RESEARCH ARTICLE

Diversity of microbial communities colonizing the walls of a Karstic cave in Slovenia

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Received 27 February 2009; revised 17 August 2009; accepted 6 September 2009. Final version published online 8 October 2009.

DOI:10.1111/j.1574-6941.2009.00789.x

Editor: Kornelia Smalla

Keywords

hypogean environments; 16S rRNA gene; diversity; Bacteria; microbial communities; cave.

Abstract

Karstic cave systems in Slovenia receive substantial amounts of organic input from adjacent forest and freshwater systems. These caves host microbial communities that consist of distinct small colonies differing in colour and shape. Visible to the naked eye, the colonies cover cave walls and are strewn with light-reflecting water droplets. In this study, the diversity of prokaryotes constituting these unusual microbial communities in Pajsarjeva jama cave was examined. A molecular survey based on small subunit rRNA diversity showed a high diversity within the Bacteria, while members of Archaea were not recovered. A total of eight bacterial phyla were detected. The application of various species richness estimators confirmed the diverse nature of the microbial community sample. Members of Gammaproteobacteria were most abundant in the clone libraries constructed and were followed in abundance by members of Actinobacteria and Nitrospira. In addition, members of Alphaproteobacteria, Betaproteobacteria and Deltaproteobacteria as well as Acidobacteria, Verrucomicrobia, Planctomycetes, Chloroflexi and Gemmatimonadetes were identified in clone libraries. The high number of clones most closely related to environmental 16S rRNA gene clones showed the broad spectrum of unknown and yet to be cultivated microorganisms inhabiting these cave systems.

Introduction

Caves are considered to be nutrient-limited ecosystems subjected to stable temperatures and high humidity. Life here is supported by photosynthetic activity only in entrance corridors where light penetrates the cave. Alternatives to carbon fixation in caves include chemoautotrophy and ammonium-, nitrite-, sulphur-, manganese- or iron-oxidizing chemolithoautotrophy (Northup & Lavoie, 2001).

Extensive areas, 40% of Slovenia, are shaped by the dissolution of limestone or dolomite to a Karstic landscape (Gams, 2004). The cave biota mainly depends on the organic input from the surface. The most obvious and most direct case of the plant and animal contribution is through penetrating roots and bat guano. But mainly, the organic material enters the caves diffusely, dissolved or dispersed in the dripping waters through the ceiling or as clay deposited on the ground and walls by subterranean rivers, or as particles drifted by air currents. This amount of organic matter supports secondary development of microbial communities on cave walls. These develop as multicoloured yellow, grey, white and pink cloddy coatings up to 1 mm thick on carbonate or clay-coated walls and ceilings of the cave. When illuminated, water droplets, usually scattered on the coatings, reflect the silvery or gold-coloured light, depending on the prevalence of whit(ish) or yellow colour, respectively, in underlying coatings. This earned the bacterial coatings the name ‘cave silver’ or ‘cave gold’ among the local cavers (Megušar & Sket, 1977; Sket, 1979). The development of such microbial communities on cave walls is observed along a number of caves of the region, most often in corridors along the allogenic cave streams, but outside water. Early studies focused on one such community inhabiting cave Planinska jama located approximately 20 km south of the cave studied here. In these studies, the organic nature of these coatings was shown, and it was found that their characteristic colouration is not due to Mn or Fe salts. The microbial community, as depicted using the cultivation approach, was found to be dominated by members of Actinomycetales. The isolates obtained could not fit any of...
the species then described (P. Kovač, D. Merlak, F. Megušar, B. Sket, unpublished data). Accordingly, members of *Actinomyxetales* were most commonly isolated from cave wall colony samples of well-studied Altamira and Tito Bustillo caves (Spain). However, *Actinomyxetales* constituted only a minor portion of a broad spectrum of unknown and yet to be cultivated bacterial species when the same samples were analysed using a cultivation-independent approach (Laiz *et al.*, 2003).

We believe that characterization of such microbial communities could serve as a basis to infer the dynamics between species assemblages and energy inputs in these ecosystems. To this aim, the microbial communities found in the easily accessible Pajsarjeva jama, 20 km southwest of Ljubljana, Slovenia, were extensively sampled. This horizontal cave is 555 m long and contains a small stream exiting the underground at its entrance. It is only occasionally visited by cavers and speleologists, but pipes of a water supply for the small fish-hatchery are inserted into it. We have no data on the terrestrial fauna of the cave, except for modest colonies of subtroglophilic bats *Rhinolophus hipposideros* (Bechstein) and *Rhinolophus ferrumequinum* (Schreber). Besides some troglobionts, the fauna of the streamlet includes epigean amphipods *Gammarus cf. fossarum* Koch, numerous larvae of *Chironomidae*, less numerous larvae of *Plecoptera*, and only single specimens of *Elminitidae* (*Coleoptera*), *Trichoptera* and *Hydra* sp. This compartment of its fauna shows that the streamlet originates on the surface. We have sampled microbial communities in a totally dark section of the entrance gallery (some tens of metres) before the siphon, preventing simple access to inner parts of the cave. The aim of this study was to provide baseline descriptive data on indigenous light-reflecting microbial communities formed on cave walls.

**Materials and methods**

**Sample collection**

Cave wall microbial community samples were collected from Pajsarjeva jama (cadastral no. 115), near Ljubljana, Slovenia (45° 59′51″N, 14° 16′15″E), in March 2008. Microbial communities covering a cave wall area of approximately 2 m² situated on the right bank of the streamlet before siphon were sampled. Within this area, six samples, each covering an area of approximately 0.1 m², were taken by scraping off colonies with a sterile scalpel without touching the supporting rocks. The samples were represented by a mixture of white, yellow, grey, greyish blue and pink macroscopic colonies. Upon collection, the samples were stored on ice and processed or frozen within 2 h after collection. The temperature and pH of the stream waters were measured *in situ* using probes (WTW, Weilheim, Germany).

**Molecular techniques**

Environmental DNA was extracted from each sample using the MoBio PowerSoil™ DNA kit (MoBio) according to the manufacturer’s instructions. Amplifications of bacterial 16S rRNA gene were performed using respective environmental DNA templates, *Taq* DNA polymerase (Fermentas), primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GTT TAG TCT TGT ACG ACT T-3′) and the following programme: 94°C (5 min), followed by 25 cycles of 94°C (1 min), 50°C (45 s) and 72°C (1 min) and a final 20-min extension step of 72°C. Amplifications of archaeal 16S rRNA gene were performed using respective environmental DNA templates, *Taq* DNA polymerase (Fermentas), primers D30 (5′-ATT CCG GTT GAT CCT GC) and D56 (5′-GYT ACC TTG TTA CGA CCTT) from Arahl *et al.* (1996) and the following programme: 94°C 2 min, followed by 30 cycles of 94°C (45 s), 50°C (45 s) and 72°C (90 s) with an additional 5 s added for each cycle with a final 10-min extension step of 72°C. The second set of 16S rRNA gene PCR amplifications was performed using primers Arch21F (5′-TTC CGG TTG ATC CYG CCG GA) and Arch958R (5′-GCC GGT GAM TCC AAT T) following the protocol described by DeLong (1992).

PCR products were checked for size and concentration by gel electrophoresis and were then cloned using the pGEM®-T Easy cloning kit (Promega) and used to transform competent *Escherichia coli* JM109 cells (Promega) as specified by the manufacturer. Six clone libraries were constructed, one for each sample collected. Insert-positive plasmids were isolated from overnight liquid cultures, using the Wizard Plus SV Minipreps DNA purification kit (Promega). An aliquot of each culture was preserved in 15% w/v sterile glycerol and stored at −80°C. A total of 28, 35, 36, 38, 40 and 40 clones were sequenced from their respective libraries at MacrogenUSA (Maryland) using T7 and SP6 plasmid-specific primers and a 536F internal rRNA primer (5′-CAG CMG CCG CGG TAA TWC-3′).

**Sequence comparisons and phylogenetic analysis**

Partial sequences of clone inserts sequenced were assembled using SEQUENCHER (Gene Codes, Ann Arbor). Assembled sequences were checked for chimera by BELLEROPHON server (Huber *et al.*, 2004) and CHIMERA_CHECK v. 2.7 (Cole *et al.*, 2003). For BELLEROPHON analysis, a variety of window sizes (200–400 bp) and corrections were used. Putative chimeric sequences were excluded from further analysis and the remaining 171 sequences were included in phylogenetic analysis. Relevant sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov, BLASTN algorithm), GREENGENES (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi, “Classify” tool) and the Ribosomal Database Project.
Alignment of these and the clone sequences obtained was generated by MUSCLE (Edgar, 2004) and the quality was checked by CORE available from TCOFFEE web server (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi). Gaps and ambiguously aligned positions were excluded from our analyses. In order to choose representative subsets of 16S rRNA gene sequences for further phylogenetic analyses, neighbour-joining trees were constructed using MEGA (Kumar et al., 2008). Four different subsets of 16S rRNA gene sequences were selected to achieve good taxonomic coverage of different regions in phylogenetic trees. These subsets contained representatives of operational taxonomic units (OTUs) detected in this analysis and relevant related sequences and were Actinobacteria and Acidobacteria (40 sequences, 1188 positions), Betaproteobacteria (32 sequences, 1220 positions), Alphaproteobacteria, Gammaproteobacteria and Deltaproteobacteria (46 sequences, 1232 positions) and Planctomyces, Verrucomicrobia, Chloroflexi, Gemmatimonadetes and Nitrospirae (31 sequences, 1154 positions). These datasets were analysed by maximum parsimony (MP) using PAUP* (Swofford, 2001) and maximum likelihood (ML) using PHYML (Guindon & Gascuel, 2003). ML searches were performed by applying a general time-reversible model of sequence evolution and taking among-site variation into account using a four-category discrete approximation of a Γ distribution with a portion of invariable sites. ML and MP bootstrap support values were assessed by 1000 bootstrap replications. The heuristic branch-swapping algorithm TBR with 10 × addition sequences randomized was applied under this optimality criterion. Bayesian posterior probabilities were computed under the same ML model with MRBAYES 3.0b4 (Huelsenbeck & Ronquist, 2001) with a Metropolis-coupled Markov chain Monte Carlo algorithm (Larget & Simon, 1999) by running four chains for $10^6$ generations, taking samples every 100 generations. The initial 6% of the trees was discarded as ‘burn-in’ to ensure that the chains had reached stationarity. From the resulting 9400 trees, posterior probabilities were assessed for individual clades based on their observed frequencies.

The sequences reported in this study were submitted to GenBank with accession numbers FJ535064–FJ535113.

Biodiversity estimates

Rarefaction curves and different biodiversity indices were estimated from our sequence data using DOTUR (Schloss & Handelsman, 2005). Clone sequences obtained in this study were aligned using MUSCLE (Edgar, 2004) and the respective distance matrices were generated under the Kimura two-parametric evolutionary model in PHYLIP (Felsenstein, 1993). The resulting matrices were used as input for DOTUR in order to (1) assign sequences to OTUs using the furthest neighbour algorithm at an evolutionary distance of 3% and (2) generate rarefaction curves and species richness estimates.

Results and discussion

Sample characteristics and microscopy

The light-reflecting cave wall microbial communities were observed throughout the visited gallery of Pajsarjeva jama cave at both its entrance and in the deeper parts, and covered extensive areas of the walls and ceiling (Fig. 1a). The observed microbial growth (Fig. 1b) was approximately 1 mm thick. Under low magnifications of the dissecting

![Fig. 1.](image-url)
microscope (Olympus SZX 12), the sample was heterogeneous in nature and was composed of cauliflower-like, flat, adhered and thread-like forms. These were sulphur yellow, white, pink, grey or greyish blue in colour with droplets of water scattered on the surface, causing the characteristic light reflection. In order to recover as much prokaryotic diversity as possible, an area covering approximately 2 m² was extensively sampled using sterile scalpels. At the time of sampling, the air temperature, which is relatively constant all year round, was 12 °C and the relative humidity was 100%. The pH of the water droplets from the sampling site was 7.0.

Optical microscopy of the cave microbial mat showed a wide range of prokaryotic microbial morphologies. Branching filaments, resembling members of phylum Actinobacteria, were most frequently observed in the sample. Other characteristic morphologies included coccoid and bacillary forms, presumed to represent prokaryotic cells. These observations were confirmed by scanning electron microscopy, which once again confirmed the domination of branching filaments (Fig. 1c). In these terms, the morphology observed was similar to the morphology of yellow colonies found on the cave walls of the Altamira cave (Cuezva et al., 2009). The latter were also found to adsorb the water vapour and retain it for long periods of time, thus acting as water condensation points (Cuezva et al., 2009).

Overall phylogenetic diversity and species richness in cave wall microbial community samples

The total environmental DNA extracted from cave wall microbial community was used as a template in order to amplify SSU rDNAs. Although we have used two different combinations of Archaea-specific primers, we were unable to amplify any 16S rRNA gene sequences of archaeal origin. Therefore, only bacterial 16S rRNA gene sequences were amplified. This contrasts with the findings reported from the Spanish Altamira cave, where novel lineages of low-temperature Crenarchaeota were shown to constitute metabolically active components of yellow-, grey- and white-coloured cave wall colonies (Gonzales et al., 2006).

In order to minimize potential PCR bias, six gene libraries were constructed. A total of 171 high-quality partial (~800 bp) sequences were compared with GenBank sequences using the BLASTN algorithm. A wide diversity was encountered with members of eight different phyla identified in the cave wall microbial community sample. Phylogenetic relatives were (in order of abundance) as follows: (1) Proteobacteria, (2) Actinobacteria, (3) Nitrospira, (4) Acidobacteria, (5, 6) Chloroflexi and Gemmatimonadales, (7) Verrucomicrobia and (8) Planctomycetales as summarized in Fig. 2.

In order to estimate the level of phylotype redundancy (rarefaction curves) and to calculate various species richness indices at different levels of evolutionary distance, DOTUR was used. We have considered three levels of OTUs defined at evolutionary distances of 3%, 5% and 20% as rough approximations to the species, genus and phylum levels. Rarefaction curves are presented in Fig. 3. Although there was a trend towards stationarity at 20% evolutionary distance, none of the rarefaction curves reached a clear plateau, showing that cave samples are very diverse at the genus level and beyond. At 3% evolutionary distance, a total of 50 OTUs were detected in six libraries constructed. Although the richness estimators were reported to stabilize at a smaller sampling effort than rarefaction curves (Kemp & Aller, 2004), a similar trend was observed in Chao1 richness estimate collector’s curves (Fig. 3). The level of diversity encountered reflected in the biodiversity estimates calculated. Chao1 estimate is based on the presence of singletons and doubletons, which frequently account for most of the phylotypes observed in 16S rRNA gene libraries (Bohannan & Hughes, 2003). The values obtained for Chao1 and ACE estimators overlapped, considering their respective confidence intervals, which were quite large at the 3% and 5% evolutionary distance. Consistent with other estimates, the Shannon index, used to compare OTU richness in different samples, was high (over 3) at 3% evolutionary distance (Table 1).

Bacterial community composition

**Actinobacteria and Acidobacteria**

The various phylotypes retrieved were ascribed to Actinobacteria and Acidobacteria (Fig. 4). Phylotypes related to
Actinobacteria represented 16.3% of the sequences obtained and were the second most retrieved group from the cave wall microbial community sample. The sequence similarities of our clone library sequences to sequences listed in GenBank were between 88% and 93%, indicative of novel, previously undescribed Actinobacteria phylotypes. The majority of Actinobacteria-related sequences from Pajsarjeva jama clone libraries were affiliated with sequences recovered from Alpine dolomite rocks (clone 2PJ5M4, 88% sequence similarity, 9% of sequences recovered) and were followed in

### Table 1. Diversity indices for cave biofilm samples estimated at different levels of difference in evolutionary distances as implemented in <i>mothur</i> (Schloss & Handelsman, 2005)

<table>
<thead>
<tr>
<th>Diversity indices</th>
<th>3% evolutionary distance difference ± CI (5%)</th>
<th>5% evolutionary distance difference ± CI (5%)</th>
<th>20% evolutionary distance difference ± CI (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simpson</td>
<td>0.07</td>
<td>0.08</td>
<td>0.14</td>
</tr>
<tr>
<td>Shannon</td>
<td>3.19 ± 0.19</td>
<td>3.05 ± 0.19</td>
<td>2.32 ± 0.16</td>
</tr>
<tr>
<td>ACE</td>
<td>69.4 ± 13.2</td>
<td>67.0 ± 14.9</td>
<td>20.2 ± 1.8</td>
</tr>
<tr>
<td>Chao1</td>
<td>62.8 ± 20.5</td>
<td>57.3 ± 21.1</td>
<td>20.4 ± 2.7</td>
</tr>
</tbody>
</table>

When applicable, 5% confidence intervals (CI) are given.

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**Fig. 3.** Rarefaction curves (a) and Chao1 richness estimator collector’s curves (b) for Pajsarjeva jama cave wall microbial community samples. Rarefaction curves and Chao1 richness estimator collector’s curves have been calculated at evolutionary distances of 0%, 3%, 5% and 20%.

**Fig. 4.** ML phylogenetic tree showing the positions of phylotypes recovered from Pajsarjeva jama cave wall microbial community clone libraries belonging to Acidobacteria and Actinobacteria. Names in italics correspond to cultivated species, while the rest correspond to 16S rRNA gene clones. Names in bold correspond to the clones obtained in this study. Only bootstrap values > 75% are given at nodes. From top to bottom: ML, MP and posterior probability (expressed as percentage).
abundance by sequences affiliated to environmental sequences obtained from alkaline saline soils of the former lake Texcoco (clones 3PJM21, 3PJM80, 3PJM67) and sequences related to members of Amycolatopsis (clone 3PJM120) and Frankia (clone 2PJM50). It is worth mentioning that Actinobacteria from caves form a diverse group with isolates and environmental sequences affiliated to Arthrobacter, Acidimicrobidae, Actinosynemataceae, Brevibacterium, Frankia, Kocuria, Microbacteriaceae, Micrococcaeae, Nocardiaceae, Nocardioidaeae, Pseudonocardiaceae, Streptomyctaceae, Saccharothrix and Rhodococcus (Cunningham et al., 1995; Groth & Saiz-Jimenez, 1999; Groth et al., 1999, 2001; Laiz et al., 1999, 2000; Holmes et al., 2001; Schabereiter-Gurtner et al., 2002a; Northup et al., 2003).

The results obtained in this study are in accordance with the results of a previous cultivation study on microbial communities in cave Planinska jama, located approximately 20 km south of Pajsarjeva jama cave. Members of Actinomycetales were most frequently recovered within this cultivation effort and were identified as members of Brevibacterium and Proactinomycetes genera. However, their characteristics could not fit any of the described species. Further studies of Proactinomycetes isolates showed that these bluish-grey colonies were able to disintegrate CaCO₃, which is in accordance with the fact that the calcareous rock is regularly mushy under the coatings. Furthermore, a Bacillus and a Pseudomonas species were reported from the same sampling site (Mulec et al., 2002).

A common constituent of microbial communities colonizing cave walls are different types of crystals, produced by members of Actinobacteria. Because of this phenotype, it has been proposed that these bacteria and others are directly or indirectly involved in constructive biomineralization processes in caves (Laiz et al., 1999; Barton et al., 2001; Caháveras et al., 2001; Groth et al., 2001; Jones, 2001). This phenomenon was not observed in the microbial colonizations studied, and it remained unclear whether the characteristic light-reflecting water droplets observed at the surface of microbial cave wall communities of both caves studied were a condensate or an exudate of microbial community.

The phylum Acidobacteria is a recently described monophyletic phylum with only a handful of cultivated species. Members of this phylum represent another group found in caves studied are a condensate or an exudate of microbial communities; yet, their function remains unknown at present. Based on culture-independent studies, Acidobacteria formed an ecologically significant constituent of microbial communities inhabiting Palaeolithic paintings in Spanish Tito Bustillo cave (Schabereiter-Gurtner et al., 2002b) and La Garma cave (Schabereiter-Gurtner et al., 2004) as well as in biodeteriorating films in Roman catacombs (Italy) (Zimmermann et al., 2005) and Wind Cave sediment (USA) (Chelius & Moore, 2004). However, in Pajsarjeva jama microbial community clone libraries, this phylum was only moderately abundant and represented 10.5% of the clone sequences obtained. The clone sequences retrieved from cave wall microbial community DNA formed a phylogenetically diverse group, represented by nine phylotypes. Seven novel phylotypes sharing sequence similarities with known sequences in GenBank of < 95% were described. This suggests that the gene libraries constructed in this study represent the Acidobacteria community, which is different in structure from the communities studied so far.

The Acidobacteria were previously divided into eight deeply branching subgroups (Hugenholtz et al., 1998) and recently expanded to 11 subgroups (Zimmermann et al., 2005). In Lower Kane Cave, a sulphidic cave system, acidobacterial populations appear to be dominated by members of subgroups 7 and 8 (Meisinger et al., 2007). Analysis of 16S rRNA gene placed Pajsarjeva jama clone sequences within acidobacterial subgroups 4 and 3. Related phylotypes were previously reported from Karstic Altamira cave, where these subgroups represented 29% and 12% of the total OTU number, respectively (Zimmermann et al., 2005). In the same study, acidobacterial OTUs belonging to subgroup 4 were affiliated with grey cave wall colonies forming within Altamira cave.

**Proteobacteria**

The ecologically successful Proteobacteria were the most abundant phylum in clone libraries and represented 51.4% of the sequences obtained. Important differences were observed in the relative distribution and abundance of the different proteobacterial subdivisions. Gammaproteobacterial phylotypes were by far the most abundant in the clone libraries constructed and represented 31.5% of all sequences recovered (Fig. 5). A vast majority of these sequences (23.4%) branched within purple sulphur bacteria and therefore might be involved in oxidation of hydrogen sulphide in anoxic parts of the microbial colonization. These sequences are represented by phylotypes 3PJM14, 3PJM42 and 3PJM72, and were closely related (96–99% sequence similarity) to environmental sequences recovered from Oregon Caves National Monument (clone ORCA-17N118, DQ823220). Another group of phylotypes within Gammaproteobacteria was found to be related to Xanthomonadales. Bacteria from this order frequently impair yellow colouration due to their production of carotenoids. Besides, Xanthomonadales were found to be exclusively associated with yellow colonizations developing on the walls of Altamira cave (Portillo et al., 2008), and thus might contribute to yellow colonizations developing on sampling sites in this study. The phylotypes recovered in this study formed a
diverse group related to environmental sequences recovered from soil, freshwater of cultivated denitrifying species.

The abundance of typical terrestrial taxa, such as Alphaproteobacteria, was low in the libraries constructed. Only a handful of phylotypes could be affiliated with this subdivision. The environmental sequences represented by phylotypes 3PJM100 and 3PJM8 were closely related to soil surrounding iron–manganese nodules (clone JH-WH224, EF492889; 99% sequence similarity). Another group of clones, represented by phylotype 3PJM78, was found to be distantly related to sequences recovered from tall grass prairie soil (clone FFCH1553, EU133452, 95% sequence similarity). Another group of clones, represented by phylotype 3PJM78, was found to be distantly related to sequences recovered from tall grass prairie soil (clone FFCH1553, EU133452, 95% sequence similarity). A similar pattern of abundance was observed in Deltaproteobacteria, a subdivision that encompasses anaerobic sulphate-reducing bacteria. Only three phylotypes (2PJM57, 3PJM140 and 1PJM294) were affiliated to this group and were all related to other environmental soil Deltaproteobacteria sequences with sequence similarities between 92% and 97%. Thus, their putative functional description remains elusive. The Epsilonproteobacteria were absent from the clone libraries.

In contrast with latter observations, phylotypes related to Betaproteobacteria (Fig. 6) formed a well-represented and diverse group. The dominant phylotypes belonging to this Proteobacteria subdivision were related to cave sequences either recovered from acidic, pendulous cave wall microbial mats from the Frasassi cave system (Macalady et al., 2007) or rock microbial mats from a gold mine in Poland (clone A05-1, FM253568). Except for phylotypes distantly related to methyl-tert-butyl ether-degrading strain PM1 (clone 3PJM33) and ammonia-oxidizing Nitrosomonas europaea (clone 3PJM125), we were unable to affiliate the remaining phylotypes to any cultivated species. However, these were found to be most closely related, with similarities between 94% and 99%, to sequences from soil habitats.

The fact that the identified bacterial community was dominated by the Proteobacteria is in accordance with previous cave studies. This cosmopolitan group was found to dominate in almost all cave biota studied. In Spanish Altamira cave, one of the best microbiologically studied subsurface systems, Proteobacteria were found to dominate in dripping waters, Palaeolithic painting bacterial communities (Laiz et al., 1999; Schabereiter-Gurtner et al., 2002a) and yellow, grey and white cave wall colonizations (Portillo et al., 2008, 2009). Similarly, Proteobacteria constituted up to 50% of the bacterial cave wall communities encountered in Tito Bustillo cave, Spain (Schabereiter-Gurtner et al., 2002b), and were among the dominant groups in bacterial communities encountered in sediment collected from Wind Cave, USA (Cheliu & Moore, 2004). The dominance of Proteobacteria has also been reported from the extreme
environment of sulphur caves. *Epsilon*- and *Gammaproteobacteria* were found to be important biofilm-forming groups in sulphidic springs and streams of Parker cave (Kentucky), Cesspool cave (Virginia), Lower Kane Cave (Wyoming) and Movile cave (Romania) (Angert et al., 1998; Sarbu, 2000; Engel et al., 2001, 2003). In the Frassasi cave system (Italy), sulphur-cycling Proteobacteria clades accounted for >75% of clones in limestone corroding stream microbial mats libraries (Macalady et al., 2006) and represented the dominant portion of diversity in extremely acidic, pendulous cave wall microbial mats analysed from the same cave system (Macalady et al., 2007). Although they are not commonly found in environments that are characterized by severe pH, temperature, nutrient or water tension stresses, Proteobacteria are well known for their ability to degrade a wide diversity of organic substrates (Palleroni, 1992). As observed in previous studies, enhanced nutrient availability is one possible explanation for the observed dominance of Proteobacteria. Northup et al. (2003) compared microbial communities inhabiting ferromanganese deposits in extremely oligotrophic and isolated Lechuguilla cave with microbial communities inhabiting the shallow, weekly visited Spider cave. Increased numbers of nitrogen-fixing Proteobacteria were reported from the human-impacted Spider cave. Similarly, the cultivable microbial diversity study of Kartchner Caverns (USA) showed the dominance of Proteobacteria in high human-impacted areas, while *Firmicutes*, known to resist desiccation and nutrient stress, dominated in low human-impacted areas (Ikner et al., 2006). Finally, in the highly human-impacted Lascaux cave microbial colonizations were affiliated almost exclusively to *Proteobacteria* (Bastian et al., 2009).

**Verrucomicrobia, Planctomycetes, Gemmatimonadetes, Nitrospirales and Chloroflexi**

Among the processed sequences, additional components of the bacterial cave wall microbial community belonged to the phyla *Verrucomicrobia, Planctomycetes, Gemmatimonadetes* and *Chloroflexi* (Fig. 7). Clone sequences represented by 3PJM118 were found to be distantly related to *Verrucosimicrobium spinosum*, a species inhabiting eutrophic freshwaters. Sequences reported by clone 3PJM51 were related to *Gemmatimonas aurantiaca*. Although this species remains the only cultivated member of Gemmatimonadetes, environmental sequence data indicate that this phylum is widespread in nature and has a phylogenetic breadth (19% 16S rRNA gene sequence divergence) that is greater than well-known phyla such as the *Actinobacteria* (18% divergence) (Zhang et al., 2003). Indeed, members of Gemmatimonadetes were recently reported from an earth cave in Guizhou province, China, where they represented 2.7% of the soil microbial community (Zhou et al., 2007).

Planctomycetes were represented by a single sequence 1PJ18, related to environmental sequences recovered from a hypersaline microbial mat and *Chloroflexi* by sequences 3PJM52 and 1PJM279, which branched with environmental soil sequences within genera *Chloroflexus* and *Caldilinea.*
Finally, a large group of clones was affiliated with phylum *Nitrospira*. These represented 15.2% of the clone libraries constructed (Fig. 7). The sequences phylogenetically related to sequences obtained in this study were previously described in a well-studied sulphur cave – Frassasi cave system, where they represented only a minor portion of the diversity encountered (Macalady et al., 2007; Fig. 7, prefix CV). The remaining *Nitrospira* clone sequences were found to be related to ammonia oxidizers such as *Nitrospira* spp. Although few *Nitrospira* cultures have been characterized, all known strains are obligate chemolithoautotrophs that obtain their energy for growth from the oxidation of nitrite. This process has been reported previously to contribute to cave food webs in Mexican anchialine caves (Pohlman et al., 1997), which received organic material in a manner similar to the caves studied here – from adjacent forest and aquatic systems. Furthermore, members of *Nitrospira* were found to contribute significantly to bacterial communities inhabiting rock surfaces of the Tito Bustillo cave (Schabereiter-Gurtner et al., 2002a).

**Conclusion**

Clone library sequences recovered in this study revealed diverse microbial communities. A number of phylotypes encountered were previously recovered from cave samples worldwide. Based on the physiological and biochemical characteristics of cultured isolates related to clone library sequences, the cave wall microorganisms are involved in nitrogen cycling and fertilization of organic carbon. However, it is important to be cautious in interpreting this information as closely related organisms can have very different physiologies.

Based on phylogenetic analysis, the light-reflecting cave wall microbial community was dominated by members of *Gammaproteobacteria*, *Actinobacteria* and *Nitrospira*. Thus, in its composition, the microbial community studied shared a number of similarities to microbial formations developing on the walls of human-impacted caves. However, this study also revealed the presence of several novel and possibly abundant phylotypes of *Actinobacteria*, *Acidobacteria* and members of *Nitrospira*, expanding our knowledge of the diversity of these groups and the habitats in which they occur.

**Acknowledgements**

We thank Maja Zagmajster for field assistance and Kazimir Drasˇlar and Rok Kostanjsˇek for microscopy assistance. This work was supported by Slovenian Research Agency research programs P1-0198 and P1-184. Barbara Kovče contributed to this work as an undergraduate student.

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