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

Running title: rRNA and ATP citrate lyase genes in deep-sea vent microbial communities

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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.

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

66 (Nercessian et al. 2003, Schrenk et al. 2003, Takai et al. 1999, Takai et al. 2001). In one
67 of these studies, the majority of 16S rRNA gene sequences obtained from the exterior of
68 the chimney were related to archaeal taxa previously recovered from benthic and pelagic
69 environments, including the crenarchaeal marine group I and uncultured benthic
70 Euryarchaeota. In contrast, the interior regions of the chimney were colonized by
71 methanogens, *Thermococcales*, and *Archaeoglobales*, in addition to uncultured
72 crenarchaeal phylotypes related to sequences previously isolated from deep subsurface
73 habitats (Schrenk et al. 2003). Culture independent studies of functional genes in black
74 smokers further confirmed the occurrence of thermophilic and mesophilic methanogens,
75 and revealed the presence of sulfate reducing bacteria and archaea and of methanotrophic
76 bacteria (Nakagawa et al. 2004, Nercessian et al. 2005a). Furthermore, 16S rRNA-based
77 studies of vent microbial communities indicated that *Epsilonproteobacteria* were
78 associated with sulfide structures (Hoek et al. 2003, Longnecker et al. 2001), and showed
79 that between 66 and 98% of the microorganisms associated with various types of
80 colonization substrates that were deployed in the vicinity of chimney orifices belonged to
81 this class of the Proteobacteria (Alain et al. 2004, Lopez-Garcia et al. 2003).
82 Recent work on the isolation of pure cultures from black smokers is
83 complementing the culture-independent approaches by providing physiological
84 information, for example suggesting that specific groups of organisms occupy discrete
85 temperature niches within the chimney structures. For instance, under autotrophic
86 conditions, temperatures between 30-65°C appear to best support growth of anaerobic or
87 microaerobic *Epsilonproteobacteria* (e.g., *Nautilia, Caminibacter, Hydrogenimonas*,
88 etc.), while temperatures between 50-80°C generally support growth of thermophiles of

89 the phylum *Aquificae* (e.g., *Desulfurobacterium, Thermovibrio, Persephonella*), and
90 growth temperatures >75°C for the most part select for hyperthermophilic archaea
91 (Miroshnichenko et al. 2006). In particular, several new species of *Epsilonproteobacteria*
92 have been isolated from deep-sea hydrothermal vents during the past few years (reviewed
93 in (Campbell et al. 2006, Miroshnichenko et al. 2006)). Along with the culture-
94 independent studies, physiological information derived from these pure cultures is
95 helping to establish the relevance of *Epsilonproteobacteria* as primary producers, early
96 colonizers, as well as metazoan epi- and endosymbionts at deep-sea vents (Campbell et
97 al. 2006).
98 One of the pathways for autotrophic CO_2 fixation in some anaerobic and
99 microaerobic bacteria is based on a tricarboxylic acid cycle which operates in reverse
100 (Buchanan et al. 1990). This reductive tricarboxylic acid cycle (rTCA) leads to the
101 fixation of CO_2 and to the synthesis of acetyl coenzyme A, which is carboxylated to
102 pyruvate and then used in further anabolic processes. The three key enzymes that are
103 essential to run the rTCA cycle are ATP citrate lyase, 2-oxoglutarate:ferredoxin
104 oxidoreductase and fumarate reductase. The first evidence for the occurrence of the rTCA
105 cycle in deep-sea hydrothermal vent microbial communities was obtained from
106 metagenome studies of the episymbiotic community associated with the vent polychaete
107 *Alvinella pompejana* and from rTCA-related gene surveys (Campbell et al. 2004,
108 Campbell et al. 2003). Recent reports demonstrated that pure cultures of
109 *Epsilonproteobacteria* and *Aquificae*, which include representatives of hydrothermal vent
110 bacteria, fix carbon dioxide via the rTCA cycle (Ferrera et al. 2007, Hügler et al. 2007,
111 Hügler et al. 2005, Takai et al. 2005a).

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

123 **Materials and Methods**

124 **Sample collection.** Fragments of active, high temperature black smoker chimneys were
125 collected from the "Rainbow" (36º 14'N, 33º 54'W; depth 2305 m), "Logatchev" (14º
126 45'N, 44º 58'W; depth 3000 m), and "Broken Spur" (29º 10'N, 43º 10'W; depth 3060 m)
127 vent fields on the Mid-Atlantic Ridge, during a cruise aboard R/V *Atlantis* (cruise AT 05-
128 03, July 2001), and from the East Pacific Rise (EPR; 9° 10'N, 104° 17'W; depth 2500 m)
129 during cruise AT 11-10 (April 2004). The samples were collected using the manipulator
130 of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest
131 of the dive. On the surface, samples were transferred to the ship's laboratory and
132 subsamples were either frozen at -80°C for nucleic acid extraction, or stored at 4ºC under
133 a dinitrogen atmosphere for enrichments and isolations. Sample locations are summarized
134 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, Vetriani 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 -80ºC. 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 (Miroshnichenko 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 performed in stoppered 0.5 ml glass cuvettes at 55°C. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm ($\varepsilon_{365\ nm}$ NAD(P)H = 3.4 • 10^3 M^{-1} cm^{-1}). Reactions involving benzyl viologen (BV) were followed

158 spectrophotometrically at 578 nm ($\varepsilon_{578\ nm}$ BV = 8.6 • 10^3 M^{-1} cm^{-1}).

159 ATP citrate lyase activity was determined according to (Hügler et al. 2007). 2-

160 Oxoglutarate:BV oxidoreductase, pyruvate:BV oxidoreductase, fumarate reductase,

161 malate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and

162 pyruvate dehydrogenase activities were measured according to references (Hügler et al.

163 2003, Hügler et al. 2005).

164 **DNA extraction.** Total genomic DNA was extracted from 1.7-2.1 g of four chimney

165 subsamples (Rainbow 3678 out, Logatchev 3667, Logatchev 3668, and Broken Spur

166 3675) using the UltraClean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach,

167 CA, USA) with the following protocol modifications: Bead beating was extended for 20

168 seconds and it was followed by heating at 70°C for 5 min. For subsample Rainbow

169 3678C-mid, 14.59 g was extracted using the Mega Prep UltraClean Soil DNA according

170 to the manufacturer's specifications (Mo Bio Laboratories, Solana Beach, CA, USA).

171 Cells obtained from 10 ml of pure cultures and from an enrichment culture inoculated

172 with a chimney sample from the Broken Spur site were extracted using the UltraClean

173 Microbial DNA Isolation Kit according to the protocol supplied with the kit (Mo Bio

174 Laboratories, Solana Beach, CA, USA).

175 **DNA amplification by PCR**. Archaeal and bacterial 16S rRNA genes were amplified

176 using the archaeal domain specific forward primer 16F (5'-CTGGTTGATCCTGCCAG-

177 3') and the bacterial domain specific forward primer 8F (5'-

178 AGAGTTTGATCCTGGCTCAG-3'), respectively, in combination with universal primer

179 1517R (5'-ACGGCTACCTTGTTACGACTT-3'). PCR conditions for amplification

180 reactions were as follows: 35 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC

for 30 s, and extension at 72ºC 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. (Campbell et al. 2003), while primers F2 and R5 and PCR conditions as described by Hügler et al. (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 (Qiagen, 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 to EF644685, EF644759 to 644814, and EF644827 to 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, chemolithoautorophic *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

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

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

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

271 **Phylogenetic analysis of the Rainbow microbial community.** The majority (97%;
272 34/35) of the bacterial clones retrieved from the Rainbow site were related to the

273 *Epsilonproteobacteria*, while only 3% (1/35) were related to the *Deltaproteobacteria*.
274 The bacterial 16S rRNA gene libraries from both the outside and middle sections of the
275 Rainbow chimney were dominated by *Epsilonproteobacteria*, accounting for eleven of
276 the twelve bacterial phylotypes sequenced from this site. Phylogenetic analysis placed six
277 of these phylotypes (ROB3, ROB4, ROB5, ROB6, RMB3 and RMB5, representing about
278 40% (22/55) of all the clones from the Rainbow chimney) in the *Caminibacter*/*Nautilia*
279 group (also defined as Group D; Fig. 1A). The nearest cultivated relatives to these
280 phylotypes are *C. mediatlanticus* and *Caminibacter* sp. strain TB1 (99-100% sequence
281 identity), both of which are hydrogen-oxidizing and nitrate-reducing thermophiles
282 (optimum growth temperature 50-55°C), and were isolated from the Rainbow
283 hydrothermal vent field (Voordeckers et al. 2005). Four phylotypes from the Rainbow
284 site (RMB1, RMB2, RMB4, and ROB1) clustered into a group that was related (96-99%
285 sequence identity) to epsilonprotebacterial sequences retrieved from an *in situ* growth
286 chamber deployed at the Snake Pit hydrothermal vent site on the MAR (Reysenbach et al.
287 2000), and to organisms enriched from the tubes of *Alvinella pompejana* worms
288 (Cambon–Bonavita, unpublished results). Phylotype ROB2 was related to clones
289 obtained from the microbial community associated with the invertebrate *Paralvinella*
290 *palmiformis* (90% sequence identity) (Alain et al. 2002a, Haddad et al. 1995), while the
291 closest cultured relative to this phylotype was *Sulfurospirillum halorespirans* (89%
292 sequence identity) (Luijten et al. 2003). Clone ROB7, the only Rainbow phylotype found
293 outside of the *Epsilonproteobacteria*, was related (94% sequence identity) to
294 *Desulfonauticus submarinus*, a moderately thermophilic (optimum growth temperature

295 45°C), sulfate-reducing *Deltaproteobacterium* isolated from 13°N on the EPR (Fig. 1A)
296 (Pikuta et al. 1998).

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

302 The gene encoding for the small subunit of the ATP citrate lyase, *aclB*, was
303 amplified from DNA extracted from the microbial community of the Rainbow black
304 smoker. All the Rainbow *aclB* sequences, represented by clones RaclB16, 7 and 21, were
305 placed within the *Caminibacter* cluster, and their amino acid sequence was 98-99%
306 identical to the ATP citrate lyase of *C. mediatlanticus* (Fig. 2).

307 **Phylogenetic analysis of the Logatchev microbial community.** The microbial
308 communities from the two samples collected at the Logatchev vent were similar to each
309 other, but their diversity was much higher than that of the Rainbow community, and for
310 the most part these sequences were related to mesophilic, aerobic or microaerobic
311 bacteria (Figs. 1A and B). The epsilonproteobacterial phylotypes retrieved from the
312 Logatchev black smokers (5%; 2/37) of the total bacterial clones; Fig 1B) were
313 phylogenetically distinct from the Rainbow *Epsilonproteobacteria* (Fig. 1A). The closest
314 cultured relatives to phylotypes L7B13 and L7B15 were *Sulfurovum lithotrophicum*
315 (Group F; 92% sequence identity) and *Sulfurimonas autotrophica* (Group B; 94%
316 sequence identity), respectively, two mesophilic (optimum growth temperature 25-30°C),

317 microaerobic, sulfur and thiosulfate-oxidizing bacteria isolated from the Okinawa Trough
318 (Inagaki et al. 2003, Inagaki et al. 2004).

319 The alphaproteobacterial related phylotypes retrieved from the Logatchev black
320 smokers (30% of the bacterial clones; 11/37; Fig 1B) were clustered into four main
321 groups related, for the most part, to sulfur-oxidizing bacteria (Fig. 1A). L7B6, L8B8, and
322 L8B9, accounting for approximately 10% (4/37) of the total clones from Logatchev,
323 grouped closely with the genus *Sulfitobacter* (97% sequence identity) and more distantly
324 (93-96% sequence identity) with *Marinosulfonomonas methylotropha* (Holmes et al.
325 1997). A second group of *Alphaproteobacteria* (L8B1, L8B2, and L8B4), accounting for
326 13% (5/37) of the total bacterial clones from Logatchev, were closely related (96%
327 sequence identity) to a 16S rRNA gene sequence (IndB1-38) retrieved from an inactive
328 chimney of the Kairei hydrothermal vent field on the Indian Ocean Ridge (Suzuki et al.
329 2004).

330 The gammaproteobacterial phylotypes retrieved from the Logatchev black smoker
331 samples (46%; of the bacterial clones; 17/37) were clustered into five groups and were,
332 for the most part, related to mesophilic lithotrophs (Fig. 1A). Clones L7B8, L7B11,
333 L7B12 and L8B5 were related (92-93% identity) to clones retrieved from a sediment
334 sample located above a gas hydrate deposit on the Cascadia Margin, Oregon (Knittel et
335 al. 2003). The nearest cultivated relative to these phylotypes (91-92% sequence identity)
336 was *Thioalkalispira microaerophilia,* a sulfur oxidizing lithoautotroph isolated from a
337 soda lake in Egypt (Sorokin et al. 2002). A second group of gammaproteobacterial
338 phylotypes, represented by L7B4, L7B5, and L8B6, was related to *Thiomicrospira*
339 *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 30ºC) 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

363 (the sequences of L7A1 and L7A4 were 94-97% identical to the 16S rRNA gene from
364 representatives of the ANME-2 group).

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

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

377 The genes encoding for the large and small subunits of the ATP citrate lyase, *aclA*
378 and *aclB*, were amplified from the Broken Spur enrichment culture. In line with the 16S
379 rRNA gene results, phylogenetic analysis placed the AclB sequences retrieved from the
380 Broken Spur enrichment culture, represented by clones BSEaclB2 and 5, in a cluster
381 related to the *Aquificales* (Fig. 2). The deduced amino acid sequences of the BSEaclB
382 clones were most similar to the AclB of *Persephonella marina* (87-88% identity), to that
383 of the Broken Spur chimney clones (86-89% identity to clones BSaclB9, 15, 21 and 31)
384 and to the AclB of *Desulfurobacterium thermolithotrophum* (Fig. 2). The AclA

385 sequences retrieved from the Broken Spur enrichment culture, represented by clones
386 BSEaclA2, 3, 4, and 5, were related to *Desulfurobacterium* spp. (Fig. 3).
387 The *aclA* and *aclB* genes were also amplified from DNA extracted directly from
388 the microbial community of the Broken Spur black smoker. In contrast to the Rainbow
389 community, both the *aclA* and *aclB* libraries constructed from DNA extracted from the
390 Broken Spur black smoker community were dominated by clones related to the
391 *Aquificales*, while a smaller number of clones were related to the *Epsilonproteobacteria*.
392 Phylogenetic analyses placed 86% (30/35) of the amino acid sequences deduced from the
393 *aclB* clones from Broken Spur, represented by clones BSaclB9, 15, 21 and 31, in a novel
394 cluster that is only distantly related to the ATP citrate lyase from the genera
395 *Thermovibrio, Desulfurobacterium* and *Persephonella*, within the *Aquificales* (Fig. 2).
396 The AclB amino acid sequences deduced from clones BSaclB15 and 31 were most
397 similar to the ATP citrate lyase of an uncultured member of the *Aquificales* (clone 820-
398 A8; 97% sequence identity). Only 14% (5/35) of the Broken Spur *aclB* clones were
399 related to the *Epsilonproteobacteria*: three of these clones, BSaclB7, 32 and 36, were
400 related to *Caminibacter* spp., clone BSaclB29 was related to ATP citrate lyase from
401 *Candidatus Arcobacter sulfidicus* and *Sulfurimonas autotrophica*, and clone BAaclB37
402 was related to the sequence of *Hydrogenimonas thermophila* (Fig. 2).
403 Similarly, 94% (17/18) of the *aclA* clones from Broken Spur were related to the
404 *Aquificales*, and formed two clusters represented by clones BSaclA30 and BSaclA17,
405 which shared 91 and 92% sequence identity to the AclA from *Persephonella marina*,
406 respectively (Fig. 3). Only 6% (1/18) of the Broken Spur *aclA* clones, represented by
407 clone BSaclA20, were related to the *Epsilonproteobacteria* (Fig. 3). Phylogenetic

analysis placed clone BSaclA20 in a cluster with the AclA from *C. mediatlanticus*, *C. hydrogeniphilus*, *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 and 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 epsilonprotebacterial 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, Inagaki et al. 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 and 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

454 dioxide fixation was not widespread throughout the autotrophic fraction of the Logatchev
455 community. The mesophilic, aerobic and microaerobic nature of the Logatchev bacterial
456 community implies that extensive mixing (dilution) of the high temperature, reduced
457 hydrothermal fluid with cold, oxygen-rich seawater may be occurring within the walls of
458 the chimneys investigated in our study. For comparison, in a recent study Perner et al.
459 (Perner et al. 2007) investigated high temperature fluids and chimney samples also
460 collected at the Logatchev vent site and, differently from our study, found a dominance of
461 clones related to the *Epsilonproteobacteria* (up to 49% of the bacterial clones) and a
462 smaller fraction of sequences related to other thermophilic bacteria. The differences in
463 the composition of the Rainbow and Logatchev black smoker communities described in
464 our study, and between the Logatchev chimneys investigated by Perner et al. (Perner et
465 al. 2007) and in our study, imply that the microhabitats within the chimney walls, which
466 are in part defined by redox and temperature gradients, may be highly variable from site
467 to site and from chimney to chimney within the same site. Although both Rainbow and
468 Logatchev are ultramafic-hosted vent systems, there are substantial differences in the
469 fluid chemistry at these sites. For instance, rare earth elements and transition metals (in
470 particular iron and manganese) are much more abundant in the hydrothermal fluids at
471 Rainbow than at Logatchev (Douville et al. 2002, Marques et al. 2006). Moreover,
472 geochemical differences have been reported between black smokers within the Logatchev
473 field, mostly related to subsurface mixing with seawater, which results in cooling and
474 chemical alteration of the fluids emanating from some of the chimneys (Schmidt et al.
475 2006). While the thermophilic and mesophilic nature of the Rainbow and Logatchev
476 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 and 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 and 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 CO_2 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

523 community. Our inability to detect *aclB* sequences in the Logatchev community may be
524 explained by the low relative abundance of *Epsilonproteobacteria* in this sample (5%
525 (2/37); Fig. 1B).

526 In contrast to the Rainbow community, the Broken Spur *aclA* and *aclB* libraries
527 were dominated by sequences related to the *Aquificales*, while *Epsilonproteobacteria*
528 were much less represented. In particular, our survey of the beta subunit of the ATP
529 citrate lyase from Broken Spur revealed a novel group of AclB sequences (BSaclB9, 15,
530 21 and 31) related to uncultured *Aquificales* previously detected at vents located on the
531 EPR (Fig. 2) (Campbell et al. 2004, Hügler et al. 2007). Furthermore, we detected an
532 analogous group of AclA clones (represented by BSaclA17) that did not include any
533 sequence from cultured organisms, and that formed a lineage distinct from the
534 *Persephonella marina* enzyme (Fig. 3). It is worth noting that the relative proportion of
535 *Aquificales*- and *Epsilonproteobacteria*-related sequences was highly conserved in the
536 *aclA* (94 and 6%, respectively) and *aclB* (86 and 14%, respectively) libraries from the
537 Broken Spur black smoker community. Comparative phylogenetic analyses of the 16S
538 rRNA gene and the alpha subunit of the ATP citrate lyase (AclA) from the Broken Spur
539 enrichment culture (grown under anaerobic, autotrophic conditions at 65°C) consistently
540 showed that this enrichment was dominated by an organism closely related to
541 *Desulfurobacterium* spp. (Figs. 1A and 3).

542 Overall, the ATP citrate lyase gene data for the Broken Spur natural community
543 combined with the enrichment studies, suggest that thermophilic bacteria related to the
544 *Aquificales* (*Persephonella* and *Desulfurobacterium* genera) are the dominant primary
545 producers using the rTCA for CO_2 fixation in the Broken Spur community.

Conclusions. 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 the 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 *Epsiloproteobacteria* were the main bacterial primary producers using the rTCA cycle for CO_2 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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Figure legends

Figure 1. Phylogenetic analysis of bacterial 16S rRNA gene sequences from the Rainbow (ROB# and RMB# indicate clone numbers from the outside and middle layer, respectively) and Logatchev (L7B# and L8B# indicate clone numbers from dives 3667 and 3668, respectively) black smokers, and from the Broken Spur enrichment culture (represented by Broken Spur Enrichment clone 8). 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 1000 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 (A). Frequency of bacterial 16S rRNA gene clones from the Logatchev black smokers (B).

Figure 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 (RaclB#) and Broken Spur (BSaclB#) black smokers, and from the Broken Spur enrichment culture (BSEaclB#). Percentages greater than 50% of bootstrap resampling (of 1000 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.

Figure 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 (BSaclA#) black smoker and enrichment culture (BSEaclA#). Percentages greater than 50% of bootstrap resampling (of 1000 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.

Figure 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 1000 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.

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

Running title: rRNA and ATP citrate lyase genes in deep-sea vent microbial communities

Keywords: *Epsilonproteobacteria*, *Aquificales*, ANME, ATP citrate lyase, black smoker, deep-sea vent, rTCA cycle

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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.

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

66 (Nercessian et al. 2003, Schrenk et al. 2003, Takai et al. 1999, Takai et al. 2001). In one
67 of these studies, the majority of 16S rRNA gene sequences obtained from the exterior of
68 the chimney were related to archaeal taxa previously recovered from benthic and pelagic
69 environments, including the crenarchaeal marine group I and uncultured benthic
70 Euryarchaeota. In contrast, the interior regions of the chimney were colonized by
71 methanogens, *Thermococcales*, and *Archaeoglobales*, in addition to uncultured
72 crenarchaeal phylotypes related to sequences previously isolated from deep subsurface
73 habitats (Schrenk et al. 2003). Culture independent studies of functional genes in black
74 smokers further confirmed the occurrence of thermophilic and mesophilic methanogens,
75 and revealed the presence of sulfate reducing bacteria and archaea and of methanotrophic
76 bacteria (Nakagawa et al. 2004, Nercessian et al. 2005a). Furthermore, 16S rRNA-based
77 studies of vent microbial communities indicated that *Epsilonproteobacteria* were
78 associated with sulfide structures (Hoek et al. 2003, Longnecker et al. 2001), and showed
79 that between 66 and 98% of the microorganisms associated with various types of
80 colonization substrates that were deployed in the vicinity of chimney orifices belonged to
81 this class of the Proteobacteria (Alain et al. 2004, Lopez-Garcia et al. 2003).
82 Recent work on the isolation of pure cultures from black smokers is
83 complementing the culture-independent approaches by providing physiological
84 information, for example suggesting that specific groups of organisms occupy discrete
85 temperature niches within the chimney structures. For instance, under autotrophic
86 conditions, temperatures between 30-65°C appear to best support growth of anaerobic or
87 microaerobic *Epsilonproteobacteria* (e.g., *Nautilia, Caminibacter, Hydrogenimonas*,
88 etc.), while temperatures between 50-80°C generally support growth of thermophiles of

89 the phylum *Aquificae* (e.g., *Desulfurobacterium, Thermovibrio, Persephonella*), and
90 growth temperatures >75°C for the most part select for hyperthermophilic archaea
91 (Miroshnichenko et al. 2006). In particular, several new species of *Epsilonproteobacteria*
92 have been isolated from deep-sea hydrothermal vents during the past few years (reviewed
93 in (Campbell et al. 2006, Miroshnichenko et al. 2006)). Along with the culture-
94 independent studies, physiological information derived from these pure cultures is
95 helping to establish the relevance of *Epsilonproteobacteria* as primary producers, early
96 colonizers, as well as metazoan epi- and endosymbionts at deep-sea vents (Campbell et
97 al. 2006).
98 One of the pathways for autotrophic CO_2 fixation in some anaerobic and
99 microaerobic bacteria is based on a tricarboxylic acid cycle which operates in reverse
100 (Buchanan et al. 1990). This reductive tricarboxylic acid cycle (rTCA) leads to the
101 fixation of CO_2 and to the synthesis of acetyl coenzyme A, which is carboxylated to
102 pyruvate and then used in further anabolic processes. The three key enzymes that are
103 essential to run the rTCA cycle are ATP citrate lyase, 2-oxoglutarate:ferredoxin
104 oxidoreductase and fumarate reductase. The first evidence for the occurrence of the rTCA
105 cycle in deep-sea hydrothermal vent microbial communities was obtained from
106 metagenome studies of the episymbiotic community associated with the vent polychaete
107 *Alvinella pompejana* and from rTCA-related gene surveys (Campbell et al. 2004,
108 Campbell et al. 2003). Recent reports demonstrated that pure cultures of
109 *Epsilonproteobacteria* and *Aquificae*, which include representatives of hydrothermal vent
110 bacteria, fix carbon dioxide via the rTCA cycle (Ferrera et al. 2007, Hügler et al. 2007,
111 Hügler et al. 2005, Takai et al. 2005a).

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

123 **Materials and Methods**

124 **Sample collection.** Fragments of active, high temperature black smoker chimneys were
125 collected from the "Rainbow" (36º 14'N, 33º 54'W; depth 2305 m), "Logatchev" (14º
126 45'N, 44º 58'W; depth 3000 m), and "Broken Spur" (29º 10'N, 43º 10'W; depth 3060 m)
127 vent fields on the Mid-Atlantic Ridge, during a cruise aboard R/V *Atlantis* (cruise AT 05-
128 03, July 2001), and from the East Pacific Rise (EPR; 9° 10'N, 104° 17'W; depth 2500 m)
129 during cruise AT 11-10 (April 2004). The samples were collected using the manipulator
130 of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest
131 of the dive. On the surface, samples were transferred to the ship's laboratory and
132 subsamples were either frozen at -80°C for nucleic acid extraction, or stored at 4ºC under
133 a dinitrogen atmosphere for enrichments and isolations. Sample locations are summarized
134 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, Vetriani 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 -80ºC. 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 (Miroshnichenko 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 performed in stoppered 0.5 ml glass cuvettes at 55°C. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm ($\varepsilon_{365\ nm}$ NAD(P)H = 3.4 • 10^3 M^{-1} cm^{-1}). Reactions involving benzyl viologen (BV) were followed

158 spectrophotometrically at 578 nm ($\varepsilon_{578\ nm}$ BV = 8.6 • 10^3 M^{-1} cm^{-1}).

159 ATP citrate lyase activity was determined according to (Hügler et al. 2007). 2-

160 Oxoglutarate:BV oxidoreductase, pyruvate:BV oxidoreductase, fumarate reductase,

161 malate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and

162 pyruvate dehydrogenase activities were measured according to references (Hügler et al.

163 2003, Hügler et al. 2005).

164 **DNA extraction.** Total genomic DNA was extracted from 1.7-2.1 g of four chimney

165 subsamples (Rainbow 3678 out, Logatchev 3667, Logatchev 3668, and Broken Spur

166 3675) using the UltraClean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach,

167 CA, USA) with the following protocol modifications: Bead beating was extended for 20

168 seconds and it was followed by heating at 70°C for 5 min. For subsample Rainbow

169 3678C-mid, 14.59 g was extracted using the Mega Prep UltraClean Soil DNA according

170 to the manufacturer's specifications (Mo Bio Laboratories, Solana Beach, CA, USA).

171 Cells obtained from 10 ml of pure cultures and from an enrichment culture inoculated

172 with a chimney sample from the Broken Spur site were extracted using the UltraClean

173 Microbial DNA Isolation Kit according to the protocol supplied with the kit (Mo Bio

174 Laboratories, Solana Beach, CA, USA).

175 **DNA amplification by PCR**. Archaeal and bacterial 16S rRNA genes were amplified

176 using the archaeal domain specific forward primer 16F (5'-CTGGTTGATCCTGCCAG-

177 3') and the bacterial domain specific forward primer 8F (5'-

178 AGAGTTTGATCCTGGCTCAG-3'), respectively, in combination with universal primer

179 1517R (5'-ACGGCTACCTTGTTACGACTT-3'). PCR conditions for amplification

180 reactions were as follows: 35 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC

for 30 s, and extension at 72ºC 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. (Campbell et al. 2003), while primers F2 and R5 and PCR conditions as described by Hügler et al. (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 (Qiagen, 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 to EF644685, EF644759 to 644814, and EF644827 to 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, chemolithoautorophic *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

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

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

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

271 **Phylogenetic analysis of the Rainbow microbial community.** The majority (97%;
272 34/35) of the bacterial clones retrieved from the Rainbow site were related to the

273 *Epsilonproteobacteria*, while only 3% (1/35) were related to the *Deltaproteobacteria*.
274 The bacterial 16S rRNA gene libraries from both the outside and middle sections of the
275 Rainbow chimney were dominated by *Epsilonproteobacteria*, accounting for eleven of
276 the twelve bacterial phylotypes sequenced from this site. Phylogenetic analysis placed six
277 of these phylotypes (ROB3, ROB4, ROB5, ROB6, RMB3 and RMB5, representing about
278 40% (22/55) of all the clones from the Rainbow chimney) in the *Caminibacter*/*Nautilia*
279 group (also defined as Group D; Fig. 1A). The nearest cultivated relatives to these
280 phylotypes are *C. mediatlanticus* and *Caminibacter* sp. strain TB1 (99-100% sequence
281 identity), both of which are hydrogen-oxidizing and nitrate-reducing thermophiles
282 (optimum growth temperature 50-55°C), and were isolated from the Rainbow
283 hydrothermal vent field (Voordeckers et al. 2005). Four phylotypes from the Rainbow
284 site (RMB1, RMB2, RMB4, and ROB1) clustered into a group that was related (96-99%
285 sequence identity) to epsilonprotebacterial sequences retrieved from an *in situ* growth
286 chamber deployed at the Snake Pit hydrothermal vent site on the MAR (Reysenbach et al.
287 2000), and to organisms enriched from the tubes of *Alvinella pompejana* worms
288 (Cambon–Bonavita, unpublished results). Phylotype ROB2 was related to clones
289 obtained from the microbial community associated with the invertebrate *Paralvinella*
290 *palmiformis* (90% sequence identity) (Alain et al. 2002a, Haddad et al. 1995), while the
291 closest cultured relative to this phylotype was *Sulfurospirillum halorespirans* (89%
292 sequence identity) (Luijten et al. 2003). Clone ROB7, the only Rainbow phylotype found
293 outside of the *Epsilonproteobacteria*, was related (94% sequence identity) to
294 *Desulfonauticus submarinus*, a moderately thermophilic (optimum growth temperature

295 45°C), sulfate-reducing *Deltaproteobacterium* isolated from 13°N on the EPR (Fig. 1A)
296 (Pikuta et al. 1998).

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

302 The gene encoding for the small subunit of the ATP citrate lyase, *aclB*, was
303 amplified from DNA extracted from the microbial community of the Rainbow black
304 smoker. All the Rainbow *aclB* sequences, represented by clones RaclB16, 7 and 21, were
305 placed within the *Caminibacter* cluster, and their amino acid sequence was 98-99%
306 identical to the ATP citrate lyase of *C. mediatlanticus* (Fig. 2).

307 **Phylogenetic analysis of the Logatchev microbial community.** The microbial
308 communities from the two samples collected at the Logatchev vent were similar to each
309 other, but their diversity was much higher than that of the Rainbow community, and for
310 the most part these sequences were related to mesophilic, aerobic or microaerobic
311 bacteria (Figs. 1A and B). The epsilonproteobacterial phylotypes retrieved from the
312 Logatchev black smokers (5%; 2/37) of the total bacterial clones; Fig 1B) were
313 phylogenetically distinct from the Rainbow *Epsilonproteobacteria* (Fig. 1A). The closest
314 cultured relatives to phylotypes L7B13 and L7B15 were *Sulfurovum lithotrophicum*
315 (Group F; 92% sequence identity) and *Sulfurimonas autotrophica* (Group B; 94%
316 sequence identity), respectively, two mesophilic (optimum growth temperature 25-30°C),

317 microaerobic, sulfur and thiosulfate-oxidizing bacteria isolated from the Okinawa Trough
318 (Inagaki et al. 2003, Inagaki et al. 2004).

319 The alphaproteobacterial related phylotypes retrieved from the Logatchev black
320 smokers (30% of the bacterial clones; 11/37; Fig 1B) were clustered into four main
321 groups related, for the most part, to sulfur-oxidizing bacteria (Fig. 1A). L7B6, L8B8, and
322 L8B9, accounting for approximately 10% (4/37) of the total clones from Logatchev,
323 grouped closely with the genus *Sulfitobacter* (97% sequence identity) and more distantly
324 (93-96% sequence identity) with *Marinosulfonomonas methylotropha* (Holmes et al.
325 1997). A second group of *Alphaproteobacteria* (L8B1, L8B2, and L8B4), accounting for
326 13% (5/37) of the total bacterial clones from Logatchev, were closely related (96%
327 sequence identity) to a 16S rRNA gene sequence (IndB1-38) retrieved from an inactive
328 chimney of the Kairei hydrothermal vent field on the Indian Ocean Ridge (Suzuki et al.
329 2004).

330 The gammaproteobacterial phylotypes retrieved from the Logatchev black smoker
331 samples (46%; of the bacterial clones; 17/37) were clustered into five groups and were,
332 for the most part, related to mesophilic lithotrophs (Fig. 1A). Clones L7B8, L7B11,
333 L7B12 and L8B5 were related (92-93% identity) to clones retrieved from a sediment
334 sample located above a gas hydrate deposit on the Cascadia Margin, Oregon (Knittel et
335 al. 2003). The nearest cultivated relative to these phylotypes (91-92% sequence identity)
336 was *Thioalkalispira microaerophilia,* a sulfur oxidizing lithoautotroph isolated from a
337 soda lake in Egypt (Sorokin et al. 2002). A second group of gammaproteobacterial
338 phylotypes, represented by L7B4, L7B5, and L8B6, was related to *Thiomicrospira*
339 *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 30ºC) 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

363 (the sequences of L7A1 and L7A4 were 94-97% identical to the 16S rRNA gene from
364 representatives of the ANME-2 group).

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

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

377 The genes encoding for the large and small subunits of the ATP citrate lyase, *aclA*
378 and *aclB*, were amplified from the Broken Spur enrichment culture. In line with the 16S
379 rRNA gene results, phylogenetic analysis placed the AclB sequences retrieved from the
380 Broken Spur enrichment culture, represented by clones BSEaclB2 and 5, in a cluster
381 related to the *Aquificales* (Fig. 2). The deduced amino acid sequences of the BSEaclB
382 clones were most similar to the AclB of *Persephonella marina* (87-88% identity), to that
383 of the Broken Spur chimney clones (86-89% identity to clones BSaclB9, 15, 21 and 31)
384 and to the AclB of *Desulfurobacterium thermolithotrophum* (Fig. 2). The AclA

385 sequences retrieved from the Broken Spur enrichment culture, represented by clones
386 BSEaclA2, 3, 4, and 5, were related to *Desulfurobacterium* spp. (Fig. 3).
387 The *aclA* and *aclB* genes were also amplified from DNA extracted directly from
388 the microbial community of the Broken Spur black smoker. In contrast to the Rainbow
389 community, both the *aclA* and *aclB* libraries constructed from DNA extracted from the
390 Broken Spur black smoker community were dominated by clones related to the
391 *Aquificales*, while a smaller number of clones were related to the *Epsilonproteobacteria*.
392 Phylogenetic analyses placed 86% (30/35) of the amino acid sequences deduced from the
393 *aclB* clones from Broken Spur, represented by clones BSaclB9, 15, 21 and 31, in a novel
394 cluster that is only distantly related to the ATP citrate lyase from the genera
395 *Thermovibrio, Desulfurobacterium* and *Persephonella*, within the *Aquificales* (Fig. 2).
396 The AclB amino acid sequences deduced from clones BSaclB15 and 31 were most
397 similar to the ATP citrate lyase of an uncultured member of the *Aquificales* (clone 820-
398 A8; 97% sequence identity). Only 14% (5/35) of the Broken Spur *aclB* clones were
399 related to the *Epsilonproteobacteria*: three of these clones, BSaclB7, 32 and 36, were
400 related to *Caminibacter* spp., clone BSaclB29 was related to ATP citrate lyase from
401 *Candidatus Arcobacter sulfidicus* and *Sulfurimonas autotrophica*, and clone BAaclB37
402 was related to the sequence of *Hydrogenimonas thermophila* (Fig. 2).
403 Similarly, 94% (17/18) of the *aclA* clones from Broken Spur were related to the
404 *Aquificales*, and formed two clusters represented by clones BSaclA30 and BSaclA17,
405 which shared 91 and 92% sequence identity to the AclA from *Persephonella marina*,
406 respectively (Fig. 3). Only 6% (1/18) of the Broken Spur *aclA* clones, represented by
407 clone BSaclA20, were related to the *Epsilonproteobacteria* (Fig. 3). Phylogenetic

analysis placed clone BSaclA20 in a cluster with the AclA from *C. mediatlanticus*, *C. hydrogeniphilus*, *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 and 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 epsilonprotebacterial 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, Inagaki et al. 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 and 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

454 dioxide fixation was not widespread throughout the autotrophic fraction of the Logatchev
455 community. The mesophilic, aerobic and microaerobic nature of the Logatchev bacterial
456 community implies that extensive mixing (dilution) of the high temperature, reduced
457 hydrothermal fluid with cold, oxygen-rich seawater may be occurring within the walls of
458 the chimneys investigated in our study. For comparison, in a recent study Perner et al.
459 (Perner et al. 2007) investigated high temperature fluids and chimney samples also
460 collected at the Logatchev vent site and, differently from our study, found a dominance of
461 clones related to the *Epsilonproteobacteria* (up to 49% of the bacterial clones) and a
462 smaller fraction of sequences related to other thermophilic bacteria. The differences in
463 the composition of the Rainbow and Logatchev black smoker communities described in
464 our study, and between the Logatchev chimneys investigated by Perner et al. (Perner et
465 al. 2007) and in our study, imply that the microhabitats within the chimney walls, which
466 are in part defined by redox and temperature gradients, may be highly variable from site
467 to site and from chimney to chimney within the same site. Although both Rainbow and
468 Logatchev are ultramafic-hosted vent systems, there are substantial differences in the
469 fluid chemistry at these sites. For instance, rare earth elements and transition metals (in
470 particular iron and manganese) are much more abundant in the hydrothermal fluids at
471 Rainbow than at Logatchev (Douville et al. 2002, Marques et al. 2006). Moreover,
472 geochemical differences have been reported between black smokers within the Logatchev
473 field, mostly related to subsurface mixing with seawater, which results in cooling and
474 chemical alteration of the fluids emanating from some of the chimneys (Schmidt et al.
475 2006). While the thermophilic and mesophilic nature of the Rainbow and Logatchev
476 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 and 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 and 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 CO_2 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

523 community. Our inability to detect *aclB* sequences in the Logatchev community may be
524 explained by the low relative abundance of *Epsilonproteobacteria* in this sample (5%
525 (2/37); Fig. 1B).

526 In contrast to the Rainbow community, the Broken Spur *aclA* and *aclB* libraries
527 were dominated by sequences related to the *Aquificales*, while *Epsilonproteobacteria*
528 were much less represented. In particular, our survey of the beta subunit of the ATP
529 citrate lyase from Broken Spur revealed a novel group of AclB sequences (BSaclB9, 15,
530 21 and 31) related to uncultured *Aquificales* previously detected at vents located on the
531 EPR (Fig. 2) (Campbell et al. 2004, Hügler et al. 2007). Furthermore, we detected an
532 analogous group of AclA clones (represented by BSaclA17) that did not include any
533 sequence from cultured organisms, and that formed a lineage distinct from the
534 *Persephonella marina* enzyme (Fig. 3). It is worth noting that the relative proportion of
535 *Aquificales*- and *Epsilonproteobacteria*-related sequences was highly conserved in the
536 *aclA* (94 and 6%, respectively) and *aclB* (86 and 14%, respectively) libraries from the
537 Broken Spur black smoker community. Comparative phylogenetic analyses of the 16S
538 rRNA gene and the alpha subunit of the ATP citrate lyase (AclA) from the Broken Spur
539 enrichment culture (grown under anaerobic, autotrophic conditions at 65°C) consistently
540 showed that this enrichment was dominated by an organism closely related to
541 *Desulfurobacterium* spp. (Figs. 1A and 3).

542 Overall, the ATP citrate lyase gene data for the Broken Spur natural community
543 combined with the enrichment studies, suggest that thermophilic bacteria related to the
544 *Aquificales* (*Persephonella* and *Desulfurobacterium* genera) are the dominant primary
545 producers using the rTCA for CO_2 fixation in the Broken Spur community.

Conclusions. 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 the 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 *Epsiloproteobacteria* were the main bacterial primary producers using the rTCA cycle for CO_2 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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Figure legends

Figure 1. Phylogenetic analysis of bacterial 16S rRNA gene sequences from the Rainbow (ROB# and RMB# indicate clone numbers from the outside and middle layer, respectively) and Logatchev (L7B# and L8B# indicate clone numbers from dives 3667 and 3668, respectively) black smokers, and from the Broken Spur enrichment culture (represented by Broken Spur Enrichment clone 8). 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 1000 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 (A). Frequency of bacterial 16S rRNA gene clones from the Logatchev black smokers (B).

Figure 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 (RaclB#) and Broken Spur (BSaclB#) black smokers, and from the Broken Spur enrichment culture (BSEaclB#). Percentages greater than 50% of bootstrap resampling (of 1000 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.

Figure 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 (BSaclA#) black smoker and enrichment culture (BSEaclA#). Percentages greater than 50% of bootstrap resampling (of 1000 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.

Figure 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 1000 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.

Table 1. Locations of sampling stations and clonal libraries.

Vent	Cruise/Dive #	Latitude	Longitude	Depth (m)	Temperature (°C)[b]	Library[a]	
						16S rRNA gene	acl
Rainbow, MAR	R/V *Atlantis* Cruise AT 05-03 DSV *Alvin* Dive 3678	36°N14'	33°W54'	2305	158	ROB RMB ROA	RaclB
Logatchev, MAR	R/V *Atlantis* Cruise AT 05-03 DSV *Alvin* Dive 3667-3668 (Irina2 vent)	14°N45'	48°W58'	3000	348	L7B L7A L8B	ND[d]
Broken Spur, MAR	R/V *Atlantis* Cruise AT 05-03 DSV *Alvin* Dive 3675	29°N10'	43°W10'	3060	ND	BSE	BSEaclA BSEaclB BSaclA BSaclB
9ºN, EPR	R/V *Atlantis* Cruise AT 11-10 DSV *Alvin* Dives 3999, 4002, 4008	9ºN50'	104°W17'	2500	339[c]	-	-

[a] See Material and Methods for clone naming

[b] Fluid temperature measured with DVS *Alvin* HighT probe at chimney orifice

[c] Fluid temperature at P vent on Dive 4008

[d] *aclB* gene fragment not detected by PCR

Table 2. Specific activities (nmol min-1 (mg cell protein)-1) of enzymes of the reductive TCA cycle in *C. mediatlanticus*[a]

Enzyme activity tested	*Caminibacter mediatlanticus*
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)	7800
Malate dehydrogenase (NADH)	4080
2-Oxoglutarate dehydrogenase (NAD)	n.d.
2-Oxoglutarate dehydrogenase (NADP)	n.d.
Pyruvate dehydrogenase (NAD)	n.d.
Pyruvate dehydrogenase (NADP)	n.d.

[a] Specific activities (nmol min-1 (mg cell protein)-1) of enzymes of the reductive TCA cycle. Mean values were obtained from at least five measurements. Standard errors were less than ± 20 %. n.d., no activity detected, detection limit < 1 nmol min-1 (mg cell protein)-1.

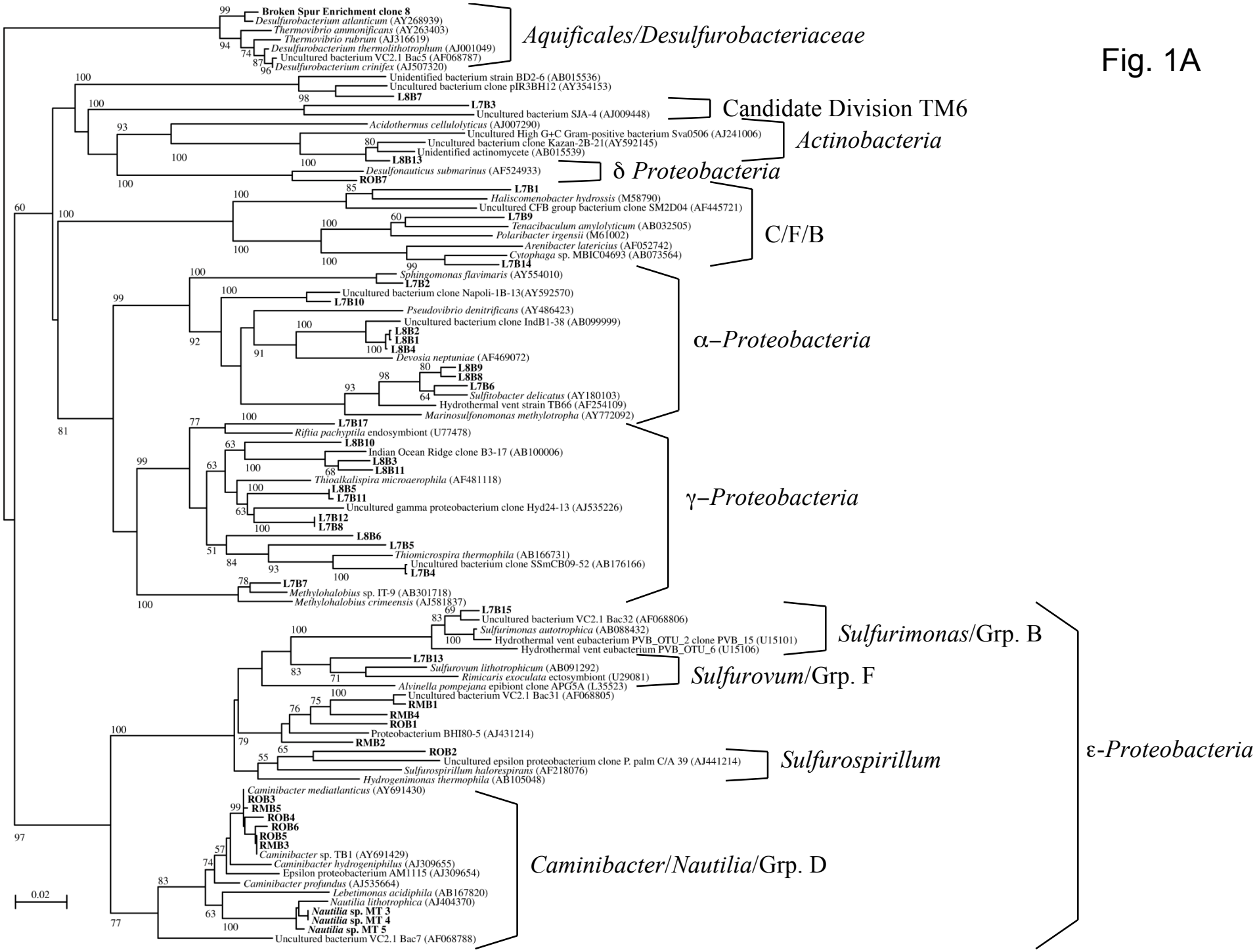
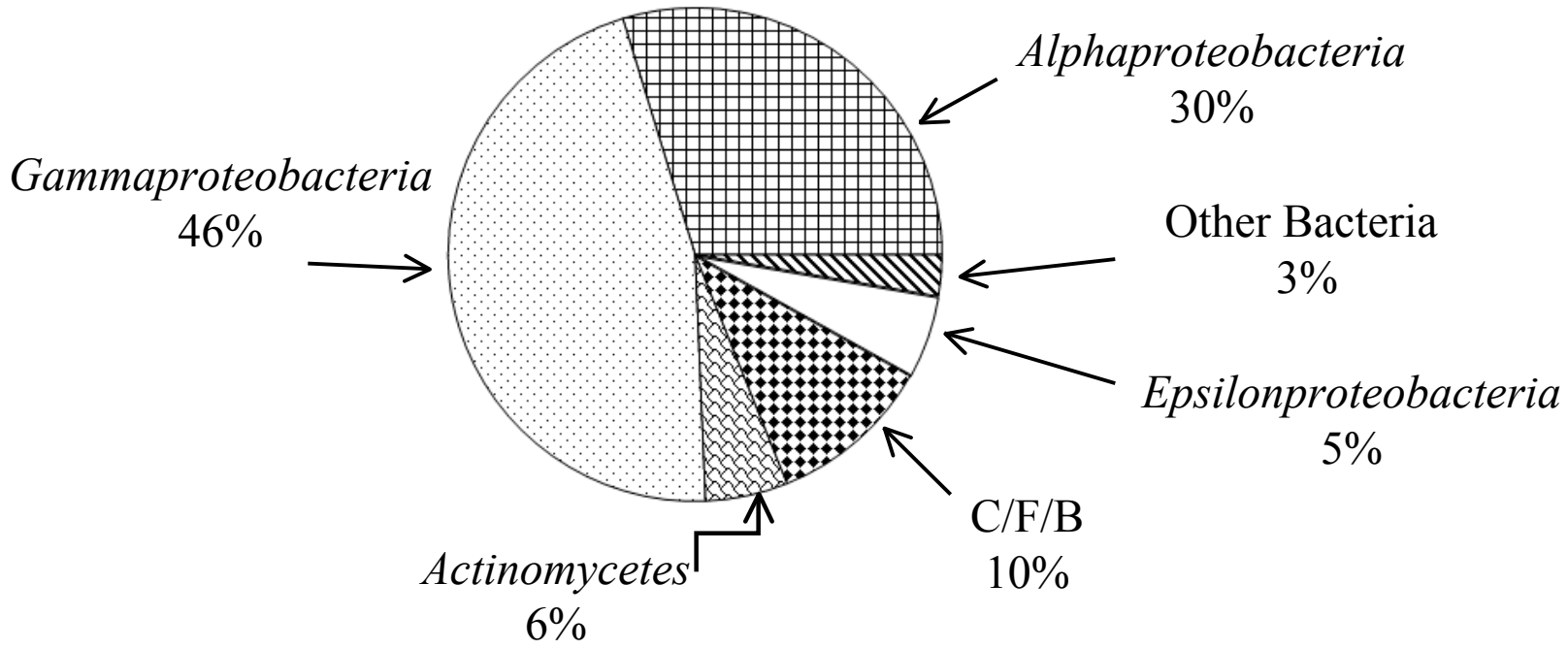
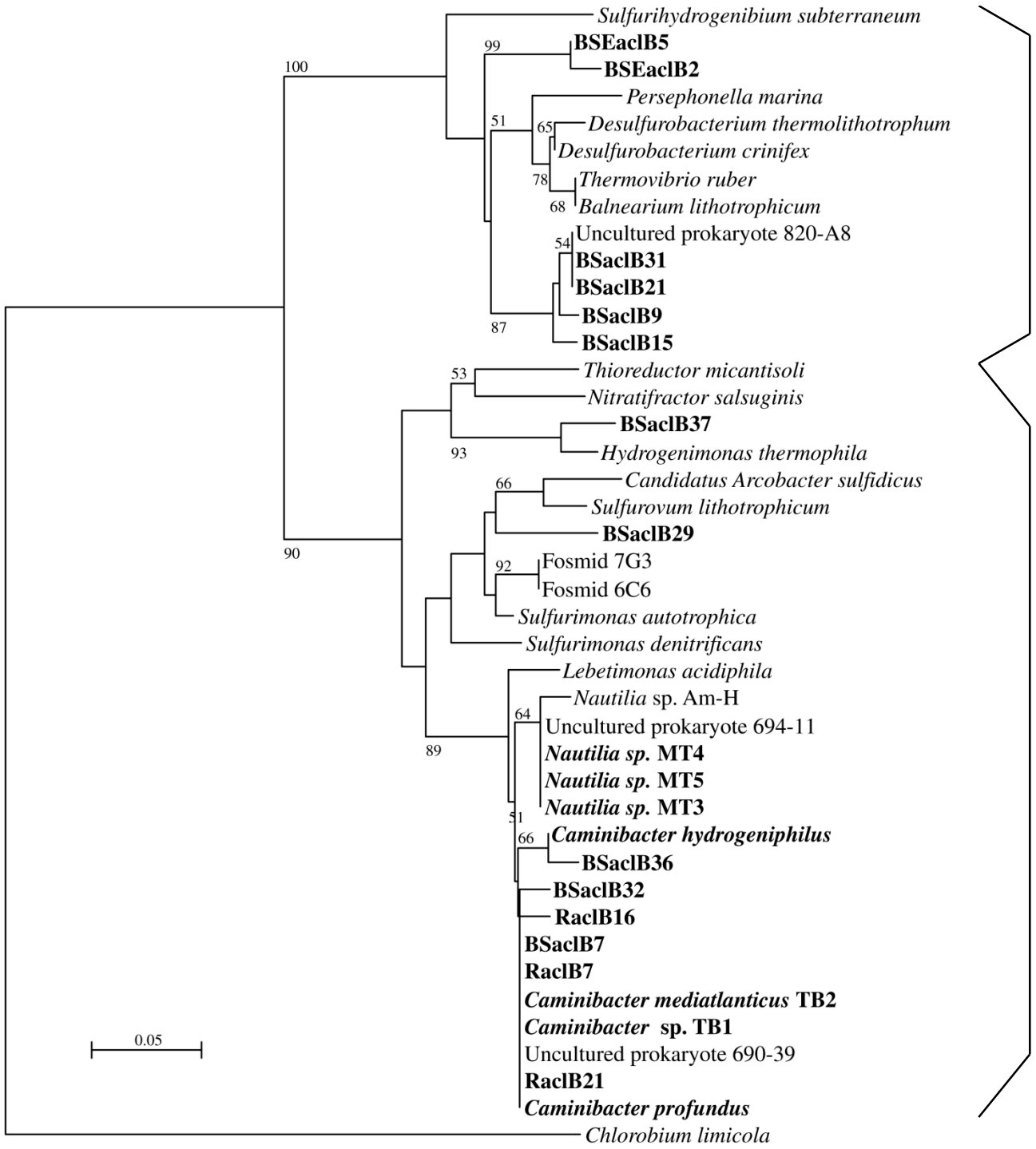

Broken Spur Enrichment clone 8
Desulfurobacterium atlanticum (AY268939)
Thermovibrio ammonificans (AY263403)
Thermovibrio rubrum (AJ316619)
Desulfurobacterium thermolithotrophum (AJ001049)
Uncultured bacterium VC2.1 Bac5 (AF068787)
Desulfurobacterium crinifex (AJ507320)
Aquificales/Desulfurobacteriaceae
Unidentified bacterium strain BD2-6 (AB015536)
Uncultured bacterium clone pIR3BH12 (AY354153)
L8B7
L7B3
Uncultured bacterium SJA-4 (AJ009448)
Candidate Division TM6
Acidothermus cellulolyticus (AJ007290)
Uncultured High G+C Gram-positive bacterium Sva0506 (AJ241006)
Uncultured bacterium clone Kazan-2B-21(AY592145)
Unidentified actinomycete (AB015539)
L8B13
Actinobacteria
Desulfonauticus submarinus (AF524933)
ROB7
δ Proteobacteria
L7B1
Haliscomenobacter hydrossis (M58790)
Uncultured CFB group bacterium clone SM2D04 (AF445721)
L7B9
Tenacibaculum amylolyticum (AB032505)
Polaribacter irgensii (M61002)
Arenibacter latericius (AF052742)
Cytophaga sp. MBIC04693 (AB073564)
L7B14
C/F/B
Sphingomonas flavimaris (AY554010)
L7B2
Uncultured bacterium clone Napoli-1B-13(AY592570)
L7B10
Pseudovibrio denitrificans (AY486423)
Uncultured bacterium clone IndB1-38 (AB099999)
L8B2
L8B1
L8B4
Devosia neptuniae (AF469072)
L8B9
L8B8
L7B6
Sulfitobacter delicatus (AY180103)
Hydrothermal vent strain TB66 (AF254109)
Marinosulfonomonas methylotropha (AY772092)
α–Proteobacteria
L7B17
Riftia pachyptila endosymbiont (U77478)
L8B10
Indian Ocean Ridge clone B3-17 (AB100006)
L8B3
L8B11
Thioalkalispira microaerophila (AF481118)
L8B5
L7B11
Uncultured gamma proteobacterium clone Hyd24-13 (AJ535226)
L7B12
L7B8
L8B6
L7B5
Thiomicrospira thermophila (AB166731)
Uncultured bacterium clone SSmCB09-52 (AB176166)
L7B4
L7B7
Methylohalobius sp. IT-9 (AB301718)
Methylohalobius crimeensis (AJ581837)
γ–Proteobacteria
L7B15
Uncultured bacterium VC2.1 Bac32 (AF068806)
Sulfurimonas autotrophica (AB088432)
Hydrothermal vent eubacterium PVB_OTU_2 clone PVB_15 (U15101)
Hydrothermal vent eubacterium PVB_OTU_6 (U15106)
Sulfurimonas/Grp. B
L7B13
Sulfurovum lithotrophicum (AB091292)
Rimicaris exoculata ectosymbiont (U29081)
Alvinella pompejana epibiont clone APG5A (L35523)
Sulfurovum/Grp. F
Uncultured bacterium VC2.1 Bac31 (AF068805)
RMB1
RMB4
ROB1
Proteobacterium BHI80-5 (AJ431214)
RMB2
ROB2
Uncultured epsilon proteobacterium clone P. palm C/A 39 (AJ441214)
Sulfurospirillum halorespirans (AF218076)
Hydrogenimonas thermophila (AB105048)
Sulfurospirillum
Caminibacter mediatlanticus (AY691430)
ROB3
RMB5
ROB4
ROB6
ROB5
RMB3
Caminibacter sp. TB1 (AY691429)
Caminibacter hydrogeniphilus (AJ309655)
Epsilon proteobacterium AM1115 (AJ309654)
Caminibacter profundus (AJ535664)
Lebetimonas acidiphila (AB167820)
Nautilia lithotrophica (AJ404370)
Nautilia sp. MT 3
Nautilia sp. MT 4
Nautilia sp. MT 5
Uncultured bacterium VC2.1 Bac7 (AF068788)
Caminibacter/Nautilia/Grp. D
ε-Proteobacteria
0.02

Fig. 1A

Fig. 1B


Alphaproteobacteria
30%
Gammaproteobacteria
46%
Other Bacteria
3%
Epsilonproteobacteria
5%
C/F/B
10%
Actinomycetes
6%


Sulfurihydrogenibium subterraneum
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BSEaclB2
Persephonella marina
Desulfurobacterium thermolithotrophum
Desulfurobacterium crinifex
Thermovibrio ruber
Balnearium lithotrophicum
Uncultured prokaryote 820-A8
BSaclB31
BSaclB21
BSaclB9
BSaclB15
Thioreductor micantisoli
Nitratifractor salsuginis
BSaclB37
Hydrogenimonas thermophila
Candidatus Arcobacter sulfidicus
Sulfurovum lithotrophicum
BSaclB29
Fosmid 7G3
Fosmid 6C6
Sulfurimonas autotrophica
Sulfurimonas denitrificans
Lebetimonas acidiphila
Nautilia sp. Am-H
Uncultured prokaryote 694-11
Nautilia sp. MT4
Nautilia sp. MT5
Nautilia sp. MT3
Caminibacter hydrogeniphilus
BSaclB36
BSaclB32
RaclB16
BSaclB7
RaclB7
Caminibacter mediatlanticus TB2
Caminibacter sp. TB1
Uncultured prokaryote 690-39
RaclB21
Caminibacter profundus
Chlorobium limicola
100
99
51
65
78
68
54
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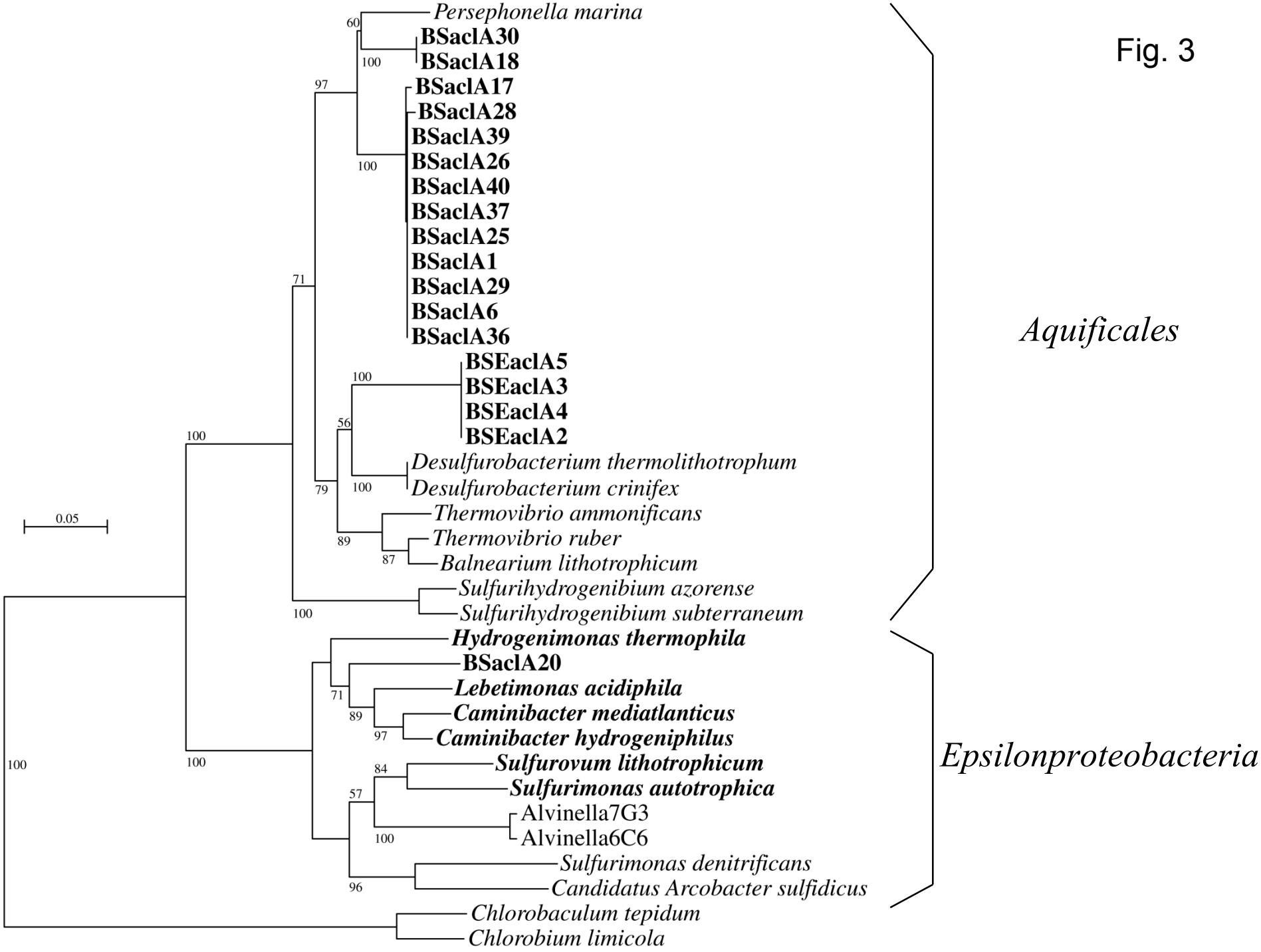
90
92
64
89
51
66
0.05
Aquificales
Epsilonproteobacteria

Fig. 2

Fig. 3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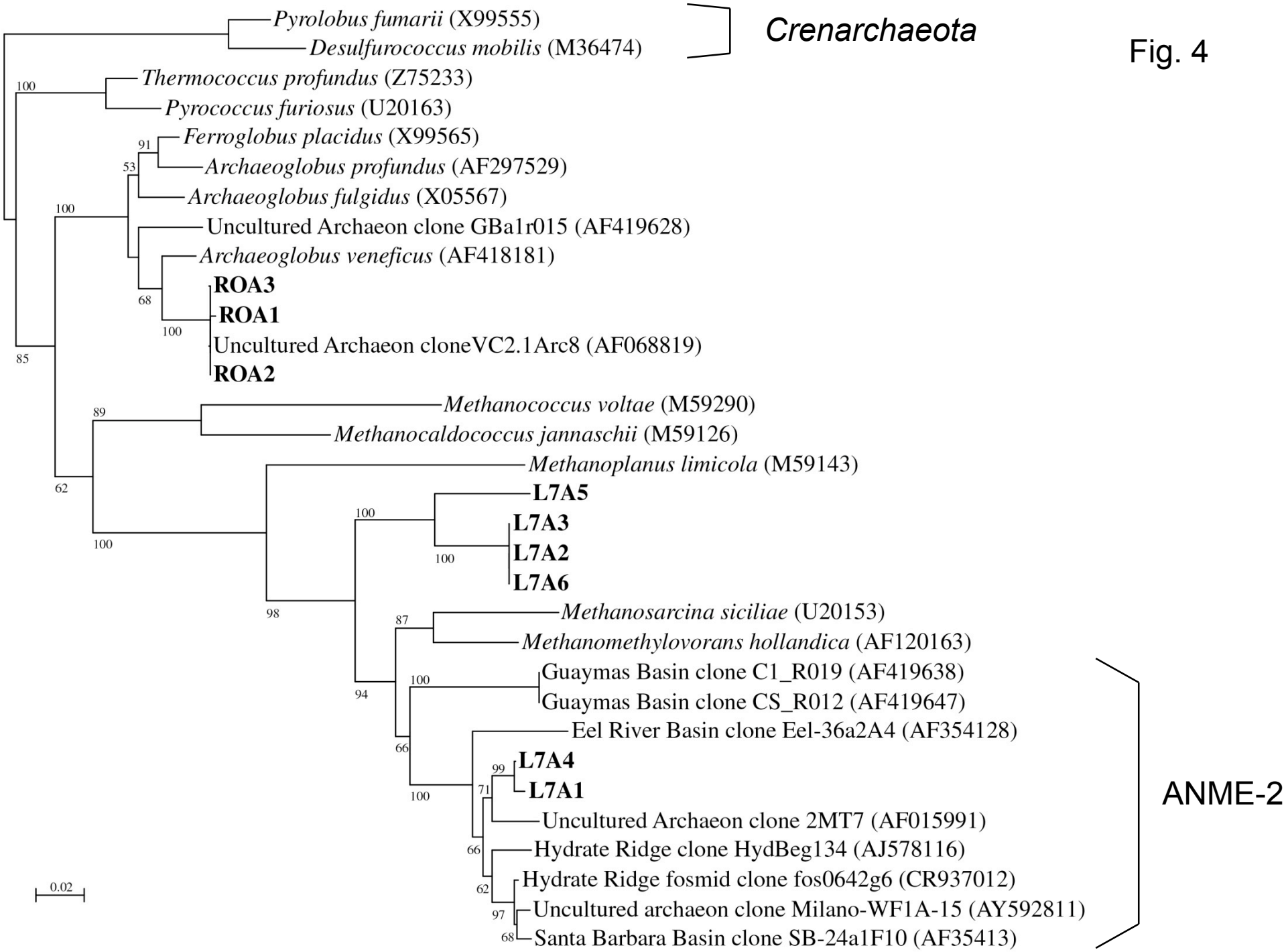
BSaclA1
BSaclA29
BSaclA6
BSaclA36
BSEaclA5
BSEaclA3
BSEaclA4
BSEaclA2
Desulfurobacterium thermolithotrophum
Desulfurobacterium crinifex
Thermovibrio ammonificans
Thermovibrio ruber
Balnearium lithotrophicum
Sulfurihydrogenibium azorense
Sulfurihydrogenibium subterraneum
Hydrogenimonas thermophila
BSaclA20
Lebetimonas acidiphila
Caminibacter mediatlanticus
Caminibacter hydrogeniphilus
Sulfurovum lithotrophicum
Sulfurimonas autotrophica
Alvinella7G3
Alvinella6C6
Sulfurimonas denitrificans
Candidatus Arcobacter sulfidicus
Chlorobaculum tepidum
Chlorobium limicola
0.05
Aquificales
Epsilonproteobacteria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Crenarchaeota
Thermococcus profundus (Z75233)
Pyrococcus furiosus (U20163)
Ferroglobus placidus (X99565)
Archaeoglobus profundus (AF297529)
Archaeoglobus fulgidus (X05567)
Uncultured Archaeon clone GBa1r015 (AF419628)
Archaeoglobus veneficus (AF418181)
ROA3
ROA1
Uncultured Archaeon cloneVC2.1Arc8 (AF068819)
ROA2
Methanococcus voltae (M59290)
Methanocaldococcus jannaschii (M59126)
Methanoplanus limicola (M59143)
L7A5
L7A3
L7A2
L7A6
Methanosarcina siciliae (U20153)
Methanomethylovorans hollandica (AF120163)
Guaymas Basin clone C1_R019 (AF419638)
Guaymas Basin clone CS_R012 (AF419647)
Eel River Basin clone Eel-36a2A4 (AF354128)
L7A4
L7A1
Uncultured Archaeon clone 2MT7 (AF015991)
Hydrate Ridge clone HydBeg134 (AJ578116)
Hydrate Ridge fosmid clone fos0642g6 (CR937012)
Uncultured archaeon clone Milano-WF1A-15 (AY592811)
Santa Barbara Basin clone SB-24a1F10 (AF35413)
ANME-2
0.02

Fig. 4

Table S1. Number of clones examined for each library

Library	Clones subjected to direct PCR for the detection of insert-containing plasmids	Clones subjected to RFLP analysis	Clones sequenced
RMB	15	10	5
ROB	25	25	8
ROA	25	20	4
L7B	25	19	17
L7A	25	21	6
L8B	25	18	13
RaclB	30	ND[a]	30
BSaclB	35	ND	35
BSaclA	40	ND	18
BSE	20	13	5
BSEaclB	8	ND	2
BSEaclA	20	ND	5

[a] ND: RFLP screening was not done.