bands M1A, M2A, M3A, and M4A) were identical to one another and distinctly different from that of the lithified morphological structures. However, the cyanobacterial community profiles of mat samples 7–9 contained four of the six bands from the lithified morphological structure community (bands R1A, R2A, R3A, and R6A), as well as at least one band (band M1A) from the non-lithified mat community (see Fig. 3), and thus appeared to contain components of both lithified and non-lithified mat communities.

Subsequent phylogenetic analyses and BLAST (Altschul et al., 1990) search indicated that the sequences recovered from all the DGGE band cutouts were most closely related to either cul-

FIG. 2. Summary DGGE profile of the cyanobacterial community from the top section of a ridged cone, represented by the six discernible bands R1A, R2A, R3A, R4A, R5A, and R6A. This DGGE gel was run under 40–70% denaturing gradient conditions. The community profiles of all top, middle, and bottom of flat-topped columns, ridged cones, and columnar cones (81 profiles in all) were entirely identical and are not shown.
tured cyanobacteria or uncultured cyanobacterial clones detected in other hot springs (Fig. 4). Phylogenetic trees from both neighbor-joining and maximum parsimony analyses were similar in the placement of the DGGE cutout sequences and their close relatives. Bands M1A, M2A, M3A, and M4A, which represented the cyanobacterial in the non-lithified mat, shared a most recent common ancestor with other uncultured cyanobacterial clones from Yellowstone hot springs (with bootstrap values of up to 97). In contrast, the cyanobacterial community in lithified morphological structures was polyphyletic. Band R1A was most closely related to uncultured hot springs clones detected in cyanobacterial mats, while the closest known, cultured relatives of band R2A belong to genera from the filamentous Oscillatoriales—Leptolyngbya, Phormidium, Plecctonema, and Oscillatoria. In addition, results from BLAST and pairwise base differences by PAUP indicated that band R2A was 98% identical to clone 1, the closest known relative of Phormidium autumnale (see Norris et al., 2002). Bands R3A, R4A, and R5A shared most recent common ancestors with two separate but strongly supported monophyletic groups, which consist of Synechococcus species as well as uncultured cyanobacterial clones. Band R6A was not closely related to any sequences from the GenBank (Benson et al., 2003) database (<72% similarity), and branched separately from other known cyanobacteria.

DISCUSSION

Cyanobacteria are the principal components in the ecology of lithified morphological structures and mats in Yellowstone hot springs (Walter et al., 1976). Our data show that (1) the cyanobacterial species inhabiting the three lithified morphological types were identical, (2) there were no differences in cyanobacterial community composition in the vertical layers of lithified structures, and (3) the cyanobacterial community inhabiting all three morphological structure types were in fact distinctly different from the cyanobacterial species inhabiting the immediate surrounding non-lithified mats.

Our study indicated that cyanobacterial composition did not differ in vertical distribution along the lithified structures, even though the differences in light and water flow along this gradient have previously been shown to affect stromatolite morphology (Cady and Farmer, 1996;...
FIG. 4. Strict consensus tree of three equally parsimonious trees from maximum parsimony analysis [from PAUP version 4.0b10 (Swofford, 2002)] of partial 16S rDNA of bands excised from cyanobacterial community DGGE profiles, in comparison with that of other related cyanobacteria. Bands from lithified morphological structures (R1A, R2A, R3A, R4A, R5A, and R6A) are in bold, and labeled; bands from non-lithified mats (M1A, M2A, M3A, and M4A) are indicated in bold, and with * symbols. The Genbank (Benson et al, 2003) accession number of each taxa is in parentheses. Bootstrap values (500 replicates) are placed at the nodes of the branches if they were >50.
Jones et al., 2002). Previous microscopic and cultural studies by Walter et al. (1976) noted that different Phormidium species dominated columnar cones and columns, which implies that each morphological structure type may be characterized by a unique microbial assemblage. By contrast, our data show that the cyanobacterial community is more phylogenetically heterogeneous than microscopic observations suggest and that the cyanobacterial community composition among three morphological structure types is identical. The latter suggests that morphological structure types are influenced more by environmental factors than the composition of cyanobacterial species inhabiting them. Phylogenetic analysis indicated that the majority of the cyanobacteria inhabiting lithified structures within the mat were more heterogeneous than in the immediate surrounding non-lithified mats, and at least one of these cyanobacteria, represented by band R2A, appeared to be closely related to members of LPP group-B, which includes Phormidium. It is likely that band R2A (see Fig. 4) may indeed be a Phormidium-like species, the dominant species observed in coniform stromatolites (Walter et al., 1976; Jones et al., 2001, 2002), which is capable of forming columnar cones in the laboratory (Walter et al., 1976).

The techniques used in this study were qualitative; hence it is not possible to infer population numbers from our data. Nevertheless, our results indicated the consistent presence of six species, comprising at least one Oscillatorian species, which may belong to the genus Phormidium, three Synechococcus-like species, as well as two cyanobacterial species of unknown taxonomy, in the three types of lithified coniform structures. In contrast, the cyanobacterial community inhabiting the surrounding mats only contained an assemblage of four closely related Synechococcus-like species.

Interestingly, three mat samples (DGGE profiles 7–9, Fig. 2) consisted of certain cyanobacteria inhabiting both lithified morphological structures and non-lithified mat. We hypothesize that these samples of non-lithified mat contained microscopic “clumps” of filaments from which stromatolitic morphological structures would later form (Walter et al., 1976; Walter, 1983). The presence of species from both non-lithified mats and morphological structural communities in these samples may thus indicate a cyanobacterial community in the early stages of transition from non-lithified mat to lithified coniform morphological structure. We are currently studying cyanobacterial diversity during various stages of coniform formation, with measurements of hot spring outflow rates and chemical gradients to study the process of cyanobacterial colonization and their impact on the formation of lithified structures.

CONCLUSION

Our data show that while cyanobacterial communities inhabiting three distinct lithified coniform morphological structures were identical, they were entirely different from those within the immediately surrounding non-lithified mats from which they developed. This suggests greater phylogenetic diversity in these systems than implied by earlier culture-based and microscopic studies. These data may also aid the interpretation of the fossil record, where lithified and non-lithified mat formations have been observed in fossils of Precambrian origin (Hofmann et al., 1999). Finally, combining genetic studies with other methods, such as detection of cyanobacterial biomarkers in stromatolitic fossils (e.g., Brocks et al., 1999; Summons et al., 1999), should provide further insights into the ecology of microorganisms inhabiting biogenic Conophyton on early Earth.

ACKNOWLEDGMENTS

We would like to thank Tracy Norris for assistance in DNA extraction protocols, Christopher Baysdorfer for use of sequencing equipment at California State University, Hayward, and Nancy Hinman and employees of the National Park Service at Yellowstone for their housing and technical support. This study was funded in part by a research associateship from the National Research Council.

ABBREVIATIONS

DGGE, denaturing gradient gel electrophoresis; LPP, Lyngbya-Phormidium-Plectonema; PCR, polymerase chain reaction.
REFERENCES


Add the following text:

Address reprint requests to:

Dr. Evan Lau
Department of Organismic and Evolutionary Biology
Harvard University
16 Divinity Avenue
Cambridge, MA 02138

E-mail: elau@oeb.harvard.edu

LAU ET AL.