

ϵ -Proteobacterial diversity from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge

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Abstract

The prokaryotic phylogenetic diversity was determined for a sample associated with an in situ growth chamber deployed for 5 days on a Mid-Atlantic Ridge hydrothermal vent (23°22'N, 44°57'W). The DNA was extracted from the sample and the 16S rDNA amplified by PCR. No *Archaea* were detected in the sample. Eighty-seven clones containing bacterial 16S rDNA inserts were selected. Based on restriction fragment length polymorphism analysis, 47 clones were unique, however, based on comparative sequence analysis some of these were very similar, and thus only 22 clones were selected for full sequence and phylogenetic analysis. The phylotypes were dominated by ϵ -*Proteobacteria* (66%). The remainder formed a novel lineage within the *Proteobacteria* (33%). One clone formed a distinct deeply branching lineage, and was a distant relative of the *Aquificales*. This report further expands the growing evidence that ϵ -*Proteobacteria* are important members in biogeochemical cycling at deep-sea hydrothermal ecosystems, participating as epibionts and free living bacteria. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Deep-sea hydrothermal vents are characterized by sharp physical and chemical gradients that support the growth of a diverse microflora including hyperthermophiles, psychrophiles, chemolithotrophs, heterotrophs, anaerobes and aerobes [1]. Phylogenetically diverse microorganisms have been isolated from deep-sea hydrothermal vents using enrichment culturing procedures [2], however, due to the inherent biases associated with this approach, little is known about the in situ microbial diversity at deep-sea vents. Molecular phylogenetic methods are now widely used to assess the microbial diversity in environmental samples [3,4]. Like enrichment culturing, these approaches also have biases [5] but they do detect a much greater diversity of organisms that have escaped selective culture media [3].

The molecular phylogenetic diversity based on the small subunit rRNA (16S rRNA) has been determined for microbial mats associated with deep-sea hydrothermal vents [6–13], and shallow marine thermal springs [14,15]. These limited studies illustrate that the phylogenetic diversity at deep-sea hydrothermal vents is much higher than that estimated by culture-based enrichments. Here we describe the bacterial phylogenetic diversity associated with the deployment of an in situ growth chamber at a Mid-Atlantic Ridge (MAR) vent site. A wide diversity of bacterial sequences of novel, as yet uncultivated ϵ -*Proteobacteria* and a novel proteobacterial lineage were detected.

2. Materials and methods

2.1. Sample collection

An in situ growth chamber ('vent cap', VC) [9] was deployed at 3500 m on a deep-sea hydrothermal vent along the MAR at 23°22'118"N, 44°56'984"W ('Snake Pit') using the submersible DSV *Le Nautilus* during the 'Microsmoke' cruise in November 1995. The vent cap

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was deployed on an active sulfide chimney ('Chi') for 5 days. During the same cruise a vent cap was also deployed on a different chimney called 'Les Ruches' [9]. Prior to deployment on 'Chi', the hydrothermal fluid temperature at the orifice of the chimney was measured to be 70°C. The temperature was monitored within the growth chamber throughout the deployment. Samples were prepared as described previously [9].

2.2. DNA extraction, amplification and cloning of 16S rDNA genes

DNA was extracted from the sample according to the protocol of Charbonnier et al. [16]. The bacterial and archaeal 16S rDNA were amplified as described previously [9]. PCR products were cloned as described previously [15], and insert-containing clones were screened by restriction fragment length polymorphism (RFLP) analysis using the enzymes *MspI*, *HindPII* and *BstUI*.

2.3. 16S rDNA sequencing and analysis

Both strands of at least one representative of each unique phylotype were sequenced completely. Sequencing procedures were as described previously [17] with the exception of the three Texas Red-labeled primers used for sequencing: 8–27 *Escherichia coli* numbering forward primer (5'-Texas Red-AGA GTT TGA TCC TGG CTC AG-3'), 515–533 *E. coli* numbering forward primer (5'-Texas Red-GTG CCA GCA GCC GCG GTA A-3') and 1474–1492 *E. coli* numbering reverse primer (5'-Texas Red-GGT TAC CTT GTT ACG ACT T-3') [18].

Phylogenetic analysis of 16S rDNA was determined by maximum likelihood [19], parsimony [20] and neighbor-joining methods [21]. Additionally, secondary structural models of the 16S rRNA were compared with the 16S rRNA secondary structures of the close relatives [22]. Secondary structures were constructed using the W3 mfold server (<http://www.ibc.wustl.edu/~zucker/rna>) [23] and optimized with established 16S rRNA secondary structures obtained from the Boulder/Halifax database of SSU rRNA, LSU rRNA and group I Introns webpage (<http://pundit.icmb.utexas.edu>) [24].

2.4. Nucleotide sequence accession number

Partial and full 16S rDNA sequences of the VC1.2 clones were deposited to GenBank under the accession numbers AF367481–AF367502.

3. Results and discussion

3.1. Sample analysis

During the 5-day deployment of the vent cap, the tem-

Table 1
Summary of the 16S rRNA sequences identified in VC1.2

Cluster ^a	% ^b	Phylotype	Clones ^c
A	27.5	VC1.2-C110	18
		VC1.2-C142	3
		VC1.2-C106	1
		VC1.2-C101	1
		VC1.2-C174 ^d	1
B	35.4	VC1.2-C126	24
		VC1.2-C132	2
		VC1.2-C149 ^d	1
		VC1.2-C150	1
		VC1.2-C151	1
		VC1.2-C168 ^d	1
		VC1.2-C1D	1
C	27.4	VC1.2-C118	20
		VC1.2-C104	1
		VC1.2-C107	1
		VC1.2-C113	1
		VC1.2-C102	1
D	5.7	VC1.2-C121	4
		VC1.2-C157 ^d	1
E	1.1	VC1.2-C131	1
F	1.1	VC1.2-C169 ^d	1
G	1.1	VC1.2-C115	1

^aPhylogenetic cluster (see Fig. 1).

^bCluster percentages in the clone library.

^cNumber of clones corresponding to the type sequence based on direct sequence comparison or inferred from RFLP profiles.

^dIndicates partial sequence.

perature inside the chamber fluctuated between 13 and 30°C (average of 20°C, data not shown). These temperatures are lower than the fluid temperature recorded before the deployment at the vent orifice by the submersible temperature probe (70°C). It is likely that once the hydrothermal fluids entered the vent cap chamber, the fluid was either diluted by the surrounding cold seawater or the distance to the chamber is sufficient to drop the temperature of the fluid significantly. Additionally, the temperature within the chamber was significantly lower than that reported for a different deployment in the same vent field [9], and therefore each deployment supported very different microbial communities.

Several approaches to amplify archaeal 16S rDNA from the DNA sample proved unsuccessful. Only bacterial 16S rDNA products were obtained. The products were cloned, and 87 insert-containing clones were selected from three independent PCR amplifications and cloning experiments. Forty-seven phylotypes (unique RFLP profiles, OTUs) were detected by RFLP analysis. It is unlikely that this is a true representation of the diversity in the sample, as identical RFLP patterns do not necessarily correspond to identical sequence types [25]. The 16S rDNA of one representative of each phylotype was sequenced. Three phylotypes appeared to be chimeras and were excluded from the phylogenetic analysis. Sequences that differed by less than 3% were considered as related groups. Consequently, 22 phylotypes were used in the phylogenetic analyses (Table 1).

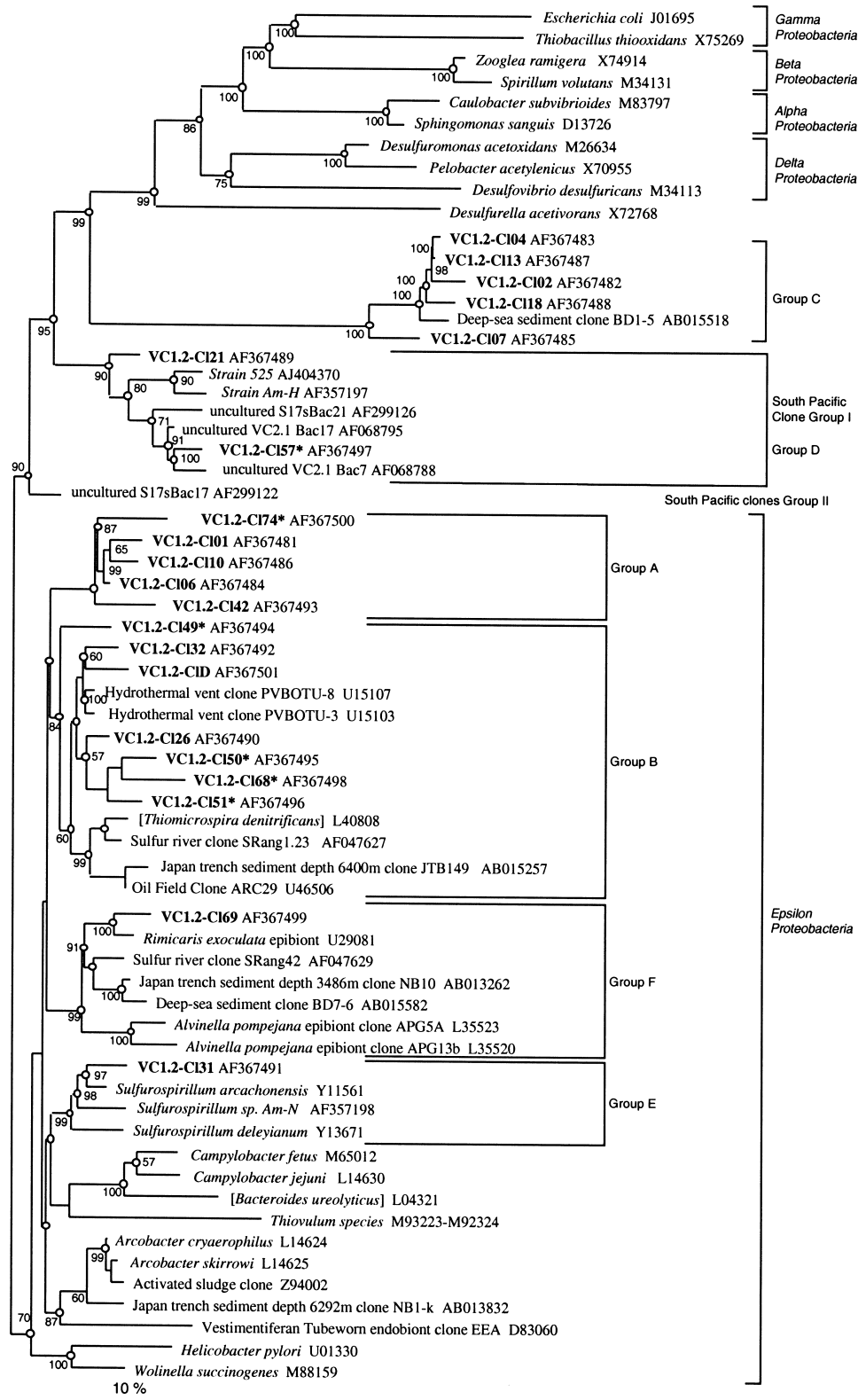


Fig. 1. Phylogenetic tree of 16S rDNA sequences obtained from the VC1.2 sample (in bold). Names between brackets correspond to misnamed genera. The topology shown is an unrooted neighbor-joining tree. Nodes marked with a circle were conserved by maximum likelihood analysis, and the bootstrap values ($\geq 50\%$) were obtained by maximum parsimony analysis for a bootstrap sampling of 100. The scale bar represents the expected percentage of changes per sequence position.

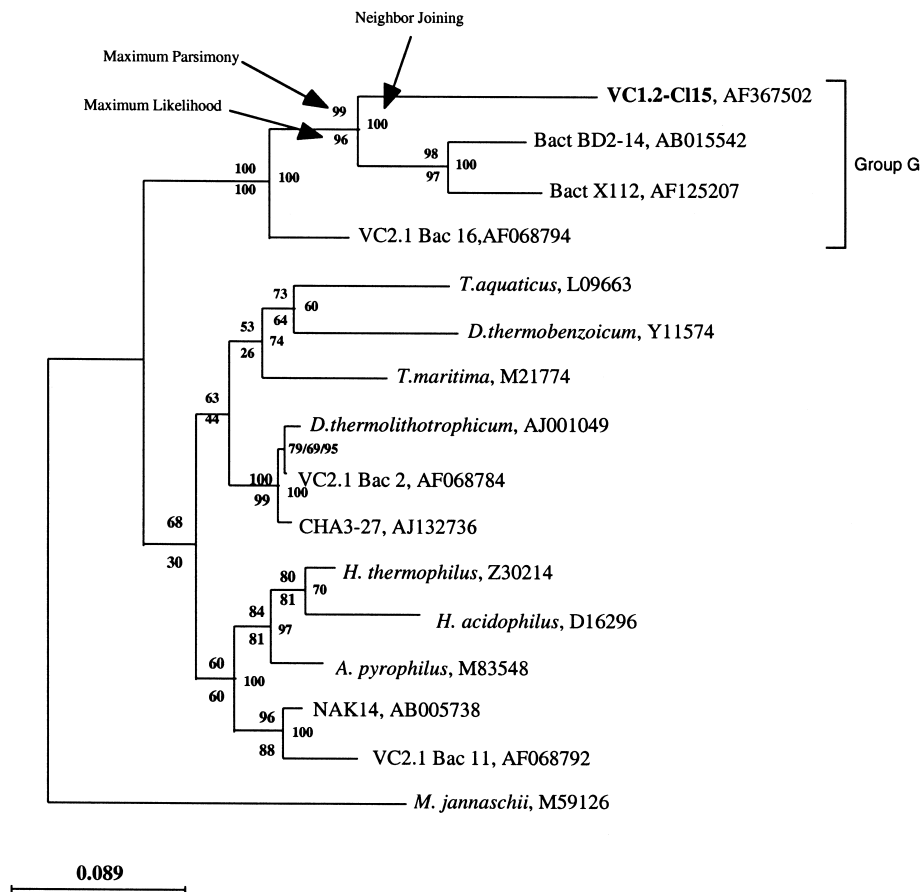


Fig. 2. Phylogenetic tree of 16S rDNA sequences obtained from VC1.2 sample (in bold). The topology shown is a consensus tree obtained using the neighbor-joining, the maximum likelihood and the maximum parsimony methods. For the neighbor-joining method, 929 sites have been retained for the analysis. The Kimura [44] or the Galtier and Gouy factor [45] were used and 1000 bootstrap replicates. For the maximum likelihood and the maximum parsimony analyses 748 sites were used in the analysis with 100 and 500 bootstrap replicates, respectively. Arrows indicate the position of bootstrap values obtained by the three methods. The scale bar represents the expected number of changes per sequence position.

3.2. Phylogenetic analyses

All the sequences could be assigned to seven phylogenetic clades (for ease of discussion these are designated A–G) (Table 1), none of the phylotypes corresponded to published 16S rRNA sequences. The majority of the sequences were associated with the *Proteobacteria* (groups A–F) (Fig. 1), of which 66% were ϵ -*Proteobacteria* and grouped with the *Campylobacteraceae* [26]. The sequence corresponding to group G is a deep lineage within the *Bacteria*, and most closely associated with the *Aquificales* (Fig. 2). Additionally, this sequence is closely related to three other sequences, one from a deep-sea hydrothermal vent sample (VC2.1Bac16, GenBank # AF068794,[9]), one from deep-sea sediments (BD2-14, GenBank # AB015542, [27]), and one from an oral cavity (clone X 112, GenBank # AF125207, Paster and Dewhirst, unpublished results).

The majority of phylotypes in our analysis (group B, 35%) were most closely related to the sequences obtained from a hydrothermal microbial mat sample collected at Loihi Seamount in the Pacific Ocean [7] and to the strain *Thiomicrospira denitrificans*. Group A (28% of the phylotypes)

formed a separate but closely associated lineage with group B, although this position was not strongly supported by high bootstrap values. Group A and B are also related to 16S rRNA sequences obtained from deep oil field samples, from microbial mats in Sulfur River Cave, USA, and from deep-sea marine sediments. Together with a sequence obtained from deep-sea sediments [27], group C (28% of the phylotypes) forms a unique lineage within the *Proteobacteria* (Fig. 1). The monophyletic position of this lineage is strongly supported by high bootstrap values and by maximum parsimony, maximum likelihood and distance analyses. Furthermore, different outgroup sequences did not change the tree topology (data not shown). Recently it was proposed that group D (6% of the phylotypes) is endemic to deep-sea hydrothermal vents [13], and several thermophilic isolates belonging to this group were described ([28], Miroshnichenko, M., Kostrikin, N., L'Haridon, S., Jeanthon, C., Stackebrandt, E. and Bonch-Osmolovskaya, E., personal communication). VC1.2-CI31 (group E, 1% of the phylotypes) clustered with sulfur reducers of the genus *Sulfurospirillum* and VC1.2-CI69 (group F, 1% of the phylotypes) was most

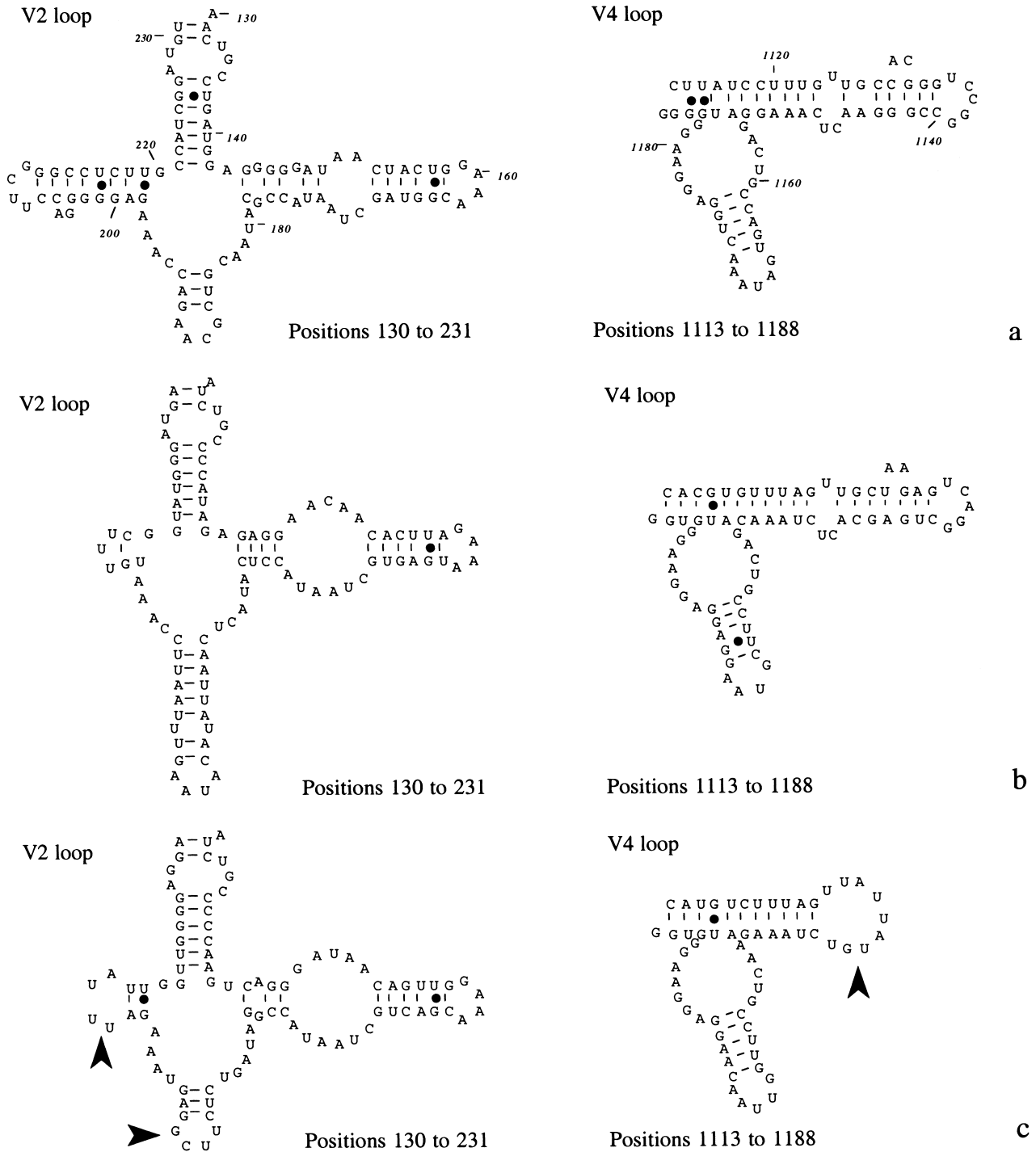


Fig. 3. Secondary structures of V2 and V4 loops of the 16S rRNA, distinguishing group C clone (c) from ϵ -Proteobacteria (*Campylobacter sputorum* (a)) and *E. coli* (b). Black arrows illustrate differences among the groups. The sequences are numbered according to the IUB nomenclature of *E. coli* 16S rRNA.

closely related to the MAR hydrothermal vent shrimp, *Rimicaris exoculata*, epibiont [29].

Four 16S rRNA secondary structural features are characteristic of the ϵ -Proteobacteria [30]. All four features were found in most of our clones (groups A, B, D, E and F). However, group C differs from the ϵ -Proteobacteria by: (i) an arrangement of two short stems at posi-

tions 184–193 and 198–219 (V2 region, Fig. 3), (ii) an adenine insertion in a loop structure corresponding to the positions 1357–1365, and (iii) a truncated stem-loop structure corresponding to positions 1127–1145 (V4 region, Fig. 3).

Although microaerophily and sulfur metabolism appear to be a common feature in the ϵ -Proteobacteria [26], phys-

iological predictions based only on the phylogenetic position of a phylotype must be made with caution. However, the phylogenetic positions of the phylotypes and in situ temperatures recorded during the deployment suggest that many of the phylotypes were mesophiles and possibly microaerophiles or aerobes capable of oxidizing reduced sulfur compounds emitted in the hydrothermal fluid or produced by sulfur and sulfate reducers. Based on the close proximity of warmer diffuse fluids, and the temperature recorded for the venting water prior to the deployment of the vent cap, it is also possible that some of the phylotypes are moderate thermophiles, and were present in the vent cap, but not actively growing. The thermophilic isolates belonging to group D ([28], Miroshnichenko, M., Kostrikina, N., L'Haridon, S., Jeanthon, C., Stackebrandt, E. and Bonch-Osmolovskaya, E., personal communication) are obligately chemolithoautotrophic bacteria (T_{opt} 41–53°C) using H_2 or formate as electron donors and elemental sulfur as an electron acceptor. Based on the strong bootstrap support and multiple phylogenetic analyses, group C may represent a new subclass of *Proteobacteria*, similar to the proposed distinction of *Desulfurella* from the δ -*Proteobacteria* [31]. The close phylogenetic relationship between the group C and one 16S rRNA sequence from deep-sea sediments (strain BD1-5) argues for a possible deep-sea origin of this group, although a deep branch within this group is represented by a phylotype obtained from 'Sulfur River Cave' in Kentucky, USA [32] (data not shown).

This report further expands the known diversity of the ϵ -*Proteobacteria* at deep-sea vents from the initial reports of the prevalence of this group associated with microbial mats and hydrothermal vent invertebrates [7,9,28,29,33]. Given the phylogenetic diversity of this group and the diversity of habitats from which they have been reported, such as coastal and abyssal marine sediments [27,34–37], boreholes and deep granitic aquifers [38,39], caves [32], oil fields [40], hydrocarbon seep sediments (O'Neil et al., unpublished results), wastewater treatment plants [41], marine-snow assemblages [42], Antarctic marine lake and fjord benthic sediments (Bowman et al., unpublished results) and endosymbiotic flora of non-hydrothermal vestimentiferan tubeworm [43], it is difficult to speculate what their role in the deep-sea hydrothermal ecosystem might be. However, ϵ -*Proteobacteria* are clearly more widely distributed in the environment than previously estimated using conventional microbiological methods. The proteobacterial phylogenetic diversity detected in this study may reflect the capacity of this group to colonize the broad range of redox and thermal niches existing at deep-sea hydrothermal vents.

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