Microbial Ecology

Epilithic and Endolithic Bacterial Communities in Limestone from a Maya Archaeological Site

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Abstract

Biodeterioration of archaeological sites and historic buildings is a major concern for conservators, archaeologists, and scientists involved in preservation of the world's cultural heritage. The Maya archaeological sites in southern Mexico, some of the most important cultural artifacts in the Western Hemisphere, are constructed of limestone. High temperature and humidity have resulted in substantial microbial growth on stone surfaces at many of the sites. Despite the porous nature of limestone and the common occurrence of endolithic microorganisms in many habitats, little is known about the microbial flora living inside the stone. We found a large endolithic bacterial community in limestone from the interior of the Maya archaeological site Ek' Balam. Analysis of 16S rDNA clones demonstrated disparate communities (endolithic: >80% Actinobacteria, Acidobacteria, and Low GC Firmicutes; epilithic: >50% Proteobacteria). The presence of differing epilithic and endolithic bacterial communities may be a significant factor for conservation of stone cultural heritage materials and quantitative prediction of carbonate weathering.

Introduction

The interaction of microorganisms and building stone is complex and has been reviewed extensively [11, 13, 23, 45–47, 64]. Visible microbial growth and associated pigments may result in undesirable aesthetic changes to historically or culturally important stone buildings and objects [5], whereas the production of organic [10, 63] or inorganic [47] acids and extracellular polysaccharides (EPS) [36, 37] may lead to deterioration of the stone. On the other hand, accumulating evidence points to a protective role for microorganisms in some situations [10, 26, 42, 55, 60].

The Maya archaeological sites in southern Mexico are among the most important cultural artifacts in the Western Hemisphere. The ruins are constructed of limestone, and deterioration is the result of cyclic changes in temperature and humidity and microbial growth [16, 24]. A small number of studies have examined the microbiota of Mayan archaeological sites. These studies have demonstrated the presence of a diverse microbial community, including heterotrophic bacteria, Cyanobacteria, algae, fungi, and lichens, on the surface of stone from the ruins [8, 12, 27, 28, 34, 35, 61, 62]. Despite the porous nature of limestone, all of these studies have been limited to examination of growth of the surface microbiota.

The presence of endolithic microorganisms has been demonstrated in a number of habitats, such as both hot and cold deserts [9, 32], the deep subsurface [2, 33], and rock from an ocean trench [19]. There have been few studies on the endolithic microbiota of culturally important structures, and these have been limited to the examination of chasmoendolithic Cyanobacteria [44, 45] and penetration of stone by fungi [3, 17, 20, 53].

The purpose of this study was to compare the epilithic and endolithic bacterial communities of limestone from the interior of the Maya site Ek' Balam. Total community DNA was extracted from stone samples, and a clone library was constructed after polymerase chain reaction (PCR) amplification. Analysis of clone sequences indicated substantial differences between the epilithic and endolithic communities.

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Methods

Samples were collected from Room 44 of the acropolis at Ek' Balam, Yucatan, Mexico (Fig. 1). Stone cores ($n = 6$) were removed from a location ca. 150 cm high on the interior of the northern wall. Electron microprobe and X-ray diffraction analyses indicated that the stone was primarily composed of calcite (>98%) and contained small amounts of magnesium, glauconite mica, K-feldspar, and Ti–Fe oxide. Samples were removed using a rock chisel sterilized with ethanol wipes and placed in sterile Whirlpak (Nasco, Fort Atkinson, WI, USA) bags on ice. In the laboratory stone samples were separated into epilithic (surface: 1 mm) and endolithic (remaining 1–2 cm) fractions in a laminar flow hood using a sterile rock chisel and hammer. Samples were then weighed in Whirlpak bags and pulverized by wrapping the bags in layers of autoclaved aluminum foil and crushing the stone with a hammer. Three epilithic and three endolithic samples were frozen in TE (100 mM Tris–HCl, 10 mM EDTA, pH 8.0) and an equal number were preserved with 1% formaldehyde.

Total numbers of bacteria in samples preserved with 1% formaldehyde were enumerated after staining with 4′,6-diamidino-2-phenylindole (DAPI) [39]. Samples were first sonicated (Branson model 2510; Danbury, CT, USA) for 5 min to detach cells from the stone. Bacteria were concentrated by filtration (15 kPa vacuum) onto 0.22-μm pore size black polycarbonate membranes (Poretics, Livermore, CA, USA), stained for 5 min with 1.0 mL of 1.0 μg/mL DAPI, and rinsed with 1.0 mL deionized water. Bacteria were enumerated using epifluorescence microscopy.

Samples frozen in TE were thawed at room temperature and DNA was extracted using the UltraClean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA). A portion of the 16S rDNA gene was amplified using the bacterial primers 341f (5′-CCTACGGGAGGC AGCAG-3′) [30] and 907r (5′-CCCCGTCAATTCATT TGAGTTT-3′) [31] as described by Schabereiter-Gurtner *et al.* [48]. PCR reactions were conducted in 50-μL volumes and contained 25 pmol of each primer, 0.2 mM of each dNTP, 5.0 μL of 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 2 U of *Taq* DNA polymerase, 4 μL of template DNA from the extractions, and deionized water. PCR was performed in a Robocycler Gradient 96 thermocycler (Stratagene, La Jolla, CA, USA) using a touchdown PCR program [48].

PCR products were cloned into the pCR 2.2-TOPO vector and transformed into competent *Escherichia coli* as described in the manufacturer's instructions (TOPO TA Cloning Kit K4500-01; Invitrogen, Carlsbad, CA). Plasmid preparation and sequencing were performed at the Dana Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility (Cambridge, MA, USA) using the McPrep Plasmid Purification system (Agencourt Bioscience, Beverly, MA, USA) and a 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) as described in the manufacturer's instructions.

We tested for Chimeric sequences using the Chimera Check program on the Ribosomal Database Project II website [7]. Unaligned sequences were compared to the National Center for Biotechnology Information database using the BLAST search program to find closely related sequences [1]. Alignments were constructed using ClustalX [59]. Phylogenetic analysis was performed using Paup 4.0 beta 10 [57]. Trees were constructed using neighbor-joining distances with 1000 bootstrap replicates. Groupings that occurred in less than 50% of replicates were excluded.

Rarefaction curves were calculated to determine if a sufficient number of clones had been sequenced to compare the two communities, and to compare the number of groups (phyla or in the case of the Proteobacteria, subphyla) found in the two communities. Rarefaction curves [21, 52] for the communities were calculated using:

$$E(\hat{S}_n) = \sum_{i=1}^{S} \left(1 - \frac{\binom{N - N_i}{N}}{\binom{N}{n}} \right) \qquad (1)$$

where

$E(\hat{S}_n)$ = the expected number of groups in a random sample of individuals
S = the total number of groups in the entire collection
N_i = the number of clones of group i
N = the total number of clones in the collection $\sum N_i$
n = value of sample size (number of clones) chosen for standardization ($n \leq N$)

Figure 1. The Acropolis at Ek' Balam, Yucatan, Mexico.

Table 1. Summary of rDNA clones identified from the epilithic community

Clone	*% Abundance*	*Putative group*	*Closest BLAST match (GenBank accession no.)*	*% Identity*	*GenBank accession no.*
EPIa06	1.6	Acidobacteria	Uncultured sludge bacterium A22 (AF234701)	97	AY674792
EPIe04	1.6	Acidobacteria	Uncultured *Holophaga* sp. clone JG30a-KF-145 (AJ536875)	97	AY674821
EPIa03	1.6	Actinobacteria	Uncultured earthworm cast bacterium clone C241 (AY154597)	95	AY674789
EPIa05	1.6	Actinobacteria	Uncultured Actinobacterium clone SBRA95 (AF383710)	94	AY674791
EPIa10	1.6	Actinobacteria	Uncultured soil bacterium clone 288-2 (AF423245)	98	AY674794
EPIa11	1.6	Actinobacteria	Uncultured Actinobacterium clone CF1 (AJ535740)	93	AY674795
EPIb01	1.6	Actinobacteria	Uncultured bacterium clone C2-K15 (AJ421197)	93	AY674796
EPIb09	1.6	Actinobacteria	Uncultured Actinobacteridae bacterium (AJ555204)	97	AY674802
EPIb12	1.6	Actinobacteria	Uncultured bacterium Q3-6C1 (AY048894)	96	AY674804
EPIc03	1.6	Actinobacteria	Uncultured Actinobacterium isolate T15 (AF544290)	91	AY674807
EPIc08	1.6	Actinobacteria	*Saccharothrix flava* (AF114808)	98	AY674810
EPId08	1.6	Actinobacteria	Uncultured Rubrobacteridae bacterium clone glen99_25 (AY150872)	91	AY674816
EPId11	1.6	Actinobacteria	Uncultured bacterium clone glen99_18 (AY150884)	96	AY674818
EPIg11	1.6	Actinobacteria	Uncultured gold mine bacterium D7 (AF337862)	94	AY674832
EPIh07	1.6	Actinobacteria	Uncultured bacterium #0649-1G9 (AF234119)	98	AY674836
EPIh11	1.6	Actinobacteria	Uncultured Acidomicrobidae bacterium clone glen99_24 (AY150886)	92	AY674839
EPIc04	1.6	CFB	Uncultured CFB group bacterium clone DC8-48-2 (AY145599)	97	AY674808
EPIe09	1.6	CFB	Uncultured soil bacterium clone 1506-2 (AF497759)	93	AY674824
EPIh10	1.6	Chloroflexi	Uncultured *Chloroflexi* bacterium (AF423364)	90	AY674838
EPIc01	1.6	Cyanobacteria	*Leptolyngbya* sp. CNP1-B3-C9 (AY239600)	91	AY674805
EPIc11	1.6	Cyanobacteria	*Acaryochloris marina* strain MBIC 11017 (AY163573)	93	AY674812
EPIg01	1.6	Cyanobacteria	Uncultured Cyanobacterium clone TAF-A34 (AY038729)	92	AY674827
EPIg10	1.6	Cyanobacteria	*Lyngbya* sp. strain PCC 7419 (AJ000714)	95	AY674831
EPIg02	4.8	Low GC Firmicutes	*Bacillus barbaricus* strain VII-B3-A2 (AJ422145)	99	AY674828
EPIf05	1.6	Nitrospirae	*Nitrospira moscoviensis* (X82558)	99	AY674825
EPIh06	1.6	Nitrospirae	Uncultured soil bacterium clone 1164-1 (AY326515)	97	AY674835
EPIe05	3.2	OP10	Uncultured candidate division OP10 bacterium clone GR25 (AY150887)	95	AY674822
EPIa02	1.6	α-Proteobacteria	Uncultured bacterium clone B79 (AF407727)	92	AY674788
EPIa09	3.2	α-Proteobacteria	Uncultured bacterium clone GIF7 (AF407198)	98	AY674793
EPIb05	1.6	α-Proteobacteria	Uncultured α-Proteobacterium clone JG34-KF-245 (AJ532704)	93	AY674798
EPIb11	1.6	α-Proteobacteria	Uncultured bacterium clone C8-K36 (AJ421177)	98	AY674803

Table 1. Continued

Clone	*% Abundance*	*Putative group*	*Closest BLAST match (GenBank accession no.)*	*% Identity*	*GenBank accession no.*
EPIc05	3.2	α-Proteobacteria	Uncultured soil bacterium clone 1152-2 (AF423211)	99	AY674809
EPIc09	1.6	α-Proteobacteria	Uncultured soil bacterium clone 178-2 (AY326611)	92	AY674811
EPId01	1.6	α-Proteobacteria	Uncultured α-Proteobacterium clone SM1E02 (AF445680)	98	AY674813
EPId02	1.6	α-Proteobacteria	*Rhizobium* sp. JH1 (AY148434)	95	AY674814
EPId09	3.2	α-Proteobacteria	*Rhodoplanes elegans* strain OK5R2b (AF487437)	97	AY674817
EPIg04	3.2	α-Proteobacteria	Uncultured α-Proteobacterium YNPRH85B (AF465655)	95	AY674829
EPIg07	1.6	α-Proteobacteria	*Nordella oligomobilis* (AF370880)	96	AY674830
EPIh01	1.6	α-Proteobacteria	Uncultured α-Proteobacterium MERTZ_OCM_99 (AF424279)	98	AY674833
EPIh05	1.6	α-Proteobacteria	Uncultured α-Proteobacterium clone E01-9C-5 (AJ581348)	90	AY674834
EPIh08	1.6	α-Proteobacteria	Uncultured bacterium clone C8-K9 (AJ421171)	97	AY674837
EPIc02	1.6	β-Proetobacteria	Uncultured soil bacterium clone 1326-2 (AF423222)	90	AY674806
EPIb02	1.6	β-Proteobacteria	Uncultured bacterium clone D34 (AY274154)	95	AY674797
EPIb06	1.6	β-Proteobacteria	β-Proteobacterium HS5/S24542 (AY337603)	97	AY674799
EPIb08	3.2	β-Proteobacteria	Uncultured soil bacterium clone 646-2 (AF423284)	98	AY674801
EPIa04	1.6	γ-Proteobacteria	Uncultured bacterium clone C7-K19 (AJ421164)	96	AY674790
EPId06	1.6	γ-Proteobacteria	*Thermomonas haemolytica* strain A50-7-3 (AJ300185)	94	AY674815
EPIe01	1.6	γ-Proteobacteria	Uncultured Proteobacterium clone OF3 (AJ538054)	99	AY674819
EPIe03	1.6	γ-Proteobacteria	Uncultured Proteobacterium clone MB7 (AF509004)	84	AY674820
EPIe07	1.6	γ-Proteobacteria	Uncultured Proteobacterium clone JG36-TzT-56 (AJ534624)	91	AY674823
EPIf10	3.2	γ-Proteobacteria	Uncultured bacterium clone JG30-KF-AS32 (AJ536881)	92	AY674826
EPIa01	1.6	δ-Proteobacteria	Unidentified eubacterium RLP10 (AF058009)	94	AY674787
EPIb07	1.6	δ-Proteobacteria	Uncultured bacterium clone CF9 (AJ538050)	92	AY674800
EPIh12	1.6	δ-Proteobacteria	Uncultured δ-Proteobacterium MERTZ_OCM_49 (AF424229)	94	AY674840

$\binom{N}{n}$ = number of combinations of n clones that can be chosen from a set of N clones, or $[N!/n!(N-n)!]$

Composition of the communities was compared using two similarity measurements. The Renkonen index [21] was calculated as

$$P = \sum \text{minimum}\,(p_{1i}, p_{2i}) \tag{2}$$

where

P = similarity between community 1 and 2

p_{1i} = proportion of group i in community 1
p_{2i} = proportion of group i in community 2.

The simplified Morisita index of similarity [18, 21] was calculated as

$$C_{\mathrm{H}} = \frac{2\sum X_{ij}X_{ik}}{\sum X_{ij}^2 X_{ik}^2} \tag{3}$$

where

C_{H} = the simplified Morisita index of similarity
X_{ij}, X_{ik} = the proportion of group i in sample j and sample k.

Table 2. Summary of rDNA clones identified from the endolithic community

Clone	*% Abundance*	*Putative group*	*Closest BLAST match (GenBank accession no.)*	*% Identity*	*GenBank accession no.*
ENDOa03	4.2	Acidobacteria	Agricultural soil bacterium clone SC-I-86 (AJ252662)	98	AY674842
ENDOc03	2.1	Acidobacteria	Uncultured *Holophaga* sp. clone JG37-AG-112 (AJ519387)	96	AY674849
ENDOd12	2.1	Acidobacteria	Bacterial species clone ii3-15 (Z95725)	95	AY674852
ENDOe03	2.1	Acidobacteria	Uncultured *Holophaga* sp. clone JG-30a-KF-86 (AJ536874)	96	AY674855
ENDOg10	4.2	Acidobacteria	Bacterial species clone ii3-36 (Z95726)	98	AY674866
ENDOa01	2.1	Actinobacteria	Unidentified eubacterium from the Amazon (U68669)	93	AY674841
ENDOb01	2.1	Actinobacteria	*Nocardiodes* sp. JS614 (AF498652)	96	AY674843
ENDOb03	8.5	Actinobacteria	Uncultured bacterium Q3-6C1 (AY048894)	98	AY674844
ENDOb11	2.1	Actinobacteria	Uncultured Rubrobacteridae bacterium clone glen99_9 (AY150882)	93	AY674846
ENDOd05	2.1	Actinobacteria	Uncultured Acidomicrobidae bacterium clone glen99_24 (AY150886)	89	AY674850
ENDOe01	2.1	Actinobacteria	Uncultured bacterium clone SL12 (AF379687)	92	AY674853
ENDOe02	2.1	Actinobacteria	Uncultured soil bacterium clone S085 (AF507693)	91	AY674854
ENDOe04	2.1	Actinobacteria	Uncultured Actinobacterium clone D064 (AF367391)	97	AY674856
ENDOe10	2.1	Actinobacteria	Uncultured Actinobacterium clone Gitt-GS-71a (AJ582207)	99	AY674858
ENDOg02	2.1	Actinobacteria	Uncultured soil bacterium clone 288-2 (AF423245)	91	AY674863
ENDOg09	10.6	Actinobacteria	Uncultured bacterium clone LO12.2 (AF357993)	98	AY674865
ENDOh01	4.2	Actinobacteria	*Nocardiodes* sp. (D87974)	98	AY674867
ENDOh12	2.1	Actinobacteria	Uncultured bacterium clone D134 (AY274140)	90	AY674870
ENDOd06	2.1	Gemmatimonadetes	Uncultured soil bacterium clone S0134 (AF507712)	87	AY674851
ENDOf06	2.1	Gemmatimonadetes	Uncultured soil bacterium clone N11.38WL (AF432632)	91	AY674861
ENDOb12	8.5	Low GC Firmicutes	*Bacillus cereus* strain KNUC51 (AY279194)	99	AY674847
ENDOe11	6.4	Low GC Firmicutes	*Bacillus barbaricus* strain VII-B3-A2 (AJ422145)	99	AY674859
ENDOh09	6.4	Low GC Firmicutes	*Bacillus thuringiensis* strain 3R2-29 (AJ581966)	98	AY674869
ENDOb04	2.1	α-Proteobacteria	Uncultured bacterium clone Alt9-K87 (AJ421908)	93	AY674845
ENDOf03	2.1	α-Proteobacteria	Uncultured α-Proteobacterium clone KCM-C-45 (AJ581616)	96	AY674860
ENDOh04	2.1	β-Proteobacteria	Uncultured eubacterium clone 1_34_417 (AJ437430)	98	AY674868
ENDOc02	2.1	γ-Proteobacteria	Uncultured Proteobacterium clone OF3 (AJ538054)	97	AY674848
ENDOe06	2.1	γ-Proteobacteria	Agricultural soil bacterium clone SC-I-87 (AJ252663)	98	AY674857
ENDOg01	2.1	γ-Proteobacteria	Bacterium ELLIN339 (AF499721)	95	AY674862
ENDOg03	2.1	δ-Proteobacteria	*Entotheonella palauensis* (AF142626)	91	AY674864

Results

Stone samples were collected from the Maya site Ek' Balam to compare the epilithic and endolithic bacterial communities. Large bacterial communities were found in both epilithic ($7.3 \times 10^7 \pm 6.8 \times 10^7$ bacteria/g) and endolithic ($2.9 \times 10^6 \pm 2.3 \times 10^6$ bacteria/g) habitats (mean ± SE, $n = 3$). To compare the taxonomic composition of the epilithic and endolithic bacterial communities, we prepared 16S rDNA clone libraries using DNA extracted from stone cores. Sequences from 110 clones (63 epilithic and 47 endolithic) were compared to the GenBank database using BLAST to determine their phylogenetic affiliation at the phylum or subphylum level. Clones were placed into groups having either 98% sequence similarity or the closest BLAST match to the same sequence (Tables 1 and 2). Rarefaction curves (Fig. 2) indicated that a sufficient number of clones had been analyzed because few new groups were likely to be found. Additionally, the epilithic community was more diverse than the endolithic community.

Among the epilithic clones, the majority of sequences were related to Proteobacteria, with the α-Proteobacteria being the most abundant (Figs. 3A and B). There was a cluster of sequences within the α-Proteobacteria primarily consisting of clones from epilithic samples. Within this cluster, two of the sequences were closely related to sequences previously obtained from stone cultural heritage materials. The closest BLAST match to clone Endob04 was from Altamira Cave, Spain (Table 2), whereas the closest BLAST match to clone Epih08 was from the wall of Llonín Cave, Spain (Table 1, [50]). A large number of clones from the epilithic community were closely related to Actinobacteria (Fig. 3C), and a number of clones from the epilithic community were closely related to the photosynthetic groups Cyanobacteria and Chloroflexi (Fig. 3D). Small numbers of clones from the epilithic community were closely related to the Nitrospirae and Candidate Division OP10 (Fig. 3D) as well as the Acidobacteria, *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, and Low GC Firmicutes (Fig. 3E).

The largest number of endolithic clones were closely related to the Actinobacteria (Table 2 and Fig. 3C). A cluster of sequences from primarily endolithic clones (Endog09 and related) within the Actinobacteria were closely related to organisms from peat soils [29]. In addition, a large percentage of the clones contained sequences similar to the Acidobacteria and the Low GC Firmicutes (Fig. 3E). A cluster of sequences (Endog10 and related) within the Acidobacteria contained clones primarily related to soil microorganisms (Fig. 3E, Table 1), whereas clones Endoe11 and Epig02 were closely related to *Bacillus barbaricus* isolated from an experimental wall painting (Fig. 3E, Tables 1 and 2; [58]). A much smaller number of clones were closely related to the Proteobacteria (Figs. 3A and B) than had been found in the epilithic community, and two of the clones were most similar to sequences of organisms from the Gemmatimonadetes group (Fig. 3E). Many of the groups found in the epilithic community were not detected in endolithic samples (i.e., Cyanobacteria, Chlorflexi, CFB, Nitrospirae, and Candidate Division OP10).

Taxonomic composition of the epilithic and endolithic communities appeared quite different (Fig. 4). Over 50% of clones from the epilithic samples were related to the Proteobacteria. Large percentages of the epilithic clones were related to the Acidobacteria, Actinobacteria, and Low GC Firmicutes. Similarity of the communities determined using the Renkonen index (RI) was 0.5, while the simplified Morisita index (SMI) returned a similarity of 0.6.

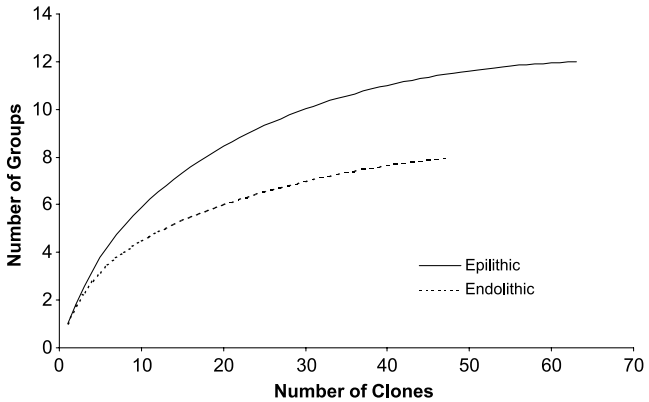

Figure 2. Rarefaction curves for the epilithic and endolithic communities. Clones were grouped by phylum and subphylum using BLAST results.

Discussion

We compared the epilithic and endolithic bacterial communities from limestone used in construction of the Acropolis at the Maya archaeological site Ek' Balam. The epilithic community was dominated by Proteobacteria with substantial numbers of Actinobacteria and the presence of photosynthetic microorganisms, whereas the endolithic community was dominated by Actinobacteria and contained large numbers of Acidobacteria and Low GC Firmicutes. Statistical indices of similarity demonstrated that the two communities differed significantly.

The Renkonen index (RI) and the simplified Morisita index (SMI) yielded different measures of

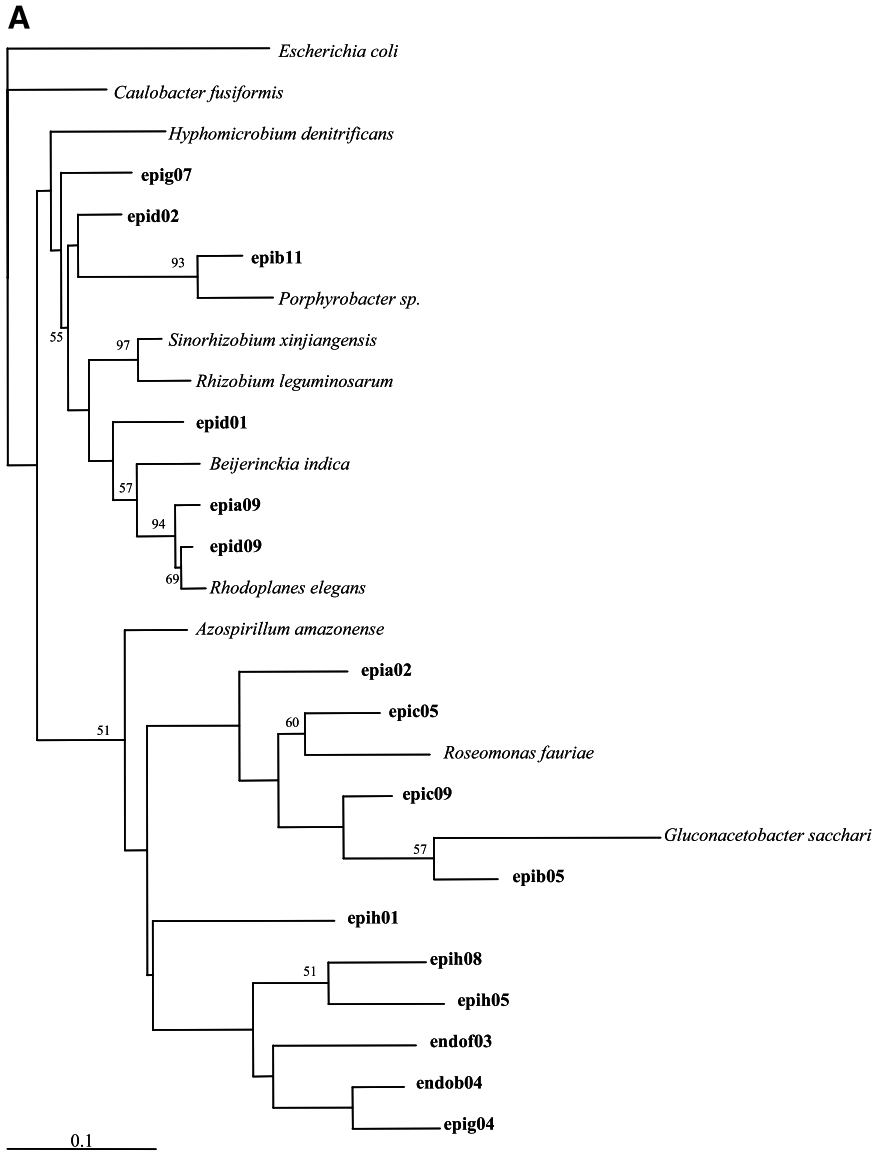

Figure 3. Phylogenetic relationships based on partial 16S rDNA sequences of epilithic and endolithic clones isolated from Ek' Balam with sequences from members of the (A) α-Proteobacteria; (B) β-, γ-, and δ-Proteobacteria; (C) Actinobacteria; (D) Nitrospirae, Chloroflexi, Candidate Division OP10, and Cyanobacteria; and (E) Acidobacteria, Gemmatimonadetes, *Cytophaga–Flavobacterium–Bacteroides*, and Low GC Firmicutes. Neighbor joining trees; bootstrap values based on 1000 replicates are indicated for branches supported by >50% of trees. Scale bar represents 0.1 nucleotide changes per position.

similarity (0.5 and 0.6, respectively). Both indices have theoretical minima and maxima of 0.0 and 1.0, but the expected maximum similarity may be lower, depending on sample size and community diversity. Extrapolating from calculations of expected maxima for different sample sizes and diversities [66], the maximum value of the RI should be in the range of 0.6–0.8, whereas the expected maximum for the SMI, which is less affected by sample size, should fall between 0.8 and 1.0. Extrapolation to small sample sizes is valid when community diversity is low (we identified 13 bacterial groups in our samples, 25% lower than the smallest diversity level examined by Wolda [66]). Because the expected maximum of the RI is substantially lower than that of the SMI, the two indices yield comparable estimates of similarity. The RI score of 0.5 and the SMI score of 0.6 each represents 60–80% of the expected maxima. Both similarity measurements clearly demonstrated that there were dissimilar epilithic and endolithic bacterial communities present on the Maya limestone.

Previous studies of microorganisms on Maya stone have been exclusively based on culture methods and microscopy. Consequently, Cyanobacteria and a few groups of readily culturable heterotrophic bacteria (e.g., *Pseudomonas* sp. and *Bacillus* sp.) were identified

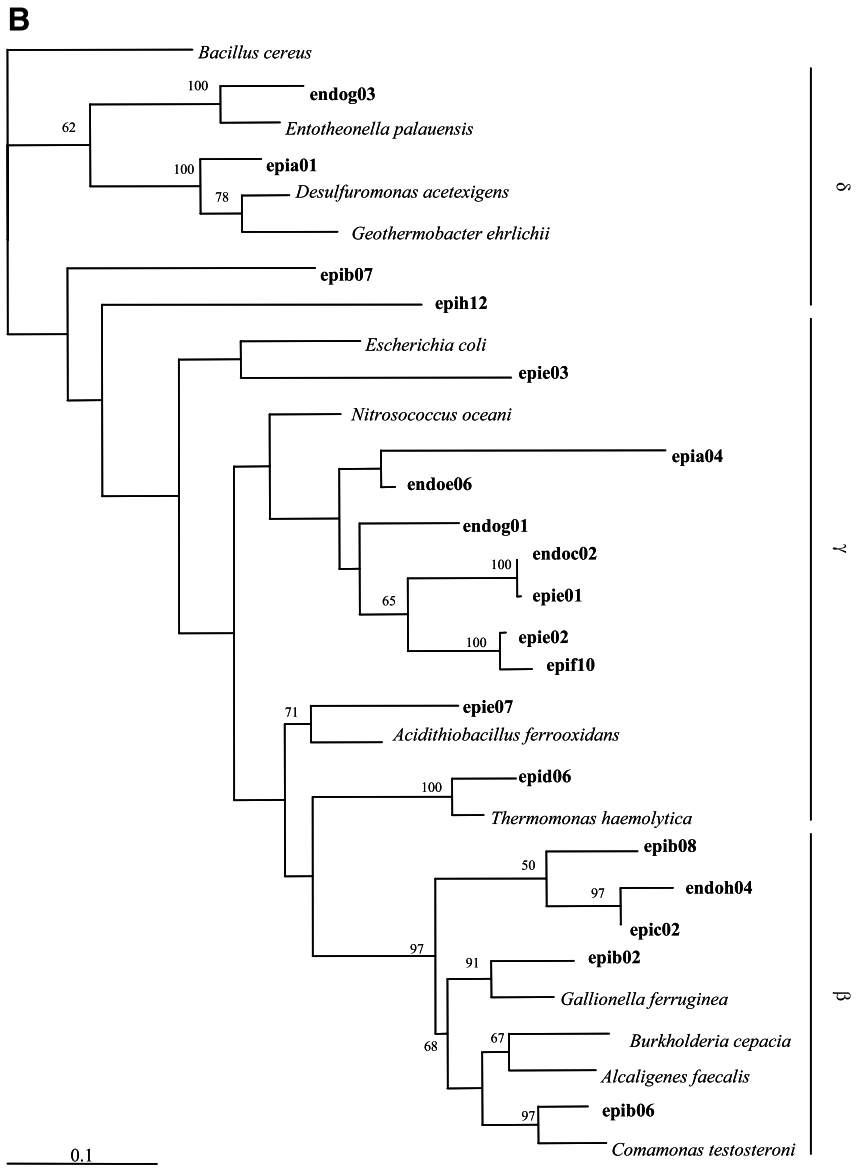

Figure 3. Continued.

as the most common organisms [12, 61]. The dominance of *Pseudomonas* and *Bacillus* is not surprising, given that these genera are readily culturable. We found that a large percentage of the clones in our study were closely related to the Proteobacteria and Low GC Firmicutes, perhaps indicating that the dominance of *Pseudomonas* and *Bacillus* in culturing studies is, in some respects, an accurate description of the bacterial community on Maya stone.

Studies using molecular techniques to examine epilithic bacteria on stone cultural heritage items have found a diverse community, including Proteobacteria, Actinobacteria, the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, and Acidobacteria [15, 43, 48]. Similarly, we found that large percentages of clones from Ek' Balam were related to the Proteobacteria, Actinobacteria, and Acidobacteria groups. Additionally, a large percentage of the endolithic clones from Ek' Balam were closely related to the Low GC Firmicutes group. Other molecular studies of bacteria on stone buildings and cultural heritage objects may have failed to detect these organisms as an important component of the stone microbiota because they have only sampled the stone surface.

The bacterial community from Ek' Balam is also similar in some respects to bacterial communities found in caves. Culture-based studies have reported large numbers of Actinobacteria in caves [14, 25]. Molecular studies of epilithic bacteria and soil from caves have shown that close to 50% of the community

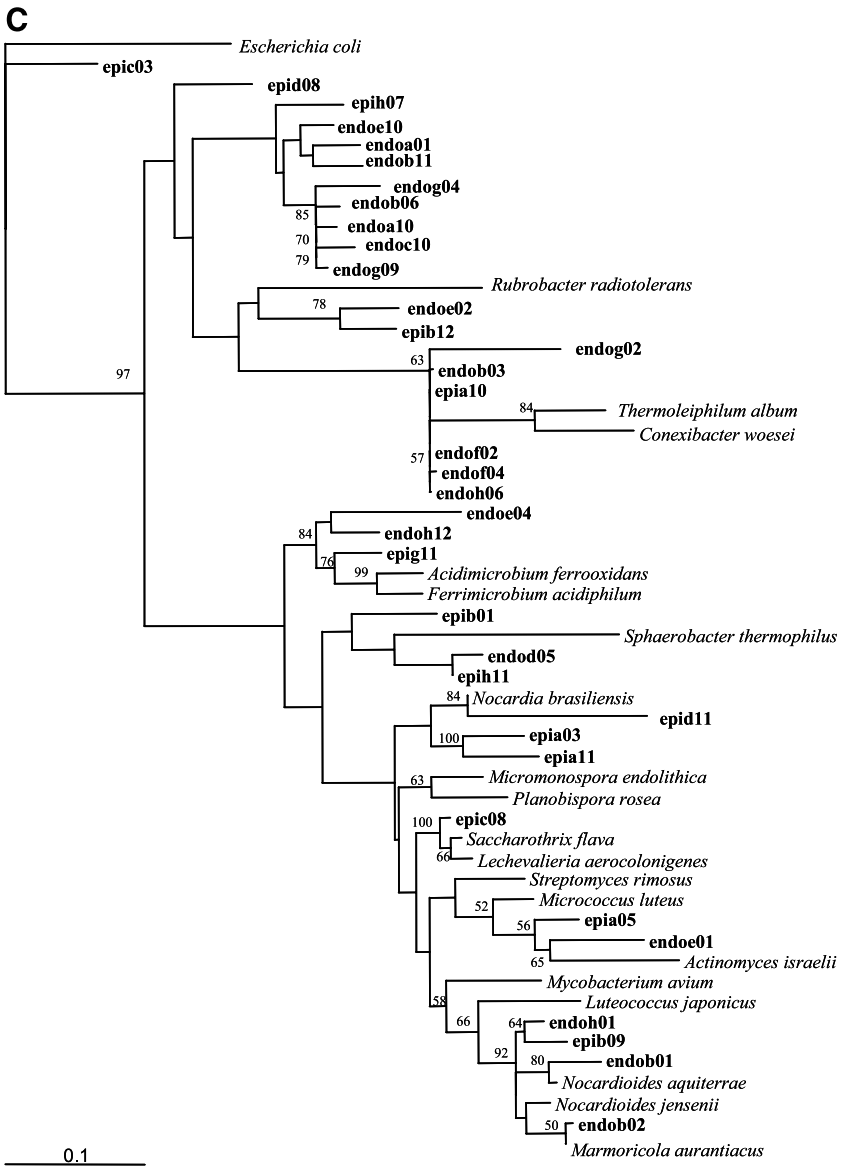

Figure 3. Continued.

is related to Proteobacteria and a large percentage of clones are closely related to Acidobacteria [49, 50]. These studies differed in the percentage of the community related to Actinobacteria (20% and 5%), and a maximum of 11% of clones were closely related to the Firmicutes. Similarities between the epilithic bacteria at Ek' Balam and the epilithic and soil bacteria in caves might be expected because the environments are comparable (i.e., rock surfaces, oligotrophic, low light levels).

Cyanobacteria have frequently been found in endolithic habitats. In extreme environments, endolithic growth provides protection from low temperature, UV radiation, and desiccation [32]. Reports of endolithic microorganisms in stone buildings and statues have detailed the growth of chasmoendolithic Cyanobacteria inhabiting cracks and fissures in the stone [44, 45]. However, we did not find any clones closely related to Cyanobacteria in our endolithic samples. On external stone and building surfaces, high light levels may favor endolithic growth by Cyanobacteria. Our samples were collected from an interior room where no direct light reached the stone surface, and light intensity may have limited the growth of endolithic Cyanobacteria.

Endolithic Cyanobacteria may cause the deterioration of culturally important stone objects. Water absorption by the biofilm matrix results in shrinking and swelling of the EPS, causing mechanical stress that opens cracks and fissures in the stone [64]. In the case

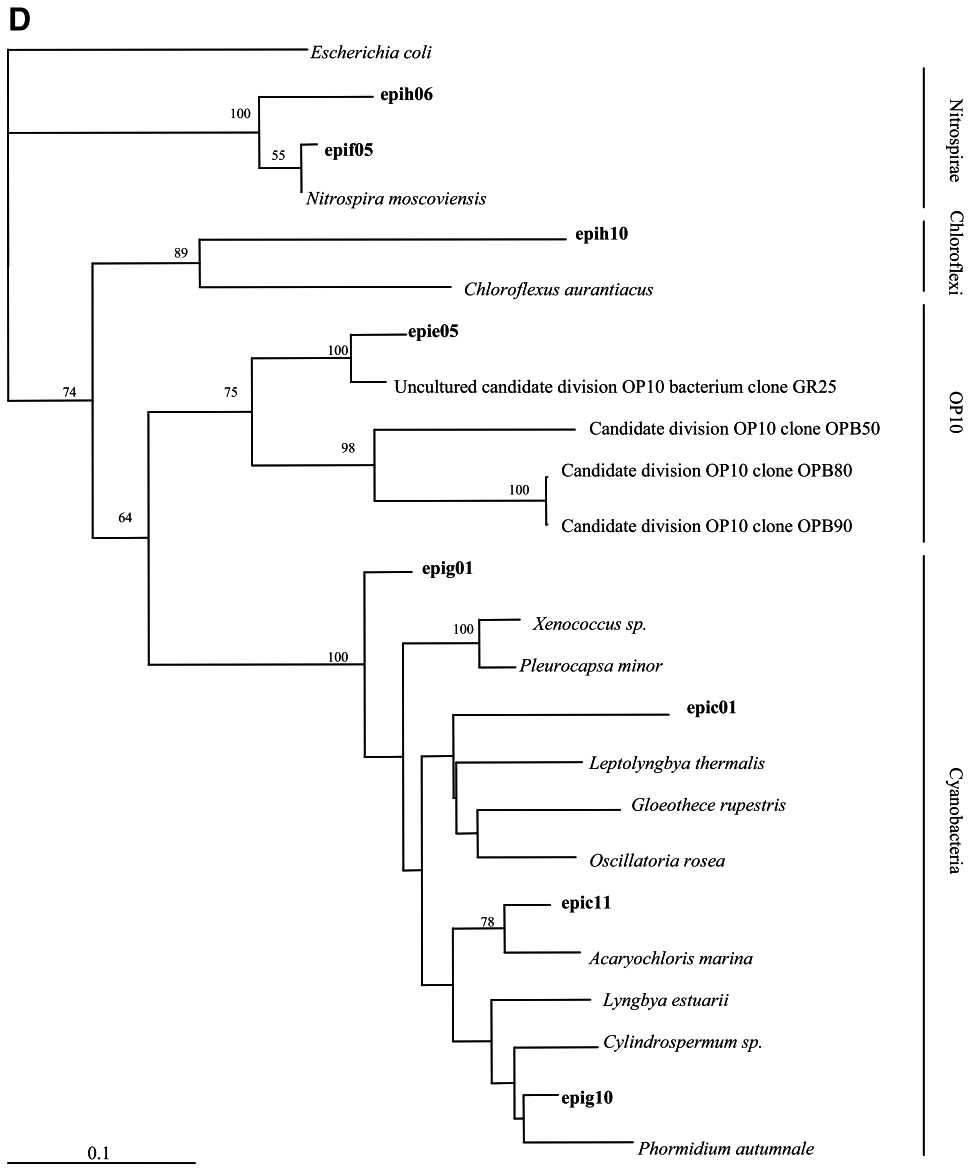

Figure 3. Continued.

of endolithic Cyanobacteria, this process may lead to the exfoliation of surface layers and crusts [22]. Conceivably, the biofilm matrix produced by the endolithic bacteria described in this study may function in a similar manner. Furthermore, much of the EPS in these previous studies may have originated from the largely ignored heterotrophic bacterial community associated with the Cyanobacteria.

The observation of a phylogenetically distinct population of microorganisms in endolithic limestone environments also affects our understanding of weathering of carbonate minerals. These minerals comprise 4% of the earth's crust and represent the most reactive mineral species [40]. Their cycling influences the chemistry of oceans [38, 51] and partially regulates local alkalinity in terrestrial environments [56], which can affect the transport of anthropogenic pollutants, especially heavy metals [41, 54]. The role of microorganisms in dissolution and precipitation of carbonate minerals is the focus of current research efforts and remains unclear. Microorganisms have been observed to accelerate the mineral dissolution rate through production of simple acids [6]. Additionally, extracellular polysaccharides in microbial biofilms can accelerate [37] or retard dissolution rates [4, 65]. These polymers may also act to stabilize secondary mineral precipitation with contaminant metal species. The observation of a distinct endolithic microbial population

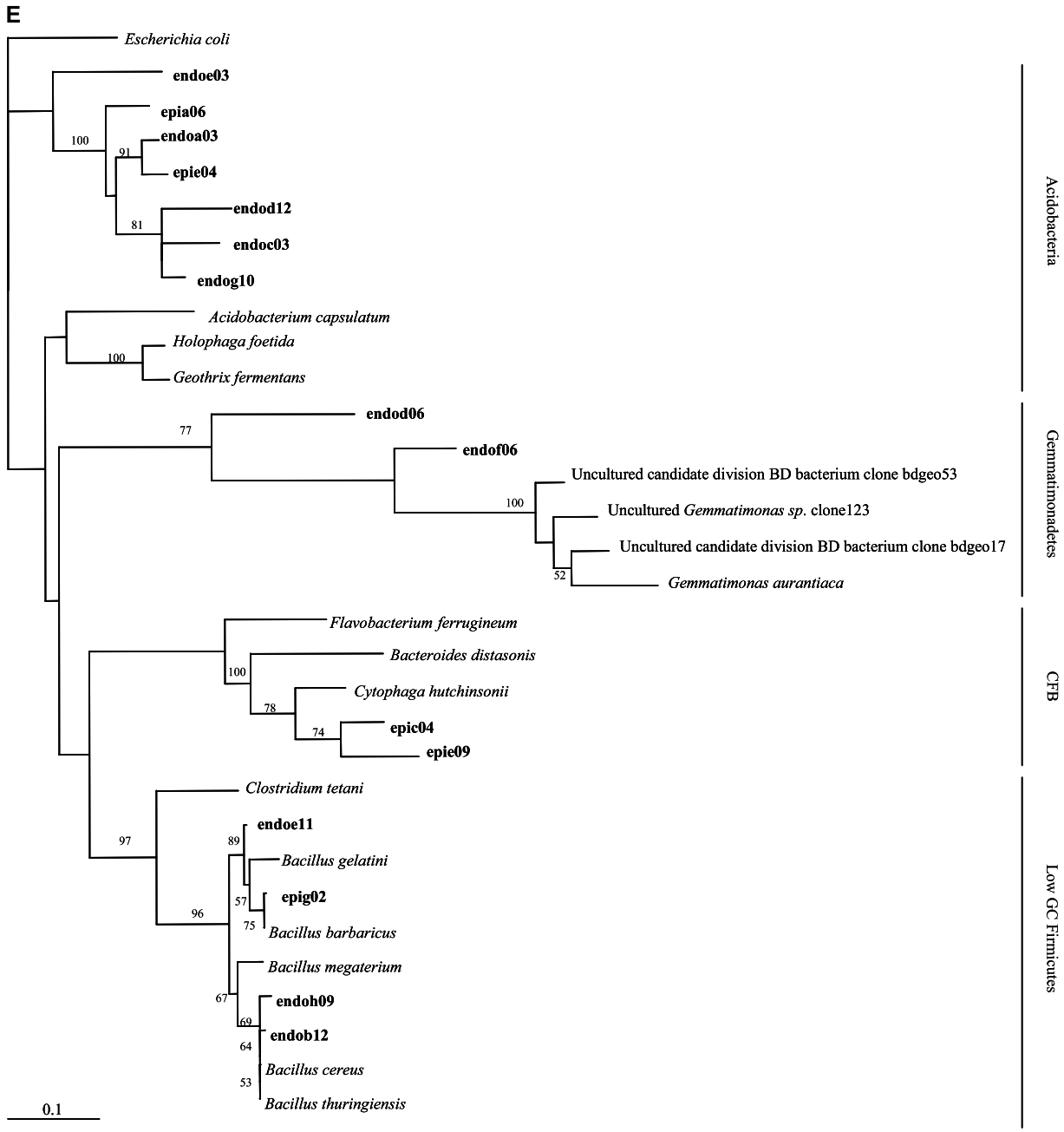

Figure 3. Continued.

affects our current understanding of limestone weathering and leads to increased complexity in geochemical modeling.

In conclusion, we have demonstrated the presence of an endolithic bacterial community in limestone from the Maya site Ek' Balam that is distinctly different from the community on the stone surface. The presence of a previously undescribed endolithic microbial community has important implications for the protection of the world's archaeological sites and historic buildings, and for our understanding of biogeochemical processes controlling dissolution of carbonate minerals.

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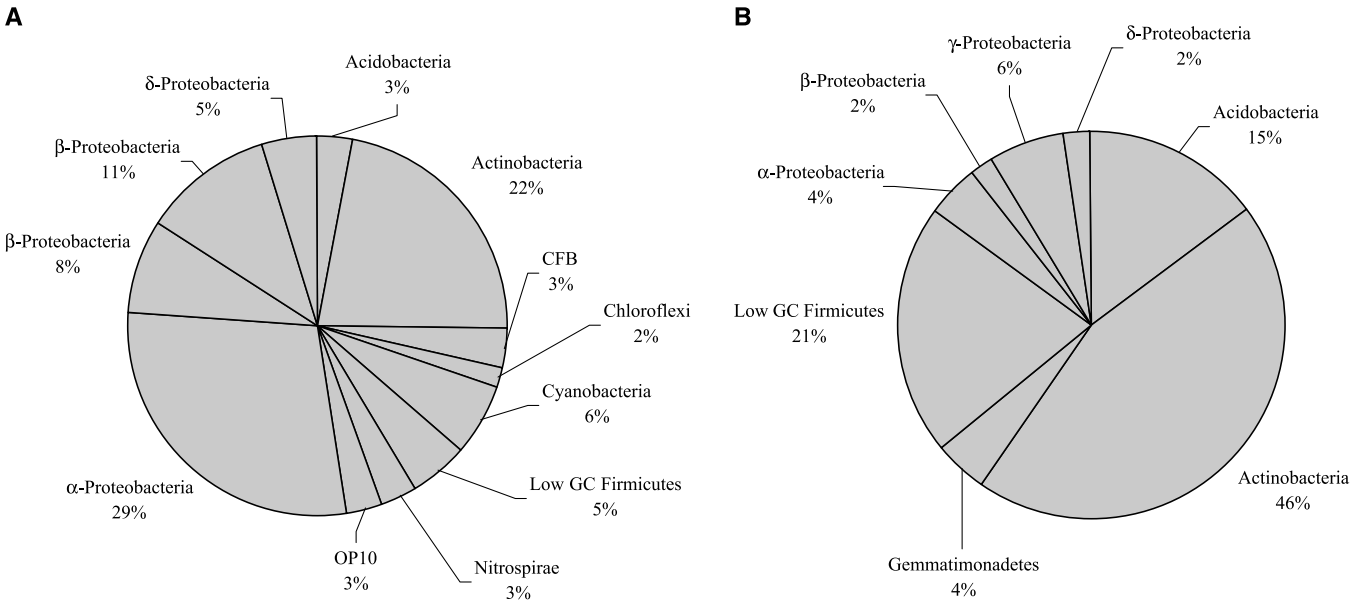

Figure 4. Percentage of clones in each group from the (A) epilithic and (B) endolithic communities.

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