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ORIGINAL PAPER

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Bacterial diversity and carbonate precipitation in the giant microbialites from the highly alkaline Lake Van, Turkey

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Abstract Lake Van harbors the largest known microbialites on Earth. The surface of these huge carbonate pinnacles is covered by coccoid cyanobacteria whereas their central axis is occupied by a channel through which neutral, relatively Ca-enriched, groundwater flows into highly alkaline (pH ~9.7) Ca-poor lake water. Previous microscopy observations showed the presence of aragonite globules composed by rounded nanostructures of uncertain origin that resemble similar bodies found in some meteorites. Here, we have carried out fine-scale mineralogical and microbial diversity analyses from surface and internal microbialite samples. Electron transmission microscopy revealed that the nanostructures correspond to rounded aragonite nanoprecipitates. A progressive mineralization of cells by the deposition of nanoprecipitates on their surface was observed from external towards internal microbialite areas. Molecular diversity studies based on 16S rDNA amplification revealed the presence of bacterial lineages affiliated to the Alpha-, Beta- and Gammaproteobacteria, the Cyanobacteria, the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, the Actinobacteria and the Firmicutes. Cyanobacteria and CFB members were only detected in surface layers. The most abundant and diverse lineages were the Firmicutes (low GC Gram positives). To the exclusion of cyanobacteria, the closest cultivated members to the Lake Van phylotypes were most frequently alkaliphilic and/or heterotrophic bacteria able to degrade complex organics. These heterotrophic bacteria may play a crucial role in the formation of Lake Van microbialites by locally promoting carbonate precipitation.

Keywords 16S rRNA · Aragonite · Alkaliphile · Biomineralization · Electron microscopy · Soda lake · Phylogenetic analysis · Stromatolite

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Introduction

Microbialites are sedimentary structures formed by mineral precipitation and/or sediment accretion induced by microbial mat communities. Laminated calcareous microbialites, or stromatolites, were once widespread in shallow marine environments (Altermann 2004; Grotzinger and Knoll 1999; Walter 1983). It is assumed that they declined as a result of the rise and diversification of metazoan grazers (Awramik 1971; Walter and Heys 1985) or due to changes in seawater composition (Grotzinger 1990; Kempe and Kazmierczak 1990; Kempe and Kazmierczak 1994). Modern stromatolites are limited to a few tropical marine and quasi-marine sites (Shark Bay, Australia, Caribbean Bahamas, central Indonesia—e.g. Dravis 1983; Kempe and Kazmierczak 1993; Logan 1961; Reid and Browne 1991; Reid et al. 2003), and to extreme environments, such as alkaline and hypersaline lakes or thermal springs, which prevent the development of grazing animals (Paerl et al. 2000) and may also hydrochemically facilitate the in situ permineralization of microbial communities (Kempe and Kazmierczak 1990).

In 1991, Kempe et al. (1991) reported the discovery of giant microbialites, up to 40 m high in the alkaline waters (pH 9.7–9.8) of Lake Van, Eastern Anatolia (Turkey). Located at 1,648 m above sea level, with a maximum depth of 450 m and a volume of 607 km^3, Lake Van is one of the largest closed water bodies known, and the largest soda lake on Earth. However, in contrast to most soda lakes, which are saline to hypersaline (5% up to saturation, ~30%) (Grant 1992; Jones et al. 1998), Lake Van has a much lower salinity (2.17%), mostly contributed by calcium, sodium, chlorine and carbonate ions, with minor contributions from sulfate, potassium and magnesium (Kempe et al. 1991). The microbialites form near shore where neutral, relatively Ca-enriched, groundwater from sublacustrine springs meets the Ca-poor, highly alkaline lake water (>2 vs. 0.204–0.176 meq Ca l^{-1}, respectively). The mixing of the two water masses induces local very high calcium carbonate supersaturation and hence precipitation, generating milky solutions in some near-shore areas. These tower-like microbialites are externally covered by mats of coccoid cyanobacteria (Fig. 1a, b) that appear to permineralize in situ with aragonite and inorganically precipitated calcite (Fig. 1c), whereas their interiors are traversed by channels transporting spring water (Fig. 1 center) (Kempe et al. 1991). The walls of the axial channels are densely covered by carbonate globules (Fig. 1d–f), which are also irregularly distributed in the organic carbon-rich aragonite and Mg silica matrix towards the external tower walls. The globules exhibit alternating dark and light concentric layers, being composed primarily of aragonite, which is sometimes replaced by Mg/Mn/Fe-enriched calcite. At micro-scale, these globules are composed by rounded to elongated nanostructures (0.05–0.15 μm in diameter) that remind the ''nanobacteria''-like morphologies observed in some stromatolites and meteorites, including the famous Martian ALH84001 meteorite and the Tatahouine meteorite (Benzerara et al. 2003; Folk 1993; Kazmierczak and Kempe 2003; Pedone and Folk 1996). Whether these nanostructures have a biogenic origin, either direct (permineralized bacteria) or indirect (nucleation on secreted or lysis-derived organics), or whether they are the product of abiotic precipitation remains undetermined (Kazmierczak and Kempe 2003; Kazmierczak et al. 2004).

The formation mechanisms of stromatolites, the best-studied microbialites, are not yet well understood (Grotzinger and Knoll 1999). For a long time, carbonate precipitation in marine stromatolites was thought to be the major outcome of cyanobacterial activity. Photosynthesis would displace the inorganic carbon species equilibrium towards CO_3^{2-}, inducing a concomitant rise in pH, and resulting in calcium carbonate supersaturation and deposition (e.g. Krumbein 1979; Merz 1992; Pentecost and Bauld 1988; Riding 1982). It has also been suggested that the negatively charged extracellular polymeric substances (EPSs) of cyanobacterial sheaths may trap Mg^{2+} and Ca^{2+} ions and template the nucleation of $CaCO_3$ crystals, although only under low dissolved inorganic carbon and high Ca^{2+} concentrations (Arp et al. 2001). However, it has been argued by some studies that aragonite precipitation is rather inhibited by cyanobacterial growth

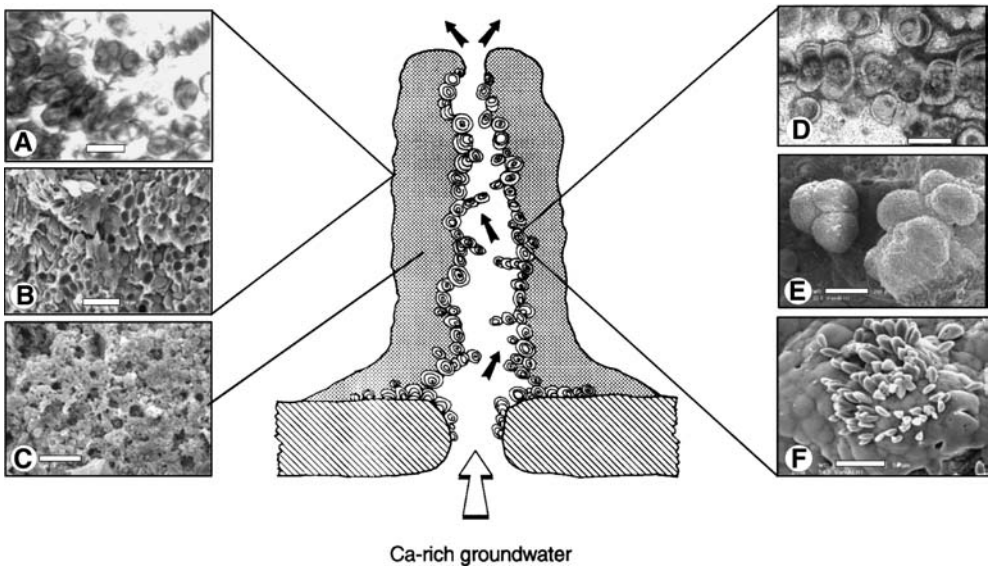

Fig. 1 Structure of pinnacle-like calcareous microbialite growing in Lake Van at inlet of Ca-rich groundwater. **a** Optical micrograph of living coccoid cyanobacteria forming a biofilm on pinnacle surface, **b** scanning electron microscopy (SEM) image of across section of the cyanobacterial biofilm, **c** scanning electron microscopy image of calcified remains of mucilage sheaths of coccoid cyanobacteria from the pinnacle wall, **d** optical micrograph (petrographic thin section) showing a group of calcareous spherulites from the pinnacle axial channel, **e** scanning electron microscopy image of similar spherulites exposed to the lumen of axial channel, **f** scanning electron microscopy image of bacteriomorphic aragonite bodies growing from the surface of a spherulite exposed to the lumen of axial channel. **a–c** sample LV60 (Tatvan Bay), **d–f** sample LV57 (Tatvan Bay). Scale bars correspond to 5 μm (**a**), 10 μm (**b**), 20 μm (**c**), 200 μm (**d, e**), and 50 μm (**f**)

as a consequence of Ca^{2+} binding to EPS and low molecular weight organic acids during early stages of marine stromatolite formation (Kawaguchi and Decho 2002; Reid et al. 2000). Recent studies suggest that heterotrophic bacteria may play a far more important and direct role in $CaCO_3$ precipitation by i) releasing the Ca^{2+} ions complexed by cyanobacterial mucilage and sheath material, and ii) serving as the dominant nucleation sites for $CaCO_3$ deposition (Chafetz and Buczynski 1992; Knorre and Krumbein 2000; Paerl et al. 2001). Experimental studies have shown that diverse heterotrophic bacteria can mediate $CaCO_3$ precipitation, including several alkaliphilic and halophilic species, and ureolytic bacilli (e.g. Hammes et al. 2003; Lee 2003; Rivadeneyra et al. 1999; Rodriguez-Navarro et al. 2003). In addition, $CaCO_3$ precipitation can be contributed by bacilli spores, which accumulate Ca^{2+} and other divalent cations in their walls (Marquis and Shin 1994), and by sulfate reducing bacteria under anoxic conditions inside microbialites (Visscher et al. 2000; Visscher et al. 1998). This would imply that cyanobacteria are not directly involved in the precipitation of micritic carbonates, although the whole process of stromatolite formation would be indirectly controlled by the cyanobacterial autotrophic primary production (Paerl et al. 2000; Paerl et al. 2001; Stolz et al. 2001).

Despite their possible essential role in the genesis of stromatolites, the diversity of heterotrophic bacteria associated with these structures is just beginning to be addressed (Burns et al. 2004), although the cyanobacterial diversity has been analyzed to some extent by classical and molecular methods (Neilan et al. 2002; Paerl et al. 2000). The microbial diversity of soda lakes has also been studied to a certain extent by classical and molecular methods (Grant et al. 1999; Humayoun et al. 2003; Jones et al. 1998; Ma et al. 2004; Rees et al. 2004), but the microbial communities specifically inhabiting microbialites in soda lakes remain to be described. To our knowledge, the only available report on the microbial diversity associated to large microbialite structures in comparable environments corresponds to the Ikaite tufa columns, which are very particular structures where alkaline water (pH~10.4) springs out into a quasi-neutral pH marine environment (Stougaard et al. 2002).

In this work, we combine scanning and transmission electron microscopy observations (SEM, TEM) with cultivation-independent molecular methods to characterize the bacterial diversity associated with Lake Van carbonates both at the surface and inside microbialites. Our results show a large diversity of alkaliphilic, likely heterotrophic, bacteria that may play an essential role in carbonate deposition and the genesis of Lake Van giant microbialites.

Table 1 Description of microbialite samples from Lake Van analyzed in this study

Name	Depth (m)	Description
LV9	19	Pinnacle surface layer with living cyanobacteria and $CaCO_3$ formed by calcified cyanobacteria and putative heterotrophic bacteria
LV60	22	Pinnacle surface layer with living cyanobacteria and $CaCO_3$ formed by calcified cyanobacteria and putative heterotrophic bacteria
LV57	17	Brown-blackish spherulite area exposed to the lumen of a pinnacle axial canal
d LV57-1	17	Brown-blackish spherulite area exposed to the lumen of a pinnacle axial canal
LV-57-3	17	Internal, ochre area of old (hundreds of thousand years) spherulite masses towards the lumen of a pinnacle axial canal

Materials and methods

Sample collection and preparation

Samples were collected from the Lake Van (Eastern Anatolia, Turkey) during the summer 1989 by diving (S. Kempe, G. Landmann, A. Lipp). The microbialite fragments used for this study were let dry in a field laboratory and then stored desiccated and protected from humidity in plastic containers. The samples analyzed were carefully dissected to separate different internal and external areas in microbialite sections (Table 1). Sample fractions (~50–100 µl volume) for microbiological surveys were selected from the cleanest protected areas with the help of sterile tools under a laminar flux chamber. The same fractions used for microbial analyses were used for microscopy observations.

Electron microscopy observations, X-Ray diffraction analyses

Samples were ground in ethanol and dry powders were loaded on hollowed aluminum plates. Powder X-ray diffraction (XRD) data were collected with a Phillips PW1710 diffractometer with Co-K_α radiation operating at 40 kV, 30 mA in step scan mode, between 3° and 90° 2θ with a 0.04° 2θ step, and a counting time of 15 s per step. For SEM observations, millimeter-sized microbialite fragments were mounted on aluminum stubs covered with carbon conductive adhesive tape and carbon-coated. Observations were conducted on a JEOL JSM6301-F microscope operated at 5 kV accelerating voltage and a sample-to-objective working distance of about 15 mm. For TEM observations, samples were ground in ethanol in agate mortar and one drop of the liquid suspension was deposited on a 200 mesh copper grid covered with formvar/carbon film. Samples were observed with a JEOL 2000 EX transmission electron microscope operating at 200 kV.

Nucleic acid extraction and construction of 16S rDNA libraries

Prior to nucleic acid extraction, dry samples were rehydrated with sterile phosphate saline buffer (130 mM NaCl, 10 mM phosphate buffer, pH 7.7, PBS). In order to minimize possible extraction biases, DNA used in this study was extracted by two methods, the SoilMaster DNA extraction kit (Epicentre) and a modified classical phenol-chloroform extraction. For the latter, samples were subjected to six freezing/thawing cycles in liquid nitrogen to facilitate cell lysis. Subsequently, 80 μg ml^{-1} proteinase K, 1% SDS, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% hexadecyltrimethylammonium bromide (CTAB) (final concentrations) were added sequentially. Lysis suspensions were incubated overnight at 55°C. Lysates were extracted once with hot phenol (65°C), once with phenol–chloroform–isoamylalcohol, and once with chloroform–isoamyl–alcohol. Nucleic acids were concentrated by ethanol precipitation. 16S rRNA genes were amplified by PCR using different combinations of the bacterial-specific primers B-27F (AGAGTTTGATCCTGGCTCAG), 63F (CAGGCCTAACACATGCAAGTC) and CYA106F (CGGACGGGTGAGTAACGCGTGA) and the prokaryote- and bacterial-specific reverse primers 1492R (GGTTACCTTGTTACGACTT) and CYA1380R (TAACGACTTCGGGGCGTGACC). Polymerase chain reactions (PCR) were performed under the following conditions: 30 cycles (denaturation at 94°C for 15 s, annealing at 50–55°C for 30 s, extension at 72°C for 2 min) preceded by 2 min denaturation at 94°C, and followed by 7 min extension at 72°C. Dimethyl sulfoxide was sometimes added to a final concentration of 3–5% to the PCR reaction mix. rDNA clone libraries were constructed using the Topo TA Cloning system (Invitrogen) following the instructions provided by the manufacturers. After plating, 250 positive transformants were screened by PCR amplification of inserts using M13R and T7 flanking vector primers.

Phylogenetic analyses

A total of 153 expected-size amplicons from seven different rDNA libraries (74 from surface samples and 79 from internal microbialite areas) was partially sequenced (Genome Express). The closest relatives to these sequences were identified in databases by BLAST (Altschul et al. 1997) and retrieved from GenBank (http://ncbi.nlm.nih.gov/). Sequences were automatically aligned using ClustalX (Thompson et al. 1997). A preliminary phylogenetic analysis of all partial sequences was done by distance methods (neighbor-joining, NJ), allowing the identification of identical or nearly identical sequences and the selection of representative clones for subsequent analysis. We selected 46 representative clones (Table 2) that were fully sequenced and aligned automatically using the program BABA (H. Philippe, personal communication) to a 16S rRNA gene alignment containing ~17,000 sequences. The multiple alignment was then manually edited using the program ED from the MUST package (Philippe 1993). Final phylogenetic trees included our sequences together with their closest relatives in GenBank and some representative cultivated species. Ambiguously aligned positions and gaps were removed from the analysis, resulting in a total of 1,097 and 1,162 conserved positions for the sequence subset sample covering most bacterial groups (Fig. 6) and that of Gram-positive bacteria (Fig. 7), respectively. The maximum likelihood (ML) trees were done using TREEFINDER (Jobb 2002) applying a general time reversible model of sequence evolution (GTR), and taking among-site rate variation into account by using an eight-category discrete approximation of a Γ distribution (invariable sites are included in one of the categories). Maximum likelihood bootstrap proportions were inferred using 1,000 replicates. Phylogenetic trees were viewed using the program TREEVIEW (Page 1996). The sequences reported in this study were submitted to GenBank with accession numbers AY642541to AY642586 (see also Figs. 6, 7).

Results

In order to correlate fine-scale mineral analysis to the microbial diversity in Lake Van microbialites, the cleanest representative fragment samples were selected from internal and external regions (Table 1). The chosen $CaCO_3$ fragments were small (~50–100 μl volume), and were split in two fractions to carry out, in parallel, microscopic and mineralogical analyses and molecular diversity surveys. Combining geological and microbiological data with a relatively high spatial resolution would hopefully help to explain the process of microbialite formation.

Electron microscopy and X-ray diffraction (XRD) analyses of microbialite samples

X-ray diffraction analyses of selected samples from the microbialite external surface (LV60) and the internal area (LV57 and LV57-3) (Table 1) showed very similar patterns (Fig. 2). The diffractograms corresponding to the two internal samples were nearly identical, and therefore only LV57-3 is shown in Fig. 2. The vast majority of peaks corresponded to aragonite. However, the (104) peak of calcite appeared in the internal samples, which was undetectable in LV60. These observations are consistent with previous data showing that aragonite dominated in microbialites but that it was often replaced by calcite in the interior of some carbonate globules (Kazmierczak and Kempe 2003).

Scanning electron microscopy observations of surface microbialite samples showed a variety of bacterial morphotypes including filamentous and coccoid microorganisms (Fig. 3). Some of these corresponded to

Table 2 Phylogenetic affiliation of bacterial phylotypes obtained from Lake Van microbialites. CFB, *Cytophaga-Flexibacter-Bacteroides* group

Clone	Length (bp)	Number of similar[a] sequences	Phylogenetic ascription	Closest 16S-rDNA BLAST hit in database, environmental origin (Accession number)	Percentage of identity (BLAST)
LV9-22	1,416	1	Actinobacteria	Uncultured actinobacterium 0649-1G9, Australian arid soil (AF234119)	90
LV9-CY3-8	1,218	3	Cyanobacteria	*Dermocarpa* sp. MBIC10004 (AB058202)	94
LV9-CY3-16	1,219	2	Cyanobacteria	*Stanieria cyanosphaera* (AF132931)	94
LV60A-11	1,450	1	Firmicutes	*Bacillus* sp. 19498, Sevilla tomb paintings (AJ315066)	96
LV60A-12	1,433	1	Gammaproteobacteria	*Acinetobacter* sp. phenon 2 strain type LUH5832 (= *Acinetobacter schindleri*) (AJ278311)	99
LV60-2	1,384	2	Gammaproteobacteria	*Terrahaemophilus aromaticivorans*, petroleum sludge (AB098612)	99
LV60-6	1,374	2	Gammaproteobacteria	Enrichment culture bacterium LB-P, wastewater treatment bioreactor (AF538773)	98
LV60-7	1,368	2	Betaproteobacteria	Arsenite-oxidizing bacterium BEN-4, Australian gold mining environement (AY027504)	99
LV60-9	1,341	2	CFB	*Hongiella mannitolivorans* IMSNU 14012, intertidal flat sediment, Korea (AY264838)	88
LV60-10	1,389	2	Firmicutes	*Bacillus* sp. LMG 20241, mural paintings of St Martin's church, Greene-Kreiensen, Germany (AJ316313)	99
LV60-21	1,332	5	Alphaproteobacteria	Clone 59H11, soil (AF245037)	96
LV60-24	1,343	1	Cyanobacteria	*Nostoc* sp. strain 152 (AJ133161)	95
LV60-26	1,467	4	Firmicutes	Clone WCHB1-21, hydrocarbon- and chlorinated-solvent-contaminated aquifer (AF050580)	94
LV60-27	1,375	1	Gammaproteobacteria	*Lysobacter* sp. Dae16 (AB166878)	95
LV60-48	1,385	1	Firmicutes	*Bacillus* sp. IDA4921, soil (AY289500)	98
LV60-52	1,375	1	CFB	Isolate CS57, Antarctic cryoconite hole (AY124340)	92
LV60-54	1,359	2	CFB	*Hongiella mannitolivorans* IMSNU 14012, intertidal flat sediment, Korea (AY264838)	88
LV60-60	1,379	1	Firmicutes	*Alkaliphilus crotonoxidans*, methanogenic environment (AF467248)	95
LV60-63	1,355	2	Firmicutes	*Clostridium bartlettii* strain WAL 16138 (AY438672)	98
LV60-CY1-1	1,307	1	Cyanobacteria	*Nostoc* sp. strain 152 (AJ133161)	95
LV60-CY1-5	1,306	2	Firmicutes	*Bacillus* sp. IDA4917, soil (AY289504)	97
LV60-CY1-6	1,328	2	Firmicutes	Lake Bogoria isolate 9B1, soda lake (X92167)	97
LV60-CY1-8	1,320	1	Firmicutes	Lake Bogoria isolate 9B1, soda lake (X92167)	96
LV60-CY1-10	1,281	2	Firmicutes	*Clostridium bartlettii* strain WAL 16138 (AY438672)	97
LV60-CY1-13	1,302	4	Firmicutes	*Bacillus* sp. IDA3504, soil (AJ544784)	98
LV60-CY1-19	1,329	1	Firmicutes	Clone PeM05, insect larva hindgut (AJ576377)	91
LV60-CY1-22	1,258	1	Alphaproteobacteria	*Sphingomonas stygia*, deep-sea sediments (AB025013)	97
LV57-5	1,371	2	Firmicutes	*Bacillus* sp. N-1, alkaliphilic species (AB043851)	98
LV57-6	1,356	2	Alphaproteobacteria	Isolate PI_GH2.1.D5, Ghana tropical forest soil (AY162047)	99
LV57-7	1,428	14	Firmicutes	Glacial ice bacterium G50-TS4 (AF479357)	97
LV57-11	1,370	1	Alphaproteobacterium	*Phenylobacterium* sp. Slu-01 (AB166881)	96
LV57-13	1,411	1	Gammaproteobacteria	*Pseudomonas stutzeri* strain 24a43, marine sediments (AJ312175)	99
LV57-17	1,411	17	Gammaproteobacteria	*Acinetobacter* sp. 11, soil phenanthrene-degrading strain (AY177359)	99
LV57-39	1,442	4	Firmicutes	*Bacillus acidogenesis*, acid Sphagnum bog (AF547209)	95
LV57-1-1	1,437	6	Firmicutes	*Planococcus alkanoclasticus*, hydrocarbon degrading species isolated from intertidal beach sediment (AF029364)	98
LV57-1-7	1,440	2	Firmicutes	*Bacillus* sp. JMM-4, arsenate-respiring isolate (AY032601)	96
LV57-1-15	1,335	1	Alphaproteobacteria	*Brevundimonas bullata* strain MBIC2745 (AB023428)	99
LV57-1-17	1,331	1	Alphaproteobacteria	*Rhizobium* sp. SH1707neu (AY141985)	99
LV57-1-22	1,393	2	Actinobacteria	Isolate Ellin5273, Australian pasture soil (AY234624)	94
LV57-1-23	1,404	2	Gammaproteobacteria	*P. anguilliseptica* strain BI, cyanophycin-degrading species isolated from pond sediment (AF439803)	98

Table 2 (Contd.)

Clone	Length (bp)	Number of similar[a] sequences	Phylogenetic ascription	Closest 16S-rDNA BLAST hit in database, environmental origin (Accession number)	Percentage of identity (BLAST)
LV57-1-27	1,426	1	Firmicutes	Unidentified bacterium F6, Hailaer soda lake (China) (AF275705)	98
LV57-1-29	1,359	2	Alphaproteobacteria	Clone 60-2, Californian agricultural soil (AF423281)	95
LV57-1-35	1,380	1	Betaproteobacteria	EHg5, methylotrophic strain from soil contaminated by chemical waste, Portugal (AY436796)	98
LV57-1-38	1,327	2	Alphaproteobacteria	*Fulvimarina litoralis* strain HTCC2156, marine (AY178863)	96
LV57-3-33	1,419	19	Firmicutes	*Bacillus* sp. 2b-2, alkaliphilic strain (AB043857)	98
LV57-3-55	1,360	1	Actinobacteria	*Dietzia psychralcaliphila* JCM 10987 (AB159036)	99

[a]Sequences 98–100% identical

"fresh" microbes, that is, microorganisms displaying intact cell walls and no obvious mineralization structures (Fig. 3a, c). However, we also observed mineralized cells and filaments (Fig. 3b, d) that, in some cases, appeared clearly covered by aragonite rounded nanoprecipitates. In contrast, bacteriomorphs towards the lumen of pinnacles always exhibited a mineralized aspect (Fig. 1f). Transmission electron microscopy analyses on the same samples further confirmed this observation. "Fresh", mineral-free, cells corresponding to cyanobacteria and other prokaryotes were seen exclusively in the outmost layer, sometimes in intimate association with mineral particles (Fig. 4a, b). Coexisting with them, organic filamentous structures covered by nanoprecipitates were also present (Fig. 4c). In the inner microbialite region, only bacteriomorphs wrapped with nanoprecipitates were apparent (Fig. 4d). These nanoprecipitates were widespread in both, external and internal regions. Electron diffraction demonstrated that they correspond to well-crystallized nanometer-sized (~30–100 nm range) spherical aragonite crystals (Fig. 4e–g) as previously suggested (Kazmierczak and Kempe 2003) (Fig. 4e–g).

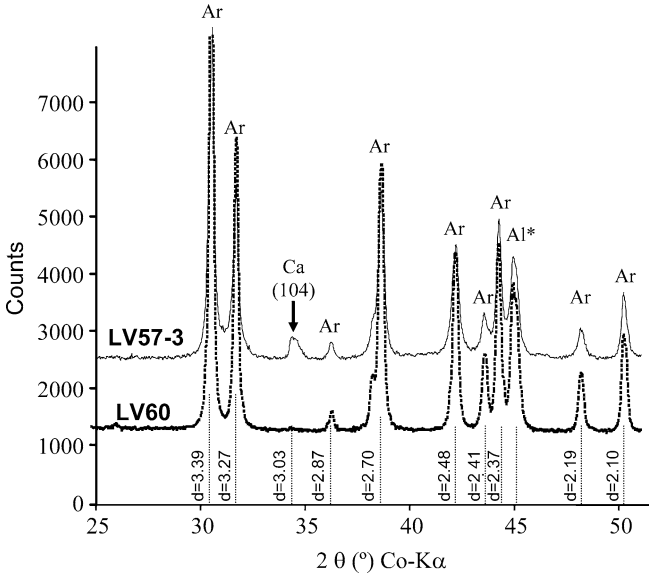

Fig. 2 X-ray diffraction (XRD) pattern of Lake Van microbialite surface (LV60) and internal (LV57-3) carbonates showing peaks characteristic of aragonite (Ar) and calcite (Ca). *The aluminium Al peaks are associated with the sample holder

Bacterial diversity in Lake Van microbialites

Nucleic acids were extracted from various surface and internal samples (Table 1) using different methods to minimize possible extraction biases due both, to the nature of the mineral substrate itself and to the fact that samples had been collected ~15 years ago. Despite this, we were able to amplify bacterial 16S rRNA genes using different bacterial-specific primer combinations. We also tried to amplify archaeal 16S rRNA genes using different archaeal-specific pairs and PCR conditions, including nested-PCR attempts. However, we failed to amplify them, suggesting that archaea, if present in microbialite samples, were not major components of the autochthonous microbial community. A preferential conservation of spore-forming or resistant bacteria over archaea would not explain this difference alone, since most archaea have highly resistant S-layers. Indeed, as we will see below, a large diversity of non-spore forming bacterial lineages was also observed.

From the bacterial amplicons obtained, we constructed seven 16S-rDNA libraries, screened about 250 clones, and sequenced partially 153 clone inserts that distributed in a number of bacterial divisions (Table 2 and Fig. 5). Most sequences affiliated to the Firmicutes (low GC Gram positive bacteria) both in the internal and external samples, but several others affiliated to the Gamma-, Alpha- and Beta- subdivisions of the Proteobacteria, the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, the Cyanobacteria and the Actinobacteria (high GC Gram positives) (Fig. 5). Some differences were observed in the relative abundance of sequences from external (LV9 and LV60) versus internal (LV57, LV57-1 and LV57-3) sample libraries. Whereas gammaproteobacteria were the second most represented group in

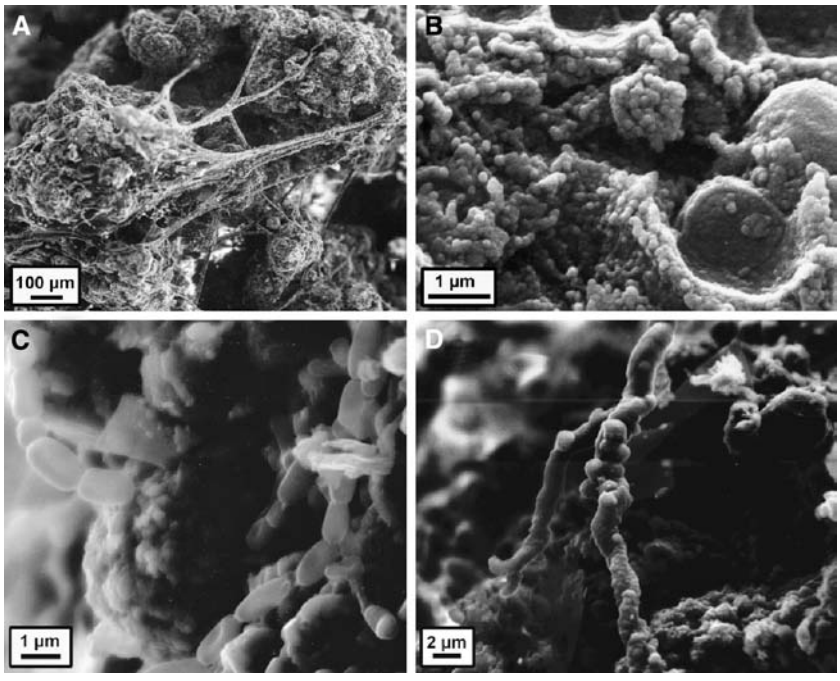

Fig. 3 Scanning electron microscopy images of Lake Van microbialite surface samples. **a** filamentous bacteria showing multiple attachment points to the substrate, **b** carbonate nanoprecipitates associated to lithified probable coccoid cyanobacteria (*CYA*), **c** microbial filaments, **d** lithified microbial filament

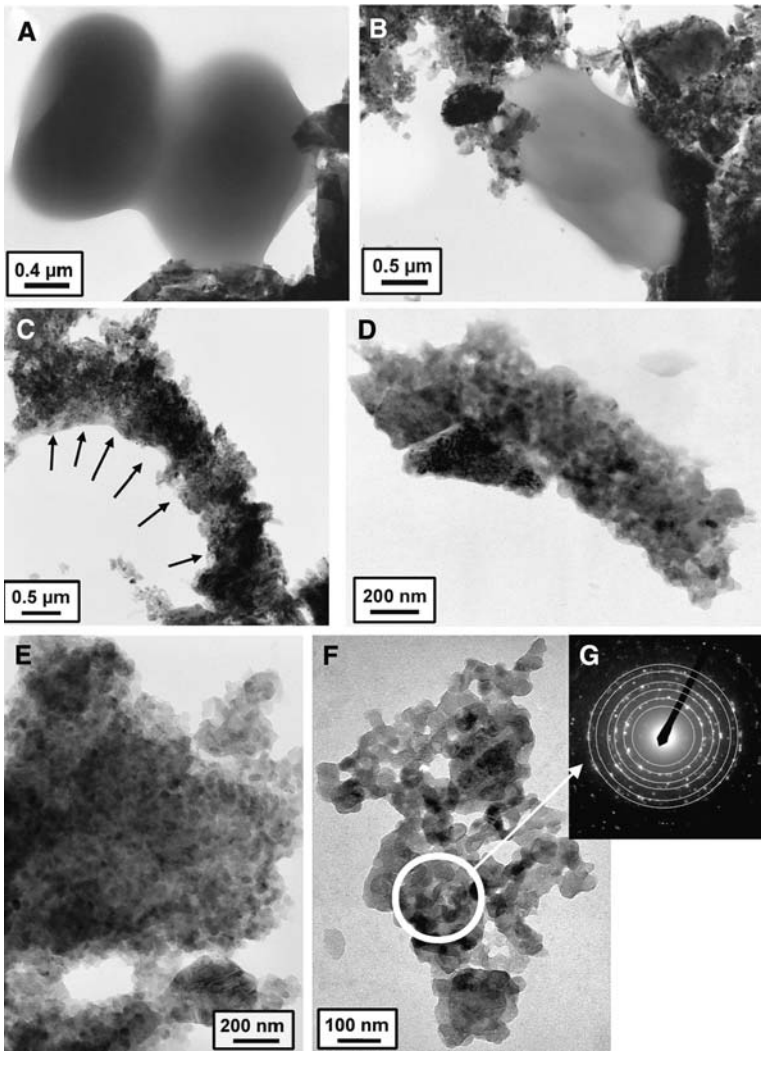

Fig. 4 Transmission electron microscopy (TEM) images of Lake Van microbialite samples. **a** coccoid cyanobacteria under division (LV60, surface), the attachment to the substrate can be observed, **b** non-fossilized microorganism intimately associated to carbonate nanoprecipitates (upper left and right, LV60), **c**, **d** mineralized microbial filaments covered by nanoprecipitate clusters (C, LV60; D, LV57-3, internal region), **e**, **f** carbonate nanoprecipitates (LV57-3), **g** electron diffraction from the area encircled in **f**. The powder pattern results from the diffraction of several single nanoprecipitates and was unambiguously indexed as aragonite ($a = 4.96$ Å, $b = 7.967$ Å, $c = 5.74$ Å, $\alpha = \beta = \gamma = 90°$). Interatomic distances between circles and indexing as aragonite: 4.98 Å (100), 3.96 Å (020), 3.27 Å (021), 2.88 Å (002), 2.37 Å (112), 2.14 Å (131)

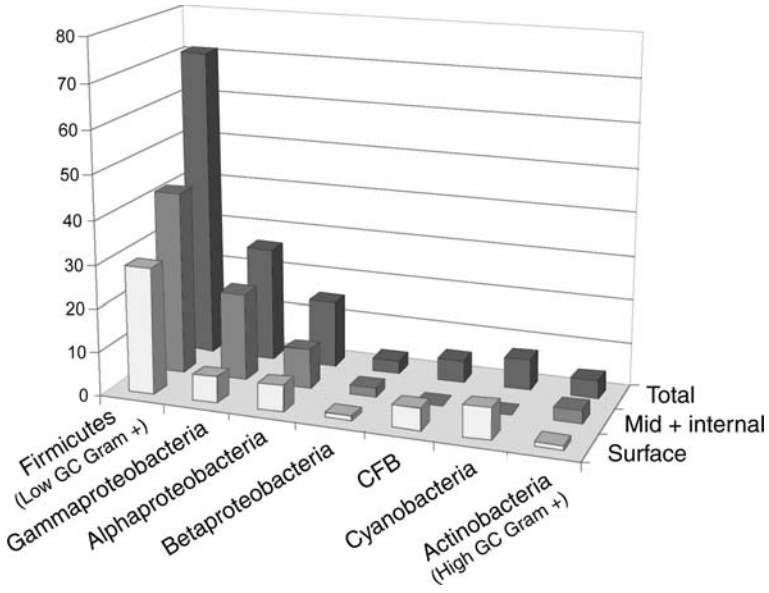

Fig. 5 Taxonomic distribution of bacterial clones in 16S rDNA libraries of surface (LV60, LV9) and internal (LV57, LV57-1, LV57-3) samples from Lake Van microbialites

internal microbialite libraries, they displayed very similar abundances to alphaproteobacteria, cyanobacteria and CFB members in surface libraries. Surface samples exhibited a relative larger diversity compared to the inner carbonates. Thus, cyanobacteria and CFB sequences were only identified on surface layers. We selected 46 bacterial clones as representatives of the diversity observed in Lake Van samples, which were fully sequenced in order to perform improved phylogenetic analysis (Table 2 and Figs. 6, 7). In general, the closest relatives to our sequences in databases, particularly those belonging to the Gram positive bacteria, corresponded to known alkaliphilic species or to sequences retrieved from soda lakes or from mural paintings. In addition to cyanobacteria, which had been also identified microscopically in samples, many sequences affiliated to clones retrieved from polluted soils or marine sediments (Table 2). Altogether, this suggests that the diversity identified was also indigenous to Lake Van samples, and not the product of later contamination.

Cyanobacterial sequences were identified only in surface samples. The sequences identified clustered within the Nostocales and the Pleurocapsales (Fig. 6). Both groups contain only coccoid cyanobacteria, which correlates well with microscopy observations (Kempe et al. 1991) (Figs. 3b, 4a). Notably, the cyanobacterial phylotypes retrieved from LV9 (19 m depth) ascribed to the order Pleurocapsales, whereas those from LV60 (22 m depth) belonged to the Nostocales. This difference might reflect a depth zonation that could be related to the specific adaptation of either lineage to slightly different light intensities and/or wavelengths. Sequences clustering within the CFB group were also identified only in surface samples (Fig. 6). Known Cytophagales, including the genera *Chitinophaga* and *Hongiella*, to which the two closest cultivated species to our sequences belonged, are gliding bacteria that degrade long polymers, particularly cellulose or chitin. They are frequent in shallow marine environments living on sewage plants or crustacean skeletons (Reichenbach 1999b; Yi and Chun 2004). Given the co-localization of CFB and cyanobacterial phylotypes on Lake Van microbialite surface, it would be thus tempting to speculate that CFB members are feeding on cyanobacterial EPSs.

Phylotypes belonging to the Alpha-, Beta- and Gammaproteobacteria were identified both in surface and internal samples (Table 2, Fig. 6). Members of these subdivisions are metabolically very versatile, so it is difficult to make predictions about the physiology of Lake Van members especially when they branch far from known cultivated species. The alphaproteobacterial sequences found were usually close to well known heterotrophic genera usually found in soil, or to uncultured organisms detected also in soils or sediments. Within the Betaproteobacteria, phylotypes related to a methylotrophic strain and to an arsenite-oxidizer were detected. Also the closest relatives to our gammaproteobacterial sequences were well-known degraders, often of xenobiotic or complex compounds (e.g. *Pseudomonas*, *Terrahaemophilus*). This suggests that several of the Lake Van phylotypes could correspond to bacteria decomposing derived cyanobacterial compounds. This idea is further supported by the identification of clones very closely related to *P. anguilliseptica* BI, which extracellularly degrades cyanophycin, a cyanobacterial nitrogen reserve polymer (Obst et al. 2004; Picossi et al. 2004), and to *Lysobacter* species (Table 2 and Fig. 6). The genus *Lysobacter* encompasses lytic strains of gliding heterotrophs that can induce the lysis of a wide spectrum of cyanobacteria and that show a remarkable resistance to alkaline conditions (Reichenbach 1999a).

A few Lake Van phylotypes ascribed to the Actinobacteria, or high GC Gram positives (Table 2, Fig. 7).

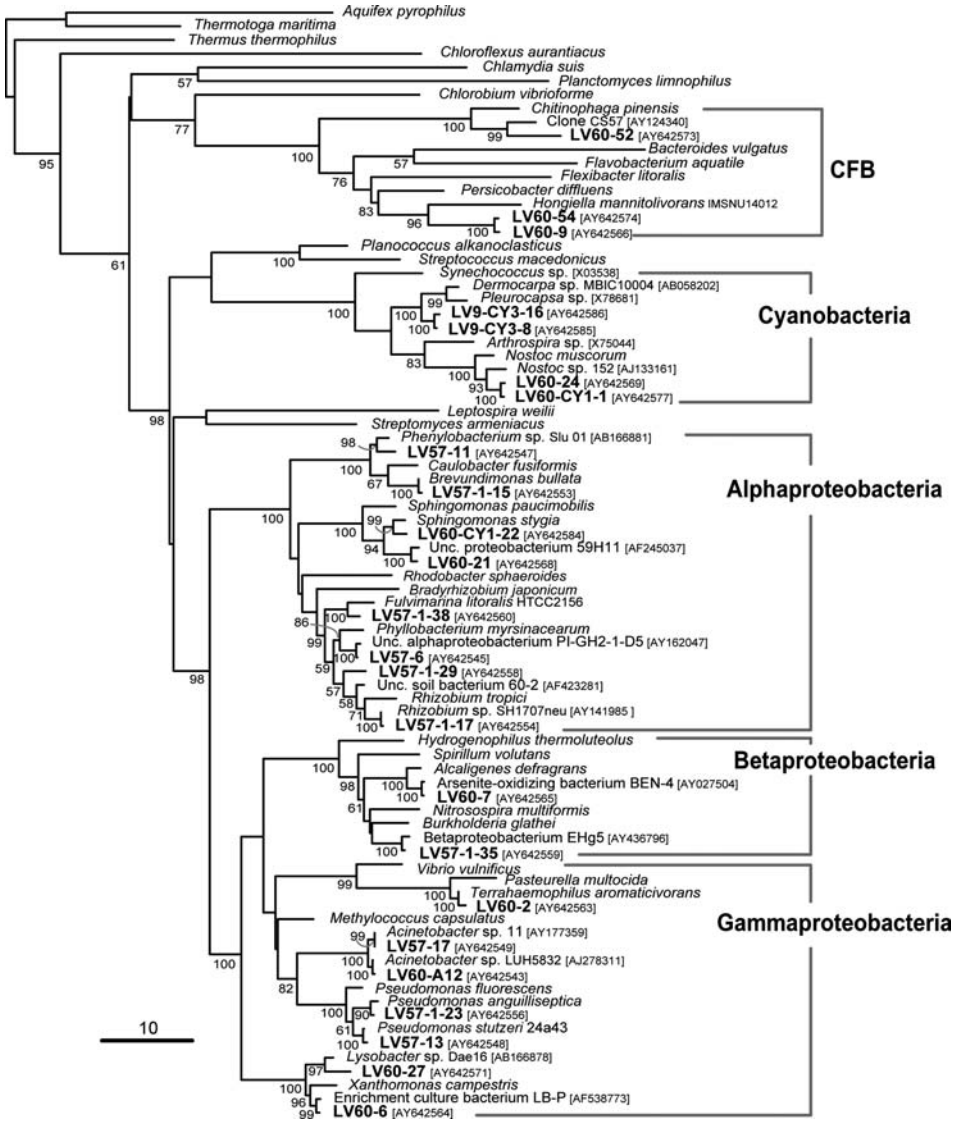

Fig. 6 Maximum likelihood (ML) phylogenetic tree of Lake Van microbial phylotypes affiliating to major bacterial groups (other than Gram positive divisions). *Numbers at nodes* are bootstrap values. The *scale bar* represents the number of substitutions per 100 positions per a unit branch length. Accession numbers of bacterial isolates and environmental sequences are given *within brackets*

Two of them were related to environmental sequences and isolates from soil, and another was nearly identical to the 16S rRNA gene of *Dietzia psychralcaliphila*, an alkaliphilic microorganism feeding on hydrocarbons (Yumoto et al. 2002). Species of the genus *Dietzia* have been isolated previously from soda lakes (Duckworth et al. 1998). However, the most represented clones in all the lake Van libraries were by far the low GC Gram positive bacteria (Firmicutes) (Fig. 5). Not only were they very abundant in our libraries, but they were also the most diverse (Fig. 7), suggesting that their overrepresentation in clone libraries may also correspond to the actual situation in nature. A few phylotypes were distantly related to environmental clones from hydrocarbon-polluted aquifers or from hindgut larvae (a worm species was found living on Lake Van microbialites (Kempe et al. 1991), one clone was related to the alkalitolerant, endospore-forming *Alkaliphilus crotonoxidans* (Cao et al. 2003), and a few sequences were related to *Clostridium bartlettii*. However, the vast majority of Gram positive Lake Van members were close relatives of different alkaliphilic hydrocarbon-degrading *Planococcus* and *Bacillus* strains or related environmental sequences retrieved from soda lakes (Bogoria, Hailaer) (Table 2, Fig. 7). They were both, abundant and diverse. All *Bacillus* species produce highly resistant endospores that accumulate Ca^{2+} in their walls (Marquis and Shin 1994; Muliukin et al. 2002). One of our clones, LV60–10, was very closely related to *Bacillus* sp. isolated from monuments and to the *Bacillus* sp. SG-1 whose dormant spores induce Mn(II) oxidation (Francis and Tebo 2002) at the outermost spore coat (exosporium) (Francis et al. 2002). The fact that most *Bacillus* relatives to our phylotypes are alkaliphilic or alkalitolerant suggests that the Lake Van clones were thriving in this alkaline biotope. Indeed, Gram positive bacteria are abundantly identified in soda lakes (Jones et al. 1998; Ma et al. 2004; Rees et al. 2004).

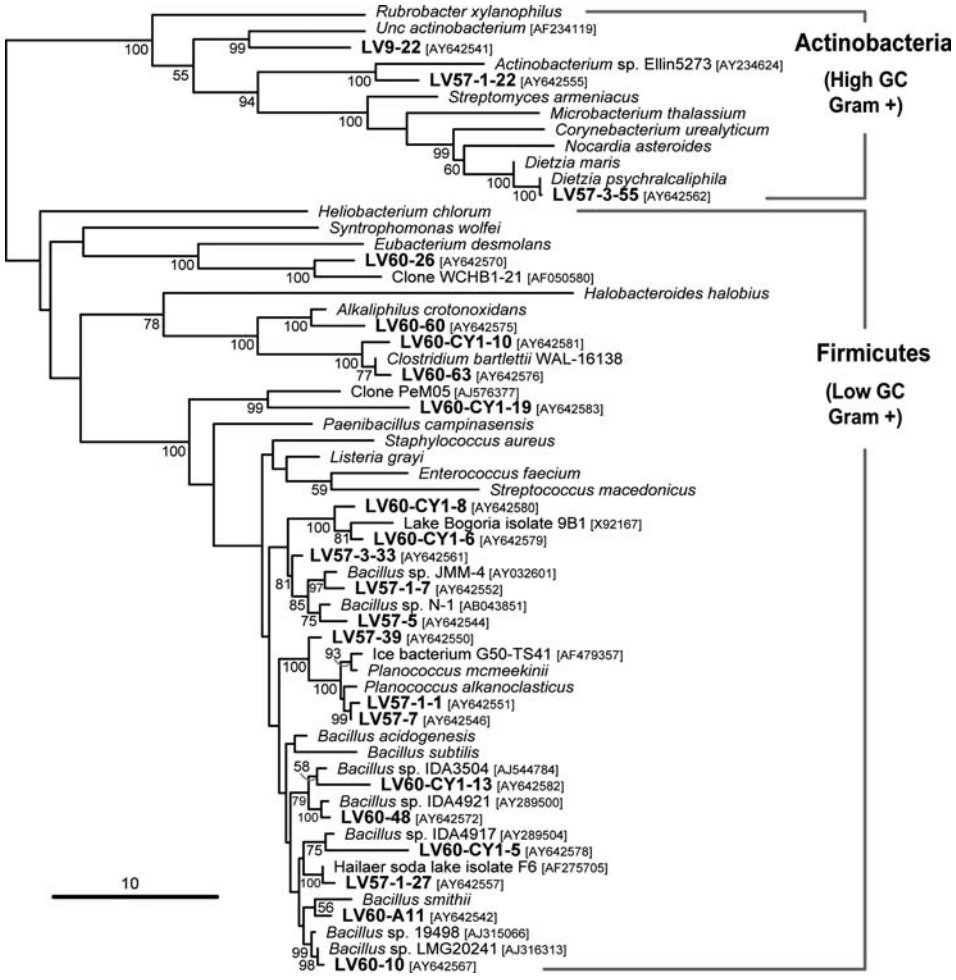

Fig. 7 Maximum likelihood phylogenetic tree of Lake Van clones affiliating to Gram positive bacteria. *Numbers at nodes* are bootstrap values. The *scale bar* represents the number of substitutions per 100 positions per a unit branch length. Accession numbers of bacterial isolates and environmental sequences are given *within brackets*

Discussion

Fine-scale mineralogical and microscopic observations of Lake Van microbialites unambiguously confirm previous studies suggesting that these giant fabrics are largely integrated by rounded aragonite nanostructures (Kazmierczak and Kempe 2003). These aragonite nanoprecipitates are very often seen in close association to "fresh" or mineralized cells (Figs. 3, 4). A variety of bacterial morphotypes at different mineralization stages was observed, which was a first suggestion of the existence of a certain bacterial diversity associated to these microbialites. Non-mineralized cells were observed only on surface samples. These included typical coccoid cyanobacterial morphologies, but also other bacterial morphologies. Mineralized cells with aragonite crystals deposited on their surface were observed both in the surface and the interior of the microbialite structure, suggesting a progressive calcification from the external towards the internal part of the towers.

16S rRNA-based molecular surveys of the diversity associated to different regions of the microbialite structure showed a wide variety of bacteria present both, in surface and internal samples (Figs. 5, 6, 7). It cannot be excluded that biases in the diversity found exist due to the fact that samples were collected ~15 years ago, yet the diversity retrieved was large. In addition, closest relatives to Lake Van sequences corresponded to alkaliphilic species or phylotypes identified in soda lakes, soils or shallow marine sediments, which strongly suggests that they are part of the indigenous microbialite microbial community. The fact that DNA was extracted also from internal samples indicates that at least a portion of the bacteria detected within microbialites were alive, either dormant or metabolically active.

As in the case of marine stromatolites, the primary producers in Lake Van microbialites are cyanobacteria. Cyanobacteria colonize various extreme environments and thrive well from neutral to alkaline pH (Paerl et al. 2000). Their ability to fix HCO_3^- is accompanied by a rise in pH, which further increases productivity as CO_2 is more easily pumped. This explains that cyanobacteria-dominated eutrophic systems become extremely alkaline (López-Archilla et al. 2004). Apart from cyanobacteria, most lineages detected in the microbialites correspond very likely to heterotrophic bacteria feeding on long hydrocarbons and polymers, as can be hypothesized from the metabolic activities of very closely-related cultivated members. In addition, Gram positive sporulating bacteria appear to be diverse and abundant both at the outer layer and the inside of microbialites. The role of heterotrophic

bacteria releasing Ca^{2+} accumulated in cyanobacterial sheaths and mucilage has been documented, as well as the induction of calcification by Gram positive bacilli and bacilli spores rich in Ca^{2+}. The role of spores in calcification might be found to involve more than a simple passive phenomenon with a preferential heterogeneous nucleation of $CaCO_3$. For instance, dormant spores from some strains have been shown to promote Mn(II) oxidation (Francis and Tebo 2002). Interestingly, aragonite is substituted by a Mn-enriched calcite in Lake Van globules. It would therefore, be interesting to investigate whether some *Bacillus* spp. spores play a role in manganese cycling within the microbialites.

Although the precise formation of Lake Van microbialites, as that of marine stromatolites, remains to be elucidated, we suggest that an important community of bacterial heterotrophs depending on cyanobacterial primary production may contribute decisively to the genesis of these structures. Given the large diversity of bacteria also inside the microbialites, a biogenic contribution to the formation of Lake Van globules is supported over an exclusively abiotic process. Indeed, organic molecules could be responsible for the unusual morphology (in size and shape) of the aragonite nanoprecipitates, similar to what was previously suggested for other systems (Benzerara et al. 2003). This might eventually have implications for understanding the origin of similar globules found in the Martian meteorite ALH84001 (Kazmierczak and Kempe 2003). However, whether the aragonite nanoprecipitates forming Lake Van globules are the result of the direct permineralization of dead cells and colonies or whether they originate from the precipitation of carbonates in the presence of organic molecules outside living cells is yet to be determined. The observation of aragonite nanoprecipitates adjacent to ''fresh'' cells would argue for an externally-induced precipitation, but both direct and indirect biogenic processes could co-occur. As more microbial diversity studies on marine stromatolites will come to light, it will be possible to compare them with contemporary soda lake microbialites. It is likely that the genesis of Lake Van microbialites and marine stromatolites share similar mechanisms, despite their different physico-chemical settings. Understanding them should help to improve our knowledge about ancient microbialites and, perhaps, also about the nature of their original natural environment.

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