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Microbial Diversity of Cryptoendolithic Communities from the McMurdo Dry Valleys, Antarctica

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In the McMurdo Dry Valleys of Antarctica, microorganisms colonize the pore spaces of exposed rocks and are thereby protected from the desiccating environmental conditions on the surface. These cryptoendolithic communities have received attention in microscopy and culture-based studies but have not been examined by molecular approaches. We surveyed the microbial biodiversity of selected cryptoendolithic communities by analyzing clone libraries of rRNA genes amplified from environmental DNA. Over 1,100 individual clones from two types of cryptoendolithic communities, cyanobacterium dominated and lichen dominated, were analyzed. Clones fell into 51 relatedness groups (phylotypes) with ≥98% rRNA sequence identity (46 bacterial and 5 eucaryal). No representatives of *Archaea* were detected. No phylotypes were shared between the two classes of endolithic communities studied. Clone libraries based on both types of communities were dominated by a relatively small number of phylotypes that, because of their relative abundance, presumably represent the main primary producers in these communities. In the lichen-dominated community, three rRNA sequences, from a fungus, a green alga, and a chloroplast, of the types known to be associated with lichens, accounted for over 70% of the clones. This high abundance confirms the dominance of lichens in this community. In contrast, analysis of the supposedly cyanobacterium-dominated community indicated, in addition to cyanobacteria, at least two unsuspected organisms that, because of their abundance, may play important roles in the community. These included a member of the α subdivision of the *Proteobacteria* that potentially is capable of aerobic anoxygenic photosynthesis and a distant relative of *Deinococcus* that defines, along with other *Deinococcus*-related sequences from Antarctica, a new clade within the *Thermus-Deinococcus* bacterial phylogenetic division.

The McMurdo Dry Valleys region of South Victoria Land, Antarctica, is one of the largest ice-free regions of the Antarctic continent. The climate of the Dry Valleys is characterized by extreme cold and dryness (28). In winter, a period of continuous darkness, temperatures reach as low as −60°C, and winds of up to 100 km/h sweep through the valleys. During summer, average daily air temperatures range from −35°C to +3°C, with oscillations of up to 15°C due to cloud cover occurring on the order of minutes throughout the day. Continuous summertime sunshine can raise the internal temperature of rocks above the freezing point, up to 10°C above the ambient temperature. In addition to the cold, the region is also one of the driest on the planet. In 1986, only 16 snowfall events (the only form of precipitation available) were recorded, most during the summer (28). However, rocks wetted by snowmelt retain liquid water internally for several days. The combination of such rapid oscillations in temperature and wetting-drying cycles contributes to the creation of an extremely hostile living environment that has been proposed to be a potential analogue for environmental conditions on Mars (27).

Life in the Dry Valleys has been selected to occupy habitats that offer protection from the desiccating surface. Battleship Promontory (76°55′S, 160°55′E; altitude, 1,600 to 1,650 m) is a large sandstone outcrop in the Convoy Range of the McMurdo Dry Valleys. At Battleship Promontory and other locations throughout the Dry Valleys, cryptoendolithic microorganisms colonize the pore spaces of exposed sandstone rocks, forming stratified microbial communities (17). Despite the fact that they occur worldwide, cryptoendolithic communities have been studied little, and their contribution to local and global geochemistry is poorly understood.

Previous studies characterized the Antarctic endolithic communities by microscopy and by laboratory cultivation of constituent organisms (3, 16, 21, 34, 35). Two classes of communities have been defined on the basis of conspicuous primary producers, lichens and cyanobacteria (16). Most of these autotrophs identified microscopically have proven refractory to cultivation and so have been described principally by morphology (16, 30). In contrast, studies of the heterotrophic microorganisms associated with these communities have been based primarily on laboratory cultivation (21, 35). Such studies have resulted in large collections of cultivars, but few have been characterized by molecular phylogenetic analysis (33). Moreover, most microorganisms are refractory to routine laboratory cultivation; therefore, prevalent organisms may not have been detected (3). Thus, information about the compositions and

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potential biochemical activities of the original communities cannot be extrapolated from culture-based studies.

To gain a better understanding of the kinds of organisms that constitute Antarctic cryptoendolithic communities, we conducted a culture-independent analysis of the phylogenetic compositions of two such communities by examining small-subunit (SSU) rRNA genes amplified from community DNA. Similar rRNA-based molecular surveys have advanced the understanding of the diversity of the natural microbial world through the identification of many novel organisms, including representatives of major phylogenetic groups never detected by cultivation. The present study revealed the occurrence and abundance of many novel organisms, including some that, based on their high abundance, are predicted to play important roles in Antarctic cryptoendolithic communities.

MATERIALS AND METHODS

Sample collection. Samples of Beacon sandstone from the McMurdo Dry Valleys were collected by one of us (E.I.F.), by colleagues, and by Seán Turner (Indiana University, Bloomington) during the 1985-1986 field season as previously described (16); these samples previously were studied extensively (16, 30). Samples were frozen in the field and stored at −20°C until processed.

Environmental genomic DNA extraction. Total community genomic DNA was extracted from cryptoendolithic communities by physical, enzymatic, and chemical disruption of cells as previously described (22). Briefly, friable surface layers of sandstone (a few millimeters) were obtained by abrasion with a flame-sterilized wire brush on a drill bit. Sterile forceps were used to remove the underlying laminae of endolith-containing sandstone (ca. 0.5 to 1 cm, which included all pigmented layers), which were then crushed by using a sterile plastic pipette in a sterile microcentrifuge tube. Approximately 0.5 g of crushed sandstone was resuspended in modified 2× buffer A (200 mM Tris [pH 8.0], 50 mM EDTA [pH 8.0], 200 mM NaCl, 2 mM sodium citrate, 10 mM $CaCl_2$) containing 2% sodium pyrophosphate and 5 mg of lysozyme/ml, and the suspension was incubated for 30 min at 37°C. Proteinase K (to 2 mg/ml) and sodium dodecyl sulfate (to 0.3% [wt/vol]) were added, and the mixture was incubated for a further 30 min at 50°C. Samples were reciprocated on a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, Okla.) at low speed for 2 min in the presence of 15% (vol/vol) phenol, 2% (wt/vol) sodium dodecyl sulfate, and approximately 0.5 g of acid-washed zirconium beads (0.1-mm diameter). Following reciprocation, samples were examined under a microscope to verify the disruption of all cells. Aqueous phases from the first extraction were reextracted once each with equal volumes of phenol-chloroform (1:1) and then chloroform alone. Nucleic acids were precipitated from the aqueous phases with 0.3 M sodium acetate and 50% isopropanol, washed with 70% ethanol, dried in air, and resuspended in 10 mM Tris (pH 8.0) at a concentration of approximately 50 ng/μl.

PCR amplification and rDNA clone libraries. SSU rRNA genes (rDNA) were amplified by PCR with purified environmental genomic DNA as a template and a combination of universal or phylogenetic-group-specific oligonucleotide primers. Each PCR mixture contained 1 to 50 ng of environmental DNA, PCR buffer II (Perkin-Elmer), 2.5 mM $MgCl_2$, 1 mg of bovine serum albumin/ml, 0.2 mM each deoxynucleoside triphosphate, 50 pmol each forward and reverse primers, and 0.025 U of AmpliTaq Gold (Perkin-Elmer) in a final volume of 20 μl. PCR amplifications were carried out under empirically determined optimum conditions with a gradient of annealing temperatures. An initial denaturation step of 12 min at 94°C was followed by 30 cycles of PCR that consisted of 30 s at 94°C, 30 s at the empirically derived annealing temperatures, and 45 s at 72°C. An extension step of 10 min at 72°C was added after the last cycle to facilitate A tailing and subsequent cloning of amplified products.

The primers used in this study were 27F (specific for bacteria) (5′-AGAGTT TGATCCTGGCTCAG-3′), 21Fa (specific for archaea) (5′-TTCCGGTTGATC CYGCCGGA-3′), 333Fa (specific for archaea) (5′-TCCAGGCCCTACGGG-3′), 515F (universal) (5′-GTGCCAGCMGCCGCGGTAA-3′), 82Fe (specific for eucarya) (5′-GAADCTGYGAAYGGCTC-3′) (10), Cyano356F (specific for cyanobacteria) (5′-GTGGGGAATTTTCCGCAA-3′), 958Ra (specific for archaea) (5′-YCCGGCGTTGAMTCCAATT-3′), 1391R (universal) (5′-GACGG GCGGTGWGTRCA-3′), and 1492R (universal) (5′-GGTTACCTTGTTACGA CTT-3′).

rRNA genes were amplified from each sample with combinations of the universal and phylogenetic-group-specific primers, and clone libraries were constructed when an amplification product was detected by agarose gel electrophoresis. In total, 11 different libraries were constructed for this study. For the cyanobacterium-dominated community, we constructed two universal libraries with primer pairs 515F-1492R (FBP1) and 515F-1391R (FBP3), two libraries specific for bacteria with 27F-1492R (FBP2) and 27F-1391R (FBP4), and one library specific for cyanobacteria with 356Fcya-1391R (FBP5). For the lichen-dominated community, we constructed four universal libraries, two each with primer pairs 515F-1492R (BPS and BPU) and 515F-1391 (TBP and TLT), and one library specific for eucarya with 83Fe-1391R (BPE) from two separate sandstone samples. A final library was constructed with archaeon-specific primer pair 333Fa-1391R at a less-than-optimal annealing temperature (45°C).

To construct environmental rDNA clone libraries, PCR-amplified products were cloned by using a TOPO TA cloning kit (Invitrogen) as specified by the manufacturer. Plasmid DNAs containing inserts were initially screened by restriction fragment length polymorphism (RFLP) analysis (13) with *Msp*I and *Hin*PII restriction endonucleases (New England Biolabs). rDNA clones containing unique restriction fragment length patterns were sequenced with twofold coverage by using an ABI373 Stretch automated sequencer (BigDye-Terminator cycle sequencing ready reaction FS kit; PE Applied Biosystems).

Phylogenetic analysis methods. Unique rDNA sequences initially were compared to a current database of genetic sequences (GenBank) by using the BLAST (Basic Local Alignment Search Tool) network service (2) to determine their approximate phylogenetic affiliations. Chimeric sequences were identified by using secondary-structure analyses and the CHECK_CHIMERA software program of the Ribosomal Database Project (25). Sequences showing a high degree of identity (≥99%) to common contaminants of rRNA-based molecular surveys (41) were excluded from all further analyses. The remaining environmental rDNA sequences were then aligned to other known SSU rRNA sequences by using the ARB software package (38). A total of 1,334 homologous nucleotide positions were included in the alignments for phylogenetic analyses by using the bacterial mask of Lane (24). Evolutionary distance, maximum-parsimony, and maximum-likelihood analyses were performed on the alignments as described previously (5). Distance and maximum-parsimony analyses were conducted by using test version 4.0b8 of PAUP* (40). Maximum-likelihood analyses were calculated by using fastDNAml, available through the Ribosomal Database Project (25). To determine the validity of inferred topologies, bootstrap analysis with resampling was performed on 100 replicates in each analysis. Consensus trees were determined from nonresampled data by majority rule.

Cultivation and phylogenetic analysis of cryptoendolithic cyanobacteria. With flame-sterilized forceps, approximately 50 mg of sandstone containing the cyanobacterium-dominated community was scraped into a sterile microcentrifuge tube. Approximately 5 mg of sandstone was spread directly on SNAX agar, a nitrogen-containing oligotrophic medium commonly used to cultivate cyanobacteria (42). The remaining sandstone material was placed into sterile filtered seawater and mixed thoroughly by vortexing, and serial dilutions of the suspension were plated on SNAX agar. Petri plates were incubated at 4°C under constant illumination until green colonies were visible (up to 4 weeks for the suspension material). Green cyanobacterial colonies were picked and replated on fresh plates to obtain isolated colonies. For PCR amplification, individual sandstone grains from the culture plates or single cyanobacterial colonies were placed in separate microcentrifuge tubes containing 2× buffer A, and genomic DNA was extracted as described above for environmental samples. SSU rRNA genes were amplified as described above with a cyanobacterium-specific forward primer (356FCya) and a universal reverse primer (1391R). PCR products were directly sequenced and phylogenetically analyzed as described above.

Nucleotide sequence accession numbers. Sequences from these experiments were deposited in GenBank under accession numbers AY250846 to AY250898. Individual accession numbers are shown in Tables 1 and 2.

RESULTS

SSU rDNA clone libraries. Several distinct classes of cryptoendolithic communities in Antarctic sandstone have been identified by microscopy and culture-based analysis (reviewed in reference 30). In order to determine the organism compositions of two kinds of previously characterized Antarctic cryptoendolithic communities with cultivation-independent methods, we prepared SSU rDNA clone libraries from total community genomic DNA. Microscopic examination of the communities indicated that one was dominated by lichens,

TABLE 1. Summary of rDNA clones identified from the lichen-dominated cryptoendolithic community

Clone	% Abundance[a]	Putative group[b]	Closest BLAST match (GenBank accession no.)	% Identity	GenBank accession no.
BPE012	29.2	*Fungi*	*Texosporium sancti-jacobi* (U86696)	97	AY250846
BPE016	1.2	*Fungi*	*Bullera unica* (D78330)	98	AY250847
TLT108	<1	*Fungi*	*Geomyces pannorum* (AB015785)	98	AY250893
BPE026	22.3	*Chlorophyta*	*Trebouxia jamesii* (Z68700)	99	AY250848
BPS021	<1	*Chlorophyta*	Chlorophyte isolate BC98 (AJ302940)	95	AY250850
BPU108	21.7	Chloroplasts	*Koliella sempervirens* chloroplast (AF278747)	90	AY250850
BPU107	6.2	CFB	*Cytophagales* strain MBIC41487 (AB022889)	95	AY250857
BPU241	2.0	Actinobacteria	*Blastococcus* sp. strain BC521 (AJ316573)	92	AY250860
TLT170	1.2	Actinobacteria	*Microsphaera multipartita* (Y08541)	97	AY250897
BPS013	<1	Actinobacteria	*Sporichthya polymorpha* (AB025317)	93	AY250949
TLT173	<1	Actinobacteria	*Rhodococcus* sp. strain LG3 (AJ007003)	95	AY250898
TBP017	2.0	Mitochondria: *Chlorophyta*	*Chaetosphaeridium globosum* mitochondrion (AF494279)	91	AY250889
BPU274	2.0	Mitochondria: *Chlorophyta*	*Spirogyra* sp. strain CLP-2000 mitochondrion (AF191801)	88	AY250862
TBP051	1.2	Mitochondria: *Chlorophyta*	*Chaetosphaeridium globosum* mitochondrion (AF494279)	87	AY250890
BPS153	2.0	α-*Proteobacteria*	Uncultured eubacterium WD2105 (AJ292609)	95	AY250855
BPU225	1.2	α-*Proteobacteria*	Uncultured bacterium clone cvf67053 (AY100556)	92	AY250859
BPS147	<1	α-*Proteobacteria*	*Sphingomonas subterranea* (AB025014)	99	AY250854
BPU105	<1	α-*Proteobacteria*	Uncultured α-proteobacterium clone W2b-8C (AY192273)	94	AY250856
BPU264	<1	α-*Proteobacteria*	Uncultured α-proteobacterium clone W2b-8C (AY192273)	94	AY250861
BPS093	<1	γ-*Proteobacteria*	*Acinetobacter* sp. strain OM-E81 (AB020207)	99	AY250852
TBP077	<1	γ-*Proteobacteria*	*Acinetobacter johnsoni* (AY167841)	92	AY250891
TLT148	<1	*Planctomycetales*	Uncultured eubacterium 2112 (AJ292683)	95	AY250896
TLT128	2.0	?	Uncultured soil bacterium clone C06 (AF507679)	87	AY250894
BPS103	<1	?	Uncultured eubacterium WD272 (AJ292684)	95	AY250853
TLT134	<1	?	Uncultured bacterium RO_clone_12B (AY159191)	91	AY250895

[a] Abundance is based on the number of clones whose RFLP patterns were identical or whose sequence was ≥98% identical to that of the clone listed. Approximately 700 individual clones were screened.
[b] CFB, *Cytophagales-Flavobacteria-Bacteroides* group.

including *Trebouxia* sp. photobionts, while the other type of endolithic community was dominated by cyanobacteria morphologically similar to *Gloeocapsa* spp. and *Hormathonema* spp. (16). To minimize potential biases in the representation of sequences in these clone libraries, we constructed 11 independent libraries with different combinations of universal and phylogenetic-group-specific oligonucleotide primers. In total, over 1,100 clones from both communities were sorted into unique classes based on RFLP analysis, and all clones with unique RFLP patterns were fully or partially sequenced.

A comparison of the sequences indicated that clones from all libraries fell into 51 distinct relatedness groups with ≥98% sequence identity (phylotypes), 46 belonging to *Bacteria* and 5 belonging to *Eucarya*. This level of rRNA sequence identity (≥98%) corresponds approximately to the species level (37). No archaeal sequences were identified, despite attempts to enrich for archaeal rRNA genes with archaeon-specific PCR primers. Comparisons of clone libraries prepared from the same environmental DNA with different sets of PCR primers indicated similar ratios of predominant sequences. A total of 25 phylotypes were identified from the lichen-dominated community, and 26 were identified from the cyanobacterium-dominated community. This study certainly did not exhaust the diversity available in the libraries, since many cloned rRNA genes were encountered only once.

All but four of the sequence types were clearly associated with known phylogenetic groups. Of the 51 sequence types, 46 (90%) were ≥90% identical to rDNA sequences available in GenBank, 25 (49%) were ≥95% identical (approximately genus level [37]), and 6 (12%) were ≥98% identical. None of the sequences was identical to a known rRNA sequence. Among the approximately 1,100 clones analyzed, we identified 14 as chimeras generated during PCR amplification. Two sequences detected only once each were presumed to be contaminants, based on their high degree of identity (≥99%) to common contaminants of rRNA-based molecular surveys (41), and were excluded from our analyses. Despite the large diversity of sequences that we encountered, the dominant populations of both endolithic communities were relatively simple.

Lichen-dominated community. Five rDNA clone libraries (four universal and one eucaryal) were constructed from two different samples of the lichen-dominated community, and a total of 672 clones were screened. RFLP and sequence analyses indicated that these clones included 25 unique phylotypes, summarized in Table 1. All of the sequences were different (<98% rRNA sequence identity) from those identified in the cyanobacterium-dominated community.

The quantitative distributions of the different phylotypes in the universal clone libraries are diagrammed in Fig. 1A. Three phylotypes were seen to dominate these libraries, accounting for more than 70% of the clones. Comparisons of these rRNA sequences with other sequences in public databases by using BLAST analysis (2) indicated that these clones were representatives of fungi (BPE012; 29% of clones), of chloroplasts (BPU108; 22% of clones), and of green algae (BPE026; 22% of clones) (Table 1 and Fig. 1A). Both BPE012 and BPE026 were very closely related (≥98% sequence identity) to rRNA sequences in public databases: BPE012 was 98% identical to the nuclear rRNA gene sequence of the ascomycete fungus *Texosporium sancti-jacobi*, and BPE026 was >99% identical to the nuclear rRNA gene sequence of the green alga *Trebouxia jamesii*. Both of these organisms have been identified as com-

TABLE 2. Summary of rDNA clones identified from the cyanobacterium-dominated cryptoendolithic community

Clone	% Abundance[a]	Putative group[b]	Closest BLAST match (GenBank accession no.)[c]	% Identity	GenBank accession no.
FBP255	31.1	α-*Proteobacteria*	*Blastomonas ursincola* (AB024289)	94	AY250869
FBP195	<1	α-*Proteobacteria*	Uncultured sheep mite bacterium Llangefni 28 (AF290483)	91	AY250863
FBP380	<1	α-*Proteobacteria*	Glacial ice bacterium M3C1.8K-TD9 (AF479381)	93	AY250878
FBP492	<1	α-*Proteobacteria*	*Amaricoccus tamworthensis* (U88044)	97	AY250888
FBP256	30.0	*Cyanobacteria*	LPP group cyanobacterium QSSC8cya (AF170758)	97	AY250870
FBP290	<1	*Cyanobacteria*	LPP group cyanobacterium QSSC8cya (AF170758)	97	AY250874
FBP341	<1	*Cyanobacteria*	*Plectonema* sp. strain F3 (AF091110)	95	AY250877
FBP403	<1	*Cyanobacteria*	LPP group cyanobacterium QSSC8cya (AF170758)	96	AY250881
FBP266	26.0	*Thermus-Deinococcus*	Uncultured Antarctic soil bacterium clone bh6.6 (AF419205)	90	AY250871
FBP269	1.3	*Actinobacteria*	*Rubrobacter radiotolerans* (U65647)	94	AY250873
FBP211	1.1	*Actinobacteria*	*Arthrobacter agilis* (X80748)	99	AY250864
FBP391	1.1	*Actinobacteria*	Uncultured high-GC-content gram-positive bacterium Ipha7 (AF10979)	90	AY250879
FBP218	<1	*Actinobacteria*	*Sporichthya polymorpha* (AB025317)	95	AY250865
FBP234	<1	*Actinobacteria*	Uncultured bacterium clone MB-A2-100 (AY093455)	93	AY250866
FBP304	<1	*Actinobacteria*	*Sporichthya polymorpha* (AB025317)	95	AY250876
FBP402	<1	*Actinobacteria*	*Frankia* sp. strain G10—Namibia (X92365)	96	AY250880
FBP406	<1	*Actinobacteria*	*Sporichthya polymorpha* (AB025317)	94	AY250882
FBP417	<1	*Actinobacteria*	*Frankia* root nodule symbiont FE37 (AF063641)	93	AY250883
FBP460	<1	*Actinobacteria*	*Frankia* sp. strain AcN14a (M88466)	93	AY250884
FBP468	<1	*Actinobacteria*	*Sporichthya polymorpha* (AB025317)	94	AY250885
FBP483	<1	*Actinobacteria*	Uncultured eubacterium Hb7-K1 (AJ298576)	96	AY250887
FBP267	1.6	GNS	Uncultured bacterium clone glen99_18 (AY150884)	96	AY250872
FBP471	<1	GNS	Uncultured bacterium clone B-06 (AB081823)	95	AY250886
FBP292	1.3	CFB	Bacterium strain 47077 (AF227830)	89	AY250875
FBP241	<1	*Acidobacteria*	Bacterium clone 11–25 (Z95709)	91	AY250867
FBP249	1.6	?	Uncultured soil bacterium clone S0202 (AF507699)	89	AY250868

[a] Abundance is based on the number of clones whose RFLP patterns were identical or whose sequence was ≥98% identical to that of the clone listed. Approximately 500 individual clones were screened.
[b] GNS, green nonsulfur bacteria; CFB, *Cytophagales-Flavobacteria-Bacteroides* group.
[c] LPP, *Leptolyngbya-Phormidium-Plectonema* group.

ponents of lichen symbioses (14) and presumably constitute the dominant lichens in the community. Clone BPU108 was not closely related to any sequence in GenBank but was most similar to the plastid rRNA gene sequence from unicellular algae (~90% sequence identity to the *Koliella sempervirens* chloroplast rRNA gene) and grouped with them in the phylogenetic tree shown in Fig. 2. The similarly high abundances of this plastid rRNA gene sequence and of the *T. jamesii*-related nuclear rRNA phylotype suggest that these sequences are derived from the same organism and represent the nuclear and corresponding chloroplast rRNA genes. The fourth most abundant phylotype, clone BPU107, accounted for approximately 6% of clones in the universal libraries and belongs to the bacterial phylogenetic order *Cytophagales*. BPU107 is not

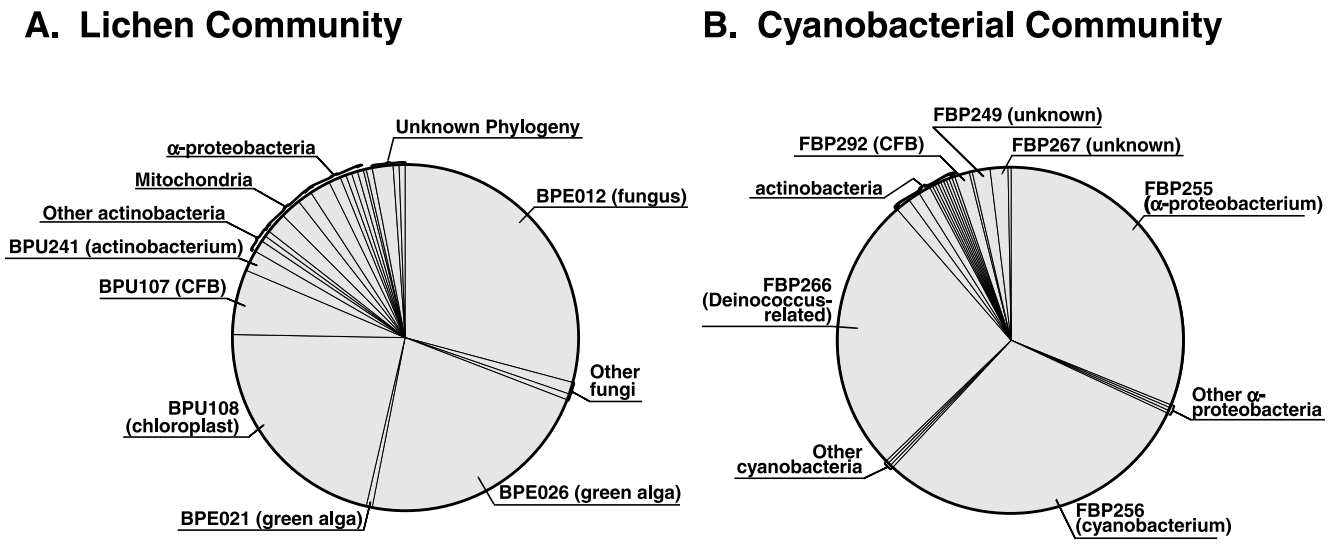

FIG. 1. Phylotypes in clone libraries. Diagrams show the relative abundance of SSU rDNA phylotypes in clone libraries from the lichen-dominated community and the cyanobacterium-dominated community. CFB, *Cytophagales-Flavobacteria-Bacteroides* group.

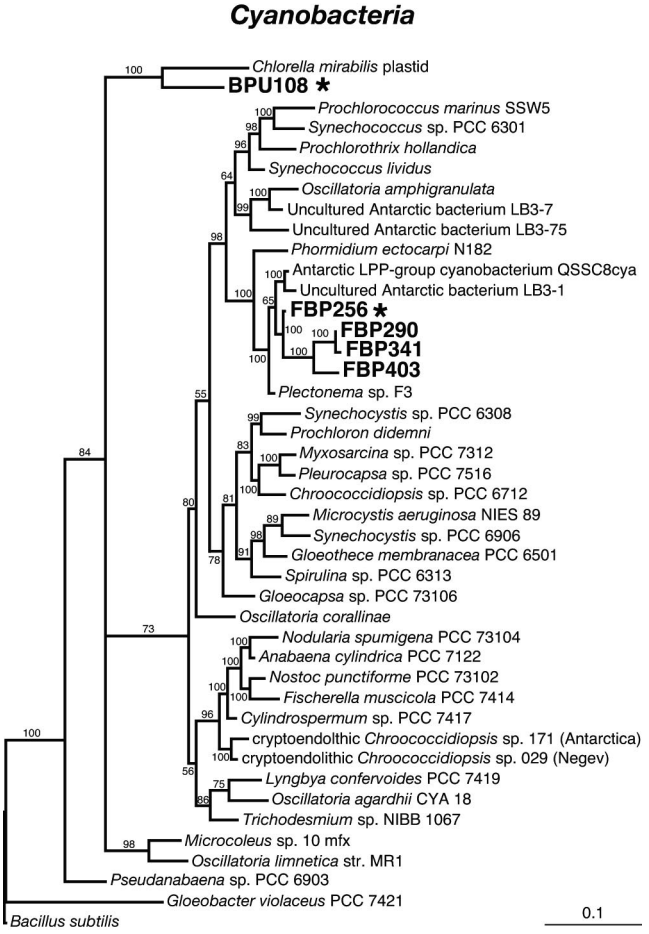

FIG. 2. Phylogenetic tree of cyanobacterial and chloroplast SSU rRNA sequences. Maximum-likelihood analysis illustrates the relationship of selected cryptoendolithic SSU rDNA clones to representative cyanobacterial and chloroplast sequences. Clones from this study are indicated in larger, bold type. Clones marked with an asterisk indicate abundant phylotypes in the clone library (Tables 1 and 2). Sequences from two molecular surveys of McMurdo Dry Valleys lake ice covers (19, 32) and Antarctic sublithic soils (36) are indicated by the designations LB3 and QSSC, respectively. Bootstrap values, given as percentages of 100 replicate trees, are indicated for branches supported by more than 50% of the trees. The scale bar represents 0.1 nucleotide change per position.

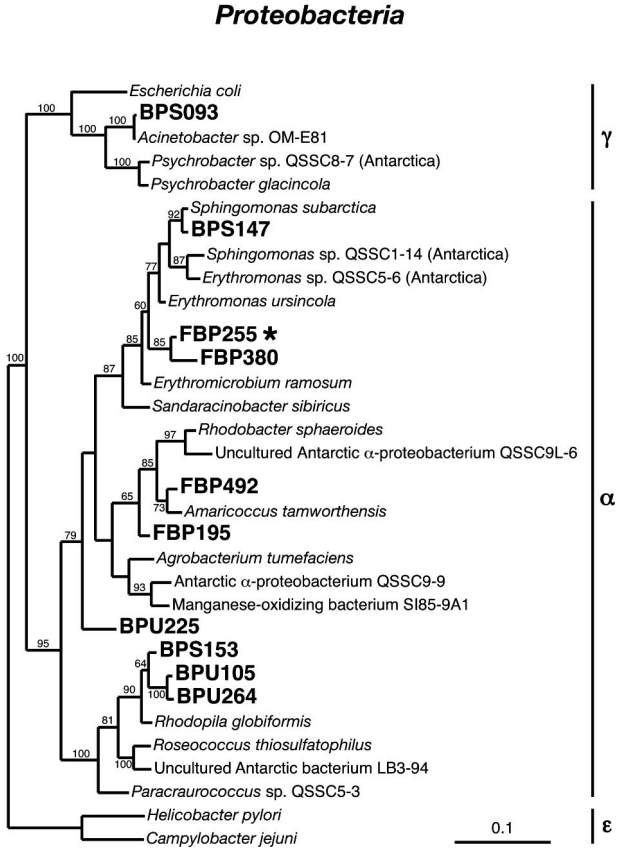

FIG. 3. Maximum-likelihood analysis illustrating the relationship of selected cryptoendolithic SSU rDNA clones to representative sequences associated with members of the *Proteobacteria*. Clones from this study are indicated in larger, bold type. Clones marked with an asterisk indicate abundant phylotypes in the clone library (Tables 1 and 2). Sequences from a molecular survey of Antarctic sublithic soils (36) are indicated by the designation QSSC. Bootstrap values, given as percentages of 100 replicate trees, are indicated for branches supported by more than 50% of the trees. The scale bar represents 0.1 nucleotide change per position.

closely related to any known rRNA sequence, having only 95% sequence identity to *Cytophagales* sp. strain MBIC4147 (Table 1).

Several bacterial groups accounted collectively for a significant fraction of the total number of clones in libraries obtained from the lichen-dominated community, although individual phylotypes were not particularly abundant. Four phylotypes belonging to the class *Actinobacteria* represented approximately 4% of clones, but only a few individual sequences were encountered. Similarly, six α-proteobacterial phylotypes accounted for about 5% of clones. Three sequences (clones TBP017, BPU274, and TBP051) appeared to be related to mitochondrial rRNA sequences but had low levels of nucleotide sequence identity to existing sequences (<90%) and generated very long branches and spurious results in phylogenetic analyses (data not shown). For this reason, these sequences were not included in the phylogenetic tree of proteobacterial sequences shown in Fig. 3. Two phylotypes represented by single clones were related to the γ-*Proteobacteria* and the *Planctomycetales* (Table 1 and Fig. 3 and 4). Finally, three sequences identified in the universal libraries (clones BPS103, TLT128, and TLT134) grouped phylogenetically with bacterial rRNA sequences (data not shown) but did not consistently group with specific bacterial taxa. Analysis of these sequences suggests that these rRNA gene clones are not PCR-generated chimeras or artifacts. Identification and analysis of additional relatives of these sequences will be required to determine whether they belong to known bacterial groups or, rather, represent currently unrecognized phylogenetic groups.

We did not encounter any archaeal rRNA genes in libraries derived from amplifications with universal PCR primers. Consequently, we attempted to detect archaea by analysis of one library prepared from lichen-dominated community DNA with archaeon-specific oligonucleotide primers at a low stringency. All clones obtained from this library were of bacterial origin;

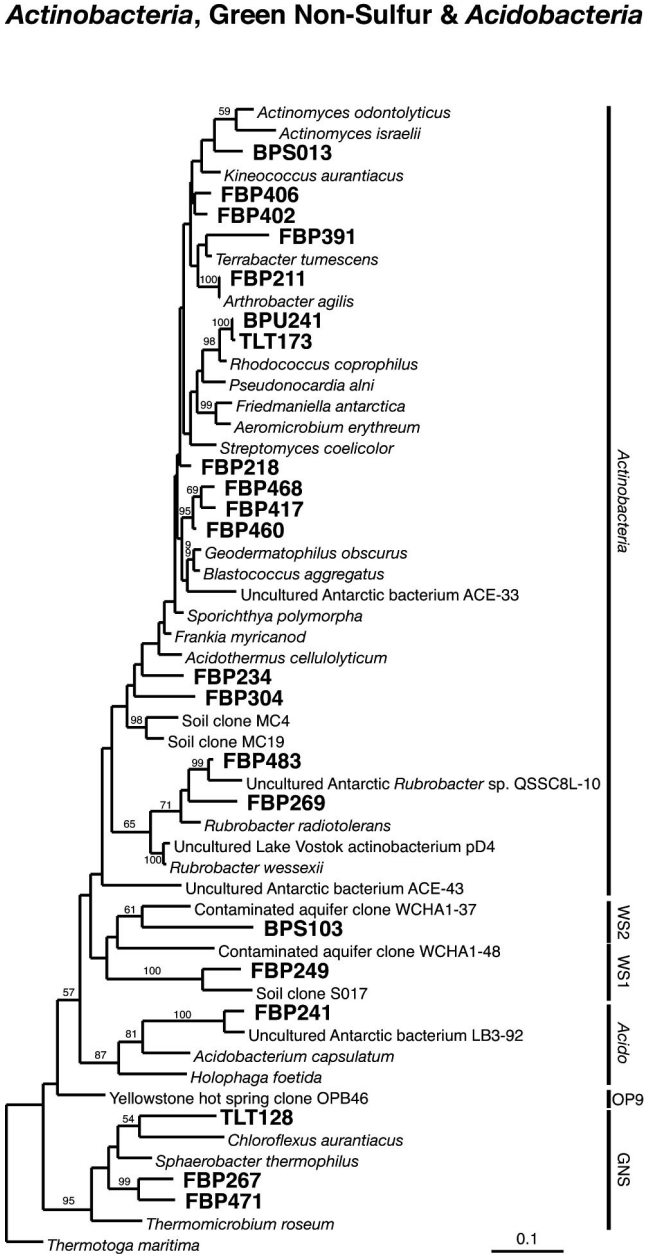

FIG. 4. Molecular phylogenetic analysis of SSU rRNA clones related to members of the *Actinobacteria*, green nonsulfur bacteria, and *Acidobacteria*. Maximum-likelihood analysis illustrates the relationship of selected cryptoendolithic SSU rDNA clones to representative actinobacterial, green nonsulfur bacterial (GNS), and acidobacterial (*Acido*) sequences. Clones from this study are indicated in larger, bold type. Sequences from two molecular surveys of McMurdo Dry Valleys lake ice covers (19, 32) and Antarctic sublithic soils (36) are indicated by the designations LB3 and QSSC, respectively. Bootstrap values, given as percentages of 100 replicate trees, are indicated for branches supported by more than 50% of the trees. The scale bar represents 0.1 nucleotide change per position. WS1, WS2, and OP9, candidate phylogenetic groups (13, 22).

however, they were presumably generated by mispriming during PCR amplification at the lower annealing temperature. We conclude that archaea are absent from this ecosystem or are present at only very low levels. Although this clone library yielded a few unique bacterial rDNA phylotypes, the abundance of the corresponding organisms in the community cannot be evaluated from this library because of the selective nature of the primer set.

Cyanobacterium-dominated community. Five clone libraries (two universal, two bacterium specific, and one cyanobacterium specific) were prepared from a sample of the cyanobacterium-dominated community. Several attempts were made to prepare eucaryal and archaeal libraries from this community, but PCR amplification with PCR primers specific for eucarya or archaea yielded no detectable products. We identified 26 unique phylotypes among 480 clones from these libraries analyzed by the RFLP method (Table 2).

Three phylotypes accounted for over 87% of the clones in the libraries (Table 2; summarized in Fig. 1B), regardless of the primer combinations used for PCR amplification. Clone FBP256 represented approximately 30% of the clones (Table 2 and Fig. 1B) and represents a cyanobacterium with >97% nucleotide sequence identity to previously identified cyanobacterial sequences from Dry Valleys lake ice covers and Antarctic sublithic soils (19, 32, 36). Figure 2 shows a phylogenetic tree indicating the relationship of FBP256 to representatives of the cyanobacteria. FBP256 consistently associates at high bootstrap values with filamentous cyanobacteria, such as *Phormidium* spp. In particular, FBP256 was most closely related to the cultivated cyanobacteria *Plectonema* spp. (97% rRNA sequence identity). These results were unexpected given the coccoid morphologies previously described for cyanobacteria in this community (16). FBP255 (~31% of clones) (Table 2 and Fig. 1B) is a representative of the α-*Proteobacteria*. The phylogenetic tree shown in Fig. 3 indicates that FBP255 associates with known aerobic anoxygenic phototrophs (43, 44) and is most closely related to *Blastomonas ursincola* (94% rRNA sequence identity). Finally, clone FBP266 accounted for approximately 26% of clones analyzed for the cyanobacterium-dominated community libraries (Table 2 and Fig. 1B). BLAST analysis (2) suggested that the microbe represented by clone FBP266 is related to known *Deinococcus* spp., but only distantly (ca. 90% nucleotide sequence identity). Phylogenetic analysis revealed that FBP266 consistently formed an independent clade within the *Thermus-Deinococcus* bacterial group. Figure 5 shows a representative phylogenetic tree in which FBP266 and several environmental sequences form this new clade related to *Deinococcus*. All of the environmental sequences associated with FBP266 in this new clade were identified from Antarctic microbial communities.

Other phylotypes identified in the clone libraries from the cyanobacterium-dominated community each accounted for less than 2% of the total number of clones and included representatives of *Actinobacteria*, green nonsulfur bacteria, *Acidobacteria*, and *Cytophagales* (Table 2 and Fig. 1B and 4). One phylotype (FBP249; <2% of the total clones) did not appear to group consistently with known bacterial phylogenetic taxa. Sequence analysis suggested that FBP249 was neither a PCR-generated chimera nor the result of some other artifact. Furthermore, this sequence type was isolated from independent libraries constructed with different combinations of PCR primers. These data suggest that FBP249 may represent a previously undetected phylogenetic group of *Bacteria*. These find-

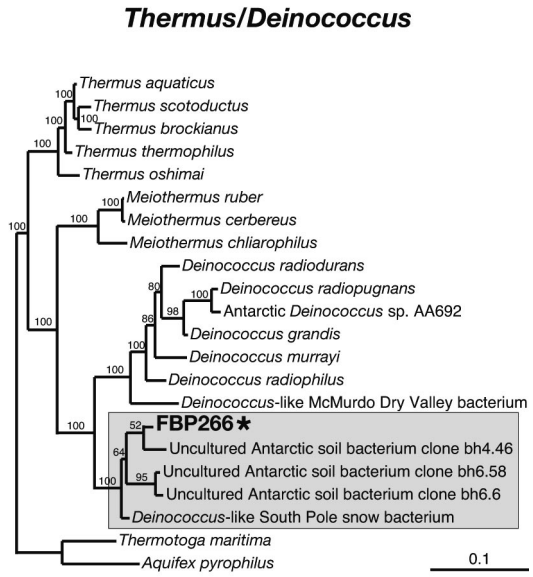

FIG. 5. Phylogenetic tree of *Thermus-Deinococcus* SSU rRNA sequences. Maximum-likelihood analysis illustrates the evolutionary relationship of the cryptoendolithic SSU rDNA clone FBP266 to representative *Thermus* sp., *Deinococcus* sp., and environmental 16S rRNA sequences. Clone FBP266 from this study is indicated in larger, bold type. The asterisk by clone FBP266 indicates that it is an abundant phylotype in the clone library (Table 2). Sequences from a molecular survey of McMurdo Dry Valleys soils (Shravage et al., unpublished) are indicated by the designation "bh." The unpublished 16S rDNA sequence for *Deinococcus* sp. strain AA692, a *Deinococcus* relative isolated by Hirsch and colleagues from a sample of the lichen-dominated community at Battleship Promontory in the McMurdo Dry Valleys (21), was kindly provided by E. Stackebrandt and P. Hirsch (personal communication). The box indicates the new clade of *Deinococcus*-related sequences defined by FBP266, the South Pole snow *Deinococcus*-related sequence, and the Antarctic soil clones. All of the sequences in this proposed new clade have been found exclusively in Antarctic microbial communities. Bootstrap values, given as percentages of 100 replicate trees, are indicated for branches supported by more than 50% of the trees. The scale bar represents 0.1 nucleotide change per position.

ings will need to be confirmed by the identification of other representatives of the putative group.

Several bacterial groups (e.g., *Actinobacteria* and *Proteobacteria*) were represented by several distantly related sequence types. For instance, individual phylotypes belonging to the class *Actinobacteria* never accounted for more than 1% of the clones. However, we identified 12 distinct actinobacterial phylotypes that collectively amounted to over 6% of the clones (Table 2 and Fig. 1B) and were phylogenetically distributed in that taxon. This result suggests a broad diversity and potentially important roles of actinobacteria in the cyanobacterium-dominated community.

Previous culture-based and microscopic characterizations of the cyanobacterium-dominated community had indicated that it was composed primarily of two cyanobacteria, *Gloeocapsa* spp. and *Hormathonema* spp., based on morphological descriptions (16). However, our analysis identified only one major and several minor cyanobacterial sequence types, which phylogenetic analysis revealed to be closely related to each other (Fig. 2). To ensure that we had properly disrupted samples during DNA extraction, we microscopically examined cell lysates obtained from the DNA extraction protocol and found no intact cellular structures (data not shown). We also constructed a cyanobacterium-specific rDNA library with one cyanobacterium-specific primer and one universal primer for PCR amplification. Analysis of 96 clones from this library showed that all had the same RFLP patterns as clone FBP256, the dominant cyanobacterial phylotype identified in the universal and bacterium-specific clone libraries. Sequencing of several randomly selected clones from the cyanobacterium-specific clone library showed that all were ≥99% identical to clone FBP256.

Cultivation of cyanobacteria. Previous microscopic observations of the cyanobacterium-dominated community had identified the occurrence of two principal and several minor species of cyanobacteria in the community. The dominant cyanobacteria, *Gloeocapsa* spp. and *Hormathonema* spp., were described mainly on the basis of coccoid morphology (16). However, the dominant cyanobacterial sequence type identified in our study (clone FBP256) is phylogenetically most closely related to the filamentous cyanobacteria *Phormidium* spp. and *Plectonema* spp. (Fig. 2). In order to resolve this apparent discrepancy between in situ morphological observations and our phylogenetic analyses, we derived cultures from the cyanobacterium-dominated community. Previous studies of Antarctic sublithic soil communities had found that cyanobacteria related to FBP256 grew optimally on oligotrophic, nitrogen-containing medium (36). We therefore cultured cyanobacteria from the cyanobacterium-dominated community on oligotrophic SNAX agar incubated under constant illumination at 4°C. Small amounts of pulverized sandstone crystals were plated directly on SNAX agar. After 2 weeks of incubation, green colonies were observed growing directly on the sandstone crystals (data not shown). In parallel, pulverized sandstone crystals from frozen rock samples were vortexed in phosphate-buffered saline, and the liquid supernatant was plated on SNAX agar. After 1 month, green cyanobacterial colonies began appearing on the plate. These colonies were picked and replated on fresh SNAX agar, and single colonies were isolated. Microscopic analysis indicated that the cultures were not axenic. Replated colonies grew well at both 4 and 15°C but failed to grow at 25°C (data not shown), suggesting that these cyanobacteria may be truly psychrophilic, as defined by Morita (29). In contrast, the cyanobacteria *Chroococcidiopsis* spp., also isolated from Antarctic cryptoendolithic communities, are capable of growing at temperatures above 25°C (31).

Cyanobacterial cells from these colonies were coccoid and measured approximately 5 μm in diameter (Fig. 6). SSU rRNA genes amplified with cyanobacterium-specific primers and sequenced from the colonies were 100% identical to FBP256. This result confirms that we had cultured the cyanobacterium corresponding to the dominant cyanobacterial rDNA in our clone libraries and that, despite their coccoid morphology, the organisms were most closely related to filamentous oscillatorian cyanobacteria, such as *Phormidium* spp.

DISCUSSION

Endolithic microbial communities occur worldwide and are thought to play an important role in global processes, such as the weathering of rocks and the cycling of nutrients and ele-

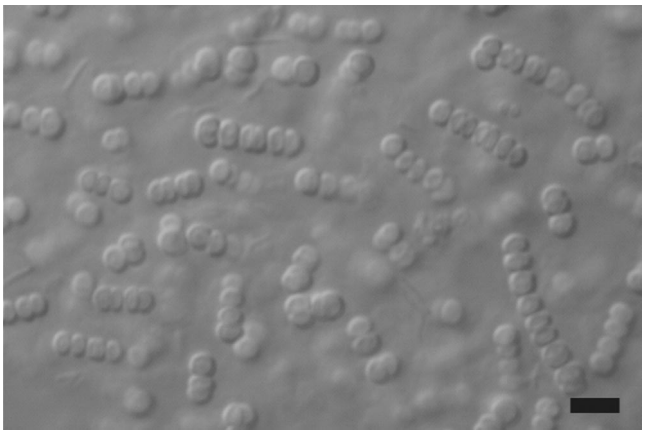

FIG. 6. Photomicrograph of cyanobacterial cells isolated from colonies growing on oligotrophic SNAX agar at 4°C under constant illumination. Scale bar, 10 μm.

ments. Nevertheless, little is known about the compositions and dynamics of these communities. To date, the best-studied examples of endolithic communities are those of the polar deserts of the McMurdo Dry Valleys in Antarctica, where such communities constitute the major forms of life. These communities and their environmental settings have been studied by microscopic observations to characterize morphologically distinct primary producers (16) and by laboratory cultivation to study heterotrophic constituents of the communities (21, 34, 35). This study is the first rRNA-based molecular survey of endolithic communities made independent of laboratory cultivation. Our results indicate that these communities are indeed diverse, as predicted from morphological analyses. Based on the frequencies of rRNA genes in our clone libraries, however, the communities seem to be dominated by relatively small numbers of particularly abundant organisms. These abundant organisms do not necessarily correspond to those identified by morphological analysis or cultivation. Because of their abundance, the organisms represented by the abundant rRNA sequences are expected to be significant primary producers in these ecosystems.

We emphasize, however, that the frequencies with which different rRNA genes are obtained in PCR-based libraries must be interpreted cautiously. Organisms and their relative abundances as detected by PCR need to be confirmed with other methods. The relative ratios of rRNA genes in clone libraries do not necessarily correspond to the abundances of the represented organisms in the original ecosystem. We acknowledge that factors such as differential efficiencies of PCR amplification or cloning (39) or different numbers of rRNA operons in different organisms (23) can influence the proportions of rRNA phylotypes in clone libraries. Nonetheless, it is likely that we have identified the most abundant organisms in these ecosystems.

For both types of communities examined, our rRNA-based molecular surveys suggest a lower diversity of the primary producers, as reflected by the abundances of rRNA genes in our clone libraries, than has been proposed from morphological studies of these communities (16, 30). Two principal classes of endolithic communities in the McMurdo Dry Valleys were defined by previous studies on the basis of the morphologically identifiable primary producers: lichen- and cyanobacterium-dominated communities (16, 30). Lichen-dominated communities are the most prevalent in the Dry Valleys. Because these lichens colonize the interstitial spaces of the rock substrate, have no epilithic morphology, and do not form mating structures, morphology-based identification of these organisms was not possible. Several species of filamentous fungi have been cultivated from these communities (1), but no rDNA sequences from these cultivars are yet available in public sequence databases. In this study, we identified only one principal fungal phylotype from two different lichen-dominated samples. This endolithic fungal sequence was closely related (>97% nucleotide sequence identity) to rDNA sequences of epilithic lichens (*Buellia* spp.) similar to those found in the Dry Valleys (15, 20). These results support the previously suggested hypothesis that the endolithic lichens are the same as the epilithic lichens, but with different cellular morphologies (30).

Morphological and physiological characterizations of the endolithic lichen-dominated community suggested the presence of many species of chlorophycean algae. These photobionts were morphologically distinctive and were identified as *Trebouxia* spp. and *Pseudotrebouxia* spp. (4, 30). As in the case of the lichen mycobionts, our molecular analysis revealed only one dominant green alga rDNA sequence. This sequence was nearly identical (>99% nucleotide sequence identity) to the published rDNA sequence of the lichen photobiont *T. jamesii* (7). We also identified only one class of chloroplast rDNA sequence, presumably that of the *T. jamesii*-related photobiont. The various photobionts identified by previous microscopic studies may be morphological variants of a single organism or may be present in our samples at levels represented by less than a few percent of the total rDNA sampled. Local small-scale heterogeneity in sampling sites may also account for variability in different studies.

Previous morphological studies of the Dry Valleys cyanobacterium-dominated communities suggested the presence of several different species of cyanobacteria, including *Hormathonema* spp., *Gloeocapsa* spp., *Anabaena* spp., *Aphanocapsa* spp., and *Lyngbya* spp. (16, 30). These organisms were often present in different combinations but were not always found in all samples of the cyanobacterium-dominated communities (16, 30). We identified only one principal cyanobacterial rDNA sequence, most closely related to *Plectonema* sp. strain F3 and to other Antarctic environmental cyanobacterial sequences identified in soil and in sediments in the Dry Valleys lake ice covers (32, 36). Unfortunately, a comparison of these molecular results with observations from previous studies of Antarctic cryptoendolithic communities is complicated by the absence of rDNA sequence data for most of the organisms identified previously by morphology. For example, there are no rDNA sequences for relatives of *Hormathonema* spp., only one rDNA sequence for *Gloeocapsa* spp., and a few sequences closely related to *Chroococcidiopsis* spp. in public sequence databases. No close relatives of these organisms were detected in our molecular surveys.

Because of their conspicuous nature, it has been presumed that photosynthesis by lichens and cyanobacteria provides the main sustenance of Antarctic endolithic communities. The high abundances of lichen and cyanobacterial rDNA se-

quences that we observed are consistent with leading roles for these organisms in these ecosystems. In addition to the sequences of known photosynthetic organisms, we identified in the cyanobacterium-dominated community other kinds of organisms that, because of the abundances of their rDNA sequences, were likely to contribute substantially to primary productivity in this community. Two such abundant sequences are a representative of the *Thermus-Deinococcus* bacterial phylogenetic group and a representative of the α-*Proteobacteria*.

Several rDNA sequences and cultured examples of *Deinococcus*-like organisms were previously obtained from Antarctic settings, including South Pole snow (9), Dry Valleys soils (9; B. V. Shravage, M. S. Patole, and Y. S. Shouche, unpublished data), and cryptoendolithic communities (21, 34). Members of the family *Deinococcaceae* are best known for their ability to withstand extremely large amounts of ionizing radiation-induced damage to their DNA (6). However, studies have suggested that this ability may be incidental and a consequence of an adaptation to repair DNA damage induced by desiccation (26). Such properties would be advantageous to organisms living in the desiccating environment of the Dry Valleys and have indeed been shown to exist in one cyanobacterium isolated from Antarctic cryptoendolithic communities (8). Nevertheless, the high abundance of *Deinococcus*-like rDNA sequences seen in the cyanobacterium-dominated community was completely unexpected. This high abundance strongly suggests that the representative organisms are engaged in primary productivity. In the Antarctic cryptoendolithic communities, photosynthesis is probably the main or only source of primary productivity (30). The results of analyses of our clone libraries might therefore suggest that the organisms represented by clone FBP266 possess phototrophic properties. However, no phototrophic examples of the *Thermus-Deinococcus* phylogenetic group are known. Various investigators were able to culture *Deinococcus*-like organisms from samples of the lichen-dominated community (21, 34). Preliminary molecular characterization of these organisms (*Deinococcus* sp. strain AA692; P. Hirsch and E. Stackebrandt, personal communication) places them well within the *Deinococcus* clade (Fig. 5), most closely related to *Deinococcus radiopugnans* (>96% rDNA sequence identity). Despite being termed *Deinococcus*-like, the organisms represented by FBP266 are only distantly related (ca. 90% rDNA sequence identity) to cultivated examples of *Deinococcus* spp. and so are likely to have many physiological differences from the cultured organisms.

Our molecular surveys revealed the unexpected presence of other potential phototrophs that were not identified in previous morphological or cultivation-based studies of the cyanobacterium-dominated community. One of the most abundant rDNA sequences in clone libraries prepared from the cyanobacterium-dominated community belongs to a member of the α-Proteobacteria most closely related to *B. ursincola* (94% rDNA sequence identity; approximately genus-level variation [18, 37]) and other aerobic, anoxygenic phototrophs (44). Related sequences have also been identified in Antarctic sublithic soil communities (QSSC clones; Fig. 3) (36). Members of this group, such as *Erythromonas ursincola*, are typically found in freshwater environments and are strictly aerobic (44). Cultivated representatives of these organisms contain bacteriochlorophyll *a* incorporated into a photochemically active reaction center and light-harvesting complexes. Laboratory cultures of these organisms seem unable to use light as their sole source of energy (44). However, the considerable abundance of α-proteobacterial sequences related to clone FBP255 in clone libraries prepared from the endolithic cyanobacterium-dominated community suggests that the corresponding organisms engage in primary productivity and may be phototrophic. The occurrence in our clone libraries of approximately equal numbers of rRNA genes of one each of a specific cyanobacterium, a specific α-proteobacterium, and a specific *Deinococcus*-like representative indicates approximately equivalent biomasses of the organisms and suggests that the representative organisms may be involved in a tightly regulated, syntrophic relationship. Further studies of additional examples of the cyanobacterium-dominated community will be needed to confirm the ubiquity of these organisms and to determine the character of their relationships.

Although our clone libraries were dominated by small numbers of particular rDNA clones, less abundant sequences represented a high degree of diversity. Collectively from these cryptoendolithic communities, we identified a total of 16 distinct phylotypes belonging to the bacterial class *Actinobacteria*. Phylogenetic analysis showed that the sequences were related to those of a broad variety of organisms within the class *Actinobacteria* (Fig. 4). The diversity of the sequences suggests a potentially broad diversity of physiologies and metabolic properties for the organisms indicated by the sequences. Most of the rRNA sequences that we detected are not clearly related at the species or genus levels to those of any cultured actinobacteria (rRNA sequence identity, usually <95%) (Tables 1 and 2). One sequence, however, is nearly identical (99%) to the rDNA sequence of *Arthrobacter agilis*. This close rDNA relatedness indicates that the properties of *A. agilis* would be a useful guide in culture-based or other studies of this environmental organism. Indeed, previous cultivation-based studies of these cryptoendolithic communities identified several isolates preliminarily identified as *A. agilis* (21, 34). Unfortunately, of the 1,500 isolates obtained by several investigators, most of which were probably representatives of the class *Actinobacteria*, only a few were characterized (33, 34, 35), and only one rDNA sequence derived from the collection is currently available (33). Although the absence of rDNA sequence information for these organisms does not permit a direct comparison of our cultivation-independent molecular surveys to culture-based studies, it is clear from both approaches that a wide variety of actinobacteria are present in these communities. Such organisms occur widely in soils, where they are considered to play important roles as saprophytic heterotrophs.

This study is far from exhaustive in the identification of the organisms and the extent of phylogenetic diversity in these endolithic communities. Many of the rDNA sequences were encountered only once in the study; therefore, much novelty remains to be identified. Indeed, several sequences from the lichen-dominated community (BPS103, TLT128, and TLT134; Table 1) and one of the sequences from the cyanobacterium-dominated community (FBP249; Table 2) are not specifically affiliated with any bacterial phylogenetic group and may indicate new candidate phylogenetic groups. Although this study does not fully characterize the potential diversity of these Antarctic ecosystems, we believe that we have identified, by their

abundances, the main contributors to the flow of carbon and energy. The sequences that we have identified will provide information for tools such as fluorescent probes (11, 12) and PCR primers with which to study the corresponding organisms further in their natural setting.

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