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Endolithic Bacterial Communities in Dolomite and Limestone Rocks from the Nanjiang Canyon in Guizhou Karst Area (China)

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Endolithic Bacterial Communities in Dolomite and Limestone Rocks from the Nanjiang Canyon in Guizhou Karst Area (China)

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Phylogenetic diversities of the endolithic bacterial communities in dolomite and limestone rocks from a karst canyon (Nanjiang Canyon), China, were analyzed based on the 16S rRNA gene analysis. In the dolomite endolithic bacterial communities, members of *Cyanobacteria* were the most abundant followed in abundance by members of *Alphaproteobacteria*, *Acidobacteria,* and *Actinobacteria*. Members of *Betaproteobacteria*, *Deltaproteobacteria*, *Bacteriodetes*, *Verrucomicrobia*, and *Chloroflexi* were also present. Large percentages of bacterial clones in the limestone were related to the *Actinobacteria*, *Alphaproteobacteria*, and *Cyanobacteria*. In addition, members of *Deltaproteobacteria*, *Bacteriodetes*, *Chloroflexi*, *Acidobacteria*, *Firmicutes*, *Planctomycetes*, and Candidate division TM7 were identified. Slight differences in endolithic bacterial abundance and community structure existed between the dolomite and limestone rocks. These rock microorganisms are inferred to have played an important role in the formation of Karst soil from carbonate rocks during a long geological history.

Keywords 16S rRNA gene analysis, bacterial community, diversity, endolithic, karst

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INTRODUCTION

The endolithic environment in rocks, the tiny pore and crack space in rocks, protects microorganisms from a number of physical stresses such as desiccation, rapid temperature variations, and UV radiation (Friedmann 1982; Hughes and Lawley 2003). Communities of microorganisms that inhabit endolithic environments include autotrophic and heterotrophic bacteria, fungi, algae, and lichens (Golubic et al. 1975; Sigler et al. 2003). Endolithic microorganisms have been observed not only in a variety of extreme terrestrial ecosystems but also in temperate environments. Additionally, endoliths have been detected inhabiting a variety of rock types ranging from hard granite to porous rocks such as limestone, sandstone, and gypsum (Dong et al. 2007; Sigler et al. 2003). Endoliths are primary producers in hyper-arid environments, where plants are rare or infrequently encountered (Friedmann and Ocampo 1976). Endolithic microorganisms have also been implicated in geobiological processes such as bioweathering of rocks (Budel et al. 2004). Papida et al. (2000) demonstrated that a mixed microbial population exacerbated physical weathering of limestone and dolomite.

Microbial weathering of rock is widely thought to occur through the actions of organic and inorganic acids produced as metabolic by-products of microorganisms (Lian et al. 2010; Sand and Bock 1991). In addition to metabolic acids, extracellular polysaccharides (EPS) can also increase the dissolution rate of calcium carbonate, suggesting that they may also cause deterioration of stone materials (Perry et al. 2004). In addition, water absorption by the biofilm matrix results in shrinking and swelling of the EPS, causing mechanical stress that opens cracks and fissures in the stones (Warscheid and Braams 2000).

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Many previous investigations of endolithic microbial communities utilized culture-dependent techniques in which standard morphological characteristics were used to identify community members (de la Torre et al. 2003; Friedmann 1982; Friedmann et al. 1988; Friedmann and Ocampo 1976; Giovannoni et al. 1988; Hirsch et al. 1988; Siebert et al. 1996). These studies have usually focused on pigmented microorganisms, oxygenic phototrophs such as green algae, cyanobacteria, and filaments of fungi as partners of lichen symbiosis (de la Torre et al. 2003; Friedmann et al. 1988; Friedmann and Ocampo 1976; Giovannoni et al. 1988).

It is generally assumed that a variety of heterotrophic organisms rapidly follows phototrophs after their invasions. The advent of molecular tools to resolve community molecular diversity in culture-independent studies has allowed determination of greater diversity. Molecular methods are now successfully applied to characterize endolithic communities such as cyanobacterial population in dolomite rocks in Switzerland (Sigler et al. 2003), cryptoendolithic community in the McMurdo Dry Valleys in the Antarctica (de la Torre et al. 2003), endolithic cyanobacteria in soil gypsum (Dong et al. 2007), endolithic community in dolomite rock in the central Alps (Horath and Bachofen 2009), and microbial population in rocks of the Rock Mountains (Garcia-Pichel et al. 2001; Norris and Castenholz 2006; Walker and Pace 2007). However to date, no study has examined the endolithic community in karst environment using culture-independent methods.

Guizhou, a province in southwest China, is one of the three largest developing karst areas in the world. The carbonate rock area is 130,000 km^2, covering 73.8% of total land surface of the province. The weathering of carbonate rocks influences the geochemical compositions of rocks and soils, the atmosphere, and organisms, and the transfer process of matter and energy in the karst environment. Weathering is closely related to a series of environmental problems in carbonate rock areas. For example, environmental pollution in karst area, erosion by water, ecodegradation, and regional climate change would be directly or indirectly related to the weathering of carbonate rocks (Lian et al. 2008).

The rock microorganisms play an important and unprecedented role in the carbonate rock weathering during the long geological history. Despite the importance of karst environments and the role of microorganisms in carbonate rock weathering and soil formation, our knowledge of the endolithic microbial diversity and community structure in carbonate rock is still limited. The aim of the present study was therefore to investigate the endolithic bacterial diversity in dolomite and limestone rocks using molecular techniques. Our results indicated that the photosynthetic *Cyanobacteria* and heterotrophic *Proteobacteria* and *Actinobacteria* were predominant and significant components in the dolomite and limestone endolithic community.

MATERIALS AND METHODS

Site Description and Sample Collection

Nanjiang Canyon (26°56′N, 106°58′E), a typical karst canyon, is located in Kaiyang County of Guizhou Province, southwest China (Figure 1). The climate is humid and often influenced by subtropical monsoon. It is neither hot in summer nor cold in winter. The average annual precipitation in the last ten years is 1108 mm and most rainfall (more than 78%) is concentrated in wet season ranging from April to September. The maximum and minimum monthly mean precipitation is 203.1 mm in July and 21.4 mm in December. The average annual temperature is 14.8°C, and the highest and lowest monthly mean temperature is 23.2°C and 3.9°C in July and January, respectively.

The dolomite and limestone rocks in the region are weathered and porous. The area is mostly soil-covered, but in many places bare dolomite and limestone escarpments are present beneath an overlying layer of vegetation and accumulated organic matter. Within this area (Figure 1), three Triassic dolomite and three Triassic limestone samples up to a depth of ∼1 cm were collected using a sterile rock chisel and placed in sterile bags on ice. All samples were collected in September 2009.

DNA Extraction, PCR Amplification, and Cloning

Genomic DNA was extracted from 0.5 to 1.0 g of each crushed rock sample using the UltraClean^{TM}soil DNA Isolation kit (MoBio, USA) according to the manufacturer's protocol. Extracted DNA was stored in 50 μL of 10 mM Tris buffer at −20°C.

PCR amplification of the bacterial 16S rRNA gene was performed using the universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). All reactions were carried out in a 50-μL reaction mixture, containing 5 μL of 10×PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH8.3), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.2 mM of each primer, 1-3 μL of DNA template, 2.5 U of *Taq* DNA polymerase (Takara, Japan). PCR was run under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 circles of 1 min denaturation at 94°C, 1 min annealing at 52°C, 2 min extension at 72°C, and a final extension step of 10 min at 72°C. PCR-amplified products from three independent PCRs were pooled to reduce the chances of PCR artifacts (Kanagawa 2003) and purified by agarose gel electrophoresis with PCR purification kit (E.Z.N.A. Gel Extraction kit, OMEGA, USA).

The purified products were ligated into the pGEM-T Easy vector (Promega, USA) and then transformed into competent *Escherichia coli* JM109 cells (Promega, USA), which allows blue-white screening on Luria-Bertani (LB) plate containing Ampicillin (100 μg ml^{-1}), X-gal (20 mg ml^{-1}), and IPTG (40 mM). Six clone libraries were constructed, three for dolomite (D1, D2, and D3) and three for limestone samples (L1, L2 and L3).

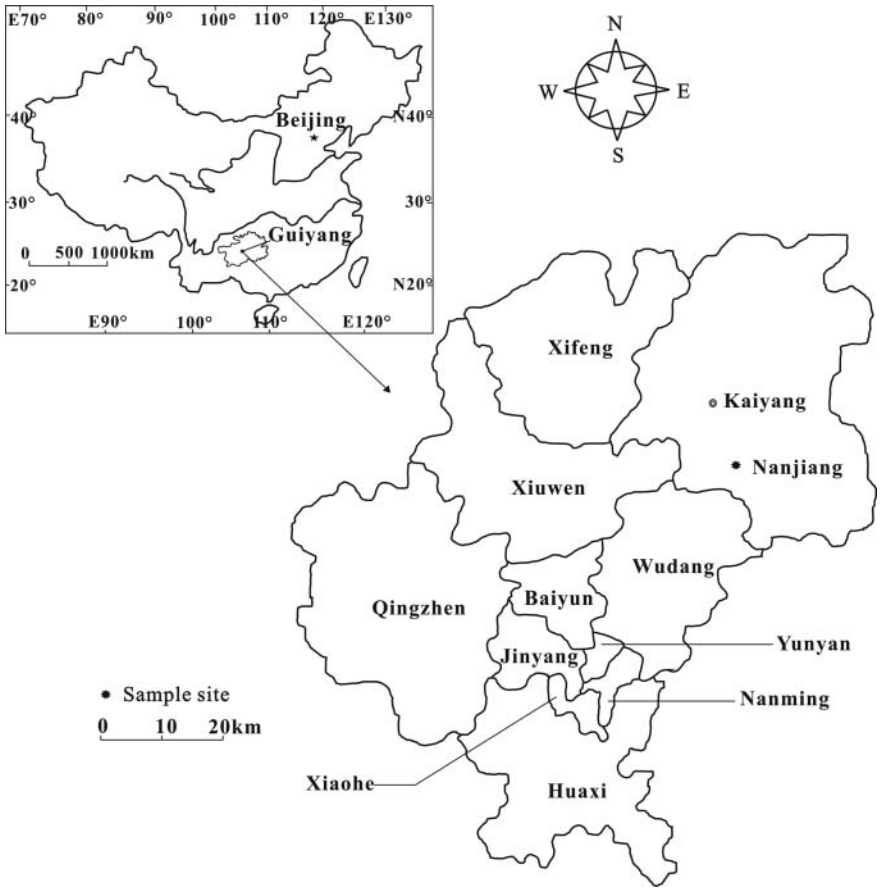

FIG. 1. Location map of the Nanjiang Canyon in Kaiyang County of Guizhou Province, southwest China.

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RFLP Analysis and Sequencing

Approximately 150 clones from each library were identified by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified plasmids with the *MspI* and *AfaI* restriction enzymes. The RFLP patterns were compared visually and clones showing identical RFLP patterns were grouped into the same operational taxonomic units (OTUs). The three dolomite libraries and three limestone libraries resulted in 48 and 42 different OTUs, respectively. One representative clone of each OTU was sequenced using an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co. Ltd, China).

Phylogenetic Analysis

DNA sequences were analyzed by the programs Bellerophon (Huber et al. 2004) and CHIMERA_CHECK (Cole et al. 2003) to remove chimeric artifacts. Clones were considered as the same phylotype if they were $\geq$97% similar to one another over the region of the 16S rRNA gene sequenced (Stackebrandt et al. 1993). As a result, 44 phylotypes from the dolomite libraries and 36 phylotypes from the limestone libraries were generated. A total of 80 sequences were compared with known sequences in the NCBI database (http://www.ncbi.nih.gov/) by Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). The sequences were aligned using Clustal X software (Thompson et al. 1997), and phylogenetic trees were constructed with the Mega 3.0 program package (Kumar et al. 2004) using the neighbor-joining method. Bootstrap confidence values were obtained with 1000 replicates. The trees were constructed by calculating the Kimura distance (Kimura 1980).

Statistical Analysis

The rarefaction curves were calculated using the software "Analytic Rarefaction 1.3" provided by Steven M. Holland (http://www.uga.edu/strata/software/index.html). The coverage of the libraries was defined to be C = 1-(n/N), where n is the number of OTUs, N is the total number of clones examined, C is the percent coverage (Good 1953; Shuang et al. 2009). Shannon index (H) was calculated with the equation $H = -\sum pi \ln pi$, where pi is the number of clones in the OUT group divided by the total number of clones in the clone library (Hill et al. 2003).

Nucleotide Sequence Accession Numbers

The nucleotide sequences reported in this study have been deposited in GenBank under accession numbers HM224415 to HM224448 and HM241096 to HM241132.

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RESULTS

Bacterial Diversity

Rarefaction curves were obtained by plotting the number of phylotypes observed against the number of clones sequenced (not shown in the text). The decrease in the rate of new phylotype detection indicated that the major part of the diversity in the six libraries was covered. In the dolomite endolithic community, the coverage value of the D1, D2, and D3 clone libraries was 74.0%, 74.0% and 74.7%, respectively, and the Shannon diversity index was 3.41, 3.40 and 3.34, accordingly. In the limestone endolithic community, the coverage value of the L1, L2 and L3 clone libraries was 79.3%, 78.7% and 80.0%, respectively, and the Shannon diversity index was 3.18, 3.19, and 3.11, accordingly. The results showed that all the clone libraries had a high degree of diversity, and the dolomite community was slightly more diverse than the limestone community.

Phylogenetic Affiliation of Sequences from Dolomite Rocks

Within 44 phylotypes from the three dolomite rocks, the percentages of sequence similarity to database sequences ranged from 86% and 99%. Up to 25 phylotypes (57%) were within the species level (more than 97% sequence identity), 15 phylotypes (34%) were in the range between 90% and 97% sequence identity, while 4 phylotypes (9%) were less than 90% similar to the closest relatives in the GenBank database. Based on the BLAST results (Table 1) and phylogenetic analysis (Figure 2), all phyloptyes were assigned to seven phylogenetic phyla of the domain Bacteria: *Proteobacteria*, *Cyanobacteria*, *Acidobacteria*, *Actinobacteria* (high G+C Gram-positive bacteria), *Bacteriodetes*, *Chloroflexi* (green nonsulfur bacteria), and *Verrucomicrobia*. The relative abundances of different phylogenetic groups present in each clone library are shown in Table 3.

One third of the obtained clones originated from phototrophic organisms (*Cyanobacteria* and *Chloroflexi*). The oxygenic phototrophic bacterial group of *Cyanobacteria* was the most abundant in the dolomite endolithic bacterial community (10 out of 44 phylotypes) (Figure 2) and represented 29.3% of the total clones (Table 3). Nine cyanobacterial phylotypes were related to cultivated bacteria (89–98% identity) including *Phormidium autumnale*, *Anabaena oscillarioides*, *Scytonema* sp., *Chroococcidiopsis* sp., *Leptolyngbya* sp., *Calothrix* sp., two *Nostoc* sp., and *Brasilonema octagenarum*. Only one phylotype was closely (98% identity) related to uncultured cyanobacterium clone F3Baug.33 (GQ417856) obtained from biological degreasing systems (GenBank description). Two phylotypes accounting for 2.7% of the clone libraries were related to the green nonsulfur phototrophic bacterial group of the *Chloroflexi* phylum. The phylotype DOL124 was closely related to (97% identity) the uncultured bacterial clone Dolo-23 (AB257647), which was previously recovered from the endolithic dolomite rock in the central Alps (Horath and Bachofen 2009). Another phylotype DOL108 was closely (97% identity) related to the uncultured *Chloroflexi* bacterium clone g15 (EU979024) from rhizosphere soil (GenBank description).

Among the heterotrophic species, *Proteobacteria* were predominant in the studied dolomite endolithic community. Fourteen phylotypes representing 30.7% of the clones were included in the phylum *Proteobacteria*, clustering within three subdivisions of *Alpha-*, *Beta-*, and *Deltaproteobacteria*. Important differences were observed in the relative distribution of the different proteobacterial subdivisions (Table 3). Alphaproteobacterial phylotypes were the second most abundant in the entire clone libraries (9 out of 44 phylotypes) and represented 22.7% of the total dolomite clone population (Table 3).

Most clone sequences represented by phylotype DOL68 and DOL126 were related to bacteria from Yellowstone National Park (AF445712, GenBank description) and from endolithic dolomite rock in the central Alps (Horath and Bachofen 2009). One third of the clones represented by three phylotypes within this group branched within *Sphingomonadaceae*. The bacteria related to these three phylotypes were *Sphingomonas* sp. MTR-71 (DQ898300), *Sphingomonas asaccharolytica* (NR_029327), and Sphingomonadaceae bacterium Gsoil 359 from soil of the ginseng field (AB245346, GenBank description), respectively.

Other phylotypes within *Alphaproteobacteria* were closely related to *Brevundimonas* (99% identity) from semi-coke (EF540454, GenBank description), *Methylobacterium* (98% identity) from plant phyllosphere (Knief et al. 2008), and uncultured bacteria from soil (EF540430, GenBank description) and Lake Tanganyika anoxic hypolimnion (FJ849190, GenBank description).

The abundance of *Betaproteobacteria* was low in the libraries. One phylotype of DOL16 could be affiliated with this subdivision, which was 97% similar to *Janthinobacterium* sp. (EU274637). Within *Deltaproteobacteria*, two phylotypes were identified as *Stigmatella koreensis* (98% identity) and *Cystobacter ferrugineus* (99% identity), respectively. The phylotype of DOL88 was distantly (86% identity) related to *Pelobacter acidigallici* (NR_026154). Another group of clones, represented by phylotype DOL9, was related to (95% identity) environmental sequences recovered from forest soil (DQ451526) (GenBank description).

The remaining phyla comprised 37.4% of the total number of clones recovered from the three dolomite libraries. Seven phylotypes belonged to the phylum *Acidobacteria* representing 12% of the total clone population. The sequences in this group were related to uncultured bacteria from various hot spring environments (GenBank description). Five phylotypes were affiliated with the *Actinobacteria* phylum and represented 11.6% of the total clone population. The phylotype DOL65 was closely related to (98% identity) uncultured actinobacterium clone (AB257641) from the endolithic dolomite rock in the central Alps (Horath and Bachofen 2009). The closest BLAST match to phylotype DOL99 was from a Karstic cave wall biofilm in Slovenia (Pasic et al. 2010).

TABLE 1
Phylogenetic affiliations of 16S rDNA clones obtained from dolomite rocks

Type (accession no.)	% Abundance[a]	Closest NCBI-BLAST match (accession no.)	% Identity
Acidobacteria			
DOL19 (HM224420)	2.7	Bacterial species clone RB41 (Z95722)	89
DOL20 (HM224421)	0.4	Uncultured bacterium clone PK33 (AY555783)	92
DOL49 (HM224426)	0.9	Uncultured bacterium clone SedUMA20 (FJ849538)	94
DOL77 (HM224433)	2.0	Uncultured bacterium clone WC3_138 (GQ264044)	98
DOL86 (HM224438)	4.4	Uncultured Acidobacteria bacterium OTU8 (AM902627)	98
DOL89 (HM241105)	0.9	Uncultured Acidobacteria bacterium clone HAVOmat69 (EF032757)	98
DOL90 (HM241101)	0.7	Uncultured bacterium clone P958 (GQ214125)	93
Actinobacteria			
DOL48 (HM224425)	0.9	Uncultured bacterium clone AK4AB2_02H (GQ396924)	98
DOL65 (HM224429)	5.6	Uncultured actinobacterium clone: Dolo_16 (AB257641)	98
DOL67 (HM241100)	3.3	Uncultured bacterium clone p7k15ok (FJ478516)	97
DOL99 (HM224440)	1.1	Uncultured actinobacterium clone 2PJM54 (FJ535083)	92
DOL117 (HM224445)	0.7	Uncultured bacterium clone 1-9F (EU289467)	96
Bacteroidetes			
DOL56 (HM224427)	4.9	Uncultured bacterium clone (DQ256316)	96
DOL85 (HM224437)	0.2	Uncultured Bacteroidetes clone BuhC-66 (FM866274)	92
DOL130 (HM224447)	0.9	Uncultured Bacteroidetes bacterium clone g25(EU97903 4)	98
DOL150 (HM241103)	2.2	Uncultured soil bacterium clone M15_Pitesti (DQ378235)	96
Chloroflexi			
DOL108 (HM224443)	0.7	Uncultured Chloroflexi bacterium clone g15 (EU979024)	97
DOL124 (HM241102)	2.0	Uncultured Chloroflexus sp. clone: Dolo_23 (AB257647)	97
Cyanobacteria			
DOL18 (HM224419)	2.9	*Nostoc* sp. Al3 (AM711531)	92
DOL33 (HM224422)	3.3	*Nostoc* sp. 152 (AJ133161)	98
DOL35 (HM224423)	0.9	*Phormidium autumnale* Arct-Ph5 (DQ493873)	89
DOL63 (HM224428)	2.2	Uncultured cyanobacterium clone F3Baug.33 (GQ417856)	98
DOL73 (HM224431)	5.3	*Anabaena oscillarioides* BO HINDAK 1984/43 (AJ630428)	90
DOL84 (HM224436)	7.1	*Scytonema* sp. U-3-3 (AY069954)	90
DOL96 (HM241104)	0.7	*Brasilonema octagenarum* UFV-OR1 (EF150855)	98
DOL97 (HM241099)	2.9	*Chroococcidiopsis* sp. CC1 16S (DQ914863)	97
DOL111(HM224444)	3.6	*Leptolyngbya* sp. CENA103 (EF088339)	97
DOL146 (HM224448)	0.4	*Calothrix* sp. PCC 7101 (AB325535)	98
Verrucomicrobia			
DOL103 (HM224441)	4.7	Bacterium Ellin506 (AY960769)	95
DOL104 (HM224442)	0.9	Spartobacteria bacterium Gsoil 144 (AB245342)	87
Alphaproteobacteria			
DOL3 (HM224415)	3.6	*Sphingomonas* sp. MTR-71 (DQ898300)	95
DOL7 (HM224416)	0.7	*Brevundimonas* sp. d1M (EF540454)	99
DOL15 (HM224417	0.7	*Methylobacterium* sp. F50 (AM910541)	98
DOL43 (HM224424)	2.7	*Sphingomonas asaccharolytica* strain Y-345 (NR_029327)	98
DOL68 (HM224430)	6.7	Uncultured alpha proteobacterium clone SM2B06 (AF445712)	97
DOL75 (HM224432)	0.7	Sphingomonadaceae bacterium Gsoil 359 (AB245346)	97
DOL79 (HM224434)	0.7	Uncultured soil bacterium clone MK34a (EF540430)	99
DOL81 (HM224435)	2.0	Uncultured bacterium clone EpiUMA10 (FJ849190)	96
DOL126 (HM241098)	5.1	Uncultured alpha proteobacterium clone Dolo_14 (AB257639)	97
Betaproteobacteria			
DOL16 (HM224418)	0.7	*Janthinobacterium* sp. (EU274637)	97
Deltaproteobacteria			
DOL9 (HM241096)	3.8	Uncultured bacterium clone FAC87 (DQ451526)	95
DOL88 (HM224439)	0.9	*Pelobacter acidigallici* strain DSM 2377 (NR_026154)	86
DOL91 (HM241097)	1.8	*Stigmatella koreensis* strain KYC-1019 (EF112185)	98
DOL129 (HM224446)	0.7	*Cystobacter ferrugineus* strain Cb fe13 (AJ233900)	99

[a]The frequency of the clones is given as the number of clones of one sort of phylotype divided by the total number of clones in the three dolomite libraries.

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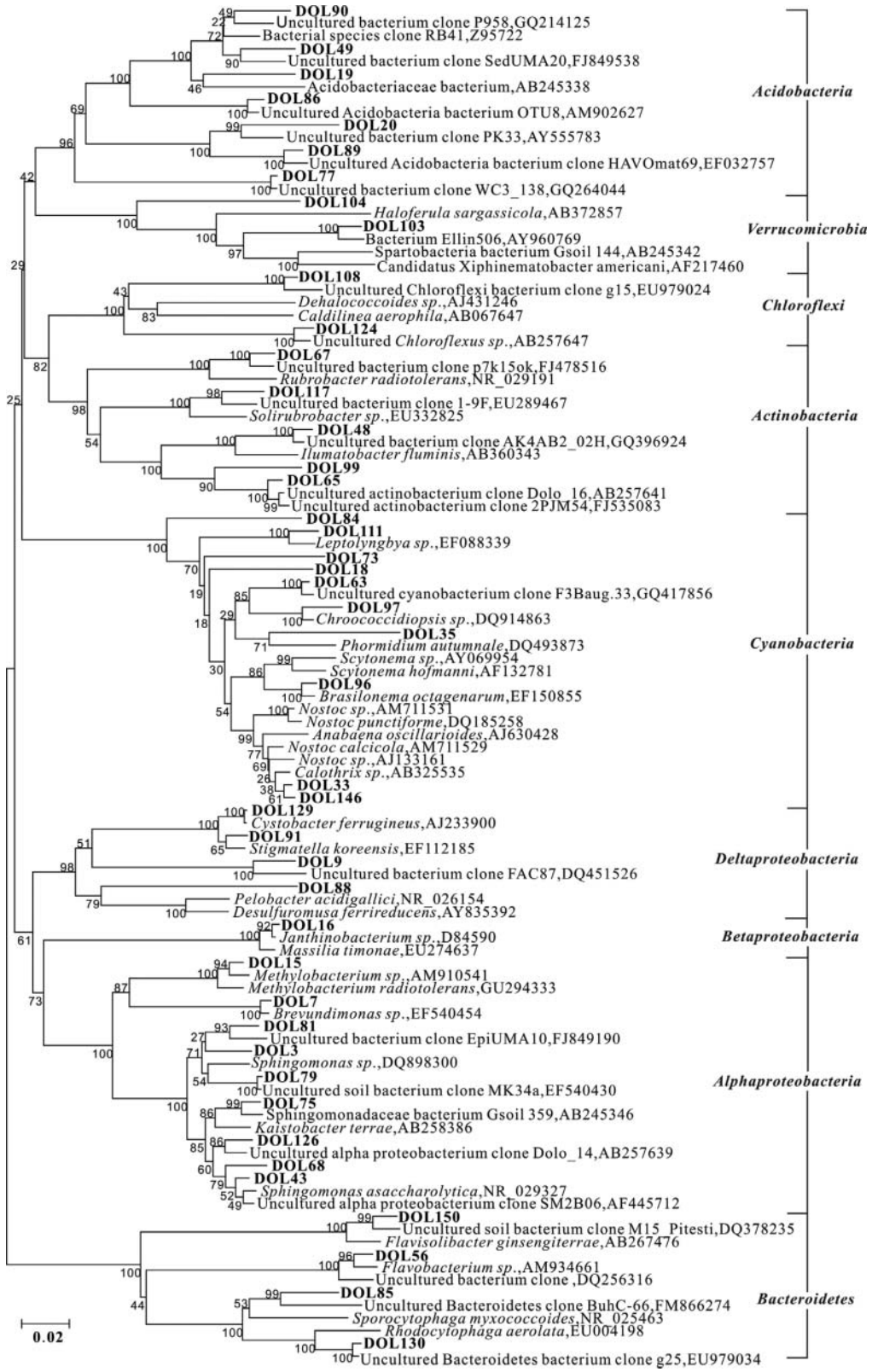

FIG. 2. Phylogenetic relationship based on 16S rRNA gene sequences of endolithic clones isolated from dolomite rocks (in **bold type**) with closely related sequences from the GenBank database. Neighbor joining trees; bootstrap values (1,000 replicates) are shown at the nodes.

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Four phylotypes were assigned to the *Bacteroidetes* phylum. Within this cluster, the sequences were related to uncultured bacteria from subsurface water, uranium mill tailings, rhizosphere soil, and oil-polluted soil (GenBank description). The *Verrucomicrobia* group was minor in all samples. Only two phylotypes fell into the category and they were related to uncultured bacteria from soil environment (GenBank description).

Phylogenetic Affiliation of Sequences from Limestone Rocks

A total of 36 sequences from the limestone libraries were subjected to BLAST search against GenBank. Fifty-six percent of the phylotypes showed more than 97% sequence similarity to their nearest database entries. Approximately 38% of the sequences had a similarity level of 90–97%, and for the remaining 6% the similarity levels were less than 90% (Table 2). The results of phylogenetic analysis are shown in Figure 3. Phylogenetic analyses placed the 36 phylotypes in the following 9 groups of the domain Bacteria: *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Bacteriodetes*, *Chloroflexi*, *Acidobacteria*, *Planctomycetes*, *Firmicutes*, and Candidate division TM7. Among them, the *Proteobacteria* was the largest group, followed by *Actinobacteria* and *Cyanobacteria*. The phylogenetic composition of 16S rDNA clones in each clone library is shown in Table 3.

Eight phylotypes, accounting for 29.8% of the total clones in the endolithic limestone libraries, were included in the phylum *Proteobacteria*, clustering with two subdivisions of *Alpha-* and *Deltaproteobacteria*. Six phylotypes, accounting for 16.7% of the clone libraries, were affiliated to *Alphaproteobacteria*. More than one third of the *Alphaproteobacterial* clones represented by phylotype LIM17 were related to (97% identity) uncultured alphaproteobacterium clone Dolo_14 from endolithic dolomite rock in the central Alps (Horath and Bachofen 2009). The phylotypes LIM23 and LIM106 were closely related to (98% identity) *Novosphingobium* sp. (D84626) and *Sphingomonas* sp. (FJ834325). The closest BLAST match to phylotype LIM58 and LIM136 were from soil (AY234707) and urban aerosol (DQ129613) (GenBank description).

The phylotype LIM21 was remotely related (87% identity) to *Kaistobacter terrae* (AB258386). The low similarity values to the closest member in the GenBank indicated that the corresponding bacteria belonged to putatively new taxonomic group. Two phylotypes were assigned to the *Deltaproteobacteria* phylum representing 13.1% of the total clone population. Phylotype LIM4, which was the most abundant in the group and accounted for 8.7% of the clone libraries, was closely related (98% identity) to *Stigmatella koreensis* (EF112185). The closest BLAST match to phylotype LIM2 was from forest soil (DQ451526, GenBank description).

Phylogenetic analysis placed seven phylotypes (24.2% of the total clone population) within *Actinobacteria* (Figure 3 and Table 3). The majority of *Actinobacteria*-related sequences from the limestone libraries were affiliated with uncultured bacteria recovered from undisturbed tall grass prairie (GenBank description) and endolithic dolomite rock in the central Alps (Horath and Bachofen 2009). Two phylotypes were closely related to cultivated bacterial clones of genera *Kineococcus* and *Friedmanniella*.

Phototrophs such as *Cyanobacteria* (four phylotypes) and *Chloroflexi* (three phylotypes) were also identified. *Cyanobacteria* were the third most abundant in the clone libraries and represented 16.7% of the total limestone clone population (Table 3). The majority of clones in this group were closely related to (97%) *Chroococcidiopsis* sp. (DQ914863) recovered from quartz hypolithic community in China's hot and cold hyper-arid deserts (Pointing et al. 2007). The LIM128 phylotype was 97% similar to the *Coleodesmium* sp. ANT.LH52B.5 (AY493596) from Antarctic cyanobacterial community (Taton et al. 2006). The remaining clones in this group represented by phylotye LIM65 were closely related to (98% identity) *Brasilonema octagenarum* UFV-OR1 (EF150855) recovered from eucalyptus leaves (Aguiar et al. 2008). Two phylotypes within the *Chloroflexi* were closely related to uncultured bacterial clone from endolithic dolomite rock in the central Alps (Horath and Bachofen 2009) and simulated low level waste site (GenBank description). The phylotype of LIM144 was 97% similar to *Kouleothrix aurantiaca* (AB079638) isolated from activated sludge (Kohno et al. 2002).

Eight phylotypes (11.6% of the total clone population) were grouped within the phylum *Bacteroidetes*. The two cultivatable relatives were *Hymenobacter sp*. strain 29F (AY647897) from biological soil crusts in the Sonoran Desert (Nagy et al. 2005) and *Adhaeribacter aquaticus* (AJ626894) from freshwater biofilms (Rickard et al. 2004). The other phylotypes within *Bacteroidetes* were related to uncultured bacteria from various soil environments (GenBank description) and eastern Mediterranean atmosphere (Polymenakou et al. 2008). Three phylotypes were related to the *Acidobacteria* group (Table 2 and Figure 3). They were related to uncultivated bacterium from rhizosphere soil (EU979113), loess (GQ214125), and cyanobacterial mat in Hawaii volcanoes (EF032757) (GenBank description). *Firmicutes* (one phylotype), Candidate Division TM7 (one phylotype), and *Planctomycetes* (one phylotype) were less frequent in the endolithic limestone environment.

Our study found that the endolithic bacterial communities in the limestone samples were slightly different from those in the dolomite samples in terms of diversity index and phylotype composition. It is unlikely that these differences were simply due to PCR and cloning bias, but may be a result of differences in mineral composition and environmental conditions between the limestone and dolomite rocks. A better understanding of any differences in the endolithic bacterial community structure between the two rock types requires a systematic study with a larger sampling size.

TABLE 2
Phylogenetic affiliations of 16S rDNA clones obtained from limestone rocks

Type (accession no.)	% Abundance[a]	Closest NCBI-BLAST match (accession no.)	% Identity
Acidobacteria			
LIM33 (HM241101)	2.9	Uncultured bacterium clone P958 (GQ214125)	93
LIM111 (HM241121)	0.7	Uncultured Acidobacteria bacterium clone g74-MR-96 (EU979113)	98
LIM120 (HM241105)	0.7	Uncultured Acidobacteria bacterium clone HAVOmat69 (EF032757)	98
Actinobacteria			
LIM7 (HM241106)	7.3	Uncultured actinobacterium clone: Dolo_16 (AB257641)	88
LIM32 (HM241100)	7.6	Uncultured bacterium clone p7k15ok (FJ478516)	97
LIM37 (HM241113)	0.7	Uncultured bacterium clone p32k22ok (FJ478603)	98
LIM42 (HM241114)	2.2	*Kineococcus* sp. 1P02MC (EU977818)	99
LIM142 (HM241125)	0.7	Bacterium Ellin504 (AY960767)	92
LIM145 (HM241127)	0.9	Uncultured bacterium clone p35k06ok (FJ479049)	96
LIM60 (HM241128)	5.1	*Friedmanniella spumicola* strain Ben 107 (NR_024907)	97
Bacteroidetes			
LIM11 (HM241108)	2.9	Uncultured CFB group bacterium clone SM1G04 (AF445698)	91
LIM22 (HM241111)	2.9	Uncultured Bacteroidetes bacterium clone 46-2_3 (FJ517715)	91
LIM57 (HM241115)	1.3	*Hymenobacter* sp. 29F (AY647897)	98
LIM78 (HM241117)	0.7	*Adhaeribacter aquaticus* type strain MBRG1.5 (AJ626894)	94
LIM131 (HM241103)	2.9	Uncultured soil bacterium clone M15_Pitesti (DQ378235)	96
LIM140 (HM241124)	0.2	Uncultured bacterium clone FFCH5663 (EU133679)	90
LIM87 (HM241130)	0.2	Uncultured Bacteroidetes bacterium clone F15_8C_FL (EF683049)	94
LIM135 (HM241132)	0.4	Uncultured bacterium clone IYF104 (DQ984594)	94
Chloroflexi			
LIM47 (HM241102)	2.7	Uncultured Chloroflexus sp. clone: Dolo_23 (AB257647)	97
LIM144 (HM241126)	0.9	*Kouleothrix aurantiaca* strain:EJ2M-A (AB079638)	97
LIM73 (HM241129)	2.4	Uncultured bacterium clone F2_07X (GQ262975)	98
Cyanobacteria			
LIM31 (HM241099)	8.9	*Chroococcidiopsis* sp. CC1 16S (DQ914863)	97
LIM84 (HM241118)	3.6	*Chroococcidiopsis* sp. BB79.2 (AJ344552)	93
LIM128 (HM241122)	1.8	*Coleodesmium* sp. ANT.LH52B.5 (AY493596)	97
LIM65 (HM241104)	2.4	*Brasilonema octagenarum* UFV-OR1 (EF150855)	98
Firmicutes			
LIM10 (HM241107)	2.2	*Bacillus megaterium* strain PRE9 (EU880506)	99
Planctomycetes			
LIM15 (HM241109)	0.4	Uncultured Planctomycetales bacterium clone SM2F01 (AF445727)	97
TM7			
LIM109 (HM241120)	4.9	Uncultured bacterium clone 2C228685 (EU800550)	93
Alphaproteobacteria			
LIM17 (HM241098)	6.0	Uncultured alpha proteobacterium clone Dolo_14 (AB257639)	97
LIM21 (HM241110)	3.6	*Kaistobacter terrae* (AB258386)	87
LIM23 (HM241112)	2.4	*Novosphingobium* sp. S23435 (D84626)	98
LIM58 (HM241116)	0.9	Bacterium Ellin6055CENA103 (AY234707)	97
LIM106 (HM241119)	0.9	*Sphingomonas* sp. BH3 (FJ834325)	98
LIM136 (HM241123)	2.9	Uncultured bacterium clone AKIW742 (DQ129613)	94
Deltaproteobacteria			
LIM2 (HM241096)	4.4	Uncultured bacterium clone FAC87 (DQ451526)	95
LIM4 (HM241097)	8.7	*Stigmatella koreensis* strain KYC-1019 (EF112185)	98

[a]The frequency of the clones is given as the number of clones of one sort of phylotype divided by the total number of clones in the three limestone libraries.

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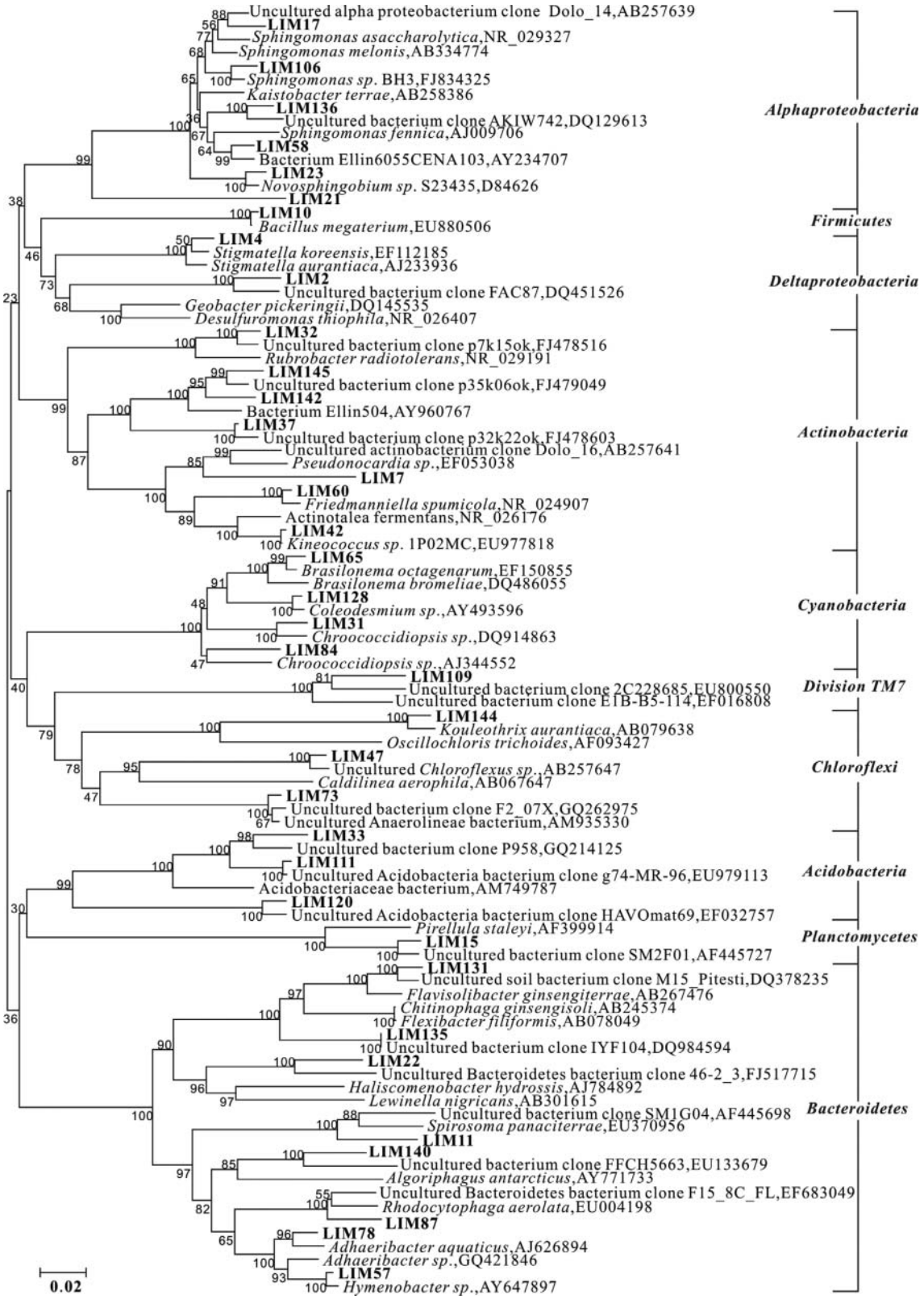

FIG. 3. Phylogenetic relationship based on 16S rRNA gene sequences of endolithic clones isolated from limestone rocks (in **bold type**) with closely related sequences from the GenBank database. Neighbor joining trees; bootstrap values (1,000 replicates) are shown at the nodes.

TABLE 3
Phylogenetic compositions of dolomite and limestone endolithic communities

	% of rRNA clones in community							
	Dolomite				Limestone			
Taxon	D1	D2	D3	Avg	L1	L2	L3	Avg
Acidobacteria	11.3	10.7	14.0	12.0	2.7	5.3	4.7	4.2
Actinobacteria	11.3	10	13.3	11.6	26.7	21.3	24.7	24.2
Bacteroidetes	9.3	7.3	8.0	8.2	10.0	12.7	12.0	11.6
Chloroflexi	2.0	3.3	2.7	2.7	8.0	5.3	4.7	6.0
Cyanobacteria	30.0	33.3	24.7	29.3	13.3	18.0	18.7	16.7
Firmicutes	0	0	0	0	3.3	2.0	1.3	2.2
Planctomycetes	0	0	0	0	0	0	1.3	0.4
TM7	0	0	0	0	4.0	6.0	4.7	4.9
Verrucomicrobia	4.0	6.0	6.7	5.6	0	0	0	0
Alphaproteobacteria	24.7	20.7	22.7	22.7	20.0	15.3	14.7	16.7
Betaproteobacteria	1.3	0.7	0	0.7	0	0	0	0
Deltaproteobacteria	6.0	8.0	8.0	7.3	12.0	14.0	13.3	13.1
Total	100	100	100	100	100	100	100	100

D1–D3 represents three dolomite clone libraries constructed. L1–L3 represents three limestone clone libraries. Avg represents the average percentage of each group in the three dolomite or limestone libraries.

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DISCUSSION

Diversity of Autotrophic Bacteria

Cyanobacteria are probably the most investigated type of rock microorganism. Epithic and endolithic cyanobacterial biofilms, as well as crusts of cyano-lichens can be found in cold and hot deserts, temperate regions, semi-deserts, savannas, rain forests, and even polar regions (Gorbushina 2007). Several studies have documented endolithic cyanobacteria in dolomite or limestone rock. Diels (1914) and Jaag (1945) found cyanobacteria in European Dolomite site, and Ferris and Lowson (1997) as well as Gerrath et al. (1995) reported their presence in limestone of the Niagara escarpment, all of which were classified by culture-dependent techniques through which standard morphological characteristics were used to identify community members. Only a few of those genera have been confirmed with molecular methods because they are easy to cultivate, but are the rare ones in nature. Recently, the utility of the molecular approach to investigate endolithic cyanobacterial communities in dolomite has been demonstrated (Horath and Bachofen 2009; Sigler et al. 2003).

By using cultured-independent techniques, we found 10 phylotypes (29.3% of total clone population) of cyanobacteria in the dolomite endolithic community and 4 phylotypes (16.7% of total clone population) in the limestone libraries. The cyanobacterial sequences included *Phormidium autumnale*, *Anabaena oscillarioides*, *Scytonema* sp., *Chroococcidiopsis* sp., *Leptolyngbya* sp., *Calothrix* sp., *Nostoc* sp., *Coleodesmium* sp., and *Brasilonema octagenarum*. In previously studied endolithic environments, the predominance of similar organisms, including the genera *Gloeocapsa*, *Chroococcidiopsis*, *Nostoc*, *Leptolyngbya* and *Scytonema*, suggests that stresses common to endolithic environments worldwide have selected for a niche-specific assemblage of tolerant organisms (Sigler et al. 2003).

Therefore, it is no surprise that the majority of the organisms detected in our study (Tables 1 and 2) are most similar to those observed previously in environments characterized by similar selective pressures such as nutrient availability, and osmotic- and UV intensity-related stress. In the dolomite libraries, clones related to *Scytomema* sp. were most numerous, followed by *Nostoc* sp., *Anabaena oscillarioides*, *Leptolyngbya* sp., and *Chroococcidiopsis* sp.. In the limestone libraries, clones related to *Chroococcidiopsis* sp. accounted for 75% of the total cyanobacterial clones. *Scytomema* and *Nostoc* have been shown to contain multiple UVB-protective compounds such as mycosporine-like amino acids (Böhm et al. 1995), carotenoids, and other uncharacterized pigments (Kumar et al. 1996).

Nostoc and *Chroococcidiopsis* have been previously noted for their outstanding tolerance to dry conditions (Billi et al. 2000). In particular, after long periods of desiccation, *Chroococcidiopsis* possess the ability to regain photosynthetic capacity within minutes following rewetting (Hawes et al. 1992). *Nostoc* has been shown in vitro to resist water loss at potentials of 400 MPa (Potts 1994). Their exceptional ability to tolerate these stresses is the possible explanation for the dominance of *Scytomema*, *Nostoc*, and *Chroococcidiopsis* in the *Cyanobacteria* group of the studied dolomite and limestone samples.

The ability to fix carbon dioxide, and in some cases atmospheric dinitrogen (N_2), gives the *cyanobacteria*, in particular, a clear competitive advantage over heterotrophic bacteria in

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colonizing the outer few millimeters of exposed rocks (Sigler et al. 2003). Cyanobacteria are considered the first colonizers and provide the main sustenance of endolithic communities, although under certain circumstances oligotrophic heterotrophic microbes (bacteria and fungi) can develop without the need for nutrients from excreted metabolites or cyanobacterial biomass (Albertano and Urzi 1999; Crispim and Gaylarde 2005).

The green nonsulfur phototrophs such as chloroflexi were also identified in our study. They were originally thought to live only in extreme environments such as hot spring (Boomer et al. 2002; Hanada et al. 2002; Pierson and Castenholz 1974), but now they were also found in temperate and even cold environments, such as wastewater treatment systems (Beer et al. 2002; Bjornsson et al. 2002), the deep ocean (Giovannoni et al. 1996), subsurface soil at a depth of 188 m (Chandler et al. 1998), as well as endolithic systems (Horath and Bachofen 2009; Papineau et al. 2005; Walker and Pace 2007). Our sequence data confirm the presence of several green nonsulfur strains in the endolithic communities of dolomite and limestone rocks of the Guizhou Karst region.

Diversity of Heterotrophic Bacteria

Little is known about the diversity of the heterotrophic bacterial communities accompanying the phototrophs. Our results showed that in spite of the hostile environment, the heterotrophic endolithic population was quite diverse and consisted of many different species. The dolomite and limestone libraries yielded 32 and 29 different heterotrophic bacteria phylotypes, respectively. Phylogenetic analysis of the 16S rRNA gene showed that heterotrophic *Alphaproteobacteria* were the second most abundant group, after the cyanobacterial group, in the studied dolomite and limestone clone libraries. The results were similar to those for central Alps dolomite endolithic microbial community within which heterotrophic *Alphaproteobacteria* were also an important component (Horath and Bachofen 2009). Although *Proteobacteria* are not commonly found in environments that are characterized by severe pH, temperature, nutrient or water tension stresses, they are well known for their ability to degrade a wide diversity of organic substrates (Pasic et al. 2010). As observed in previous studies, the availability of organic carbon from autotrophs is one possible explanation for the observed presence of heterotrophic *Proteobacteria*.

The group of *Actinobacteria* made up 24.2% of all clones found in the limestone libraries, with a similar occurrence in rock varnish of the Whipple Mountains (Kuhlman et al. 2006), in the Rocky Mountains (Walker and Pace 2007), and in dolomite rock of the Central Alps (Horath and Bachofen 2009). An explanation for the high fraction of *Actinobacteria* in Guizhou karst region could be due to their strong cell wall, the capability of forming spores, and their high GC-content. These characteristics would allow their survival in harsh environments. Our results confirmed that members of the *Proteobacteria* and *Actinobacteria* are ecologically significant constituents of carbonate rocks. However, caution must be exercised to infer any physiological functions based on relatedness of clone sequences to known cultures. Future work is needed to determine the functions of the two important groups in karst environments.

Role of Microbial Weathering of Carbonate Rocks in Soil Formation

Although the carbonate rock is not suitable for the survival of heterotrophic microorganisms, autotrophic microorganisms may be able to survive through photosynthesis and N fixation, and heterotrophic microbes adapt survival strategy through symbiosis with autotrophic microorganisms or intercepting small soil particles in which nutrients are occasionally brought in from air flow and rainwater (Lian et al. 2010; Viles and Gorbushina 2003). The rock microorganisms are thus of collaboration or symbiosis, and different from soil microorganisms commonly found in the relationship of competition or predation. The main purpose of different microbial taxa is to retain water and gain limited trace nutrients to sustain life activity and population continuity (Gorbushina et al. 2003; Sterflinger 2000).

Normally, carbonate rocks are enriched in Ca, Mg and depleted in Si, Al, and Fe, but inorganic substances in soils are mainly composed of Si, Al, Ca, Mg, and Fe. Therefore, pure carbonate rocks generally cannot be weathered to supply a large number of soil nutrients. Nonetheless natural carbonate rocks contain certain impurities to form muddy carbonates or mixed rock types, and therefore microbial weathering of impure carbonates may be important in soil formation in karst regions.

The microorganisms could erode carbonate rocks through the chemical degradation (organic acids secreted by microbial metabolism to promote calcium carbonate dissolution and weathering), the biological effect (mineral particles are broken due to microbial growth such as fungal hyphae interspersed to mineral particles, which generates more easily eroded surface), and enhanced erosion by metabolites or enzymes (microorganisms secrete enzymes such as carbonic anhydrase enzymes, etc.) (Chen et al. 2008; Dou and Lian 2009; Lian et al. 2008).

Microbial weathering of carbonate rocks produces residual minerals, secondary minerals, and organic components over a long time duration, providing a source of soil materials in the karst areas. In addition, autotrophic microorganisms can fix N and C elements from the air, and become the main producers of organic matter for microbial communities in carbonate rocks (Cao and Yuan 1999; Gorbushina 2007). Furthermore, microorganisms can also capture, intercept or absorb dust and soil particles brought in by air flow and rain, and use these particles to maintain limited life activities, producing more soil materials. Accumulation of these materials from diverse processes would lead to the progressive rock fragmentation and the formation of soil particles, which in turn develops a diversity of microbial populations. In summary, rock microorganisms play an important role in the formation of karst soil from carbonate rock weathering over the long geological history.

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