

Culture dependent and independent analyses of 16S rRNA and ATP citrate lyase genes: a comparison of microbial communities from different black smoker chimneys on the Mid-Atlantic Ridge

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Abstract The bacterial and archaeal communities of three deep-sea hydrothermal vent systems located on the Mid-Atlantic Ridge (MAR; Rainbow, Logatchev and Broken Spur) were investigated using an integrated culture-dependent and independent approach. Comparative molecular phylogenetic analyses, using the 16S rRNA gene and the deduced amino acid sequences of the alpha and beta subunits of the ATP citrate lyase encoding genes were carried out on natural microbial communities, on an enrichment culture obtained from the Broken Spur chimney, and on novel chemolithoautotrophic bacteria and reference strains originally isolated from several different deep-sea vents. Our data showed that the three MAR hydrothermal vent chimneys investigated in this study host very different microbial assemblages. The microbial

community of the Rainbow chimney was dominated by thermophilic, autotrophic, hydrogen-oxidizing, sulfur- and nitrate-reducing *Epsilonproteobacteria* related to the genus *Caminibacter*. The detection of sequences related to sulfur-reducing bacteria and archaea (*Archaeoglobus*) indicated that thermophilic sulfate reduction might also be occurring at this site. The Logatchev bacterial community included several sequences related to mesophilic sulfur-oxidizing bacteria, while the archaeal component of this chimney was dominated by sequences related to the ANME-2 lineage, suggesting that anaerobic oxidation of methane may be occurring at this site. Comparative analyses of the ATP citrate lyase encoding genes from natural microbial communities suggested that *Epsilonproteobacteria* were the dominant primary producers using the reverse TCA cycle (rTCA) at Rainbow, while *Aquificales* of the genera *Desulfurobacterium* and *Persephonella* were prevalent in the Broken Spur chimney.

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Introduction

The steep chemical (redox, pH) and temperature gradients present at deep-sea hydrothermal vents provide numerous unique niches that microorganisms can colonize. In particular, the walls of black smoker chimneys are characterized by the rapid transition from reduced, high temperature conditions (in the interior section of the chimneys) to more oxidized, low temperature ones (at the interface between the chimneys and seawater). This transition occurs as a continuum when the hot, reduced

hydrothermal fluids percolate from the interior conduits towards the outside walls of the chimneys, transporting hydrogen, reduced sulfur species and carbon dioxide, among other compounds. The flux rate and the chemical composition of the fluids (which in turn affect the mineral composition of the chimney) are likely to influence the formation of the chemical and temperature gradients. When the reduced fluids mix with oxidized chemical species (e.g., oxygen, nitrate and sulfate) diffusing inward from ambient seawater, microorganisms take advantage of the available redox potentials and convert chemical energy into ATP that can be used for carbon dioxide fixation and other anabolic processes. The integration of whole cell hybridization with 16S rRNA gene surveys revealed that there was a transition from higher density, mixed bacterial and archaeal communities near the exterior of the chimney to lower density, archaea dominated communities in the interior of the structure (Schrenk et al. 2003). Surveys of the diversity of archaea associated with active black smokers showed that the communities in the chimney structure consisted, for the most part, of hyperthermophilic archaea and of several new archaeal groups, and that the various phylotypes were differently distributed within the chimney structure, possibly as a function of the thermal and redox gradients (Nercessian et al. 2003; Schrenk et al. 2003; Takai et al. 1999, 2001). In one of these studies, the majority of 16S rRNA gene sequences obtained from the exterior of the chimney were related to archaeal taxa previously recovered from benthic and pelagic environments, including the crenarchaeal marine group I and uncultured benthic Euryarchaeota. In contrast, the interior regions of the chimney were colonized by methanogens, *Thermococcales*, and *Archaeoglobales*, in addition to uncultured crenarchaeal phylotypes related to sequences previously isolated from deep subsurface habitats (Schrenk et al. 2003). Culture independent studies of functional genes in black smokers further confirmed the occurrence of thermophilic and mesophilic methanogens, and revealed the presence of sulfate reducing bacteria and archaea and of methanotrophic bacteria (Nakagawa et al. 2004, Nercessian et al. 2005a). Furthermore, 16S rRNA-based studies of vent microbial communities indicated that *Epsilonproteobacteria* were associated with sulfide structures (Hoek et al. 2003; Longnecker et al. 2001), and showed that between 66 and 98% of the microorganisms associated with various types of colonization substrates that were deployed in the vicinity of chimney orifices belonged to this class of the Proteobacteria (Alain et al. 2004; Lopez-Garcia et al. 2003).

Recent work on the isolation of pure cultures from black smokers is complementing the culture-independent approaches by providing physiological information, for example suggesting that specific groups of organisms

occupy discrete temperature niches within the chimney structures. For instance, under autotrophic conditions, temperatures between 30–65°C appear to best support growth of anaerobic or microaerobic *Epsilonproteobacteria* (e.g., *Nautilia*, *Caminibacter*, *Hydrogenimonas*, etc.), while temperatures between 50–80°C generally support growth of thermophiles of the phylum *Aquificae* (e.g., *Desulfurobacterium*, *Thermovibrio*, *Persephonella*), and growth temperatures >75°C for the most part select for hyperthermophilic archaea (Miroshnichenko et al. 2006). In particular, several new species of *Epsilonproteobacteria* have been isolated from deep-sea hydrothermal vents during the past few years (reviewed in Campbell et al. 2006; Miroshnichenko et al. 2006). Along with the culture-independent studies, physiological information derived from these pure cultures is helping to establish the relevance of *Epsilonproteobacteria* as primary producers, early colonizers, as well as metazoan epi- and endosymbionts at deep-sea vents (Campbell et al. 2006).

One of the pathways for autotrophic CO₂ fixation in some anaerobic and microaerobic bacteria is based on a tricarboxylic acid cycle which operates in reverse (Buchanan et al. 1990). This reductive tricarboxylic acid cycle (rTCA) leads to the fixation of CO₂ and to the synthesis of acetyl coenzyme A, which is carboxylated to pyruvate and then used in further anabolic processes. The three key enzymes that are essential to run the rTCA cycle are ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase and fumarate reductase. The first evidence for the occurrence of the rTCA cycle in deep-sea hydrothermal vent microbial communities was obtained from metagenome studies of the episymbiotic community associated with the vent polychaete *Alvinella pompejana* and from rTCA-related gene surveys (Campbell et al. 2003, 2004). Recent reports demonstrated that pure cultures of *Epsilonproteobacteria* and *Aquificae*, which include representatives of hydrothermal vent bacteria, fix carbon dioxide via the rTCA cycle (Ferrera et al. 2007; Hügler et al. 2005, 2007; Takai et al. 2005a).

Here we present a study of the microbiology of chimney structures from three hydrothermal vent sites along the Mid-Atlantic Ridge (MAR): two ultramafic-hosted systems, Rainbow and Logatchev, and one basalt-hosted system, Broken Spur. For the first time, we carried out a comparative analysis between 16S rRNA genes and the alpha and beta subunits of ATP citrate lyase, *acIA* and *acIB*, retrieved from natural microbial communities and from reference strains isolated from several different vent sites (including the MAR, the East Pacific Rise, the Central Indian Ridge, the Okinawa Trough and the Mariana Arc). Our results revealed significant differences in the composition of the microbial communities of the three MAR sites, identified the dominant primary producers that use the

rTCA cycle in these communities, and further defined the phylogeny of the ATP citrate lyase genes.

Materials and methods

Sample collection

Fragments of active, high temperature black smoker chimneys were collected from the “Rainbow” (36°14'N, 33°54'W; depth 2,305 m), “Logatchev” (14°45'N, 44°58'W; depth 3,000 m), and “Broken Spur” (29°10'N, 43°10'W; depth 3,060 m) vent fields on the Mid-Atlantic Ridge, during a cruise aboard R/V *Atlantis* (cruise AT 05–03, July 2001), and from the East Pacific Rise (EPR; 9°10'N, 104°17'W; depth 2,500 m) during cruise AT 11–10 (April 2004). The samples were collected using the manipulator of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest of the dive. On the surface, samples were transferred to the ship's laboratory and subsamples were either frozen at –80°C for nucleic acid extraction, or stored at 4°C under a dinitrogen atmosphere for enrichments and isolations. Sample locations are summarized in Table 1.

Enrichments, isolations and reference strains

Primary enrichment cultures were initiated by adding about 1 ml of inoculum (prepared by resuspending approximately

1 g of chimney sample in 1 ml of anaerobic artificial seawater) to 10 ml of modified SME media that had been prepared as previously described (Stetter et al. 1983; Vetrani et al. 2004). Incubation temperatures were 55°C for the isolation of *Nautilia* spp. from EPR samples, and 65°C for the Broken Spur enrichment culture. Long-term stocks were prepared by supplementing 50 ml of DSMO (Fisher Scientific, Pittsburgh, PA, USA) to 1 ml of culture, and stored at –80°C. The reference strains used in this study included: *Caminibacter mediatlanticus* DSM 16658, *Caminibacter* sp. strain TB1 (Voordeckers et al. 2005), *Caminibacter hydrogeniphilus* DSM 14510 (Alain et al. 2002b), *Caminibacter profundus* DSM 15016 (Mirshnichenko et al. 2004), *Hydrogenimonas thermophila* JCM 11971 (Takai et al. 2004b), *Lebetimonas acidiphila* DSM 16356 (Takai et al. 2005b), *Sulfurimonas autotrophica* DSM 16294 (Inagaki et al. 2003), and *Sulfurovum lithotrophicum* JCM 12117 (Inagaki et al. 2004).

Preparation of cell extracts and enzyme assays

Caminibacter mediatlanticus DSM 16658 was used as a reference strain for activity assays of enzymes involved in the reductive TCA cycle. Cell extracts of *C. mediatlanticus* were prepared using a mixer mill (type MM 301, Retsch, Haare, Germany) according to Hügler et al. 2005. Protein concentrations in cell extracts were determined by the method of Bradford 1976 using bovine serum albumine as standard. Enzyme assays (0.5 ml assay mixture) were

Table 1 Locations of sampling stations and clonal libraries constructed in this study

Vent	Cruise/Dive No.	Latitude	Longitude	Depth (m)	Temperature (°C) ^b	Library ^a	
						16S rRNA gene	Citrate lyase
Rainbow, MAR	R/V <i>Atlantis</i>	36°N14'	33°W54'	2,305	158	ROB	RacIB
	Cruise AT 05–03					RMB	
	DSV <i>Alvin</i> Dive 3678					ROA	
Logatchev, MAR	R/V <i>Atlantis</i>	14°N45'	48°W58'	3,000	348	L7B	ND ^d
	Cruise AT 05–03					L7A	
	DSV <i>Alvin</i> Dive 3667–3668 (Irina2 vent)					L8B	
Broken Spur, MAR	R/V <i>Atlantis</i>	29°N10'	43°W10'	3,060	ND	BSE	BSEacIA
	Cruise AT 05–03						BSEacIB
	DSV <i>Alvin</i> Dive 3675						BSacIA
9°N, EPR	R/V <i>Atlantis</i>	9°N50'	104°W17'	2,500	339 ^c		BSacIB
	Cruise AT 11–10					–	–
	DSV <i>Alvin</i> Dives 3999, 4002, 4008						

^a See Material and methods for library and clone naming

^b Fluid temperature measured with DSV *Alvin* HighT probe at chimney orifice

^c Fluid temperature at P vent on Dive 4008

^d *acIB* gene fragment not detected by PCR

performed in stoppered 0.5 ml glass cuvettes at 55°C. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm ($\epsilon_{365 \text{ nm}} \text{ NAD(P)H} = 3.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Reactions involving benzyl viologen (BV) were followed spectrophotometrically at 578 nm ($\epsilon_{578 \text{ nm}} \text{ BV} = 8.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

ATP citrate lyase activity was determined according to Hügler et al. 2007. 2-Oxoglutarate:BV oxidoreductase, pyruvate:BV oxidoreductase, fumarate reductase, malate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and pyruvate dehydrogenase activities were measured according to references (Hügler et al. 2003, 2005).

DNA extraction

Total genomic DNA was extracted from 1.7–2.1 g of four chimney subsamples (Rainbow 3678 out, Logatchev 3667, Logatchev 3668, and Broken Spur 3675) using the UltraClean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA, USA) with the following protocol modifications: Bead beating was extended for 20 s and it was followed by heating at 70°C for 5 min. For subsample Rainbow 3678C-mid, 14.59 g was extracted using the Mega Prep UltraClean Soil DNA according to the manufacturer's specifications (Mo Bio Laboratories, Solana Beach, CA, USA). Cells obtained from 10 ml of pure cultures and from an enrichment culture inoculated with a chimney sample from the Broken Spur site were extracted using the UltraClean Microbial DNA Isolation Kit according to the protocol supplied with the kit (Mo Bio Laboratories, Solana Beach, CA, USA).

DNA amplification by PCR

Archaeal and bacterial 16S rRNA genes were amplified using the archaeal domain specific forward primer 16F (5'-CTGGTTGATCCTGCCAG-3') and the bacterial domain specific forward primer 8F (5'-AGAGTTTGATCC TGGCTCAG-3'), respectively, in combination with universal primer 1517R (5'-ACGGCTACCTTGTTACGAC TT-3'). PCR conditions for amplification reactions were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension time of 7 min during the last cycle. The *aclB* gene was amplified from pure cultures (*Nautilia* spp. and *Caminibacter* spp.), from the Rainbow and Broken Spur natural communities, and from the Broken Spur enrichment culture (Table 1) using primers 892F and 1204R and PCR conditions as described by Campbell et al. (2003), while primers F2 and R5 and PCR conditions as described by Hügler et al. (2005) were used to amplify the *aclA* gene from natural communities, from the Broken

Spur enrichment culture (Table 1) and from the following strains: *Caminibacter mediatlanticus*, *Caminibacter hydrogeniphilus*, *Hydrogenimonas thermophila*, *Lebetimonas acidiphila*, *Sulfurimonas autotrophica*, and *Sulfurovum lithotrophicum*.

Library construction, restriction fragment length polymorphism screening and sequence

The amplified 16S rRNA and *acl* gene fragments were gel-purified using the QIAGEN Gel Spin purification kit (Qia-gen, Santa Clarita, CA, USA), cloned into either pCR II or pCR4-TOPO plasmid vectors, and the ligation products were transformed into competent *E. coli* Oneshot cells (Invitrogen, Inc., Carlsbad, CA, USA). Nine environmental libraries (six 16S rRNA and three ATP citrate lyase gene libraries) were constructed from different chimney samples, and three libraries were constructed from an enrichment culture (one 16S rRNA and two ATP citrate lyase gene libraries) (Table 1). A total of 293 randomly chosen clones (160 16S rRNA gene clones and 133 ATP citrate lyase clones) were analyzed for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. Forty-one archaeal and seventy-two bacterial 16S rRNA gene clones from the environmental libraries, and thirteen bacterial clones from the Broken Spur enrichment culture were screened by restriction fragment length polymorphism (RFLP) as previously described (Reed et al. 2006). Representative clones for each library showing unique RFLP patterns were selected and their sequences (about 1,400 nucleotides) was determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). A total of sixty *aclA* and seventy-three *aclB* clones were screened by PCR, and selected inserts were sequenced (about 970 nucleotides for *aclA* fragments and about 290 nucleotides for *aclB* fragments). A summary showing the number of clones examined for each library is presented in the supplemental material (Table S1).

Phylogenetic analyses

Sequences were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). For the detection of putative chimeric sequences, both Pintail (Ashelford et al. 2005) and the Check_Chimera 2.7 program of the Ribosomal Database Project II were used (<http://rdp.cme.msu.edu/html/index.html>; Cole et al. 2003). Two bacterial phylotypes from Logatchev, represented by a single clone each, appeared to be chimeras and were eliminated from the phylogenetic analysis. The remaining 16S rRNA gene sequences were aligned using ClustalX v 1.8 (Thompson et al. 1997) and manually adjusted using Seaview (Galtier et al. 1996). Phylogenetic distances were

calculated using the Jukes-Cantor model and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier et al. 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings. ATP citrate lyase gene fragments were translated using the online tool EMBOSS Transeq (<http://www.ebi.ac.uk/emboss/transeq/>) and the amino acid sequences were aligned with ClustalX v 1.8. Phylogenetic distances were calculated using the Observed Divergence matrix and the neighbor joining method was used to evaluate tree topologies.

Nucleotide sequence accession numbers

The sequences from this study are available through GenBank under the following accession numbers: EF644656–EF644685, EF644759–EF644814, and EF644827–EF644847.

Results

Pure cultures

Enrichment cultures for thermophilic, chemolithoautotrophic, hydrogen-oxidizing organisms were obtained by inoculating 10 ml of anaerobic SME medium (Stetter et al. 1983), supplemented with 0.1% nitrate, with approximately 1 ml of slurries obtained from black smoker chimneys. The isolation (from a MAR black smoker) and characterization of *Caminibacter mediatlanticus* DSM 16658 and of *Caminibacter* sp. strain TB1, two anaerobic, chemolithoautotrophic *Epsilonproteobacteria*, was reported previously (Voordeckers et al. 2005). Three moderately thermophilic, anaerobic, hydrogen-oxidizing and nitrate-reducing, chemolithoautotrophic bacteria, designated as strains MT3, MT4, and MT5, were isolated, using the dilution to extinction technique, from the walls of active deep-sea hydrothermal vents chimneys collected at the 9°N site on the EPR. All three strains were grown at 55°C. Phylogenetic analysis of the 16S rRNA gene indicated that all three organisms belonged to the *Epsilonproteobacteria*, and that they were closely related to *Nautilia lithotrophica* (Miroshnichenko et al. 2002), with 96–98% sequence identity (Fig. 1a).

The genes encoding for either the large and/or the small subunit of the ATP citrate lyase, *aclA* and *aclB*, were amplified from the three newly isolated *Nautilia* strains (*aclB*), *Caminibacter* spp. (*aclA* and *aclB*), *Hydrogenimonas thermophila* (*aclA*), *Lebetimonas acidiphila* (*aclA*), *Sulfurimonas autotrophica* (*aclA*), and *Sulfurovum lithotrophicum* (*aclA*). Phylogenetic analyses of the amino acid sequence deduced from the *aclB* gene placed *Nautilia*

sp. strains MT3, MT4, and MT5, *C. mediatlanticus*, *Caminibacter* sp. strain TB1, *C. profundus* and *C. hydrogeniphilus* in two closely related clusters distinct from the *aclB* sequences from other *Epsilonproteobacteria* (Fig. 2). In line with the phylogenetic analysis, the *AclB* amino acid sequence of the *Caminibacter* strains was more similar to the sequences of the *Nautilia* strains (95–97% identity) than to the sequences of other vent *Epsilonproteobacteria*.

Phylogenetic analysis of the amino acid sequence deduced from the *aclA* fragment showed that the sequences of *C. mediatlanticus* and *C. hydrogeniphilus* were closely related to each other (95% identity) and more distantly related to ATP citrate lyase from *Lebetimonas acidiphila* and *Hydrogenimonas thermophila*, while the *AclA* from *Sulfurimonas autotrophica* and *Sulfurovum lithotrophicum* formed a separate cluster related to uncultured episymbionts of the vent worm *Alvinella pompejana* (Fig. 3).

Activities of enzymes of the reductive TCA cycle in *Caminibacter mediatlanticus*

In order to establish that the rTCA cycle operates in *C. mediatlanticus*, the activity of enzymes of the rTCA cycle were tested in cell extracts of this strain. The activities of all enzymes of the rTCA cycle, including that of the ATP-dependent citrate lyase (encoded by the *aclBA* genes), could be detected in *C. mediatlanticus*, indicating that a functional rTCA cycle is present in this organism (Table 2). In contrast, the activities of enzymes specific to the oxidative TCA cycle, such as the 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase, which are not shared with the rTCA, could not be detected (Table 2).

Phylogenetic analysis of the Rainbow microbial community

The majority (97%; 34/35) of the bacterial clones retrieved from the Rainbow site were related to the *Epsilonproteobacteria*, while only 3% (1/35) were related to the *Deltaproteobacteria*. The bacterial 16S rRNA gene libraries from both the outside and middle sections of the Rainbow chimney were dominated by *Epsilonproteobacteria*, accounting for 11 of the 12 bacterial phylotypes sequenced from this site. Phylogenetic analysis placed six of these phylotypes, ROB3, ROB4, ROB5, ROB6, RMB3 and RMB5, representing about 40% (22/55) of all the clones from the Rainbow chimney in the *Caminibacter/Nautilia* group (also defined as Group D; Fig. 1a). The nearest cultivated relatives to these phylotypes are *C. mediatlanticus* and *Caminibacter* sp. strain TB1 (99–100% sequence identity), both of which are hydrogen-oxidizing and nitrate-reducing thermophiles (optimum growth temperature 50–55°C), and were isolated from the Rainbow

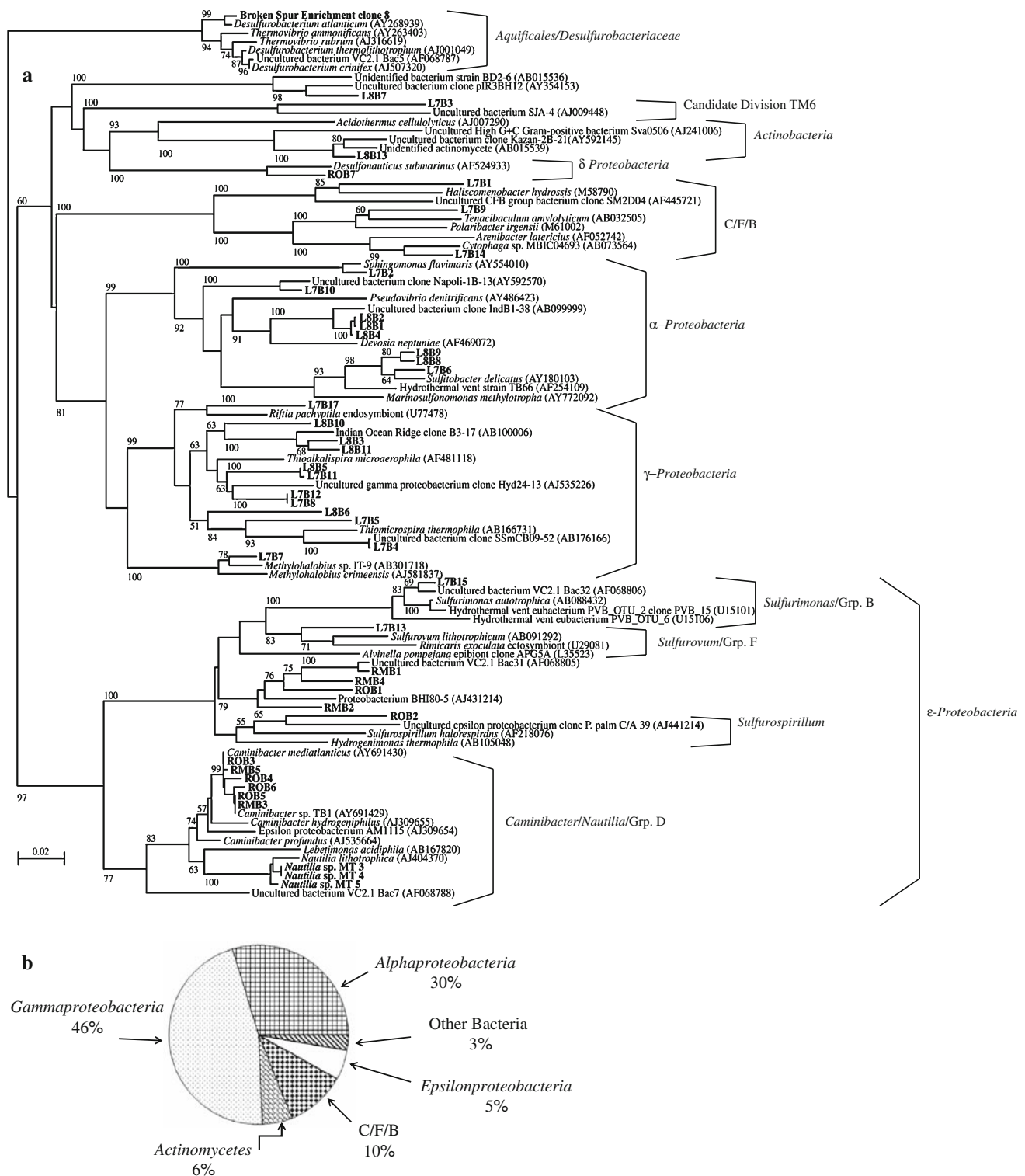


Fig. 2 Neighbor-joining phylogenetic analysis of the amino acid sequence deduced from a fragment of the gene encoding for the beta subunit of ATP citrate lyase (*aclB*) from the Rainbow (RacIB#) and Broken Spur (BSacIB#) black smokers, and from the Broken Spur enrichment culture (BSEacIB#). Percentages greater than 50% of bootstrap resampling (of 1,000 resamplings) that support each topological element are indicated near the nodes. Representative sequences obtained in this study are in **bold**. The scale bar is 5 substitutions per 100 sequence positions

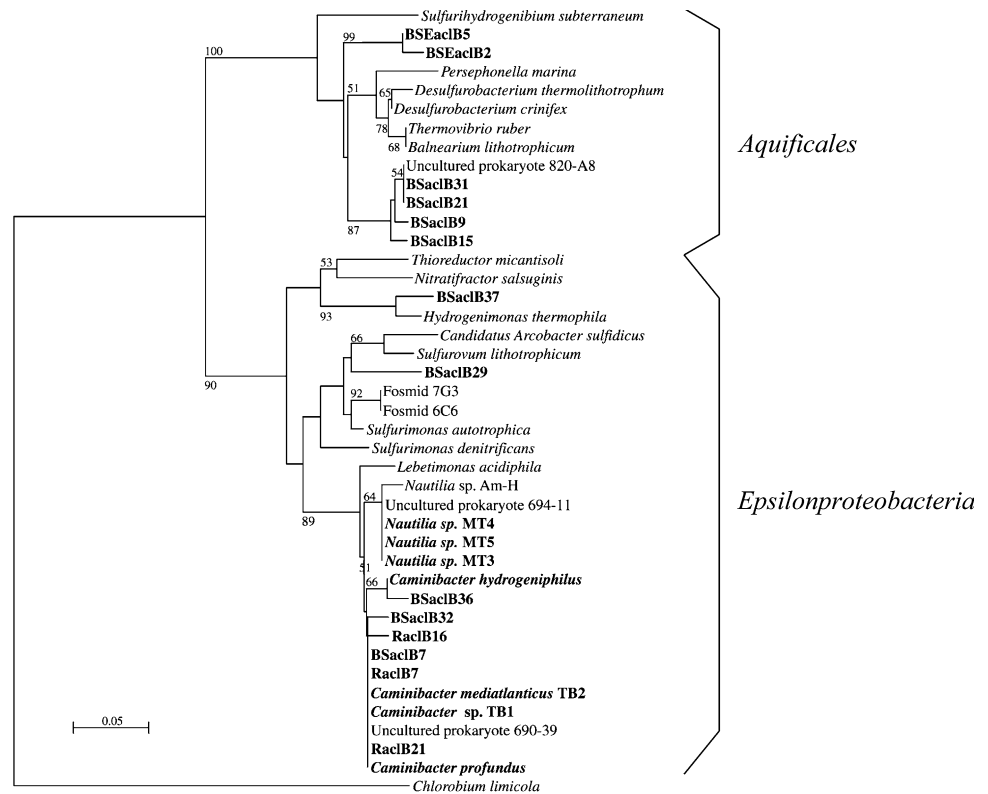
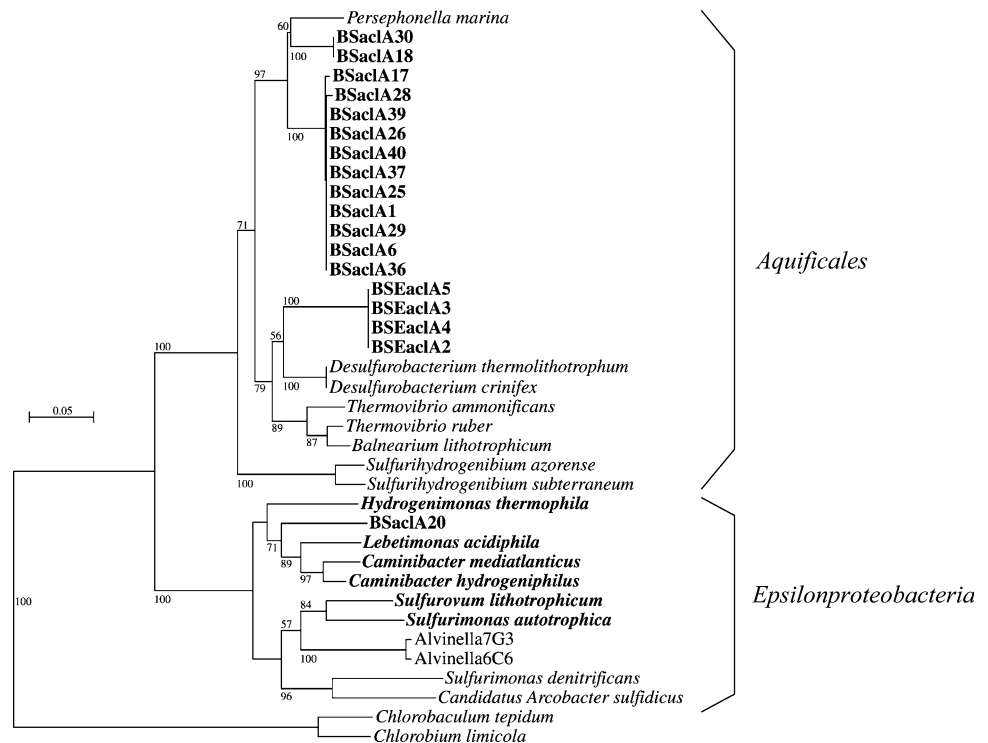


Fig. 3 Neighbor-joining phylogenetic analysis of the amino acid sequence deduced from a fragment of the gene encoding for the alpha subunit of ATP citrate lyase (*aclA*) from the Broken Spur (BSacIA#) black smoker and enrichment culture (BSEacIA#). Percentages greater than 50% of bootstrap resampling (of 1,000 resamplings) that support each topological element are indicated near the nodes. Representative sequences obtained in this study are in **bold**. The scale bar is 5 substitutions per 100 sequence positions



hydrothermal vent field (Voordeckers et al. 2005). Four phylotypes from the Rainbow site (RMB1, RMB2, RMB4, and ROB1) clustered into a group that was related (96–99% sequence identity) to epsilonproteobacterial sequences

retrieved from an in situ growth chamber deployed at the Snake Pit hydrothermal vent site on the MAR (Reysenbach et al. 2000), and to organisms enriched from the tubes of *Alvinella pompejana* worms (Cambon–Bonavita,

unpublished results). Phylotype ROB2 was related to clones obtained from the microbial community associated with the invertebrate *Paralvinella palmiformis* (90% sequence identity; Alain et al. 2002a; Haddad et al. 1995), while the closest cultured relative to this phylotype was *Sulfurospirillum halorespirans* (89% sequence identity; Luijten et al. 2003). Clone ROB7, the only Rainbow

Table 2 Specific activities [$\text{nmol min}^{-1} (\text{mg cell protein})^{-1}$] of enzymes of the reductive TCA cycle in *C. mediatlanticus*

Enzyme activity tested	<i>Caminibacter mediatlanticus</i>
Assay temperature (°C)	55
ATP citrate lyase	275
2-Oxoglutarate:BV oxidoreductase	330
Pyruvate:BV oxidoreductase	160
Fumarate reductase (BV)	710
Isocitrate dehydrogenase (NAD)	45
Isocitrate dehydrogenase (NADP)	7,800
Malate dehydrogenase (NADH)	4,080
2-Oxoglutarate dehydrogenase (NAD)	n.d.
2-Oxoglutarate dehydrogenase (NADP)	n.d.
Pyruvate dehydrogenase (NAD)	n.d.
Pyruvate dehydrogenase (NADP)	n.d.

Mean values were obtained from at least five measurements. Standard errors were less than $\pm 20\%$. n.d., no activity detected, detection limit $<1 \text{ nmol min}^{-1} (\text{mg cell protein})^{-1}$

phylotype found outside of the *Epsilonproteobacteria*, was related (94% sequence identity) to *Desulfonauticus submarinus*, a moderately thermophilic (optimum growth temperature 45°C), sulfate-reducing *Deltaproteobacterium* isolated from 13°N on the EPR (Fig. 1a; Pikuta et al. 1998).

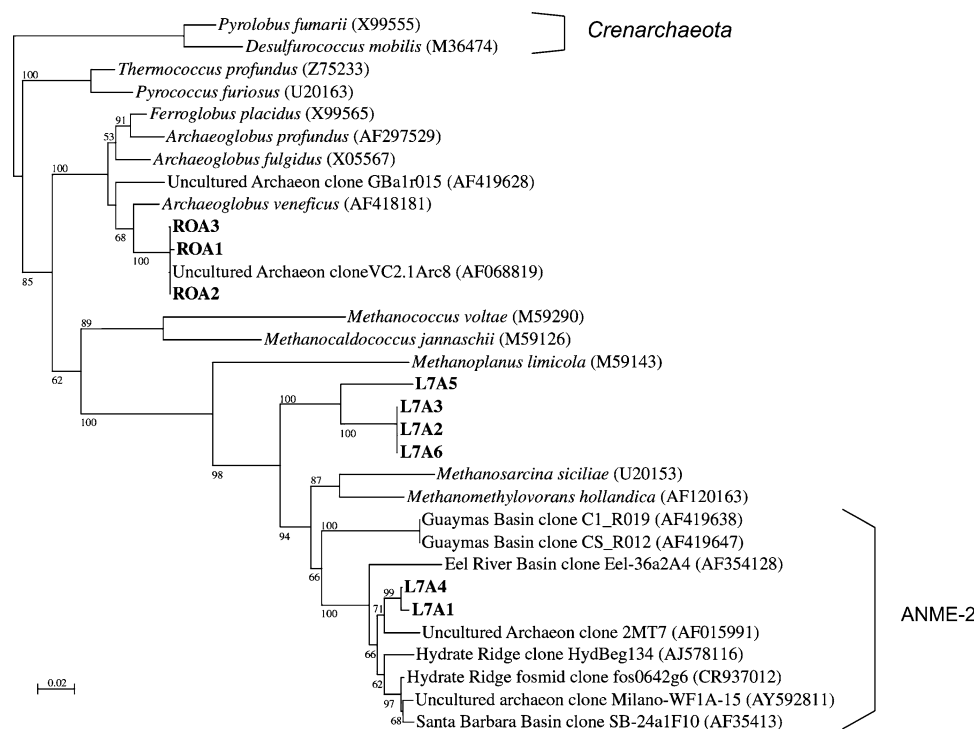
All archaeal sequences retrieved from the outside wall of the Rainbow chimney belonged to the order *Archaeoglobales*. Clones ROA1, ROA2, and ROA3 were related to *Archaeoglobus veneficus* (95–97% sequence identity; Huber et al. 1997), and clone ROA3 was the dominant phylotype, accounting for 90% (18/20) of the Rainbow archaeal library (Fig. 4).

The gene encoding for the small subunit of the ATP citrate lyase, *aclB*, was amplified from DNA extracted from the microbial community of the Rainbow black smoker. All the Rainbow *aclB* sequences, represented by clones RaciB16, 7 and 21, were placed within the *Caminibacter* cluster, and their amino acid sequence was 98–99% identical to the ATP citrate lyase of *C. mediatlanticus* (Fig. 2).

Phylogenetic analysis of the Logatchev microbial community

The microbial communities from the two samples collected at the Logatchev vent were similar to each other, but their diversity was much higher than that of the Rainbow community, and for the most part these sequences were related to mesophilic, aerobic or microaerobic bacteria (Fig. 1a,

Fig. 4 Phylogenetic analysis of archaeal 16S rRNA gene sequences from the Rainbow (ROA#) and Logatchev (L7A#) black smokers. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model. Percentages greater than 50% of bootstrap resampling (of 1,000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rRNA genes obtained in this study are in **bold**. The scale bar is 2 substitutions per 100 sequence positions



b). The epsilonproteobacterial phylotypes retrieved from the Logatchev black smokers [5% (2/37) of the total bacterial clones; Fig. 1b] were phylogenetically distinct from the Rainbow *Epsilonproteobacteria* (Fig. 1a). The closest cultured relatives to phylotypes L7B13 and L7B15 were *Sulfurovum lithotrophicum* (Group F; 92% sequence identity) and *Sulfurimonas autotrophica* (Group B; 94% sequence identity), respectively, two mesophilic (optimum growth temperature 25–30°C), microaerobic, sulfur and thiosulfate-oxidizing bacteria isolated from the Okinawa Trough (Inagaki et al. 2003, 2004).

The Alphaproteobacteria-related phylotypes retrieved from the Logatchev black smokers (30% of the bacterial clones; 11/37; Fig. 1b) were clustered into four main groups related, for the most part, to sulfur-oxidizing bacteria (Fig. 1a). L7B6, L8B8, and L8B9, accounting for approximately 10% (4/37) of the total clones from Logatchev, grouped closely with the genus *Sulfitobacter* (97% sequence identity) and more distantly (93–96% sequence identity) with *Marinosulfonomonas methylotrophica* (Holmes et al. 1997). A second group of Alphaproteobacteria (L8B1, L8B2, and L8B4), accounting for 13% (5/37) of the total bacterial clones from Logatchev, were closely related (96% sequence identity) to a 16S rRNA gene sequence (IndB1–38) retrieved from an inactive chimney of the Kairei hydrothermal vent field on the Indian Ocean Ridge (Suzuki et al. 2004).

The gammaproteobacterial phylotypes retrieved from the Logatchev black smoker samples (46% of the bacterial clones; 17/37) were clustered into five groups and were, for the most part, related to mesophilic lithotrophs (Fig. 1a). Clones L7B8, L7B11, L7B12 and L8B5 were related (92–93% identity) to clones retrieved from a sediment sample located above a gas hydrate deposit on the Cascadia Margin, Oregon (Knittel et al. 2003). The nearest cultivated relative to these phylotypes (91–92% sequence identity) was *Thioalkalispira microaerophila*, a sulfur oxidizing lithoautotroph isolated from a soda lake in Egypt (Sorokin et al. 2002). A second group of gammaproteobacterial phylotypes, represented by L7B4, L7B5, and L8B6, was related to *Thiomicrospira thermophila* (94, 90 and 92% sequence identity, respectively), a microaerobic, sulfur-oxidizing mesophile isolated from a deep-sea vent from the Mariana Arc (Takai et al. 2004a), while L7B17 was related to the endosymbiotic bacterium of the vent tubeworm, *Riftia pachyptila* (91% sequence identity) (Feldman et al. 1997). Interestingly, clone L7B7 was related (94% sequence identity) to *Methylohalobius crimeensis*, a mesophilic (optimum growth temperature 30°C) methanotroph from a hypersaline lake in Ukraine (Heyer et al. 2005), and to an isolate, *Methylohalobius* sp. strain IT-9 (93% sequence identity), from a shallow hydrothermal vent off Japan (Bodrossy et al. 1999).

Finally, three bacterial phylotypes from the Logatchev site, L7B9, L7B14 and L7B1, were related to the C/F/B group, (Fig. 1a). The closest cultured relatives to L7B9 and L7B14 were *Tenacibaculum amylolyticum* (90% sequence identity; Suzuki et al. 2001) and *Cytophaga* sp. MBIC04693 (94% sequence identity; Matsuo et al. 2003), which are both algae-associated bacteria.

The Logatchev archaeal community was more diverse than the Rainbow one, and it was dominated by members of the *Euryarchaeota*. Four of the Logatchev phylotypes, L7A2, L7A3, L7A5, and L7A6, formed a distinct cluster related to both the *Methanosarcinales* (88 and 90% sequence identity to *Methanosarcina siciliae* and *Methanomethylovorans hollandica*, respectively), and the *Methanomicrobiales* (83% sequence identity to *Methanoplanus limicola*), and accounted for about 38% (8/21) of the archaeal clones from this site (Fig. 4). Phylogenetic analysis showed that a second cluster of sequences, represented by L7A1 and L7A4, accounted for about 62% (13/21) of the archaeal clones retrieved from Logatchev. These phylotypes were related to a lineage whose organisms are involved in the anaerobic oxidation of methane (ANME-2; Orphan et al. 2001), and which were retrieved from both Eel River and Hydrate Ridge sediments (the sequences of L7A1 and L7A4 were 94–97% identical to the 16S rRNA gene from representatives of the ANME-2 group).

No *aclB* gene fragment could be amplified from the Logatchev black smoker microbial community, although PCR was repeated several times using DNA templates obtained from three independent extractions (Table 1).

Phylogenetic analysis of the ATP citrate lyase from the Broken Spur enrichment culture and bacterial community

An enrichment culture was obtained by inoculating, in anaerobic SME medium, a black smoker sample collected from the Broken Spur site on the MAR. The incubation temperature for this enrichment was 65°C. The 16S rRNA gene was amplified from the enrichment culture, cloned into *E. coli*, and several clones were screened by RFLP analysis. A single hydrogen-oxidizing, autotrophic bacterium dominated the Broken Spur enrichment culture. Its 16S rRNA gene was placed within the phylum *Aquificae*, with 98% sequence identity to *Desulfurobacterium atlanticum* (Fig. 1a; L'Haridon et al. 2006).

The genes encoding for the large and small subunits of the ATP citrate lyase, *aclA* and *aclB*, were amplified from the Broken Spur enrichment culture. In line with the 16S rRNA gene results, phylogenetic analysis placed the *AclB* sequences retrieved from the Broken Spur enrichment culture, represented by clones BSEaClB2 and 5, in a cluster related to the *Aquificales* (Fig. 2). The deduced amino acid

sequences of the BSEaClB clones were most similar to the AclB of *Persephonella marina* (87–88% identity), to that of the Broken Spur chimney clones (86–89% identity to clones BSaClB9, 15, 21 and 31) and to the AclB of *Desulfurobacterium thermolithotrophum* (Fig. 2). The AclA sequences retrieved from the Broken Spur enrichment culture, represented by clones BSEaClA2, 3, 4, and 5, were related to *Desulfurobacterium* spp. (Fig. 3).

The *aclA* and *aclB* genes were also amplified from DNA extracted directly from the microbial community of the Broken Spur black smoker. In contrast to the Rainbow community, both the *aclA* and *aclB* libraries constructed from DNA extracted from the Broken Spur black smoker community were dominated by clones related to the *Aquificales*, while a smaller number of clones were related to the *Epsilonproteobacteria*. Phylogenetic analyses placed 86% (30/35) of the amino acid sequences deduced from the *aclB* clones from Broken Spur, represented by clones BSaClB9, 15, 21 and 31, in a novel cluster that is only distantly related to the ATP citrate lyase from the genera *Thermovibrio*, *Desulfurobacterium* and *Persephonella*, within the *Aquificales* (Fig. 2). The AclB amino acid sequences deduced from clones BSaClB15 and 31 were most similar to the ATP citrate lyase of an uncultured member of the *Aquificales* (clone 820-A8; 97% sequence identity). Only 14% (5/35) of the Broken Spur *aclB* clones were related to the *Epsilonproteobacteria*: three of these clones, BSaClB7, 32 and 36, were related to *Caminibacter* spp., clone BSaClB29 was related to ATP citrate lyase from *Candidatus Arcobacter sulfidicus* and *Sulfurimonas autotrophica*, and clone BAaClB37 was related to the sequence of *Hydrogenimonas thermophila* (Fig. 2).

Similarly, 94% (17/18) of the *aclA* clones from Broken Spur were related to the *Aquificales*, and formed two clusters represented by clones BSaClA30 and BSaClA17, which shared 91 and 92% sequence identity to the AclA from *Persephonella marina*, respectively (Fig. 3). Only 6% (1/18) of the Broken Spur *aclA* clones, represented by clone BSaClA20, were related to the *Epsilonproteobacteria* (Fig. 3). Phylogenetic analysis placed clone BSaClA20 in a cluster with the AclA from *C. mediatlanticus*, *C. hydrogenophilus*, *Hydrogenimonas thermophila*, and *Lebetimonas acidiphila* (Fig. 3).

Discussion

Comparative analysis of the microbial diversity in black smokers from the MAR

Phylogenetic analysis of the 16S rRNA genes obtained from the microbial communities of the middle and outside sections of the Rainbow chimney did not show significant

differences: *Epsilonproteobacteria* dominated the 16S rRNA gene libraries derived from the microbial communities associated with both sections of the chimney, and they were distributed in two main clusters of sequences (Fig. 1a). The finding that a high proportion (40%; 22/55) of all the clones retrieved from the Rainbow chimney were closely related to hydrogen-oxidizing *Caminibacter* spp. (up to 100% sequence identity) is consistent with the previous isolation from this site of three thermophilic, chemolithoautotrophic, hydrogen-oxidizing *Epsilonproteobacteria*, *C. profundus*, *C. mediatlanticus*, and *Caminibacter* sp. strain TB1 (Miroshnichenko et al. 2004; Voordeckers et al. 2005) and with the high concentration of hydrogen measured in Rainbow hydrothermal emissions (Charlou et al. 2002). Overall, the Rainbow black smoker communities showed a very limited diversity, and all the cultured relatives to the Rainbow clones were strictly anaerobic thermophiles (e.g., *Caminibacter* spp., *Desulfonauticus submarinus*) or hyperthermophiles (e.g., *Archaeoglobus* spp.) (Figs. 1a, 2). The thermophilic and anaerobic nature of the Rainbow community implies a relatively low dilution of the reduced hydrothermal fluids with cold, oxygenated seawater within the chimney wall. In contrast, two independent studies (Lopez-Garcia et al. 2003; Nercessian et al. 2005b) showed that the microbial communities of hydrothermally influenced sediments collected within the limits of the Rainbow vent field were more phylogenetically diverse, and had a higher representation of pelagic microbial taxa than the Rainbow chimney. The sediment communities investigated in both these studies, which are likely to be less impacted by hydrothermal fluids than chimneys, included only a few epsilonproteobacterial clones, which were related to group B (which comprises members of the genus *Sulfurimonas*) and to group F (which comprises *Sulfurovum lithotrophicum*), and none of the sediment clones was related to thermophilic microorganisms.

In contrast to the Rainbow black smoker community, only 5% (2/37) of all the bacterial clones retrieved from the two Logatchev samples were related to the *Epsilonproteobacteria* (Fig. 1b), and none of these sequences were related to the *Caminibacter*/*Nautilia* group (Fig. 1a). However, similarly to the microbial communities of the Rainbow sediments (Lopez-Garcia et al. 2003; Nercessian et al. 2005b), the few epsilonproteobacterial clones retrieved from the Logatchev chimney were related to two microaerobic, mesophilic, sulfur and thiosulfate oxidizing bacteria, *Sulfurovum lithotrophicum* (group F) and *Sulfurimonas autotrophica* (group B) (Inagaki et al. 2003, 2004), which do not use hydrogen as an electron donor. The remaining sequences from the Logatchev samples were phylogenetically diverse, included a relatively large fraction of *Gamma*- and *Alphaproteobacteria* [46% (17/

37) and 30% (11/37), respectively; Fig. 1b], and none of the bacterial and archaeal clones retrieved from the Logatchev samples were related to thermophilic microorganisms (Figs. 1a, 4). Furthermore, the gene encoding for the beta subunit of ATP citrate lyase could not be amplified from this chimney, suggesting that the use of the rTCA cycle for carbon dioxide fixation was not widespread throughout the autotrophic fraction of the Logatchev community. The mesophilic, aerobic and microaerobic nature of the Logatchev bacterial community implies that extensive mixing (dilution) of the high temperature, reduced hydrothermal fluid with cold, oxygen-rich seawater may be occurring within the walls of the chimneys investigated in our study. For comparison, in a recent study Perner et al. (2007) investigated high temperature fluids and chimney samples also collected at the Logatchev vent site and, differently from our study, found a dominance of clones related to the *Epsilonproteobacteria* (up to 49% of the bacterial clones) and a smaller fraction of sequences related to other thermophilic bacteria. The differences in the composition of the Rainbow and Logatchev black smoker communities described in our study, and between the Logatchev chimneys investigated by Perner et al. (2007) and in our study, imply that the microhabitats within the chimney walls, which are in part defined by redox and temperature gradients, may be highly variable from site to site and from chimney to chimney within the same site. Although both Rainbow and Logatchev are ultramafic-hosted vent systems, there are substantial differences in the fluid chemistry at these sites. For instance, rare earth elements and transition metals (in particular iron and manganese) are much more abundant in the hydrothermal fluids at Rainbow than at Logatchev (Douville et al. 2002; Marques et al. 2006). Moreover, geochemical differences have been reported between black smokers within the Logatchev field, mostly related to subsurface mixing with seawater, which results in cooling and chemical alteration of the fluids emanating from some of the chimneys (Schmidt et al. 2006). While the thermophilic and mesophilic nature of the Rainbow and Logatchev communities, respectively, may not be directly related to the temperatures of the fluids emitted by the two chimneys (the temperature of the Logatchev fluid was higher than that of the Rainbow fluid; Table 1), it is possible that specific differences in the fluid chemistry (and therefore in the mineral composition) of the two sulfide structures may affect the temperature and redox gradients within the chimney walls. In particular, differences in the permeability of the mineral structures may influence the flux of hydrothermal fluids through the Rainbow and Logatchev chimney walls, by increasing or decreasing the amount of fluid/seawater mixing, and in turn affect the temperature and redox gradients (i.e., the availability of the

geothermal sources of energy, such as molecular hydrogen). These differences could then be reflected in the composition of the microbial communities at the two sites.

Potential for anaerobic methane oxidation in the Logatchev archaeal community

About 62% (13/21) of the archaeal clones retrieved from the Logatchev black smokers were affiliated with the ANME-2 lineage of the *Methanosarcinales*, which is involved in the anaerobic oxidation of methane (Orphan et al. 2001). While ANME-2 related sequences have been previously recovered from marine hydrothermal sediments (Teske et al. 2002) and, more recently, one sequence was recovered from hydrothermal emissions at Logatchev (Perner et al. 2007), this is the first report of abundant ANME sequences detected in a 16S rRNA gene library derived from a microbial community associated with a black smoker. Interestingly, no sequences related to SRB were detected in the Logatchev chimneys (Fig. 1a). While archaea involved in the anaerobic oxidation of methane usually occur in association with sulfate-reducing bacteria (SRB), in some instances SRB-free ANME association have also been observed (Orphan et al. 2002). However, the fact that we did not detect SRB in the Logatchev libraries does not represent evidence, per se, of the occurrence of SRB-free ANME associations in these samples. The possibility that anaerobic methane oxidation occurs within the chimney walls at Logatchev is consistent with the very high concentrations of methane (up to 3.5 mm) (Charlou et al. 1998; Schmidt et al. 2006) that have been measured in hydrothermal fluids at this site, and with the detection of methyl coenzyme M reductase encoding genes (*mcrA*) related to the ANME lineage from the Logatchev community (Reed and Vetriani, unpublished results). However, further work to assess methane oxidation rates and/or to detect molecular signatures of the ANME lineages will be necessary to test this hypothesis.

ATP citrate lyase and rTCA cycle in cultures and in vent natural communities

In this study we carried out, for the first time, a comparative survey of the *aclA* and *aclB* genes from natural vent microbial communities (Figs. 2, 3). In addition, we sequenced the *aclA* and *aclB* genes from representative *Epsilonproteobacteria* isolated from different deep-sea hydrothermal vents, further defining the phylogeny of this locus (Figs. 2, 3). Finally, we measured the activities of the enzymes of the rTCA cycle in *C. mediatlanticus* which, along with the detection of the *aclBA* genes, provided full evidence that CO₂ fixation occurs via the rTCA cycle in this strain (Table 2). This is consistent with previously

published data that showed the activity of rTCA-related enzymes in other *Epsilonproteobacteria* (Hügler et al. 2005; Takai et al. 2005a).

In line with the 16S rRNA gene analyses, all the Rainbow *aclB* sequences were closely related to *Caminibacter* spp. (Fig. 2). Combined with the enzymatic activities measured in *C. mediatlanticus* (Table 2), these results strongly suggest that members of the *Caminibacter* genus are the main primary producers in the Rainbow bacterial community. Our inability to detect *aclB* sequences in the Logatchev community may be explained by the low relative abundance of *Epsilonproteobacteria* in this sample [5% (2/37); Fig. 1b].

In contrast to the Rainbow community, the Broken Spur *aclA* and *aclB* libraries were dominated by sequences related to the *Aquificales*, while *Epsilonproteobacteria* were much less represented. In particular, our survey of the beta subunit of the ATP citrate lyase from Broken Spur revealed a novel group of *AclB* sequences (BS*aclB*9, 15, 21 and 31) related to uncultured *Aquificales* previously detected at vents located on the EPR (Fig. 2; Campbell et al. 2004; Hügler et al. 2007). Furthermore, we detected an analogous group of *AclA* clones (represented by BS*aclA*17) that did not include any sequence from cultured organisms, and that formed a lineage distinct from the *Persephonella marina* enzyme (Fig. 3). It is worth noting that the relative proportion of *Aquificales*- and *Epsilonproteobacteria*-related sequences was highly conserved in the *aclA* (94 and 6%, respectively) and *aclB* (86 and 14%, respectively) libraries from the Broken Spur black smoker community. Comparative phylogenetic analyses of the 16S rRNA gene and the alpha subunit of the ATP citrate lyase (*AclA*) from the Broken Spur enrichment culture (grown under anaerobic, autotrophic conditions at 65°C) consistently showed that this enrichment was dominated by an organism closely related to *Desulfurobacterium* spp. (Figs. 1a, 3).

Overall, the ATP citrate lyase gene data for the Broken Spur natural community combined with the enrichment studies, suggest that thermophilic bacteria related to the *Aquificales* (*Persephonella* and *Desulfurobacterium* genera) are the dominant primary producers using the rTCA for CO₂ fixation in the Broken Spur community.

Conclusion

In conclusion, comparative analyses of 16S rRNA and ATP citrate lyase genes indicated that the three MAR hydrothermal vent chimneys investigated in this study host very different microbial assemblages, probably as a consequence of differences in the fluid chemistry, mineral composition, redox and temperature gradients at the

three sites. *Caminibacter*- and *Archaeoglobus*-related sequences dominated the Rainbow chimney, suggesting that thermophilic, autotrophic hydrogen oxidation and hyperthermophilic sulfate reduction were the main energy yielding pathways in this environment. The Logatchev bacterial community included several sequences related to sulfur-oxidizing bacteria and, in general, it appeared to be mesophilic and microaerobic. The archaeal component of the Logatchev community was dominated by sequences related to the ANME-2 lineage, suggesting that anaerobic oxidation of methane may also be occurring in this environment. Finally, comparative analyses the 16S rRNA and ATP citrate lyase genes from Rainbow suggested that *Epsilonproteobacteria* were the main bacterial primary producers using the rTCA cycle for CO₂ fixation at this site, while the primary producers in the Broken Spur chimney were dominated by *Aquificales* of the genera *Desulfurobacterium* and *Persephonella*.

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