ORIGINAL ARTICLE

Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave

Yin Chen^{1,5}, Liqin Wu^{2,5}, Rich Boden¹, Alexandra Hillebrand³, Deepak Kumaresan¹, Hélène Moussard¹, Mihai Baciu⁴, Yahai Lu² and J Colin Murrell¹

¹Department of Biological Sciences, University of Warwick, Coventry, UK; ²College of Resources and Environmental Sciences, China Agricultural University, Beijing, China; ³Institute of Speleology 'Emil Racovita', Bucharest, Romania and ⁴The Group of Underwater and Speleological Exploration, Bucharest, Romania

Microbial diversity in Movile Cave (Romania) was studied using bacterial and archaeal 16S rRNA gene sequence and functional gene analyses, including ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), *soxB* (sulfate thioesterase/thiohydrolase) and *amoA* (ammonia monooxygenase). Sulfur oxidizers from both *Gammaproteobacteria* and *Betaproteobacteria* were detected in 16S rRNA, *soxB* and RuBisCO gene libraries. DNA-based stable-isotope probing analyses using ¹³C-bicarbonate showed that *Thiobacillus* spp. were most active in assimilating CO₂ and also implied that ammonia and nitrite oxidizers were active during incubations. *Nitrosomonas* spp. were detected in both 16S rRNA and *amoA* gene libraries from the 'heavy' DNA and sequences related to nitrite-oxidizing bacteria *Nitrospira* and *Candidatus 'Nitrotoga'* were also detected in the 'heavy' DNA, which suggests that ammonia/nitrite oxidation may be another major primary production process in this unique ecosystem. A significant number of sequences associated with known methylotrophs from the *Betaproteobacteria* were obtained, including *Methylotenera*, *Methylophilus* and *Methylovorus*, supporting the view that cycling of one-carbon compounds may be an important process within Movile Cave. Other sequences detected in the bacterial 16S rRNA clone library included *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes*, alphaproteobacterial *Rhodobacterales* and gammaproteobacterial *Xanthomonadales*. Archaeal 16S rRNA sequences retrieved were restricted within two groups, namely the Deep-sea Hydrothermal Vent Euryarchaeota group and the Miscellaneous Crenarchaeotic group. No sequences related to known sulfur-oxidizing archaea, ammonia-oxidizing archaea, methanogens or anaerobic methane-oxidizing archaea were detected in this clone library. The results provided molecular biological evidence to support the hypothesis that Movile Cave is driven by chemolithoautotrophy, mainly through sulfur oxidation by sulfur-oxidizing bacteria and reveal that ammonia- and nitrite-oxidizing bacteria may also be major primary producers in Movile Cave.

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Introduction

It is generally believed that all the terrestrial and aquatic environments are based on energy and organic carbon that ultimately originated from photosynthesis, that is, by converting light energy into chemical energy used to fix carbon dioxide from the atmosphere. One of the few exceptions are deep-sea hydrothermal vents (reviewed by

Nakagawa and Takai, 2008), in which chemoautotrophic bacteria that gain energy from the oxidation of chemicals such as sulfur and hydrogen are the primary producers. Other exceptions are cave ecosystems, such as Movile Cave (Romania). Unlike deep-sea hydrothermal vents, Movile Cave is located a few kilometers from the Black Sea coast. It is a unique groundwater ecosystem in that: (a) the atmosphere is rich in hydrogen sulfide, carbon dioxide and a substantial amount of methane; (b) the redox potential of the cave water (pH = 7.5) is low and it contains reduced compounds such as sulfide (0.2–0.3 mM), ammonium (0.2–0.3 mM) and methane (0.02 mM) (Sarbu, 2000). The cave is isolated from the outside world and earlier studies

Correspondence: JC Murrell, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK.
E-mail: J.C.Murrell@warwick.ac.uk

⁵These authors contribute equally to this work.

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have implied that this unique ecosystem is exclusively based on chemosynthesis, mainly by sulfur oxidation and methane oxidation (Sarbu *et al.*, 1996; Sarbu, 2000).

Movile Cave is unusual for its high productivity and fauna richness. Characterization of invertebrates has been carried out and 48 terrestrial and aquatic species have been identified, of which 33 are endemic (Sarbu, 2000). However, microbial diversity in this cave has not been studied in detail. Earlier microscopic observations have suggested that filamentous sulfur oxidizers and fungi were abundant in the microbial mat (Sarbu *et al.*, 1994). Later, Vlasceanu *et al.*, (1997) isolated a *Thiobacillus thioparus* strain, which is particularly abundant in the microbial mat and the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme in CO₂ fixation for many chemoautotrophs, has been detected for this organism (Sarbu *et al.*, 1994). Although sulfur oxidizers are thought to be the major primary producers in Movile Cave (Sarbu *et al.*, 1996; Rohwerder *et al.*, 2003), the presence of up to 1.5% (v/v) methane in the cave atmosphere suggested that methanotrophs may also be present. Hutchens *et al.*, (2004) used DNA stable-isotope probing (DNA-SIP) to analyze the methanotroph populations and a diverse range of methanotrophs belonging to *Alphaproteobacteria* and *Gammaproteobacteria* were identified.

In this study, culture-independent analyses using 16S rRNA gene sequences for bacteria and archaea were carried out to determine the microbial diversity present in this unique cave system. Analyses were also carried out using the RuBisCO gene as a functional gene marker for many chemoautotrophs, *soxB* (sulfate thioesterase/ thiohydrolase) for sulfur oxidizers, which have the Sox-dependent sulfur oxidation pathway and *amoA* (ammonia monooxygenase) for ammonia oxidizers. SIP incubations

with ¹³C-bicarbonate were also carried out to detect active CO₂-fixing bacteria in the cave.

Materials and methods

Environmental sampling and DNA extraction

Movile Cave water and mat samples were collected in September 2007. The pH of the water was 7.5. The samples were kept at 4 °C until they were shipped to the University of Warwick (UK) for further processing. A total of 50 ml water samples (which includes ~1 g floating microbial mat), well mixed by vortexing, were pelleted by centrifugation, and DNA was extracted using a method described earlier (Neufeld *et al.*, 2007), and then stored at –20 °C for later analyses.

PCR, cloning and restriction length fragment polymorphism analyses

Bacterial 16S rRNA genes were amplified using the 27F/1492R primer set (Lane, 1991). In total, 96 clones were randomly selected from this library and were sequenced using the primer 341F. Archaeal 16S rRNA genes were amplified using a semi-nested approach, with Arch21F/1492R (DeLong, 1992) for the first round and Arch21F/Arch958R for the second round (DeLong, 1992). The clones were randomly selected and analyzed by RFLP analysis using *MspI*. Representative clones from each operational taxonomic unit were sequenced. Crude DNA was also used as template to amplify *amoA*, *soxB*, form I RuBisCO (green-like and red-like) and form II RuBisCO genes. The primers used are listed in Table 1. In total, 48 clones from each library were randomly selected and sequenced using the M13F/M13R primers. All PCR products were cloned into the pCRTOP02.1 plasmid using the Invitrogen TOPO TA Cloning Kit (Invitrogen, Paisley, UK).

Table 1 Oligonucleotides used in this study

Oligonucleotides	Sequences (5'–3')	Target	References
27F	AGAGTTTGATCMTGGCTCAG	Bacterial 16S rRNA gene	Lane, 1991
1492R	TACGGYTACCTTGTTACGACTT		
341F	CCTACGGGAGGCAGCAG	For sequencing of bacterial 16S rRNA gene	Muyzer <i>et al.</i> (1993)
Arch21F	TTCCGGTTGATCCYGCCGGA	Archaeal 16S rRNA gene	DeLong, 1992
Arch958R	YCCGGCGTTGAMTCCAATT		
soxB693F	ATCGGNCARGCNTTYCCNTA	<i>soxB</i>	Meyer <i>et al.</i> (2007)
soxB1446R	CATGTCNCCNCCRTGYTG		
amoA1F	GGGGTTTCTACTGGTGGT	Bacterial <i>amoA</i>	Rotthauwe <i>et al.</i> (1997)
amoA2R	CCCCTCKGSAAGCCTTCTTC		
Arch-amoAF	STAATGGTCTGGCTTAGACG	Archaeal <i>amoA</i>	Francis <i>et al.</i> (2005)
Arch-amoAR	GCGGCCATCCATCTGTATGT		
cbbLR1F	AAGGAYGACGAGAACATC	Form I RuBisCO, red form	Selesi <i>et al.</i> (2005)
cbbLR1R	TGGTCGSGTGTTAGTTGAA		
cbbLG1F	GGCAACGTGTTCCGGSTTCAA	Form I RuBisCO, green form	Selesi <i>et al.</i> (2005)
cbbLG1R	TTGATCTCTTTCCAGGTTTCC		
cbbMf	ATCATCAARCCSAARCTSGGCCTGCGTCCC	Form II RuBisCO	Giri <i>et al.</i> (2004)
cbbMr	MGAGGTGACSGCRCCGTGRCCRGCMCGRTG		

SIP

Time-course SIP incubations were carried out. In total, 20 ml of well-mixed cave water and mat sample were put in 120 ml serum vials and incubated for 1 and 3 weeks, respectively, with ^{13}C sodium bicarbonate (Cambridge Isotope Laboratories, Hook, UK, $^{13}\text{C} > 99\%$). A control was set up for each microcosm in exactly the same way except that ^{12}C sodium bicarbonate was used. Sodium bicarbonate was added to a final concentration of 0.5 mM at day 1 of each week for each microcosm. The headspace contained regular air and no attempts were made to adjust the headspace air to that of *in situ* conditions. Owing to the production of ^{12}C - CO_2 from heterotrophs present in the samples (data not shown), the incorporation of ^{13}C carbon was not measured. After incubation, cells and mat material were pelleted by centrifugation and DNA was extracted according to the protocol described earlier (Neufeld *et al.*, 2007). Ultracentrifugation, fractionation and DNA precipitation were carried out according to the procedure described by Chen *et al.*, (2008). The 'heavy' ^{13}C -labelled DNA from the 1-week incubation (time point 1) and 3-week incubations (time point 2) was used as template to amplify 16S rRNA genes from bacteria. A clone library for *amoA* of bacteria was also constructed using the 'heavy' DNA from 3-week incubations. A total of 48 clones from each library were randomly picked up and sequenced.

DNA sequencing and phylogenetic analyses

DNA sequencing was carried by cycle sequencing with the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) using a 3730A automated sequencing system (PE Applied Biosystems). Bacterial and Archaeal 16S rRNA gene sequences were aligned using the ARB program (Ludwig *et al.*, 2004) and functional genes were aligned using the ClustalX program (Thompson *et al.*, 1997). Phylogenetic and bootstrap analyses were carried out using the Phylowin program (Galtier *et al.*, 1996). Nucleotide sequences from this study were deposited in the GenBank database under the accession numbers FJ604604–FJ604848.

Results

Microbial diversity in Movile Cave as assessed by 16S rRNA gene clone libraries

To investigate the microbial diversity in Movile Cave, a 16S rRNA clone library for bacteria and archaea, respectively, was constructed and 96 clones were randomly selected and analyzed. The results are summarized in Table 2 and Figures 1–3.

16S rRNA sequences related to known sulfur-oxidizing bacteria were detected in the crude DNA (Figure 1). These were classified into *Betaproteobacteria* (*Thiobacillus*, three clones), *Gammaproteo-*

bacteria (*Thiovirga*, seven clones; *Thiothrix*, one clone; *Thioploca*, 17 clones) and *Epsilonproteobacteria* (*Sulfuricurvum*, one clone). Sequences related to sulfur reducers (*Desulfobulbaceae*) and denitrifiers (*Denitratisoma*) were also found (Figure 2). No sequences related to known methanotrophs were detected in the crude DNA. However, a number of 16S rRNA gene sequences were associated with known methylotrophs within the *Betaproteobacteria* (Table 2), including *Methylothermus* (15 clones), *Methylophilus* (three clones) and *Methylovorus* (three clones). Other sequences detected include *Verrucomicrobia* (one clone), *Firmicutes* (two clones), *Bacteroidetes* (15 clones), *Alphaproteobacteria* from the *Rhodobacterales* (two clones) and *Gammaproteobacteria* from the *Xanthomonadales* (seven clones).

The archaeal 16S rRNA genes sequences were clustered into two major groups (Figure 3). In total, 48 16S rRNA gene clones were classified in the DHVE1 (Deep-sea Hydrothermal Vent Euryarchaeota; Takai and Horikoshi, 1999) group. A total of 42 clones were associated with the MCG (Miscellaneous Crenarchaeotic group; Inagaki *et al.*, 2003) however, these only showed moderate identity (~92%) to the 16S rRNA gene sequences from MCG. Among those, an uncultured Crenarchaeota clone (B10), which was retrieved from the water column of a sulfurous karstic lake in Spain, was the closest BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) match (Llirós *et al.*, 2008). It is to be noted that no sequences related to known sulfur-oxidizing archaea, ammonia-oxidizing archaea, methanogens or anaerobic methane-oxidizing archaea were detected in this 16S rRNA gene clone library.

Diversity of RuBisCO genes

The diversity of RuBisCO genes was analyzed using three separate primer sets targeting form I green-like, form I red-like and form II RuBisCO genes. Results are shown in Figure 4. It is to be noted that all of the sequences from the form I RuBisCO (red type) clone library showed <90% identity to sequences in the GenBank database and they formed two separate groups. In total, 30 clones were related (~88% identity) to the RuBisCO genes from *Rhodobacter azotoformans* and *Labrenzia aggregata*. The other two sequences were related (84% identity) to the RuBisCO gene from *Methylobium petroleiphilum*.

Similarly, all the clones from the form I green type RuBisCO clone library showed <90% identity to sequences in the GenBank database. They were reasonably closely related (85–88% identity) to the RuBisCO gene sequences from *Thiobacillus denitrificans* and *T. thioparus*.

The sequences from the form II RuBisCO clone library fell into two clades. In total, 11 clones had 92% identity to the form II RuBisCO gene from *T. denitrificans* and the other 25 clones were most closely related (97% identity) to the RuBisCO gene

Table 2 Classification by the RDP Classifier of 16S rRNA genes retrieved from Movile Cave crude DNA and 'heavy' DNA from ^{13}C -CO₂-SIP incubations

Phylum/order	Crude DNA	Time point 1, ^{13}C -CO ₂	Time point 2, ^{13}C -CO ₂
<i>Verrucomicrobia</i>	1		
<i>Firmicutes</i>	2	2	
<i>Bacteroidetes</i>	15	2	1
<i>Planctomycetes</i>		1	2
<i>Spirochetes</i>		1	1
<i>Nitrospira</i>		2	
<i>Proteobacteria</i>	63	34	42
<i>Alpha</i>			
<i>Caulobacteriales</i>			<i>Brevundimonas</i> (3)
<i>Rhodospirillales</i>		Unclassified <i>Rhodospirillales</i> (1)	Unclassified <i>Acetobacteraceae</i> (1)
			Unclassified <i>Rhodospirillales</i> (1)
<i>Rhodobacterales</i>	<i>Rhodobacter</i> (1)	Unclassified <i>Rhodobacteraceae</i> (1)	<i>Hyphomonas</i> (1)
	Unclassified <i>Rhodobacteraceae</i> (1)		<i>Roseovarius</i> (1)
<i>Sphingomonadales</i>		Unclassified <i>Sphingomonadaceae</i> (3)	<i>Sphingopyxis</i> (1)
<i>Rhizobiales</i>		Unclassified <i>Rhizobiales</i> (3)	<i>Devosia</i> (1)
<i>Beta</i>			
<i>Burkholderiales</i>		Unclassified <i>Incertae sedis</i> 5 (2)	Unclassified <i>Comamonadaceae</i> (1)
		Unclassified <i>Burkholderiales</i> (1)	Unclassified <i>Incertae sedis</i> 5 (4)
<i>Hydrogenophilales</i>	<i>Thiobacillus</i> (3)	<i>Thiobacillus</i> (3)	<i>Thiobacillus</i> (4)
<i>Rhodocyclales</i>	<i>Denitratisoma</i> (1)	Unclassified <i>Rhodocyclaceae</i> (4)	<i>Dechloromonas</i> (1)
<i>Methylophilales</i>	<i>Methylothera</i> (15)		
	<i>Methylophilus</i> (3)		
	<i>Methylovorus</i> (3)		
<i>Nitrosomonadales</i>		<i>Nitrosomonas</i> (1)	<i>Nitrosomonas</i> (4)
Unclassified		<i>Thiobacter</i> (8)	<i>Candidatus 'Nitrotoga arctica'</i> (13)
<i>Betaproteobacteria</i>			<i>Methyloversatilis</i> (1)
<i>Gamma</i>			
<i>Chromatiales</i>	<i>Thiovirga</i> (7)		<i>Thiovirga</i> (1)
<i>Xanthomonadales</i>	<i>Aquimonas</i> (6)	Unclassified <i>Xanthomonadaceae</i> (1)	<i>Aquimonas</i> (2)
	Unclassified <i>Xanthomonadaceae</i> (1)		
<i>Thiotrichales</i>	<i>Thiothrix</i> (1)	<i>Thioploca</i> (1)	
	<i>Thioploca</i> (17)	Unclassified <i>Thiotrichaceae</i> (2)	
	Unclassified <i>Thiotrichaceae</i> (2)		
<i>Epsilon</i>			
<i>Myxococcales</i>	<i>Byssovorax</i> (1)	Unclassified <i>Myxococcales</i> (2)	
<i>Desulfobacterales</i>	Unclassified <i>Desulfobulbaceae</i> (1)		Unclassified <i>Desulfobulbaceae</i> (1)
<i>Campylobacterales</i>	<i>Sulfuricurvum</i> (1)		
Unclassified bacteria	4	4	1
Total	85	46	47

Abbreviations: RDP, Ribosomal Database Project; SIP, stable isotope probing.

from *Halothiobacillus neapolitanus*, but they clearly formed a separate branch as supported by bootstrap analyses.

Diversity of *soxB* and *amoA* genes

On phylogenetic analysis, it was obvious that the *soxB* genes retrieved were distributed throughout the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (Figure 5). Within the *Betaproteobacteria*, four clones were closely related to *Thiobacillus* and two clones were related to the *soxB* homolog from *M. petroleiphilum*. Within the *Gammaproteobacteria*, five clones were related to *soxB* genes from *Thiothrix* and the other 10 clones

showed only 65% identity to *soxB* genes from *Halothiobacillus* species.

amoA genes from bacteria formed two separate clades: two clones were within the *Nitrosomonas europaea* clade and the other 45 clones grouped within the *Nitrosomonas oligotropha* clade (Figure 6). Using crude DNA as template with primers for *amoA* genes from archaea did not yield any PCR products of the expected sizes.

SIP analyses using ^{13}C -bicarbonate

To investigate microorganisms which were active in assimilating CO₂ in Movile Cave, SIP incubations were carried out using ^{13}C -bicarbonate.

Figure 1 Neighbor-joining phylogenetic tree of bacterial 16S rRNA sequences related to known sulfur-oxidizing and sulfur-reducing bacteria. Sequences were aligned using the ARB program and the tree was constructed by Phylowin (556 bp). Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences in bold were retrieved from the crude DNA; sequences in bold and italic were retrieved from ‘heavy’ DNA of the 1-week incubation with ^{13}C -bicarbonate; sequences in bold and underlined were retrieved from ‘heavy’ DNA of the 3-week incubation with ^{13}C -bicarbonate.

A bacterial 16S rRNA clone library was constructed using the ‘heavy’ DNA extracted after a 1-week incubation and a 3-week incubation, respectively. The results are presented in Table 2 and Figure 1.

After 1 week of incubation with ^{13}C -bicarbonate, it was found that sulfur-oxidizing bacteria from both the *Betaproteobacteria* (*Thiobacillus*, three clones; *Thiobacter*, eight clones) and *Gammaproteobacteria* (*Thioploca*, one clone; unclassified *Thiotrichaceae*, two clones) were labeled with ^{13}C . *Sulfuricurvum*-related 16S rRNA gene sequences were not found, although they were found in the 16S rRNA gene library constructed with crude DNA. It is interesting to note that 16S rRNA gene sequences related to ammonia-oxidizing bacteria (*Nitrosomonas*, one clone) and nitrite-oxidizing bacteria (*Nitrospira*,

two clones) were present after 1-week incubation with ^{13}C -bicarbonate. After 3 weeks of incubation with ^{13}C -bicarbonate, sulfur-oxidizing bacteria were still present, including *Thiobacillus* (four clones), *Thiovirga* (one clone). Four 16S rRNA clones were associated with the ammonia-oxidizing bacterium *Nitrosomonas* and 13 clones showed high identity to the 16S rRNA from *Candidatus* ‘*Nitrotoga arctica*’, a novel nitrite-oxidizing bacterium isolated from the Siberian Arctic (Alawi *et al.*, 2007).

A number of 16S rRNA sequences in the ‘heavy’ DNA clone library were associated with the *Alphaproteobacteria*, including *Rhodospirillales*, *Rhodobacterales* and *Sphingomonadales*. A few sequences related to *Bacteroidetes*, *Planctomycetes* and *Spirochetes* were also found in the ‘heavy’ DNA from both the 1-week and the 3-week incubations

Figure 2 Neighbor-joining phylogenetic tree of bacterial 16S rRNA sequences related to known ammonium- and nitrite-oxidizing bacteria and denitrifying bacteria. Sequences were aligned using the ARB program and the tree was constructed by Phylowin (672 bp). Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences in bold were retrieved from the crude DNA; sequences in bold and italic were retrieved from 'heavy' DNA of the 1-week incubation with ^{13}C -bicarbonate; sequences in bold and underlined were retrieved from 'heavy' DNA of the 3-week incubation with ^{13}C -bicarbonate.

with ^{13}C -bicarbonate. Sequences related to *Aquimonas* were also found in the DNA from the 3-week SIP incubation microcosm.

A clone library of *amoA* genes from bacteria was constructed using the 'heavy' DNA from the 3-week incubation with ^{13}C -bicarbonate. In total, 26 *amoA* clones clustered in the *N. europaea amoA* clade and the other 21 clones were associated with the *N. oligotropha* clade. It was not possible to amplify *amoA* genes from archaea using the 'heavy' DNA as template.

Discussion

It has been hypothesized earlier that the Movile Cave system is driven by chemoautotrophy (Sarbu and Kane, 1995; Sarbu *et al.*, 1996; Sarbu, 2000). The cave continuously receives a high flow rate of hydrothermal water rich in sulfide and methane (Sarbu, 2000) and sulfur oxidation was thought to be

the major energy source for primary production (Sarbu *et al.*, 1996). Earlier analyses showed that aerobic methanotrophs were also present in this cave, suggesting that methanotrophy could also be one of the major primary production processes (Hutchens *et al.*, 2004; Sarbu, 2000). Although the cave water contains high concentrations of ammonium (0.28 mM, Sarbu, 2000), nitrification as a major energy production route has not been investigated earlier. It has, however, been observed that nitrogen in the microbial mat is isotopically lighter ($\delta^{15}\text{N} = -9.1\text{‰}$) than ammonium-nitrogen in the cave water ($\delta^{15}\text{N} = +19.9\text{‰}$) (Sarbu *et al.*, 1996). Lack of further evidence made it difficult to ascertain whether ammonium uptake by microbes or nitrification was the reason for the observed lighter nitrogen isotope content in the microbial mat. Our DNA-SIP experiment using ^{13}C -bicarbonate indicated that ammonia- and nitrite oxidizers were particularly active in the assimilation of ^{13}C -labelled CO_2 during microcosm incubations, indicating that

Figure 3 Neighbor-joining phylogenetic tree of archaeal 16S rRNA gene sequences (779 bp) retrieved from Movile Cave crude DNA. Sequences were aligned using the ARB program and the tree was constructed by Phylowin. Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences retrieved from the crude DNA are highlighted in bold.

nitrification could at least partially account for the shift of nitrogen isotope content.

Further molecular-based analyses using 16S rRNA and *amoA* genes showed that *Nitrosomonas* spp. were responsible for ammonia oxidation and *Nitrospira* and *Candidatus Nitrotoga* were probably the major nitrite utilizers. The community of *Nitrosomonas* in the crude DNA is predominated by bacteria of the *N. oligotropha* clade, whereas more *amoA* gene sequences from the *N. europaea* clade were seen during SIP incubations (Figure 6). Studies have shown that ammonia availability could select against different *Nitrosomonas* clades. For example, by studying the ammonia-oxidizing bacterial community in aeration sequencing batch reactors, Otawa *et al.*, (2006) have observed that the *N. europaea* clade were dominant in reactors in which ammonium concentration was higher, whereas bacteria of the *N. oligotropha* clade were dominant in those in which the ammonium concentration was lower. It is also possible that the

toxicity of nitrite may affect the relative distribution of these two clades. For example, Limpiyakorn *et al.*, (2007) observed that the *N. oligotropha* clade comprised the majority of ammonia-oxidizing bacterial populations when nitrite did not accumulate, whereas the *N. europaea* clade became dominant when concentrations of nitrite were high (>2.1 mM). The toxic effect of nitrite may explain the increase in the relative abundance of the *N. europaea* clade during SIP incubations. The relative change of ammonium concentration during SIP incubations is probably not as dramatic as that of nitrite because of the presence of relatively high concentrations of ammonium (~0.3 mM) in the Movile Cave water. Temporary accumulation of nitrite may, however, occur during microcosm incubations: this was supported by the appearance of nitrite-oxidizing populations in the 'heavy' DNA during ¹³C-bicarbonate incubations. Clearly, in the future, quantification of ammonium, nitrite and nitrate needs to be followed during SIP incubations to fully understand the

Figure 4 Neighbor-joining phylogenetic trees of form I red-like (430 bp) ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (a), form I green-like (571 bp) RuBisCO (b) and form II RuBisCO (238 bp) (c) genes retrieved from Movile Cave crude DNA. Sequences were aligned using the ClustalX program and the trees were constructed by Phylowin. Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences retrieved from the crude DNA are highlighted in bold.

nitrogen cycling in Movile Cave and to establish the link between microbial diversity and activity.

The atmosphere in Movile Cave contains a high concentration of H_2S ($8\text{--}12\text{ mg l}^{-1}$) and highly diverse sulfur oxidizers inhabit the cave water and form a visible microbial mat. Microscopic observations indicated that the floating mat contained *Thiothrix*- and *Beggiatoa*-like filamentous bacteria (Sarbu *et al.*, 1994) and subsequently a *T. thioparus* strain was isolated from Movile Cave water (Vlasceanu *et al.*, 1997). Earlier studies have shown that aerobic and anaerobic sulfur oxidizers are abundant (up to 10^7 CFU (colony-forming unit) per gram sample) (Rohwerder *et al.*, 2003). Our molecular-based analyses confirmed the presence of filamentous sulfur-oxidizing bacteria related to *Thiothrix*, *Beggiatoa* and *Thioploca* within the *Gammaproteobacteria* (Figure 1). The 16S rRNA, *soxB* (Figure 5) and RuBisCO (Figure 4c) sequences analyses also suggest the presence of a novel group of sulfur-oxidizing *Gammaproteobacteria*, related to *Thiovirga* and *Thiofaba* within the *Halothiobacillaceae* family (Ito *et al.*, 2005; Mori and Suzuki, 2008).

They are loosely related to *Halothiobacillus* spp. in terms of 16S rRNA gene, *soxB* gene and RuBisCO gene sequence identities. They are also separated from *Thiovirga* and *Thiofaba* by their 16S rRNA identity (Figure 1). 16S rRNA gene sequences related to *T. denitrificans* and *T. thioparus* within the *Betaproteobacteria* were also abundant, which has been suggested earlier (Vlasceanu *et al.*, 1997). This group, compared with the filamentous sulfur oxidizers *Thiothrix*–*Thioploca*–*Beggiatoa*, was particularly active during SIP microcosm incubations. The presence of anaerobic sulfur oxidizers in Movile Cave has also been suggested earlier (Rohwerder *et al.*, 2003) and a 16S rRNA sequence related to *Sulfuricurvum* was detected in our crude DNA clone library, although it was not labeled during SIP incubations, probably because of the fact that the incubation was carried out aerobically, and *Sulfuricurvum* oxidizes sulfur anaerobically (Kodama and Watanabe, 2004). It is to be noted that no sequences related to known sulfur-oxidizing archaea were observed in the archaeal 16S RNA clone library, which may reflect the fact that the

Figure 5 Neighbor-joining phylogenetic tree of *soxB* (sulfate thioesterase/thiohydrolase) gene sequences (412 bp) retrieved from Movile Cave crude DNA. Sequences were aligned using the ClustalX program and the tree was constructed by Phylowin. Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences retrieved from the crude DNA are highlighted in bold.

temperature in the cave (20 °C) is constant while all known sulfur-oxidizing archaea to date are thermophilic.

One notable difference in the Movile Cave microbial community compared with other chemoautotrophically based cave systems, such as Lower Kane Cave (USA), is that *Epsilonproteobacteria* were less abundant in Movile Cave (Engel *et al.*, 2003, 2004). However, as with our study, another chemoautotrophically based cave, Frasassi Cave (Italy), was also dominated by *Gammaproteobacteria* and *Betaproteobacteria* rather than *Epsilonproteobacteria* (Macalady *et al.*, 2006). The most recent research on niche differentiation among sulfur-oxidizing bacteria within Frasassi Cave tends to suggest that *Epsilonproteobacteria* dominated when sulfide to oxygen ratios were high (Macalady *et al.*, 2008). Clearly, more study of Movile Cave is needed to

determine the distribution and niche adaption of sulfur oxidizers in the mat, in different depths of the cave water and in biofilms on the surface of the walls of the cave.

The presence of a high relative abundance of methylotroph 16S rRNA gene sequences in the crude DNA, as revealed by molecular analyses, is surprising given the fact that the cave receives a high flux of methane. It is likely that these methylotrophs feed on methanol produced by and released from methanotrophs that are consuming methane. Earlier studies showed high methane oxidation activity in the cave water and floating mats and a variety of methanotrophs have been detected in this cave (Hutchens *et al.*, 2004). In fact, methylotroph-related 16S rRNA sequences were also observed in the 'heavy' DNA from ¹³C-methane incubations and this has been interpreted as cross-feeding of carbon,

Figure 6 Neighbor-joining phylogenetic tree of bacterial *amoA* gene sequences (328 bp) retrieved from Movile Cave crude DNA and 'heavy' DNA from the 3-week stable-isotope probing (SIP) incubation with ^{13}C -bicarbonate. Sequences were aligned using the ClustalX program and the tree was constructed by Phylowin. Bootstrap values are indicated as blank circles (<50%), gray circles (50–75%) and black circles (>75%). Sequences retrieved from the crude DNA are highlighted in bold and those in bold and underlined were retrieved from 'heavy' DNA of the 3-week incubation with ^{13}C -bicarbonate.

presumably methanol or formate, from methanotrophs to methylotrophs (Hutchens *et al.*, 2004). Another possibility is that methanol may come from the degradation of chitin-like substances from cave fauna, as these compounds need to be recycled when cave fauna die, or that the methylotroph sequences that were labeled are in fact from as yet uncultured methanotrophs in the *Betaproteobacteria*. The methylotrophs were assigned to the *Methylophilaceae* family (*Betaproteobacteria*). Sequences related to *M. petroleiphilum* (a facultative methylotroph in the *Betaproteobacteria*) were also found in the *soxB* and RuBisCO clone libraries. An earlier culture-based study also showed that the numbers of culturable methylotrophs in the floating mat were around 10^6 CFU per gram dry weight of mat (Rohwerder *et al.*, 2003).

Archaeal communities in cave systems, in general, have not been studied in detail. Macalady *et al.*, (2007) showed that the dominant archaea in the wall biofilms from Frasassi Cave (another chemoautotrophy-based cave, but with a low pH) were associated with two groups, namely *Ferroplasma* and an environmental clone group with sequences from acid mine drainage and Yellowstone hot spring. No *Crenarchaeota* were found in Frasassi Cave. In contrast, the archaeal community in Movile Cave showed some similarity to deep-sea hydrothermal vent environments (Takai and Horikoshi, 1999), but differed from these in that no 16S rRNA sequences

related to known ammonia-oxidizing archaea were found in our study, and *Nitrosomonas* spp. were probably the major nitrifiers in Movile Cave, whereas it has been suggested that ammonia-oxidizing *Crenarchaea*, rather than their bacterial counterparts, are the major nitrifiers in deep-sea vents (Lam *et al.*, 2004; Nakagawa and Takai, 2008). Although it is possible that the clone library had bias because of the imperfect 16S rRNA primers for archaea, as well as bias during SIP incubations, our results imply that bacterial chemolithotrophs are probably the dominant primary producers in Movile Cave.

In addition to 16S rRNA analyses, we also used functional marker genes to characterize the microbial diversity in Movile Cave. Great care is needed in the interpretation of the results, as not all chemolithoautotrophs use RuBisCO for CO_2 fixation and at least three other CO_2 fixation pathways are known (reviewed by Nakagawa and Takai, 2008). Similarly, microorganisms also use a number of different pathways for sulfur oxidation, although the Sox pathway is the most highly characterized (Kelly *et al.*, 1997). Thus, functional gene analyses should always be used in combination with other techniques, such as 16S rRNA gene-based methods. In addition, we did not carry out functional gene analyses of *soxB* and RuBisCO for 'heavy' DNA from SIP incubations because bacteria, which contain these genes, will also be present in 'light' DNA.

Furthermore, *soxB* genes also occur in facultative bacteria that could derive most of their cell carbon from multicarbon sources.

In summary, we have studied the microbial diversity in Movile Cave and suggest that ammonia/nitrite-oxidizing bacteria may also contribute to primary productivity in this cave. Further work on the niche distribution of sulfur and ammonia/nitrite oxidizers, as well as the contribution of one-carbon compound oxidation to primary production, is needed for a full understanding of the sulfur, nitrogen and carbon cycles within Movile Cave.

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