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Epilithic Cyanobacterial Communities of a Marine Tropical Beach Rock (Heron Island, Great Barrier Reef): Diversity and Diazotrophy[▽]

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The diversity and nitrogenase activity of epilithic marine microbes in a Holocene beach rock (Heron Island, Great Barrier Reef, Australia) with a proposed biological calcification "microbialite" origin were examined. Partial 16S rRNA gene sequences from the dominant mat (a coherent and layered pink-pigmented community spread over the beach rock) and biofilms (nonstratified, differently pigmented microbial communities of small shallow depressions) were retrieved using denaturing gradient gel electrophoresis (DGGE), and a clone library was retrieved from the dominant mat. The 16S rRNA gene sequences and morphological analyses revealed heterogeneity in the cyanobacterial distribution patterns. The nonheterocystous filamentous genus *Blennothrix* sp., phylogenetically related to *Lyngbya*, dominated the mat together with unidentified nonheterocystous filaments of members of the *Pseudanabaenaceae* and the unicellular genus *Chroococcidiopsis*. The dominance and three-dimensional intertwined distribution of these organisms were confirmed by nonintrusive scanning microscopy. In contrast, the less pronounced biofilms were dominated by the heterocystous cyanobacterial genus *Calothrix*, two unicellular *Entophysalis* morphotypes, *Lyngbya* spp., and members of the *Pseudanabaenaceae* family. *Cytophaga-Flavobacterium-Bacteroides* and *Alphaproteobacteria* phylotypes were also retrieved from the beach rock. The microbial diversity of the dominant mat was accompanied by high nocturnal nitrogenase activities (as determined by in situ acetylene reduction assays). A new DGGE *nifH* gene optimization approach for cyanobacterial nitrogen fixers showed that the sequences retrieved from the dominant mat were related to nonheterocystous uncultured cyanobacterial phylotypes, only distantly related to sequences of nitrogen-fixing cultured cyanobacteria. These data stress the occurrence and importance of nonheterocystous epilithic cyanobacteria, and it is hypothesized that such epilithic cyanobacteria are the principal nitrogen fixers of the Heron Island beach rock.

In recent decades the importance and ecological significance of marine cyanobacteria have become evident, and considerable attention has been paid to planktonic nitrogen fixers, such as the genus *Trichodesmium*, and more recently also to unicellular representatives (6, 15, 55). The marine benthic cyanobacteria that often form microbial mats have received considerably less attention (23, 47), and their diversity in tropical coastal areas remains largely unexplored (23).

Sandy beaches are ideal locations for stabilization and agglutination of sand grains and for precipitation of carbonate "cement." Intertidal lithification of beach sand and gravel via carbonate cementation results in formations termed "beach rocks" (20). These features are typical of many tropical and subtropical coastal zones. Organisms inhabiting such beach rocks must be resilient to extreme stresses, such drastic salinity changes, desiccation, and highly fluctuating light and UV regimens between the periods of full submersion (high tide). Hence, these organisms must possess numerous tolerance and adaptive mechanisms (14). In the tropics, rocky shores are dominated by primary producers such as cyanobacteria. Because they are photoautotrophic, oxygen evolving, and often nitrogen fixing (diazotrophic), cyanobacteria have competitive advantages and may also constitute the energy base of intertidal benthic food webs (32).

The Heron Island beach rock consists of microbialites and micritic aragonite cements. These structures may have formed as a result of biologically induced calcification within and beneath an organic biofilm in shallow, aphotic subsurface cavities in circulating seawater near the margin of the accreting reef (51, 52, 53). The microbial communities inhabiting the rock were apparently dominated by filamentous microorganisms capable of inducing carbonate formation and deposition, including formation and deposition of cements (51, 53). Several "zones" have been distinguished on the rock surface based on the distribution of diverse algae, cyanobacteria, and fungi, and cyanobacteria are most prevalent organisms (10). More recently, fluorescence signals originating primarily from cyanobacteria were detected on the beach rock, and rapid recovery of cyanobacterial photosynthesis was evident after rehydration (44). However, the only previous detailed study examining the occurrence of cyanobacterial diversity on the Heron Island beach rock was a morphological inventory conducted in 1966 (10).

To considerably increase our knowledge about the occurrence and role of prokaryotes in the biology of a tropical beach rock, a polyphasic approach was initiated using both phenotypic characteristics (determined by direct microscopic examination) and genotypic characteristics (determined by 16S rRNA PCR-denaturing gradient gel electrophoresis [DGGE]

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analysis) of the prokaryotic community, with an emphasis on cyanobacteria. The dominant stratified microbial community on the beach rock, referred to here as the dominant mat, was investigated further by constructing a 16S rRNA genetic library and by using scanning microscopy (confocal laser scanning microscopy [CLSM] and scanning electron microscopy [SEM]) to obtain the three-dimensional distribution of the microbial community. As beach rocks are often hostile and nutrient-poor environments (54), we expected the presence of nitrogen fixers. This was investigated by cyanobacterial *nifH* gene characterization and diurnal acetylene reduction (nitrogenase activity) assays in order to estimate the potential ecophysiological importance.

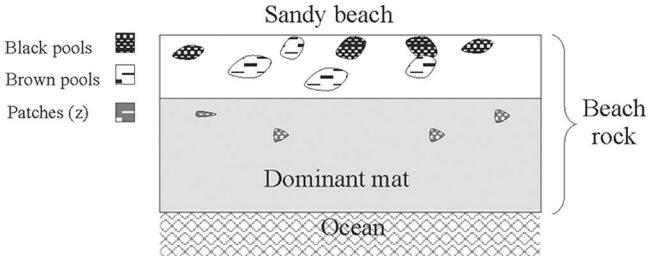

FIG. 1. Schematic drawing of a section of the Heron Island beach rock, indicating the relative locations of the main sampling sites, including black pools, brown pools, transition patches, and the dominant mat.

MATERIALS AND METHODS

Site description and sample collection. Heron Island is a carbonate sand cay that is 750 by 240 m. It belongs to the Great Barrier Reef group of islands and is located approximately 85 km northeast off the coast of Queensland (Australia) in the western Pacific Ocean. The average water temperature ranges from 20 to 26°C; the minimum water temperature is 15°C, and the maximum water temperature is 29°C. Rainfall occurs in January through March and in May and June. The beach rock is subject to a semidiurnal tidal regimen, with two high tides and two low tides each day. The mean tidal ranges are about 2 m (spring) and 1 m (neap).

Samples were collected in January 2003 from different zones (Fig. 1) of the most extensive and continuous beach rock (9 to 20 m wide and approximately 400 m long) located on the southern part of the east coast of Heron Island. The beach rock contains small pools that often retain water through the drier low tide (Fig. 1). Descriptions of the sampling site have also been given by Davies and Kinsey (11), Stephenson and Searles (48), Cribb (10), and Webb et al. (53).

Microbial biomass was collected primarily from the more pronounced microbial mat (thickness, ~2 mm) spread over and dominating the surface in middle portions of the beach rock and from differently pigmented shallow depressions or small pools ranging from 15 to 60 cm in diameter and with a maximum depth of approximately 12 cm, which were spread over the otherwise homogeneous dominant mat and were located mainly in the inner part of the beach rock (Fig. 1). The pools were covered by black-pigmented and brown-pigmented biofilms. A few samples were also retrieved from smaller transient "patches" within the dominant mat (Fig. 1). The dominant pink-pigmented mat and the pigmented biofilms in the depressions may correspond to the "*Kyrtuthrix maculans* band" and the "*Entophysalis deusta* band" described by Cribb in 1966 (10), respectively.

DNA was immediately extracted from the microbial samples, and the extracts were frozen at −70°C (see below). Cores were also collected, using a cork borer, from the microbial mats throughout diel cycles and incubated for measurement of nitrogen fixation (see below). After these analyses, the cores were placed on dry absorbent paper and stored frozen at −70°C for nucleic acid extraction or dried at room temperature for CLSM and SEM. In parallel, samples were fixed in 2.5% (final concentration) glutaraldehyde and stored at 4°C for light microscopy studies.

Light microscopy and morphological descriptions. The glutaraldehyde-fixed samples were used for light microscopy. A Zeiss Axiovert 200 microscope equipped with interference contrast was used, and images were recorded with an AxioCam HRC digital camera. Morphological identification of cyanobacteria was performed as described by Komárek and Anagnostidis (29, 30), Desikachary (12), and Silva and Pienaar (45).

CLSM and SEM. Rehydrated microbial mat cores were used for detailed examination of the three-dimensional distribution of microorganisms with a Leica confocal scanning laser microscope. A helium-neon laser generated the excitation wavelength, 633 nm. The three-dimensional images (sum of projections and stereoscopic images) were made up of several confocal optical sections through the sample obtained by computer-assisted microscopy.

Microbial mat cores were also used for SEM analyses. The cores were mounted on stubs after critical point drying, sputter coated with Au for 6 min in a E5100 high-resolution sputter coater (Polaron, England), and viewed with a Cambridge Stereoscan 260 electron microscope at 10 kV. The cyanobacteria were identified using morphological criteria (i.e., cell size and shape, filaments, and/or colonies).

DNA extraction, 16S rRNA DGGE, *nifH* DGGE, and phylogenetic affiliation. Nucleic acid extraction was initiated by freezing samples from the mat and biofilms in liquid nitrogen. This was followed by DNA extraction using a GenElute plant genomic DNA mini prep kit (Sigma). The integrity of the total DNA obtained was checked by agarose gel electrophoresis. Nucleic acid extracts were stored at −70°C until they were analyzed.

PCR amplification was performed using a hot-start *Taq* DNA polymerase (HotStar; QIAGEN), the general bacterial oligonucleotide primers B341F (with a 40-nucleotide GC clamp at the 5′ end) and 907R (35), and the cyanobacterium-specific 16S rRNA gene oligonucleotide primers CYA359F (with a 40-nucleotide GC clamp at the 5′ end) and CYA781R (36). These bacterial and cyanobacterial primer sets amplify about 585 and 446 bp, respectively. The PCR conditions used for the bacterial and cyanobacterial primer sets were those described previously by Muyzer et al. (35) and Nübel et al. (36), respectively. For PCR amplification of the *nifH* gene we used cyanobacterium-biased primers CNF and CNR described by Olson et al. (37), which amplify a fragment that is approximately 359 bp long. Primer CNF (with a 40-nucleotide GC clamp at the 5′ end [36]) and the CNR reverse primer were used for DGGE analyses. The PCR conditions comprised a denaturation step of 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. During the last cycle of both programs, the length of the extension step was increased to 7 min.

DGGE was carried out with a Dcode system (Bio-Rad) to reveal the partial 16S rRNA genes from bacterial and cyanobacterial communities present in the different samples, as well as the cyanobacterial *nifH* genes present in the dominant mat. Electrophoresis was performed at 75 V for 16 h in 0.75-mm-thick, 6% polyacrylamide gels (acrylamide/bisacrylamide ratio, 37.5:1) submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 60°C as described previously (13). Linear gradients of denaturing agents, 40 to 70% for 16S rRNA bacterial genes, 45 to 65% for 16S rRNA cyanobacterial genes, and 35 to 80% for *nifH* cyanobacterial genes, were used. After electrophoresis, the gel was stained in 1× TAE buffer containing SYBR gold nucleic acid stain (1:10,000 dilution; Molecular Probes), and the results were recorded using a fluorescent scanner (Typhoon 8600 variable mode imager; Amersham). The banding patterns revealed in the samples by the bacterial DGGE images were compared using the QuantityOne software (Bio-Rad). The number of DGGE bands present was considered to be the number of phylotypes in each sample. A dendrogram was constructed by cluster analysis using the software STATISTICA 6.0 by the City Block distance and Ward's method, taking into account the presence or absence as well as the relative contribution to the total amplified DNA of individual DGGE bands in all lanes.

In order to obtain the gene sequences of the DGGE bands, polyacrylamide fragments were excised from the gel using sterilized razor blades, resuspended in 20 μl of MilliQ water, and stored at 4°C overnight. An aliquot of the eluted DNA was used for PCR reamplification with the primers and conditions described above. The reamplified PCR products were sequenced (with the corresponding forward primer) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI PRISM model 377 (v.3.3) automated sequencer (DNA Technology, Denmark). 16S rRNA gene DGGE partial sequences were added to an alignment of about 50,000 homologous bacterial 16S rRNA gene primary structures as implemented in the ARB software package and corresponding to the database available at http//www.arb-home.de (33). In addition, the same sequences were compared with the available sequences in public databases by performing BLAST searches (2) in order to retrieve the most closely related sequences not included in the ARB database. All added sequences were automatically aligned with the alignment tool implemented in ARB and were corrected manually to improve the alignment. The backbone trees shown in Fig. 5 and 6 were initially reconstructed by using only complete or almost complete

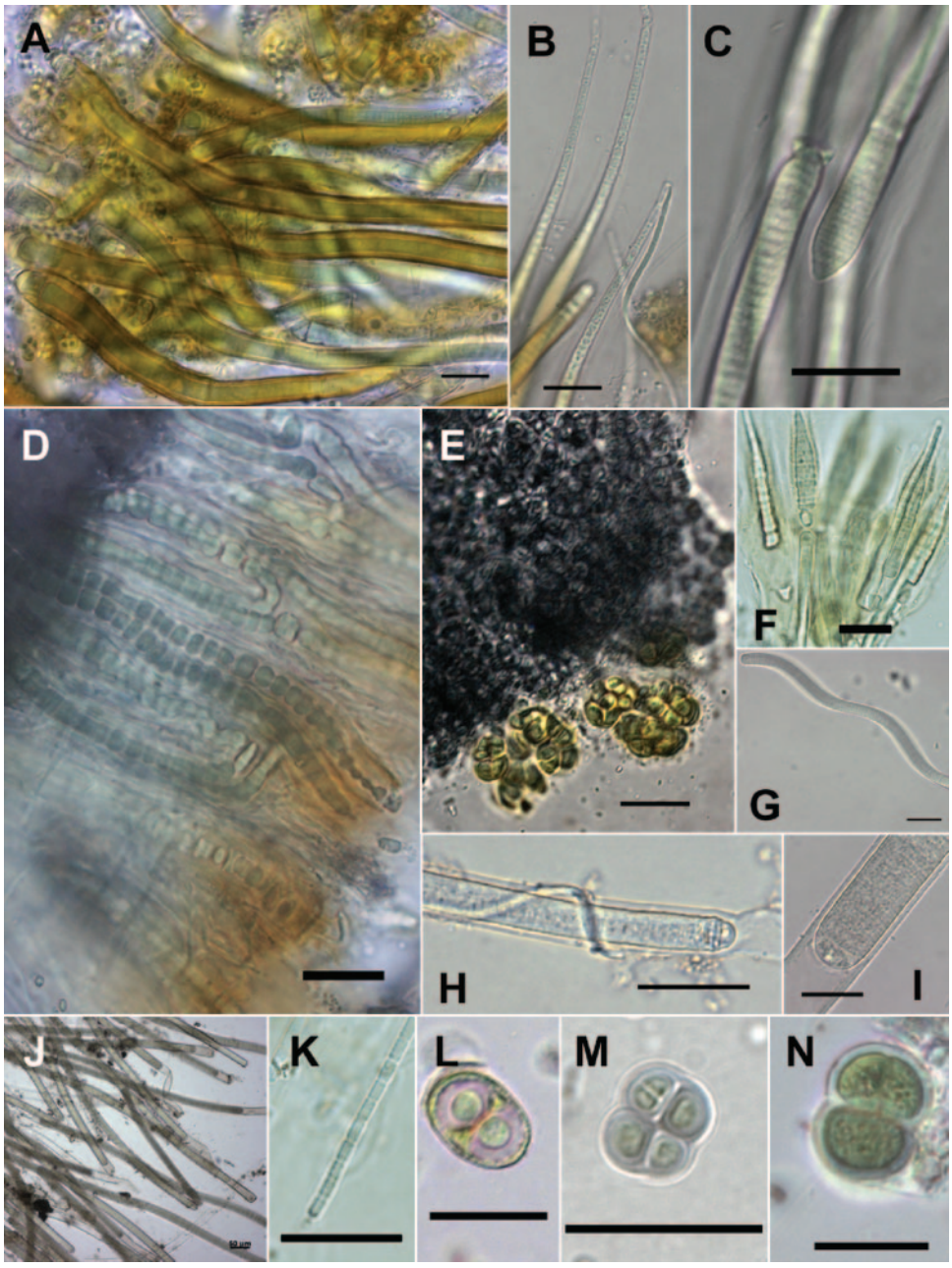

FIG. 2. Optical light microscopy micrographs of representative cyanobacterial morphotypes from the Heron Island beach rock. See Table 1. (A to G) Black-pigmented pool biofilms. (H to L) Brown-pigmented pool biofilms. (M and N) Dominant microbial mat. (A) *Calothrix* species 1. (B) Hair-like end cells of *Calothrix* species 1. (C) *Blennothrix* sp. (D) *Kyrtuthrix* sp. (E) *Enthophysalis* species 1 (brownish yellow) and 2 (blue-black). (F) *Calothrix* species 2. (G) *Arthrospira* sp. (H) *Lyngbya* species 2. (I) *Lyngbya* species 1. (J) *Lyngbya* species 1 bundle of filaments. (K) Unidentified member of the *Pseudanabaenaceae* family. (L) *Gloeocapsa* sp. (M) Unicellular cyanobacteria, possibly *Chroococcidiopsis* sp. (N) *Chroococcus* sp. Taxonomic classifications were determined as described by Komárek and Anagnostidis (29, 30) and Desikachary (12). (A to I and K to M) Scale bar = 20 μm. (J) Scale bar = 50 μm.

gene sequences. The trees were inferred using the neighbor-joining algorithm with the Jukes-Cantor correction. Then partial sequences were added to the existing trees by use of the Parsimony Tool as implemented in the ARB package (34) without allowing changes in the overall topology. Branch support was measured by bootstrap analysis using a parsimony search strategy with 1,000 bootstrap replicates. The 16S rRNA gene sequences of *Sulfolobus acidocaldarius* and one *Halobacteriaceae* strain for bacteria and *Escherichia coli* for cyanobacterial affiliations were used as outgroups. *nifH* DGGE sequences were compared with the available *nifH* sequences in public databases by performing BLAST searches (2). The novel sequences and the reference taxa were used for phylogenetic inference based on distance approximations by the neighbor-joining method and the Kimura two-parameter correction in Paup (version 4.0b10; Sinauer Associates, Inc.). A total of 1,000 bootstrap replicates were used for the tree shown in Fig. 7C. The *nifH* sequences of *Azospirillum brasilensis* and *Desulfovibrio vulgaris* were used as outgroups.

Cloning of the dominant mat, RFLP analysis, rRNA gene sequencing, and phylogenetic affiliation. Nucleic acid extraction was performed as described above. Partial 16S rRNA gene sequences from the dominant microbial mat were

TABLE 1. Taxonomic classification of the cyanobacteria found in the Heron Island beach rock, based on morphological characteristics[a]

Order	Family	Genus or species	Size	Morphology	Locations	Figure
Chroococcales (coccoid)	*Microcystaceae*	*Gloeocapsa* sp.[b]	Colonies 14 to 19 μm in diam, cells without sheaths approx 5 μm in diam	Cells spherical, colonies (two or more cells) surrounded by thick sheaths	br	2L
	Chroococcaceae	*Chroococcus* sp.[b]	Cells 10 to 14 μm in diam, sheath approx 1.5 μm thick	Cells subspherical or hemispherical, often two to four cells surrounded by sheath	m, z	2N
	Entophysalidaceae	*Entophysalis* species 1	Cells 3 to 4.5 μm in diam	Cells spherical to ellipsoidal, yellow-brown pigmented, forming large coherent colonies, sometimes in row-like arrangements surrounded by sheaths	bl, z	2E (lower part)
		Entophysalis species 2	Cells approx 2 μm in diam	Cells spherical to ellipsoidal, bluish black pigmented, forming large coherent colonies, often encountered together with *Entophysalis* species 1	bl, z	2E (upper part)
*Pleurocapsales** (coccoid)	*Xenococcaceae*	*Chroococcidiopsis* sp.[b]	Cells approx 1.4 to 4 μm in diam	Spherical to elliptical cells with distinct sheaths, single or few cells together forming colonies	m, z	2M
Oscillatoriales (trichal)	*Phormidiaceae*	*Arthrospira* sp.[b]	Filaments approx 6 to 7 μm wide, cells much shorter than wide	Filaments in loosely screw-like coils, approx 70 μm long and 15 μm wide	bl	2G
	Pseudanabaenaceae	Unidentified[b]	Cells approx 1.8 μm wide and twice as long	Trichomes constricted at cross walls, end cells rounded	bl, br	2K
	Oscillatoriaceae	*Blennothrix* sp.[b]	Trichomes approx 8 to 10 μm wide, cells much shorter than wide	Several light blue-green trichomes together in a sheath, end cells pointed	m, z, bl	2C
		Lyngbya species 1	Cells approx 17 μm wide and 1.8 μm long	Trichomes not constricted at cross walls, surrounded by sheath, open at the ends, end cells rounded	br	2I and J
		Lyngbya species 2	Cells approx 7.5 μm wide and 1.3 μm long	Trichomes not constricted at cross walls, surrounded by sheath, open at the ends; end cells rounded, slightly attenuated	br	2H
Nostocales (heterocystous)	*Scytonemataceae*	*Kyrtuthrix* sp.	Cells 4 to 5.5 μm wide and 4 to 5 μm long, heterocysts 6 to 7 μm in diam	Trichomes constricted at cross walls, heterocysts intercalary, some trichomes characteristically U-shaped and tapering towards the ends	bl	2D
	Rivulariaceae	*Calothrix* species 1	Filaments approx 16 to 17 μm wide at the base, cells approx 7 to 9 μm wide, length of cells varies along the filament, heterocysts 8 to 10 μm in diam	Basal heterocysts, long trichomes attenuated into thin hairs, single in thick brownish yellow-pigmented sheaths	bl	2A and B
		Calothrix species 2	Cells approx 7.5 μm wide and 1.5 μm long, heterocysts approx 4.5 μm in diam	Basal heterocysts, short trichomes, slightly swollen and tapering at one end, individually located in lamellate sheaths	bl	2F

[a] Organisms were classified as described by Komárek and Anagnostidis (29, 30) and Desikachary (12). The corresponding classification according to *Bergey's Manual of Systematic Bacteriology* (7) is indicated by an asterisk when the classifications differ. Location refers to the different sites where the samples were collected, as follows: z, patches within the dominant microbial mat; br, brown pool; bl, black pool; and m, cores from the dominant mat which were used to measure nitrogenase activity.
[b] Genera not previously reported from Heron Island.

PCR amplified using bacterial primers 27F and 1392R (approximately 1,365 bp) (31). The PCR products were then cloned using a TOPO TA cloning kit (Invitrogen). PCR amplification products containing the right-size insert were next digested with 1 U of restriction enzymes HaeIII and MspI (New England Biolabs) for 6 to 12 h at 37°C. Clones that produced the same restriction fragment length polymorphism (RFLP) pattern (DNA fragments of the same size) were grouped together and considered representatives of the same operational taxonomic unit (OTU). Coverage values were calculated for the library by using the relative distribution of OTUs and the equation of Good (24). Double-stranded plasmid DNAs from selected clones were purified using a QIAprep Spin mini-

prep kit (QIAGEN). Sequencing reactions and phylogenetic affiliation analysis were performed as described above.

Nitrogenase activity. Cores that were 8 mm in diameter and 2 to 3 mm thick were collected randomly from the dominant microbial mat during low tide in January 2003 using a cork borer and were incubated for measurement of the nitrogenase activity using the acetylene reduction assay (25, 50). Three cores were placed in a 9-ml glass incubation vial containing 1 ml sterile filtered seawater and 10% acetylene gas and incubated under conditions that mimicked the in situ conditions. The vials were placed in a plastic container with seawater located close to the beach rock for 2 to 3 h. Incubation was started with 2- to 5-h intervals. Following incubation, gas phase subsamples were withdrawn for analysis of the ethylene produced in each vial. Samples were stored in 5-ml Vacutainers (BD Biosciences) and analyzed using a gas chromatograph (Shimadzu GC-8A) equipped with a Porapac N column. A 100-ppm ethylene standard was used to calculate the concentrations. At each time, three replicate vials were analyzed (a total of nine cores).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers DQ072854 to DQ072861 (DGGE) and DQ072862 to DQ072868 (clones) for bacteria and DQ072869 to DQ072874 (DGGE) and DQ072875 to DQ072893 (clones) for cyanobacteria. The GenBank accession numbers of the partial *nifH* gene DGGE sequences are DQ916283 to DQ916287.

RESULTS

Cyanobacterial occurrence and morphology. As shown in Fig. 2, the pink-pigmented dominant microbial mat, covering large parts of the beach rock, and the black- and brown-pigmented biofilm communities of the small pools, also spreading within the dominant mat area (Fig. 1), harbored a variety of morphologically distinct cyanobacterial taxa (see Materials and Methods for a detailed description of the beach rock sites examined). Morphological descriptions and tentative identifications of the cyanobacteria are given in Table 1. Also shown in Table 1 are site references related to the dominant mat, the smaller black-pigmented pools, and the brown-pigmented pools. Cores from the dominant mat were also used for nitrogenase activity assays (see Fig. 7A), and the cyanobacteria identified in these samples were labeled to distinguish them from the organisms collected from the smaller patches (Fig. 1 and Table 1). Taxonomic classifications were determined as described by Komárek and Anagnostidis (29, 30), by Desikachary (12), and in *Bergey's Manual of Systematic Bacteriology* (7). The morphological characteristics used to taxonomically affiliate the cyanobacteria detected are shown in Table 1.

The most abundant cyanobacteria in the pink dominant microbial mat were the filamentous nonheterocystous genus *Blennothrix* sp. (Fig. 2C and Table 1), a unicellular morphotype tentatively referred to the genus *Chroococcidiopsis* (Fig. 2M and Table 1), and one unidentified member of the filamentous *Pseudanabaenaceae* family (Fig. 2K and Table 1). Also present in the mat, although less frequently, was the unicellular cyanobacterium *Chroococcus* sp. (Fig. 2N and Table 1). In two of the smaller patches in the microbial mat (Fig. 1) unicellular *Enthophysalis* species 1 and 2 were discovered (Fig. 2E and Table 1).

In contrast, the biofilm community present in the small black-pigmented pools (Fig. 2A to G) harbored several filamentous heterocystous cyanobacteria, such as (at least) two species of *Calothrix* (Fig. 2A, B, and F), and the genus *Kyrtuthrix* (Fig. 2D). Additionally, two unicellular species, *Entophysalis* species 1 and 2 (Fig. 2E), and three nonheterocystous filamentous species, *Arthrospira* sp. (Fig. 2G), *Blennothrix* sp. (Fig. 2C), and an unidentified member of the *Pseudanabaenaceae* family (Fig. 2K), were also identified (Table 1). In contrast, the brown-pigmented biofilms were dominated by nonheterocystous filamentous cyanobacteria and one unicellular cyanobacterium identified as *Gloeocapsa* sp. (Fig. 2L). Two species of the genus *Lyngbya* also occurred in the brown pools, *Lyngbya* species 1 (Fig. 2I and J) and *Lyngbya* species 2 (Fig. 2H), along with an unidentified member of the *Pseudanabaenaceae* family (Fig. 2K).

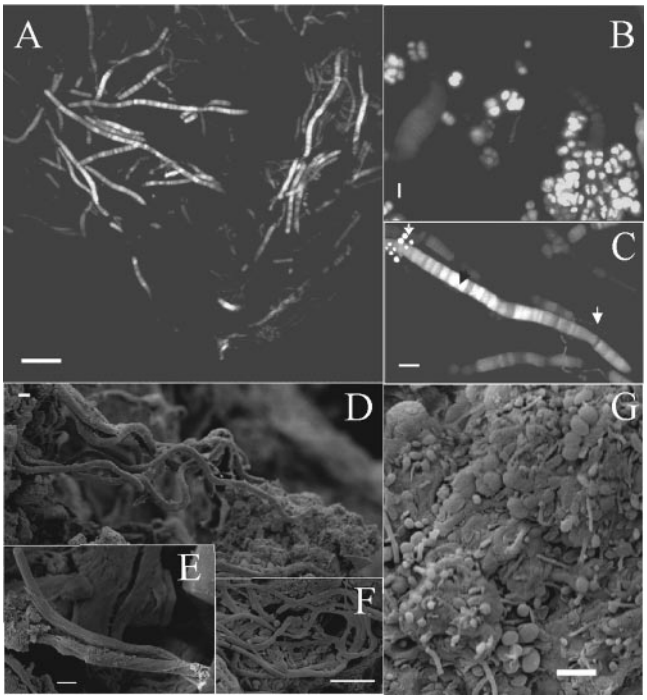

FIG. 3. Three-dimensional images of the dominant microbial mat from Heron Island beach rock viewed by CLSM (A to C) and SEM (D to G). (A and D) General view of the intermixed and randomly oriented filamentous cyanobacterial morphotypes in the dominant mat. Scale bars = 80 and 10 μm, respectively. (B and G) Detailed images of *Chroococcidiopsis*-like morphotype and other unicellular cyanobacteria, such as *Chroococcus* (left white arrow in panel C). Scale bars = 4 μm. (C, E, and F) Detailed images of filamentous cyanobacteria with different cell sizes, including *Blennothrix* (E and black arrowhead in panel C) and thin nonheterocystous filamentous cyanobacteria belonging to the *Pseudanabaenaceae* family (F and right white arrow in panel C). Scale bars = 10 μm.

Three-dimensional distribution of cyanobacteria. In order to obtain more detailed information on the spatial organization of the dominant mat, CLSM and SEM were used. The analyses showed that cyanobacteria that varied in cell size and morphology were spatially intermixed in the mat (Fig. 3). Large void spaces between the microorganisms were also apparent and were possibly air or water filled depending on whether the beach rock was desiccated (low tide) or submerged (high tide). Although the stereo images confirmed the filamentous nature of the majority of the cyanobacteria in the mat, these organisms were also associated with numerous unicellular representatives (Fig. 3). The cyanobacterial genera identified by light microscopy were confirmed by CLSM and SEM on the basis of size and morphology (Table 1). The wider dominant nonheterocystous filamentous (Fig. 3A) genus *Blennothrix* (Fig. 2C and 3E) appeared to be intermixed with species of

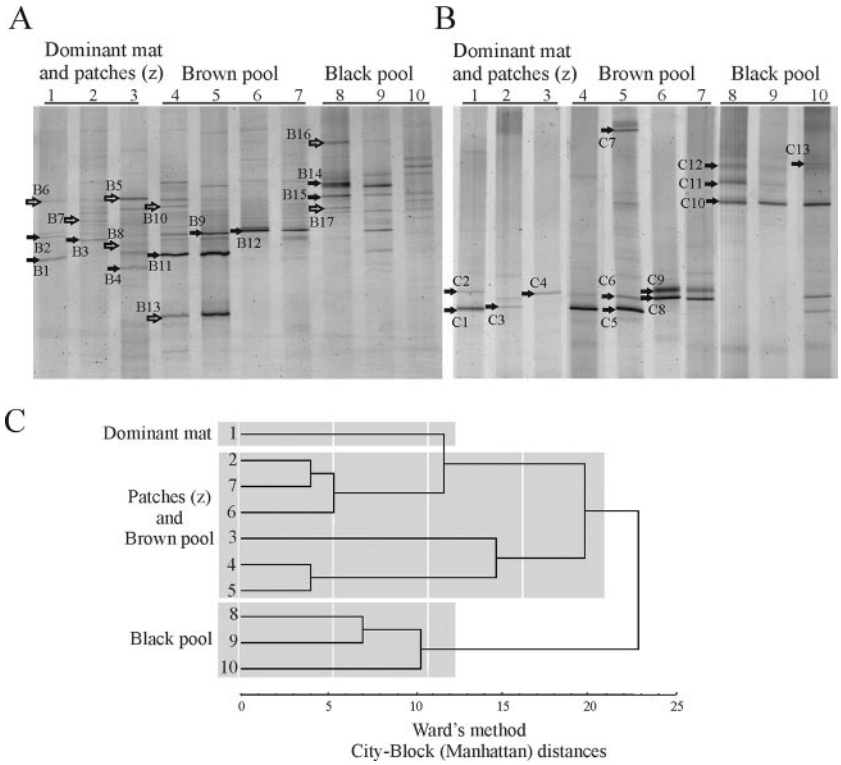

FIG. 4. Genetic fingerprints of microbes collected from the Heron Island beach rock. The partial 16S rRNA gene fragments retrieved were visualized using DGGE. Bacterium-specific (A) and cyanobacterium-specific (B) primers were used (see Materials and Methods) to amplify the gene fragments in the corresponding groups. The lanes contained samples from the dominant microbial mat (lane 1), the patches within the mat (lanes 2 and 3), the biofilms of the small brown pools (lanes 4 to 7), and the biofilms of the small black pools (lanes 8 to 10). Sequenced bands are indicated by arrows (open arrows for bacteria and solid arrows for cyanobacteria) and designations that correspond to those in Fig. 5 and 6. (C) Dendrogram showing the levels of similarity (as determined by the presence or absence and relative abundance of each DGGE band) for the bacterial community analyzed in panel A. The scale bar indicates linkage distances.

the thinner nonheterocystous family *Pseudanabaenaceae* (Fig. 2K and 3F), the unicellular genus *Chroococcidiopsis*, and possibly the genus *Chroococcus* (Fig. 2M and N and 3B and G).

Composition and genetic identification by 16S rRNA gene analyses. DGGE was used to compare the various microbial communities, specifically, the cyanobacterial assemblages from the different sites and zones on the beach rock; Fig. 4A shows the results for bacteria, and Fig. 4B shows the results for cyanobacteria. These analyses revealed several different 16S rRNA gene fragment fingerprint patterns for the three sites and the smaller patches (Fig. 1). The results of the cluster analysis of bacterial DGGE fingerprints, based on the presence or absence and relative intensities (abundance) of the observed bands, are shown in Fig. 4C. Some homogeneity between samples from the dominant mat (Fig. 4A and B, lane 1), the mat patches (lanes 2 and 3), and the brown pool (lanes 4 to 7) was evident, while the 16S rRNA gene samples from the black pools (lanes 8 to 10) were distinctly different. In addition, dissimilarities between the dominant mat (Fig. 4A and B, lane 1) and two samples collected from patches (lanes 2 and 3) were apparent. Fingerprint pattern differences were also apparent when different samples from the same site or zone were compared. For instance, all samples in Fig. 4A and B, lanes 4, 5, 6, and 7 originated from the brown pool biofilms. However, even though most bands were unique to one site or zone, some bands were shared. Cyanobacterial phylotype C6 (Fig. 4B) and bacterial phylotypes B3, B6, B11, B12, and B7 (Fig. 4A) were present in the dominant mat, in most cases in the patches (lanes 2 and 3), and in the brown pool (lanes 4 to 7). Bacterial phylotype B14 was likewise present in both the brown and black pool biofilms, while bacterial phylotypes B10 and B17 were identified in all microbial samples.

To identify the members of the populations detected using 16S rRNA gene-based DGGE analyses, sequencing of major bands was conducted. The 17 DGGE bands (Fig. 4A) obtained using universal bacterial primers and the 13 bands (Fig. 4B) obtained using cyanobacterium-specific primers were sequenced. Of the 17 bands retrieved with the bacterial primers, 9 were closely related to cyanobacteria (Fig. 4A) and 8 were closely related to bacteria (Fig. 4A). In total, 11 DGGE phylotypes (6 phylotypes obtained using bacterial primer-based DGGE and 5 phylotypes obtained using cyanobacterial primer-based DGGE) represented cyanobacteria present in the dominant mat and the small patches (Fig. 1), and 6 represented bacteria. As mentioned above, some of the partial 16S rRNA gene sequences of the bands/phylotypes were also present in the pool biofilms, which explains their scattered occurrence in the phylogenetic trees (Fig. 5 and 6). Furthermore, sequence analysis verified that the phylotypes that grouped together (Fig. 5 and 6) were common in both the cyanobacterial and

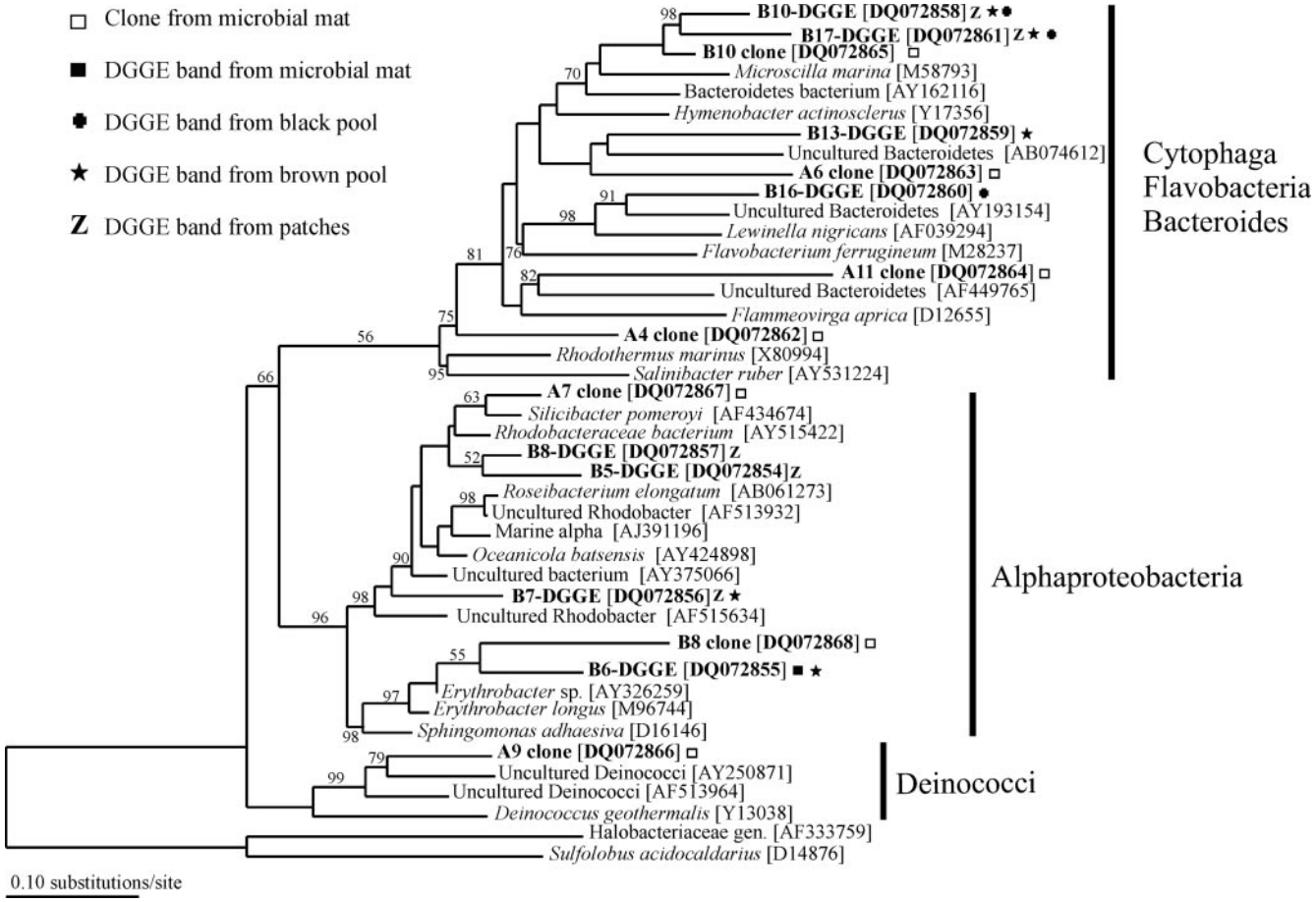

FIG. 5. Phylogenetic affiliations of the genetic fingerprints (partial 16S rRNA gene DGGE bands and clones) from the dominant mat for the eubacterial domain (excluding cyanobacteria). Sequences obtained were added to a preexisting tree with the parsimony tool as implemented in ARB and without allowing overall topology changes. Heron Island microbial sequences are indicated by bold type and designations beginning with A and B for 16S rRNA DGGE and clone (clone library) sequences. Different symbols indicate the 16S rRNA gene clone library sequences from the microbial mat and the 16S rRNA gene DGGE sequences obtained from the different areas analyzed: the dominant microbial mat, the patches within the mat, the black pools, and the brown pools. The sequences of *S. acidocaldarius* (accession no. D14876) and *Halobacteriaceae* (accession no. AF333759) were used as outgroups. Taxonomic classifications were determined as described by Komárek and Anagnostidis (29, 30) and Desikachary (12).

bacterial DGGE gel analyses and in the clone library (see below).

As expected from the morphological analyses, the most intense cyanobacterial bands in the dominant pink mat (DGGE bands B1 and C1 [Fig. 4]) corresponded to a nonheterocystous cyanobacterium closely related to *Lyngbya* phylotypes. However, this cyanobacterium was morphologically identified as a member of the genus *Blennothrix* (Fig. 2C and Table 1). Since no 16S rRNA gene sequences of *Blennothrix* are present in public databases and since both genera belong to the subfamily *Oscillatorioideae*, we concluded that the DNA sequence retrieved represents the genus *Blennothrix*. The other phylotypes obtained from the mat and the patches were affiliated with uncultured cyanobacteria closely related to the *Pseudanabaenaceae*. These cyanobacteria were benthic, moderately halophilic, and thermophilic cyanobacteria, including *Halomicronema excentricum* (DGGE bands B3, C2, C3, and C6), *Leptolyngbya* (DGGE band B2), *Phormidium* (DGGE bands B4 and C4), and *Spirulina* sp. (DGGE band B11). Several of these organisms may represent members of novel groups of cyanobacteria.

As shown by the morphological analysis (Fig. 2A, B, and 2F and Table 1), heterocystous cyanobacteria related to *Calothrix* sp. strain CCMEE 5085 (DGGE bands B14, C10, C11, and C12) dominated the DGGE fingerprints in the black pool biofilms. Although DGGE bands C11 and C12 grouped together with *Calothrix*, the branch lengths indicated a greater phylogenetic distance, and as supported by our morphological analysis, this may suggest genotypes belonging to other genera, such as *Rivularia* or *Kyrtuthrix*, whose 16S rRNA gene sequences are not in genomic databases. Sequences closely related to *H. excentricum* and to sequences from the pink mat grouped together and also with one *Synechococcaceae* phylotype and other *Pseudanabaenaceae* family phylotypes (DGGE bands C13 and B15) (Fig. 6). In the brown pool biofilms, *Oscillatoriales* phylotypes were identified as phylotypes that were related to *Lyngbya* spp. and the planktonic cyanobacteria *Trichodesmium* spp. (DGGE bands B12, C8, and C9). *Oscilla-*

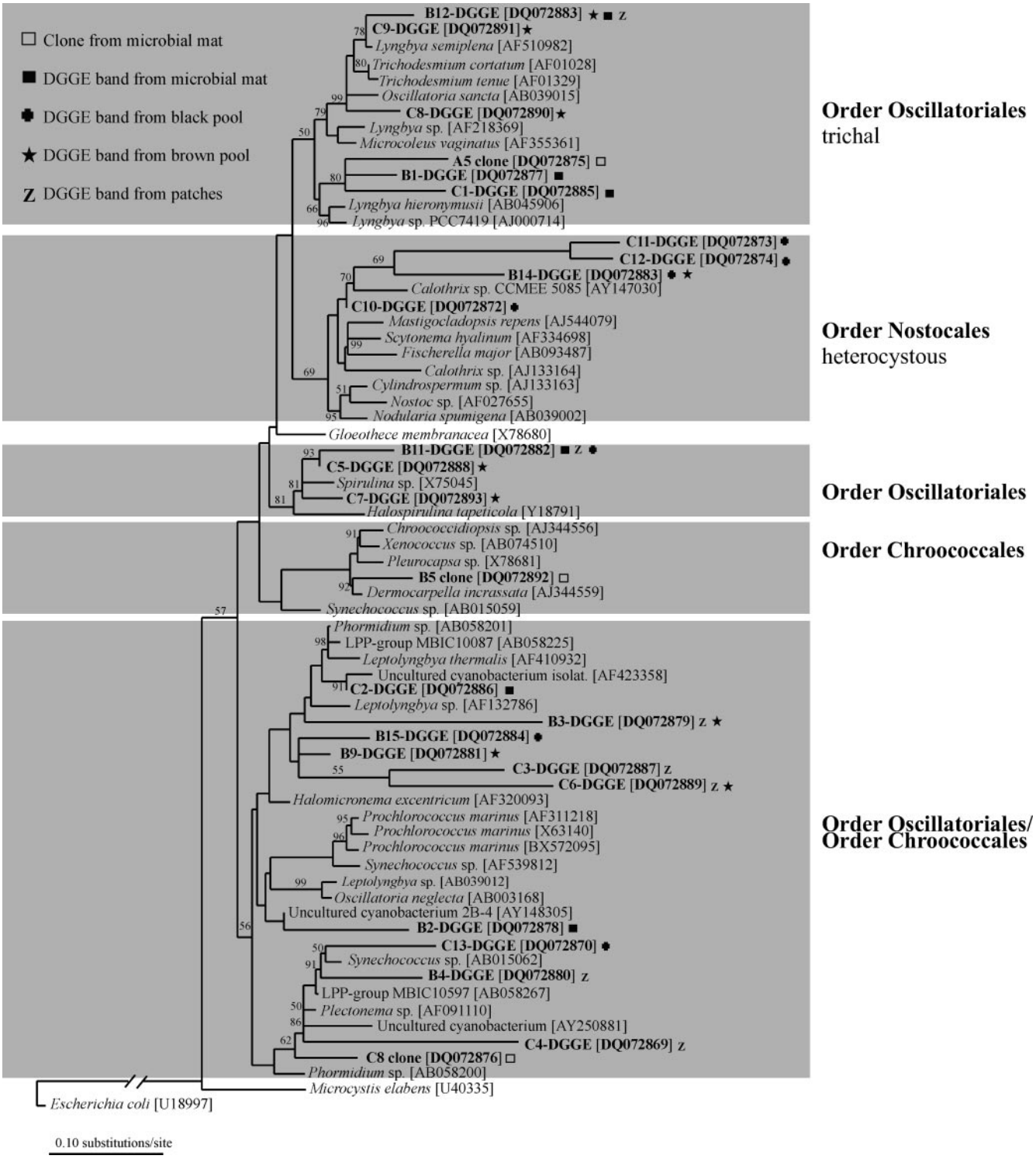

FIG. 6. Phylogenetic affiliations of the genetic fingerprints (partial 16S rRNA gene DGGE bands and clones) from the dominant mat for the cyanobacterial domain. Sequences obtained were added to a preexisting tree with the parsimony tool as implemented in ARB and without allowing overall topology changes. Heron Island microbial sequences are indicated by bold type and designations beginning with C for 16S rRNA gene DGGE and clone sequences for cyanobacteria and with B for 16S rRNA gene DGGE and clone (clone library) sequences for bacteria. Different symbols indicate the clone library sequences from the microbial mat and the 16S rRNA gene DGGE sequences obtained from the different areas analyzed: the dominant microbial mat, the patches within the mat, the black pools, and the brown pools. The sequence of *E. coli* (accession no. U18997) was used as an outgroup. Taxonomic classifications were determined as described by Komárek and Anagnostidis (29, 30) and Desikachary (12).

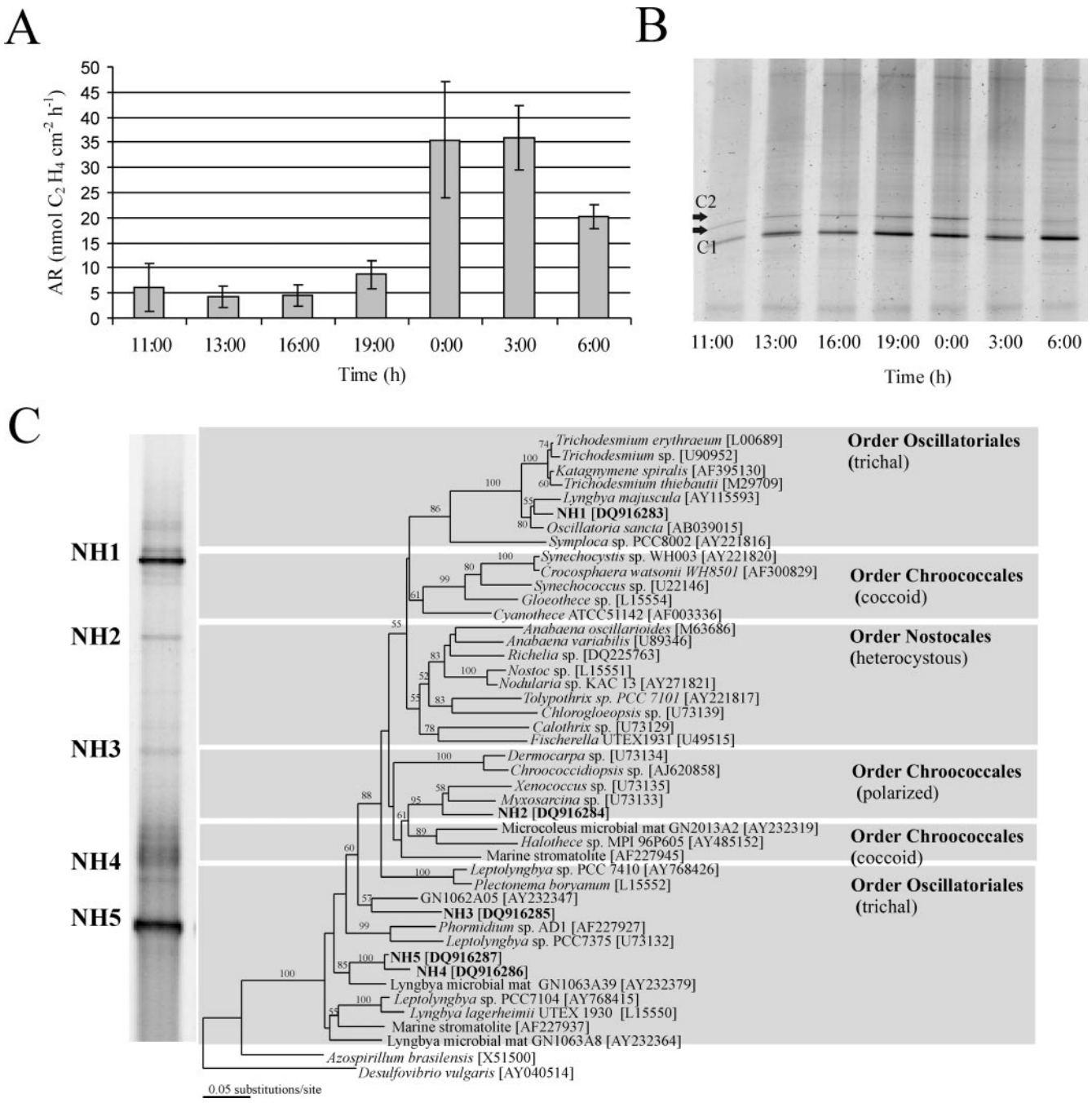

FIG. 7. (A) Diurnal variation in nitrogenase activity in the dominant mat of the Heron Island beach rock. The study was conducted in January 2003, and activities were determined by the acetylene reduction-gas chromatography assay. The error bars indicate standard deviations. AR, acetylene reduction. (B) 16S rRNA gene DGGE fingerprinting analysis of the core samples used for the acetylene reduction assay. Band C1 and C2 correspond to the identically labeled bands in Fig. 4B, lane 1, and are related to *Lyngbya* and *Leptolyngbya* phylotypes in Fig. 6, respectively. (C) Cyanobacterial *nifH* DGGE and phylogenetic affiliation of the *nifH* gene sequences from the dominant mat and their cultured and uncultured relatives (including tentative novel candidate group sequences). GenBank accession numbers are indicated in brackets. Heron Island microbial sequences are indicated by bold type and designations beginning with NH. Taxonomic classifications were determined as described by Komárek and Anagnostidis (29, 30) and Desikachary (12).

toriales phylotypes were also well represented in the dominant mat and were related to *Spirulina* sp. and *Halospirulina tapeticola* (DGGE bands B11, C5, and C7), as well as *Pseudanabaenaceae* family phylotypes (DGGE bands B9 and C6).

Other bacteria were also identified by DGGE analyses (Fig. 4A). These bacteria were allied with the *Alphaproteobacteria*, which occurred primarily in the dominant mat, and with *Cytophaga-Flavobacterium-Bacteroides* species, which were well represented in both the brown and black pools (Fig. 5). *Rhodobacteraceae* bacterium and uncultured *Rhodobacter* phy-

lotypes (DGGE bands B5, B7, and B8) in patches and *Erythrobacter* spp. (DGGE band B6) in the mat belonged to the same group as clones A7 and B8 (see below). The *Bacteroidetes* DGGE phylotypes B10 and B17 grouped together with the B10 clone; the B13 DGGE phylotype grouped together with the A6 clone; and the B16 DGGE phylotype and the A11 clone occurred in two separate groups of uncultured *Bacteroidetes* species.

16S rRNA library of the dominant mat. To obtain more phylogenetically informative sequences, longer 16S rRNA gene fragments (approximately 1,365 bp) were amplified, cloned, and analyzed by the RFLP method to identify OTUs to sequence. The sequences obtained and their closest relatives after comparison with the public database and selected sequences from the ARB database are shown for bacteria in Fig. 5 (excluding cyanobacteria) and for cyanobacteria in Fig. 6. Longer 16S rRNA gene partial fragments were successfully amplified from 32 of the clones. The RFLP analyses revealed greater diversity than that recovered in the DGGE analyses, and 11 potentially different OTUs were identified in the dominant mat. The high coverage value obtained, 90.6%, suggested that the majority of the diversity present was represented. Again, cyanobacteria dominated on the basis of total clone abundance and may have represented 44% of the total community in the mat, followed by *Cytophaga-Flavobacterium-Bacteroides* group members (31% of the total clones), which were found only in the patches (Fig. 1) using DGGE, *Alphaproteobacteria* (16% of the total clones), and some other minor bacterial groups. Clone analysis and PCR-DGGE analysis of DNA extracted from the mat using both cyanobacterial and bacterial primers resulted in common phylotypes sharing group positions in the phylogenetic trees (Fig. 5 and 6). Three different genera of cyanobacteria were identified using cloning. The A5 clone, related to *Lyngbya* spp. (Fig. 6), dominated and represented approximately 30% of the total clone library, as also shown by DGGE. This was followed by the B5 clone (10% of the total clones), the only phylotype clearly related to *Chroococcales* coccoid cyanobacteria, such as *Dermocarpella* and *Chroococcidiopsis* spp. (Fig. 6). Five percent represented the *Pseudanabaenaceae* family (Fig. 6, clone C8). However, additional morphotypes of unicellular cyanobacteria were distinguished in samples from the dominant mat and the patches (Table 1).

The rest of the 16S rRNA sequences from the clone library were more similar to other bacteria than to cyanobacteria and (all except one) to *Alphaproteobacteria* such as the *Rhodobacteriaceae* bacterium (clone A7) and *Erythrobacter* spp. (clone B8) and to the *Bacteroidetes* such as clones A4, A6, A11, and B10 (Fig. 5). Most of these sequences were affiliated with the same group of sequences as the sequences obtained in the DGGE analyses. In addition, the A9 clone was related to *Deinococcus* spp., a group of highly UV-resistant bacteria.

Nitrogenase activity and cyanobacterial *nifH* DGGE characterization. The identification on the beach rock of a range of cyanobacteria that have the potential to fix nitrogen prompted us to examine this possibility. The acetylene reduction assay and gas chromatography were used to assess nitrogenase activities in situ in cores obtained over a 24-h period from the dominant microbial mat (Fig. 1). As shown in Fig. 7A, nitrogenase activity was detected throughout the diurnal cycle, although the activities were considerably lower during the day than at night. The highest activity was 35.9 μmol C_2H_4 m^{-2} h^{-1} at 3:00 a.m. Partial 16S rRNA and cyanobacterial *nifH* DGGE profiles were generated in parallel using DNA extracted from the cores used for the nitrogenase activity measurements. The community composition of the samples was the same over the diurnal period examined (Fig. 7B), and the two principal 16S rRNA DGGE bands present, corresponding to bands C1 and C2 in Fig. 4B, grouped with the nonheterocystous *Lyngbya* phylotype and one unidentified member of the *Pseudanabaenaceae* family. Similar results were obtained in the *nifH* DGGE analysis, and the most dominant bands (bands NH1 to NH5 in Fig. 7C) were related to *Lyngbya* spp. (band NH1 in Fig. 7C), to novel members of the *Pseudanabaenaceae* family (bands NH3 to NH5 in Fig. 7C), and to unicellular cyanobacteria closely related to genera such as *Myxosarcina*, *Chroococcidiopsis*, and the halotolerant genus *Halothece* (band NH2 in Fig. 7C).

DISCUSSION

Collectively, our morphological and molecular data clearly extend the previously suggested microbial complexity (10) in the mat and biofilms covering the Heron Island beach rock, and this study is the first study based on genetic information for such a habitat. The data clearly show that each zone, composed of the dominant microbial mat, the patches, and the differently pigmented biofilms, harbors a range of different morphotypes and phylotypes, resulting in different fingerprint patterns, as demonstrated in our cluster analysis (Fig. 4C). Certain morpho- and phylotypes were shared by the sites, while others were predominantly associated with one site or zone and either absent or poorly represented in the other sites investigated. The organisms identified in the small patches spread over the beach rock clearly were similar to the organisms at the surrounding sites (mat and pools).

As suggested by Cribb in his morphological analyses (10), cyanobacteria still quantitatively (biomass) dominate the beach rock microbial community. These organisms range from unicellular to filamentous nonheterocystous and heterocystous taxa, some of which are known or potential nitrogen fixers. The dominant microbial mat is now clearly a mixed community composed of several cyanobacterial taxa. This mat was categorized as the "*Kyrtuthrix maculans* band" in 1966 (10) (see Materials and Methods), while members of this heterocystous genus were not detected in the dominant mat in our investigation (2003), although *Kyrtuthrix* was identified occasionally on the beach rock.

Rather, the majority of the cyanobacterial phylotypes in the dominant mat were identified as species of the genus *Lyngbya*, although our morphological analyses suggest that this morphotype is affiliated with the genus *Blennothrix* (sensu Anagnostidis and Komárek [3]), as species of the unicellular genus *Chroococcidiopsis*, and as species of the *Pseudanabaenaceae* family. As also shown by our genetic data, these organisms are now the most abundant phototrophs of the Heron Island beach rock. We therefore propose that the great abundance of filamentous, nonheterocystous cyanobacterial genera may be involved in the ongoing construction and/or stabilization of the organosedimentary structures, or microbialites, of Heron Island. As a

similar conclusion was drawn by Abed et al. (1), who examined a tropical benthic system in French Polynesia with a microbial origin similar to that of the Heron Island beach rock; this may suggest that there is a more general phenomenon. Although the genetic data also demonstrated the common occurrence of bacteria related to the anoxygenic photosynthetic *Alphaproteobacteria* and the *Cytophaga-Flavobacterium-Bacteroides* group, these organisms represented a much smaller biomass in the microbial communities examined. However, these organisms may still play significant roles in the biology and maintenance of the beach rock biology.

There is no doubt that the complementary techniques used here resulted in more solid diversity and distribution patterns for the microbial communities. Microscopy alone may have limited use since microorganisms may share morphological characteristics even though they have unrelated genotypes. Environmental factors may also influence morphological characteristics (29, 45). Cultured strains may have taxonomic affiliations that deviate from their true morphological and genetic identities (18) and may be polyphyletic (26, 28). On the other hand, cyanobacterial gene sequences from natural populations in the tropics often result in GenBank entries denoted "uncultured" or "unidentified"; this is particularly true for unicellular taxa and members of the understudied *Pseudanabaenaceae* family. Although molecular techniques provide excellent complementary data, deeply branching phylogenetic relationships within the cyanobacteria are still not well resolved (43). Unresolved trees may generate inconsistent results due to low branch support, long-branch attraction artifacts, and lack of a phylogenetic signal. For instance, the identities of *Blennothrix*, *Kyrtuthrix*, and *Entophysalis* could not be fully confirmed genetically as 16S rRNA gene sequences are not available yet in public databases. Similar conclusions could be drawn when *nifH* gene sequences were analyzed (see below). Together, our data confirm the presence of several tentatively novel marine cyanobacterial genera or species on the Heron Island beach rock. The three-dimensional images obtained also stressed the complexity of the cyanobacterial population covering the beach rock. Loosely organized microorganisms surrounded by void spaces may play a role in permitting the gliding movements and frequent changes in orientation typical of mat-forming cyanobacteria (19) and may allow exploitation of optimal light conditions. The voids may also ensure efficient penetration of atmospheric gases and transport of nutrients and may generate the forces needed to stabilize and provide cohesion for the beach rock mats, which are constantly subjected to strong tidal regimens and desiccation stress.

The thin (<3-μm) cyanobacterial filaments of the *Pseudanabaenaceae* family always occurred intermixed with numerous unicellular cyanobacteria and the considerably larger filamentous cyanobacteria, such as *Lyngbya* or *Blennothrix*. Indeed, members of the the *Pseudanabaenaceae* family are typical components of marine microbial mats (16, 46), and the genus *Lyngbya* is known to be one of the most common constituents of tropical stratified intertidal microbial mats (21, 38, 39, 42). Furthermore, the close relationships between some of these benthic phylotypes, such as *Blennothrix*, and known planktonic organisms, such as *Trichodesmium*, support the ecological and/or evolutionary interconnection between benthic and planktonic microbiota recently suggested (1). The morphologically complex and baeocyte-forming coccoid cyanobacterial genera *Chroococcidiopsis* and *Entophysalis*, which were present both in the dominant mat and in the pigmented biofilms, appeared to be particularly successful in colonizing the rocky carbonate substrates and are also known constituents of some other marine microbial mats (7, 8, 21, 22). Not unexpectedly, most cyanobacterial pheno- and phylotypes identified here were also related to pheno- and phylotypes observed in environments subject to osmotic and UV intensity-related stresses (1, 17, 42, 55).

Benthic cyanobacteria diversify and adapt in response to substrate availability and the external conditions, which may explain the complexity of the cyanobacterial morphotypes and distribution patterns observed. However, the differences between the cyanobacteria identified in 1966 (10) and the cyanobacteria identified in our study (2003) may reflect the different methodologies used (10). For instance, the heterocystous genus *Calothrix* dominated in both studies, with *Calothrix crustacea* and *Calothrix pilosa* present in the "*Entophysalis deusta* band" according to Cribb (10), while *Kyrtuthrix* was found only at low abundance in 2003. However, Cribb stressed that it was difficult to determine which of the numerous species in the *Kyrtuthrix* band was the most important, while in 1973 Davies and Kinsey (11) described the most distinct layer as the layer containing filamentous *Lyngbya*-type cyanobacteria. The nonheterocystous genera *Lyngbya* and *Leptolyngbya* and representatives of the *Pseudanabaenaceae* family were described as *Oscillatoria* spp. in 1966 and were reported to constitute the "*Entophysalis deusta* band" (10), while *Blennothrix* sp. was not identified. These cyanobacteria are known to be versatile and to readily cope with and adjust to fluctuating physical conditions, and heterocystous cyanobacteria are extremely well adapted to perform nitrogen fixation and oxygenic photosynthesis simultaneously (5).

The fact that the highest nitrogenase activities were recorded at night in the dominant mat (Fig. 7A) emphasized the involvement of the nonheterocystous morpho- and phylotypes, as these types use a temporal separation strategy to avoid inhibitory O_2 levels during the photoperiod (5). The nitrogenase activity recorded in the mat at night was substantial (~35 nmol C_2H_4 cm^{-2} h^{-1}) and corroborated the data for other marine intertidal (4, 40, 47, 49) and hypersaline (38) cyanobacterial mats, but the values are higher than those recorded for a similar habitat (9) and for beach rock at the nearby One Tree Island (32). The possibility that bacteria also contributed to the nitrogenase activities cannot be excluded, even though the cyanobacteria dominated on a biomass basis and the 16S rRNA and *nifH* gene sequences retrieved from the cores assayed grouped with known cyanobacterial diazotrophs, such as *Lyngbya* spp. and *Trichodesmium* spp. The genus *Lyngbya*/*Blennothrix*, together with the novel thin filamentous and unicellular cyanobacterial genera and species encountered, may therefore be major N_2 fixers on the Heron Island beach rock.

The differences in diversity between the sites on the beach rock that are close to each other may be driven by external factors. For instance, the often wet pools are a very different environment than the dry beach rock surface covered by the dominant mat. Refilling of the pools twice per day promotes the establishment of less-desiccation-stress-tolerant species and allows more continuous photosynthetic activity or other

physiological activity compared with the activity in the mat, which remains dry for several hours per day during low tide. In addition, differences in the substrate in the beach rock (i.e., porosity, cavities, and chemical composition) may shape the microbial biomass, diversity, and activity patterns (17, 48). Such microclimate differences may also promote complementary functional behaviors (e.g., efficient nutrient acquisition) in the beach rock communities. For instance, it seems logical to assume that the nocturnal nitrogen fixation in the dominant mat is complemented by nitrogen fixation during the day in the black pools as these pools are dominated by potent diazotrophs.

The difference in cyanobacterial composition between our study and the study of Cribb in 1966 may have different explanations. Seasonal or climate changes are possible, although less likely, in this comparatively stable environment. Rather, the changes observed may have been due to natural disturbances, such as tropical hurricanes (cyclones) and storm waves, predators, or anthropogenic activities in the area. For instance, successional and long-term changes in algae, cyanobacteria, and corals have previously been described on the island (27, 48). Disturbance may create empty patches, subsequently filled by new species (41). As we observed small areas or patches (Fig. 1) inhabited by cyanobacteria and bacteria similar to the organisms retrieved both from the mat and the pools, we hypothesized that these areas are transition areas where community changes are occurring. A combination of such phenomena may explain the apparently dynamic character of the microbial consortia in the beach rock documented.

In summary, our data show that the beach rock has highly varied and dynamic microbial communities dominated by nonheterocystous cyanobacteria that fix substantial quantities of nitrogen at night and suggest that this community may play a role in the maintenance and stability of the beach rock. Combined with the potential daytime nitrogen fixation by heterocystous species in the black pools, this nitrogen fixation is likely to play a significant role in the nitrogen budget of the beach rock. The fact that some of the sequences recovered are similar to sequences from hypersaline microbial mats in Guerrero Negro in Mexico (38, 39) and in salt ponds in the Bahamas (54) suggests that these phylotypes may have a worldwide distribution in benthic marine environments. It is therefore now of great interest to isolate and characterize these novel cyanobacteria in order to be able to assign proper identities and specific physiological functions.

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REFERENCES

1. **Abed, R. M. M., S. Golubic, F. Garcia-Pichel, G. F. Camoin, and S. Sprachta.** 2003. Characterization of microbialite-forming cyanobacteria in a tropical lagoon: Tikehau Atoll, Tuamotu, French Polynesia. J. Phycol. **39:**862–873.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Anagnostidis, K., and J. Komárek.** 1988. Modern approach to the classification of cyanophytes. 3. Oscillatoriales. Algolog. Stud. **50-53:**327–472.
4. **Bebout, B. M., M. W. Fitzpatrick, and H. W. Paerl.** 1993. Identification of the sources of energy for nitrogen fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. Appl. Environ. Microbiol. **59:**1495–1503.
5. **Bergman, B., J. R. Gallon, A. N. Rai, and L. J. Stal.** 1997. N_2 fixation by non-heterocystous cyanobacteria. FEMS Microbiol. Rev. **19:**139–185.
6. **Capone, D. G., J. Zehr, H. Paerl, B. Bergman, and E. J. Carpenter.** 1997. *Trichodesmium*: a globally significant marine cyanobacterium. Science **276:**1221–1229.
7. **Castenholz, R. W.** 2001. Phylum BX. Cyanobacteria, p. 473–599. *In* D. R. Boone, R. W. Castenholz, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer, New York, NY.
8. **Chacón, E., E. Berrendero, and F. Garcia-Pichel.** 2006. Biogeological signatures of microboring cyanobacterial communities in marine carbonates from Cabo Rojo, Puerto Rico. Sediment. Geol. **185:**215–228.
9. **Charpy-Roubaud, C., and A. W. D. Larkum.** 2005. Dinitrogen fixation by exposed communities on the rim of Tikehau Atoll (Tuamotu Archipelago, French Polynesia). Coral Reefs **24:**622–628.
10. **Cribb, A. B.** 1966. The algae of Heron Island, Great Barrier Reef, Australia, part I. A general account. Univ. Qld. Pap. Great. Barrier Reef Comm. Heron Isl. Res. Stn. **1:**3–23.
11. **Davies, P. J., and D. W. Kinsey.** 1973. Organic and inorganic factors in recent beach rock formation, Heron Island, Great Barrier Reef. J. Sediment. Petrol. **43:**59–81.
12. **Desikachary, T. V.** 1959. Cyanophyta, p. 686. Indian Council of Agricultural Research, New Delhi, India.
13. **Díez, B., C. Pedrós-Alió, T. L. Marsh, and R. Massana.** 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. **67:**2942–2951.
14. **Dillon, J. G., S. R. Miller, and R. W. Castenholz.** 2003. UV-acclimation responses in natural populations of cyanobacteria (*Calothrix* sp.). Environ. Microbiol. **5:**473–483.
15. **Falcón, L. I., E. J. Carpenter, F. Cipriano, B. Bergman, and D. G. Capone.** 2004. N_2 fixation by unicellular bacterioplankton from the Atlantic and Pacific oceans: phylogeny and in situ rates. Appl. Environ. Microbiol. **70:**765–770.
16. **Fourçcans, A., T. García de Oteyza, A. Wieland, A. Sole, E. Diestra, J. van Bleijswijk, J. O. Grimalt, M., Kühl, I. Esteve, G. Muyzer, P. Caumette, and R. Duran.** 2004. Characterization of functional bacterial groups in a hypersaline microbial mat community (Salins-de-Giraud, Camargue, France). FEMS Microbiol. Ecol. **51:**55–70.
17. **Garcia-Pichel, F., A. López-Cortés, and U. Nübel.** 2001. Phylogenetic and morphological diversity of cyanobacteria in soil desert crusts from the Colorado Plateau. Appl. Environ. Microbiol. **67:**1902–1910.
18. **Garcia-Pichel, F., L. Prufert-Bebout, and G. Muyzer.** 1996. Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. Appl. Environ. Microbiol. **62:**3284–3291.
19. **García-Pichel, F., M. Mechling, and R. W. Castenholz.** 1994. Diel migrations of microorganisms within a benthic, hypersaline mat community. Appl. Environ. Microbiol. **60:**1500–1511.
20. **Ginsburg, R. N.** 1953. Beachrock in South Florida. J. Sediment. Petrol. **23:**85–92.
21. **Golubic, S.** 1991. Stromatolites of Shark Bay, p. 103–130. *In* L. Margulis and L. Olendzenski (ed.), Environmental evolution. Effects of the origin and evolution of life on planet Earth. MIT Press, Cambridge, MA.
22. **Golubic, S.** 1991. Microbial mats of Abu Dhabi, p. 131–147. *In* L. Margulis and L. Olendzenski (ed.), Environmental evolution. Effects of the origin and evolution of life on planet Earth. MIT Press, Cambridge, MA.
23. **Golubic, S., T. Le Campion-Alsumard, and S. E. Campbell.** 1999. Diversity of marine cyanobacteria. Bull. Inst. Oceanogr. (Monaco) **19**(Special Issue)**:**53–76.
24. **Good, I. J.** 1953. On the population frequencies of species and the estimation of population parameters. Biometrika **40:**237–264.
25. **Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns.** 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. **43:**1185–1207.
26. **Hoffmann, L., J. Komárek, and J. Kaštovský.** 2005. System of Cyanoprokaryotes (Cyanobacteria)—state in 2004. Arch. Hydrobiol. Suppl. **159:**95–115.
27. **Hughes, T. P., and J. H. Connell.** 1999. Multiple stressors on coral reefs: a long-term perspective. Limnol. Oceanogr. **44:**932–940.
28. **Ishida, T., M. M. Watanabe, J. Sugiyama, and A. Yokota.** 2001. Evidence for polyphyletic origin of the members of the orders of Oscillatoriales and Pleurocapsales as determined by 16S rDNA analysis. FEMS Microbiol. Lett. **201:**79–82.
29. **Komárek, J., and K. Anagnostidis.** 1998. Cyanoprokaryota. 1. Teil Chroococcales, p. 548. *In* H. Ettl, G. Gärtner, H. Heynig, and D. Mollenhauer (ed.), Süsswasserflora von Mitteleuropa. Gustav Fisher Verlag, Jena, Germany.
30. **Komárek, J., and K. Anagnostidis.** 2005. Cyanoprokaryota. 2. Teil, p. 759.

In B. Büdel, G. Gärtner, L. Krienitz, and M. Schagerl (ed.), Süsswasserflora von Mitteleuropa. Elsevier GmbH, Mñchen, Germany.
31. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Chichester, United Kingdom.
32. **Larkum, A. W. D., I. R. Kennedy, and W. J. Muller.** 1988. Nitrogen fixation on a coral reef. Mar. Biol. **98:**143–155.
33. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, T. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. P. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
34. **Ludwig, W., R. Amann, E. Martinez-Romero, W. Schönhuber, S. Bauer, A. Neef, and K. H. Schleifer.** 1998. rRNA based identification systems for rhizobia and other bacteria. Plant Soil **204:**1–9.
35. **Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer.** 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 1–27. *In* D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual, vol. 3.4.4A. Kluwer Academic Publishers, Dordrecht, The Netherlands.
36. **Nübel, U., F. Garcia-Pichel, and G. Muyzer.** 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl. Environ. Microbiol. **63:**3327–3332.
37. **Olson, J. B., T. F. Steppe, R. W. Litaker, and H. W. Paerl.** 1998. N_2-fixing microbial consortia associated with the ice cover of Lake Bonney, Antarctica. Microb. Ecol. **36:**231–238.
38. **Omoregie, E., L. Crumbliss, B. Bebout, and J. Zehr.** 2004. Determination of nitrogen-fixing phylotypes in *Lyngbya* sp. and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja, Mexico. Appl. Environ. Microbiol. **70:**2119–2128.
39. **Omoregie, E., L. Crumbliss, B. Bebout, and J. Zehr.** 2004. Comparison of diazotroph community structure in *Lyngbya* sp. and *Microcoleus chthonoplastes* dominated microbial mats from Guerrero Negro, Baja, Mexico. FEMS Microbiol. Ecol. **47:**305–318.
40. **Paerl, H. W., M. Fitzpatrick, and B. M. Bebout.** 1996. Seasonal nitrogen fixation dynamics in a marine microbial mat: potential roles of cyanobacteria and microheterotrophs. Limnol. Oceanogr. **41:**419–427.
41. **Paine, R. T., and S. A. Levin.** 1981. Intertidal landscapes: disturbance and the dynamics of pattern. Ecol. Monogr. **51:**145–178.
42. **Rothrock, M. J., and F. Garcia-Pichel.** 2005. Microbial diversity of benthic mats along a tidal desiccation gradient. Environ. Microbiol. **4:**593–601.
43. **Sánchez-Baracaldo, P., P. K. Hayes, and C. E. Blank.** 2005. Morphological and habitat evolution in the Cyanobacteria using a compartmentalization approach. Geobiology **3:**145–165.
44. **Schreiber, U., R. Gademann, P. Bird, P. J. Ralph, A. W. D. Larkum, and M. Kühl.** 2002. Apparent light requirement for activation of photosynthesis upon rehydration of desiccated beachrock microbial mats. J. Phycol. **38:**1–10.
45. **Silva, M. F., and R. N. Pienaar.** 2000. Benthic marine Cyanophyceae from Kwa-Zulu Natal, South Africa, p. 456. Gebrüder Borntraeger Verlagsbuchhandlung, Berlin, Germany.
46. **Solé, A., N. Gaju, and I. Esteve.** 2003. The biomass dynamics of cyanobacteria in an annual cycle determined by confocal laser scanning microscopy. Scan **25:**1–7.
47. **Stal, L. J.** 2000. Cyanobacterial mats and stromatolites, p. 61–120. *In* B. A. Whitton and M. Potts (ed.), The ecology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
48. **Stephenson, W., and R. B. Searles.** 1960. Experimental studies on the ecology of intertidal environments at Heron Island. I. Exclusion of fish from beach rock. Aust. J. Mar. Freshw. Res. **11:**241–267.
49. **Steppe, T. F., and H. W. Paerl.** 2005. Nitrogenase activity and *nifH* expression in a marine intertidal microbial mat. Microb. Ecol. **49:**315–323.
50. **Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris.** 1967. In situ studies of N_2 fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. USA **58:**2071–2078.
51. **Webb, G. E., and B. S. Kamber.** 2000. Rare earth elements in Holocene reefal microbialites: a new shallow seawater proxy. Geochim. Cosmochim. Acta **64:**1557–1565.
52. **Webb, G. E., J. C. Baker, and J. S. Jell.** 1998. Inferred syngenetic textural evolution in Holocene cryptic reef microbialites, Heron Reef, Great Barrier Reef, Australia. Geology **26:**:355–358.
53. **Webb, G. E., J. S. Jell, and J. C. Baker.** 1999. Cryptic intertidal microbialites in beachrock, Heron Island, Great Barrier Reef: implications for the origin of microcrystalline beachrock cement. Sediment. Geol. **126:**317–334.
54. **Yannarell, A. C., T. F. Steppe, and H. W. Paerl.** 2006. Genetic variance in the composition of two functional groups (diazotrophs and cyanobacteria) from a hypersaline microbial mat. Appl. Environ. Microbiol. **72:**1207–1217.
55. **Zehr, J. P., J. B. Waterbury, P. J. Turner, J. P. Montoya, E. Omoregie, G. F. Steward, A. Hansen, and D. M. Karl.** 2001. New nitrogen-fixing unicellular cyanobacteria discovered in the North Pacific subtropical gyre. Nature **412:** 635–638.