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Novel uncultured Epsilonproteobacteria dominate a filamentous sulphur mat from the 13°N hydrothermal vent field, East Pacific Rise

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Abstract:

Rapid growth of microbial sulphur mats have repeatedly been observed during oceanographic cruises to various deep-sea hydrothermal vent sites. The microorganisms involved in the mat formation have not been phylogenetically characterized, although the production of morphologically similar sulphur filaments by a Arcobacter strain coastal marine has been documented. An in situ collector deployed for 5 days at the 13°N deep-sea hydrothermal vent site on the East Pacific Rise (EPR) was rapidly colonized by a filamentous microbial mat. Microscopic and chemical analyses revealed that the mat consisted of a network of microorganisms embedded in a mucous sulphur-rich matrix. Molecular surveys based on 16S rRNA gene and aclB genes placed all the environmental clone sequences within the Epsilonproteobacteria. Although few 16S rRNA gene sequences were affiliated with that of cultured organisms, the majority was related to uncultured representatives of the Arcobacter group (≤95% sequence similarity). A probe designed to target all of the identified lineages hybridized with more than 95% of the mat community. Simultaneous hybridizations with the latter probe and a probe specific to Arcobacter spp. confirmed the numerical dominance of Arcobacter-like bacteria. This study provides the first example of the prevalence and ecological significance of free-living Arcobacter at deep-sea hydrothermal vents.

Keywords: deep-sea hydrothermal vent, biofilm, 16S rRNA, Epsilonproteobacteria, Arcobacter, FISH, East Pacific Rise

1 Introduction

At deep-sea hydrothermal vents, steep temperature and chemistry gradients created by the mixing of anoxic hydrothermal fluids with oxygenated seawater are known to offer a variety of habitats for the growth of diverse microorganisms. The microbial communities occupy both aerobic and anaerobic environments having in situ temperatures ranging from ambient seawater temperature to temperatures greater than 110°C (Karl, 1995). Cultivation of metabolically and phylogenetically diverse thermophilic and hyperthermophilic bacteria and archaea has been extensively reported from the hottest ecological niches of the hydrothermal environment (Jones *et al.*, 1983; Harmsen *et al.*, 1997a; Takai *et al.*, 2001; Huber *et al.*, 2002). In part, this is because black smokers and related sulphide structures are the most extensively studied vent environments. Microorganisms of other thermal classes have however been isolated from these heterogeneous and fluctuating habitats although most of the microbial diversity has only been characterized by molecular methods (Takai and Horikoshi, 1999; Jeanthon, 2000; Reysenbach *et al.*, 2000; Teske *et al.*, 2002; Nercessian *et al.*, 2003).

Microbial mats growing on rocks, chimneys, sediments, or animal surfaces which are exposed to vent waters represent another hydrothermal vent habitat with associated microbial communities (Karl, 1995). Most of the reports on microbial mats involved microscopic observations (Jannasch and Wirsen, 1981; Nelson *et al.*, 1989; Taylor *et al.*, 1999), or fatty acid (Hedrick *et al.*, 1992) and phylogenetic analyses (Moyer *et al.*, 1995; Longnecker and Reysenbach, 2001; Alain *et al.*, 2004). In certain locations, the mat forming organisms were identified as large filamentous sulphur-oxidizing bacteria of the genera *Beggiatoa* and *Thiothrix* (Jannasch and Wirsen, 1981; Jannasch *et al.*, 1989; Nelson *et al.*, 1989). These bacteria are chemoautotrophic; they fix CO_2 by oxidizing hydrogen sulphide present in hydrothermal fluids. Flocculent material entrained out of the seafloor in the venting fluids and rapid growth of white filamentous bacteria have also often been observed after recent eruptive

events or at sites of extensive emission discharges at Loihi hydrothermal vents, Guaymas Basin, on the EPR and Juan de Fuca ridge (Nelson *et al.*, 1991; Haymon *et al.*, 1993; Embley *et al.*, 1995; Moyer *et al.*, 1995; Shank *et al.*, 1998; Taylor *et al.*, 1999; Embley *et al.*, 2000). Subsequent studies of the emitted particles showed that they were composed of inorganic, sulphur-rich filaments with a low organic carbon content (Nelson *et al.*, 1991). The associated microorganisms have never been isolated or phylogenetically characterized. However, the formation of filamentous sulphur of morphology nearly identical to the material discharged from diffuse-flow hydrothermal vents was documented for a sulphide-oxidizing bacterium isolated from shallow coastal marine waters (Taylor and Wirsen, 1997; Taylor *et al.*, 1999). An extensive characterization revealed that this motile vibrioid autotrophic organism clustered with members of the genus *Arcobacter* within the '*Epsilonproteobacteria*' (Wirsen *et al.*, 2002).

Molecular examination of mats attached to chimneys, animal surfaces and various sampling devices revealed also microbial communities dominated by a wide diversity of uncultured groups of '*Epsilonproteobacteria*' (Polz and Cavanaugh, 1995; Cary *et al.*, 1997; Corre *et al.*, 2001; Longnecker and Reysenbach, 2001; Huber *et al.*, 2003; Lopez-Garcia *et al.*, 2003; Alain *et al.*, 2004). Although there is generally no evidence available that they are also numerically abundant *in situ*, it is now recognized that the '*Epsilonproteobacteria*' are the predominant bacteria in the global deep-sea hydrothermal systems (Polz and Cavanaugh, 1995; Cary *et al.*, 1997; Corre *et al.*, 2001; Longnecker and Reysenbach, 2001; Huber *et al.*, 2003; Lopez-Garcia *et al.*, 2003; Alain *et al.*, 2004). Genes for key enzymes of the reductive tricarboxylic acid (rTCA) cycle, e.g. *aclB*- the β subunit of the ATP citrate lyase, were detected in cultured '*Epsilonproteobacteria*' and recovered in various hydrothermal vent habitats (Campbell *et al*., 2003; Campbell and Cary, 2004; Hügler *et al*., 2005; Takai *et al*.,

2005). These reports suggested that autotrophic CO_2 fixation via the rTCA cycle operate in diverse '*Epsilonproteobacteria*' and might sustain the predominant primary production in situ.

In this study we investigated the bacterial community from a white filamentous mat formed within a few days on a syntactic foam float associated with an *in situ* collector deployed at the 13°N hydrothermal site (EPR). The goal in this study was to determine the microbial compositions of the mat that was morphologically comparable to that observed and described by Taylor *et al*. (1999). On the basis of molecular phylogenetic surveys based on 16S rDNA and *aclB* genes and quantitative fluorescence in situ hybridization (FISH), we demonstrate the actual *in situ* dominance of yet uncultured '*Epsilonproteobacteria*' including the prevalence of *Arcobacter* relatives in the hydrothermal sulphur mat.

2 Material and methods

2.1 Hydrothermal site description

The hydrothermal sample was collected at the 13°N hydrothermal vent field on the East Pacific Rise during the oceanographic cruise PHARE conducted with the research vessel *'L'Atalante'* and the remoted operated vehicle (ROV) *'Victor 6000'*. The portion of the East Pacific Rise situated at 13°N is about 300 km south the Orozco fracture zone and 100 km north of a small transform fault located at 11°49'N. At 13°N, the dome-shaped ridge has a central rift valley varying between 200 m and more than 600 m in width and with a mean depth of about 2600 m (Hekinian and Fouquet, 1985). The volcanic activities at 13°N give rise to a wide range of hydrothermal venting structures from diffuse vents (up to 100°C) to black smokers with temperatures above 350°C. Our study focuses on the PP12 chimney of the Genesis hydrothermal site (12°48’66" N, 103°56’44" W). The chimney was colonized by dense populations of the polychaetous annelid *Alvinella pompejana* that builds tubes directly

in contact with the sulphides. Few specimens of the tube-worm *Riftia pachyptila* were also observed.

2.2 Sample collection

An *in situ* collector (Nercessian *et al.*, 2003), designed to concentrate the microorganisms discharged by hydrothermal emissions, was deployed on the PP12 chimney (Fig. 1). The *in situ* collector was brought to the deployment site into an insulated container and settled down by the arm of the ROV. Just before deployment of the *in situ* collector, the chimney's surface was scraped by the arm of the ROV during the sampling of *Alvinella pompejana* specimens. The mechanical removing of the overlying sulphide structure resulted in an increase of the vent emission. The collector was settled at the exact location where alvinellids were taken, a few centimeters above the vent emission (Fig. 1). Immediately after deployment and prior removal, discrete temperature measurements were achieved at the bottom and at the top of the collector by using the thermoprobe (Micrel, Hennebont, France) held by the teleoperated arm of the ROV. Video imagery of the *in situ* collector was acquired during successive dives. The collector was surveyed during the following *Victor* dives. At the fourth day of deployment, a thick and dense white biofilm was observed on the syntactic foam float attached to the collector (Fig. 1).

After 5 days of exposure, the *in situ* collector was transferred into enclosed containers to minimize contamination from surrounding seawater during transportation to surface. Once on shipboard, the microbial mat covering the marker was aseptically collected by scraping and immediately subsampled. Material for scanning electron microscopy (SEM) was fixed with 2.5% glutaraldehyde in 30 $g.l^{-1}$ Sea Salts (Sigma) and stored at 4°C. Aliquot for molecular and X-ray diffraction (XRD) analyses was stored in ethanol at –20°C. For FISH experiments, biomass was fixed for 3 hours at 4°C with 4% paraformaldehyde in 3X phosphate-buffered

saline (PBS) (Sambrook *et al.*, 1989).The fixation buffer was then replaced by a mixture of equal volumes of 3X PBS and 100% ethanol and the fixed sample was stored at -20°C until further use.

2.3 Scanning electron microscopy coupled (SEM) to energy-dispersive X-ray spectroscopy (EDS)

Macroscopic mat filaments were deposited on a 10-µm-pore-size membrane filter (Millipore) and fixed overnight at 5°C in 2.5% formaldehyde. After salt was eliminated, the preparation was dehydrated in increasing ethanol concentrations (50%, 70%, 90% and 100%). Samples were then critical point dried and gold coated. Observation was carried out with a Philips (XL 30-LaB6) scanning electron microscope operated at 25 kV.

The elemental composition of the sample was analyzed by energy-dispersive X-ray spectroscopy (EDS) coupled to the scanning electron microscope.

2.4 X-ray diffraction (XRD)

XRD analysis was accomplished with a Brucker D-500 goniometer coupled with the automated DACO-MP system for data acquisition. Mineral phases were identified on the diffraction spectra using the EVA program. Powder XRD scans were performed on dried crushed samples under the following conditions: Cu Kα radiation, 25 mA, 35 kV, and 5 sec per step with a step size of 0.02° (2θ). The scan range for all samples was 5-70°. Background and Kalph2 lines were removed from the diagrams. The mineralogical composition of the sample was determined by comparing sample diffraction patterns to mineral standards provided by the JCPDS files.

2.5 Nucleic acid isolation and purification

The sample was centrifuged at 4,000 x g for 10 min. The pellet was suspended in 150 µl of homogenization buffer (50 mM Tris [pH 8], 25% sucrose). Cells were lysed at room temperature with 1 $mg.ml^{-1}$ lysozyme for 10 min, followed by 10 min of incubation after addition of 1% SDS. Then, proteinase K (1 $mg.ml^{-1}$) were added to the lysis suspensions and the tubes were incubated at 50°C for 60 min. Nucleic acid extraction was performed with prewarmed (55°C) phenol-chloroform-isoamyl alcohol mixture as previously described (Teske *et al.*, 2002). Nucleic acids were precipitated with NaCl 5M (0.1 vol) and 100% ethanol (2 vol). After overnight incubation at -20°C, the precipitated DNA was centrifuged (10,000 X *g* for 30 min) and washed with 75% ethanol. Nucleic acids was dried and suspended in 50 µl of sterile water.

2.6 PCR amplifications and cloning

Small-subunit rRNA genes were amplified by PCR using universal primer 1492R (5'-CGGTTACCTTGTTACGACTT-3'), *Bacteria*-specific primer 8F (5'-AGAGTTTGATYMTGGCTCAG-3'), and *Archaea*-specific primer 4F (5'-TCCGGTTGATCCTGCCRG-3'). The final 25 µl PCR mixture consisted in 1 X buffer (Promega), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer, 1 U of *Taq* DNA Polymerase (Promega) and 2 µl of DNA. Contaminants in PCR reaction mixtures were avoided as previously described (Goldenberger and Altwegg, 1995). Polymerase chain reaction cycles were performed on a Perkin Elmer 9700 thermal cycler. After a denaturation step at 95°C for 5 min, the following conditions were repeated for 30 cycles: denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and chain extension at 72°C for 2 min. A final extension step of 8 min at 72°C was performed. Under these conditions, no PCR products were visible on 0.8% (wt/vol) agarose gels stained with ethidium bromide. To obtain visible

PCR products, primary amplifications using primer pairs 8F-1492R and 4F-1492R were stopped at 15 cycles. Aliquots (1 µl) of the primary amplifications were then used as templates for secondary amplifications. Nested PCRs were performed using forward primers 50F (5'-AACACATGCAAGTCGAACG-3') and 63F (5'-CAGGCCTAACACATGCAAGTC-3') specific for *Bacteria* and 341F (5'-CCTAYGGGGYGCASCAGGCG-3') specific for *Archaea* with universal reverse primer 1407R (5'-GACGGGCGGTGWGTRCAA-3'). The PCR mixtures and conditions were as described before except that 30 cycles were applied.

PCR detection of the ATP citrate lyase beta subunit (*acl*B) was based on previously designed primers (Campbell *et al*., 2003). The cycling parameters for amplification of *acl*B genes were that described by Campbell and Cary (2004). Negative controls were performed without DNA template or PCR products.

PCR products were cloned using the TOPO XL PCR Cloning Kit (Invitrogen) into in *Escherichia coli* TOP 10 cells. Plasmids containing 16S rRNA genes were extracted using the QIAprep Miniprep kit (Qiagen).

2.7 Restriction fragment length polymorphism (RFLP) analysis, clone sequencing, and phylogenetic analysis

Screening of clones containing 16S rRNA gene sequences was performed by restriction fragment length polymorphism (RFLP) analysis. The PCR products of the clone inserts amplified with primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') were cut with the restriction endonuclease *Bst*U1 (New England Biolabs) according to manufacturer instructions. The DNA fragments were separated by electrophoresis on 2% agarose gels run in 1 X Tris-acetate buffer (Sambrook *et al.*, 1989).

One to three clones per RFLP pattern were sequenced with a 4200 IR^2 Licor DNA sequencer, using the Thermo Sequenase Primer Cycle sequencing kit (Amersham Pharmacia Biotech) and the infrared-labeled primers M13F and M13R (MWG, Germany). DNA sequences were submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/ html/). Sequences were subjected to BLAST searches within the GenBank database (http://www.ncbi.nlm.nih.gov/) to determine 16S rRNA gene sequence similarities to cultured and not yet cultured organisms. Sequence alignments were performed using the CLUSTALW program (Thompson *et al.*, 1994) and refined manually using the SEAVIEW program (Galtier *et al.*, 1996).

Phylogenetic trees were constructed using the PHYLO-WIN program (Galtier *et al.*, 1996). Distance trees were constructed using the neighbor-joining algorithms (Saitou and Nei, 1987) with the Kimura two-parameters correction (Kimura, 1980) for nucleotides and PAM correction for proteins. Maximum-likelihood analyses on protein sequences were performed using the PHYML program (Kimura, 1980). The robustness of inferred topologies was evaluated by using a bootstrap analysis (Felsentein, 1985). The sequences obtained in this study have been deposited in the GenBank/EMBL/DDBJ database with the following accession numbers: DQ071274 to DQ071290 (16S rRNA) and AM181241 to AM181291 (*aclB*).

2.8 16S rRNA oligonucleotide probe design and optimization

The 16S rRNA oligonucleotide probe EPSI682 [S-*-Epsi-0682-a-A-19 according to Alm *et al.* (Alm *et al.*, 1996)] specific to all Epsilonproteobacterial lineages identified in this study was designed using the PROBE_DESIGN function of the ARB software package (http://www.arb-home.de). The probe EPSI682 (5'-CGGATTTTACCCCTACACM-3') was

checked for specificity using the PROBE_MATCH function in the RDP (http://rdp.c:e.msu.edu).

Dot-blot hybridization was performed to estimate the specificity of the designed probe. The probe was labeled at its 5' end with T4 polynucleotide kinase (phosphatase-free; Boehringer Mannheim Biochemicals, Meylan, France) and [γ-32P] ATP (Amersham, Les Ulis, France).

For determination of probe specificity, PCR-amplified 16S rRNA gene fragments from *Alvinella pompejana* epibiont clone APG13B (Haddad *et al.*, 1995), and environmental clones VC1.2 (Corre *et al.*, 2001) and VC2.2 (Corre, 2000) were used as targets. Total nucleic acids from *Sulfurospirillum barnesii* (DSM 10660^T), *Wolinella succinogenes* (DSM 1740^T), *Arcobacter nitrofragilis* (DSM 7299^T), *Campylobacter coli* (DSM 4689^T), *C. jejuni* (DSM 4688^T), *Thiomicrospira denitrificans* (DSM 1251^T), *Helicobacter pylori* (ATCC 43579), *Zobellia galactanivorans* (DSM 12802^T), *Escherichia coli* (DSM 3950) and *Bacillus sp*. strain SG9 (Marteinsson *et al.*, 1996) were also used.

The probe specificity was determined as previously described (Harmsen *et al.*, 1997b) with slight modifications. Denatured PCR-amplified 16S rRNA gene fragments and total nucleic acids from cultured strains were applied on nylon membrane (Hybond-N$^+$, Amersham Biosciences, Orsay, France) using a dot-blot unit (SRC 96D, MinifoldI, Schleicher and Schuell, Dassel, Germany). The dissociation temperature (Td) was determined for the [γ-P^{32}]ATP-labeled probe and hybridization was carried out as described by (Franks *et al.*, 1998). In addition to the newly designed probe, the 16S rRNA-targeting oligonucleotide probe EUB338 (S-D-Bact-0338-a-A-18) specific for *Bacteria* (Amann *et al.*, 1990) was used as control in the dot-blot analysis. Hybridization responses of the radioactively labeled probe were detected with a Posphor Imager (Molecular Dynamics, Sunnyvale, USA) and printed with Adobe (Seattle, USA) Photoshop 3.0.

Campylobacter jejuni (DSM 4688^T), *Arcobacter skirrowii* (DSM 7302^T), *Nautilia lithotrophica* (DSM 13520^T) and *Desulfurobacterium thermolithotrophum* (DSM 11699^T) were used for specificity studies in whole-cell hybridization experiments with the newly designed oligonucleotide probe ARC94 which targets the genus *Arcobacter* (Snaidr *et al.*, 1997) and the bacterial probe EUB338. Fluorescein-, Cy-3- and rhodamine-labeled and unlabeled oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Cells were fixed for 3 hours in 4% paraformaldehyde in 1X PBS (3X PBS for marine strains) at 4°C, washed in 1X (or 3X) PBS and stored at –20°C in 50% (vol/vol) ethanol-1X PBS. FISH analysis was performed as previously described (Harmsen *et al.*, 1997b). The optimal formamide concentration of probe EPSI682 was evaluated by varying the formamide concentration from 0 to 60%.

2.9 Fluorescence in situ hybridization (FISH) on the hydrothermal mat sample

To quantify the amount of cells hybridizing with probes EPSI682, ARC94, EUB338 and ARCH945, 250 µl of mat sample fixed in 3X PBS containing 4% paraformaldehyde were centrifuged 6 min at 3,500 X g. The pellet was resuspended in 50 µl of the hybridization buffer used above with 250 ng of each labeled probe used and incubated at 46°C for 3 h. Cells were then washed for 30 min with 5 ml of prewarmed (48°C) wash-buffer before being filtered onto 0.2-µm-pore-size Nucleopore polycarbonate filters (Costar, Dutscher, France). The cells were viewed and counted with an Olympus BX60 epifluorescence microscope equipped with a U-MWIB (for fluorescein) and U-MWG (for rhodamine) filter cubes (Olympus). A minimum of 25 cells per field, 15 fields per filter and a minimum of three filters per probe tested were counted.

3 Results

3.1 Habitat characterization of the mat-forming organisms

In order to better understand microbial diversity, dynamics and colonization processes at vents, an *in situ* collector was deployed on the chimney PP12 at the Genesis site of the 13°N vent field (EPR). The deployment site was selected based on the maximum temperature observed in the venting fluid (< 100°C). The collector was placed a few centimeters above the vent orifice within the zone of active mixing of diffuse flow and seawater (Fig. 1a). The temperatures recorded in the shimmering vent fluid near the collector ranged between 11°C and 45°C, in the range of, or exceeding temperatures recorded a few days earlier (Alain *et al.*, 2004). No temperature measurements were performed near the float, but given that the distance between the collector and the float was about 15 cm, we can reasonably assume that average temperatures at which the biofilm formed were below 20°C. Chemical analyses had also previously shown that diffuse-flow fluids of this chimney contained high sulphide levels and negligible amounts of iron (Alain *et al.*, 2004). At the fourth day of deployment, a thick and dense white biofilm visible on video imagery was observed on the syntactic foam float attached to the collector and at the collector's base (data not shown). The collector was recovered after 5 days incubation (Fig. 1b). The *in situ* collector had been colonized by several specimens of juvenile and adult *Alvinella* worms in their freshly secreted organic tubes. The white mat that remained on the marker was collected for further detailed analyses.

Scanning electron microscopy (SEM) analysis revealed that the mat consisted in a dense network of cells (mainly rods) embedded within a mucous matrix (Supplementary Fig. 1a and 1b). Cells were interconnected by the matrix throughout the biofilm.

The elemental microanalysis of the mat was investigated by electron probe analysis (EDS) and X-ray diffractometry (XRD). The SEM-EDS analysis indicated that the network of microorganisms was coated with elemental sulphur. XRD diagrams confirmed the dominance

of elemental sulphur (with 2 θ peaks at about 23.1°, 25.9°, 27.8°, 28.7°, 28.95°and 31.4°) (data not shown). No traces of sulphide minerals, such as pyrite, were detected.

3.2 Molecular characterization of the mat-associated microbial communities

The genetic diversity of the microbial assemblages associated with the mat sample was analyzed by constructing 16S rRNA. Regardless of the PCR conditions used, no archaeal 16S rRNA genes could be amplified from the DNA extractions. 16S rRNA gene amplification products were obtained using two different bacterial primer pairs. A total of 78 clones containing full-length inserts (59 clones from the library obtained using 50F-1407R and 19 clones from the library generated with primers 63F-1407R) were categorized by RFLP. The clones were then grouped into distinct clusters on the basis of their restriction pattern. Verification of the RFLP clone family groupings was obtained by comparing full 16S rRNA sequences of up to three representative clones from each family. Sequences that were ≥ 97% similar to each other were grouped as a sequence type (Table 1). None of the sequences were identified as chimeras from RDP analysis. Homologous coverage values (Singleton *et al.*, 2001) of the libraries generated using primers 50F-1407R ($C_{'50F'}$ = 93.2 %) and 63F-1407R ($C_{'60F'}$= 94.7%) indicated that a significant part of their 16S rRNA gene diversity was assessed.

All clone sequence types were affiliated to the *'Epsilonproteobacteria'* (Fig. 2; Table 2). A majority of the clones (~80%) had only 92% sequence identity with an uncharacterized *Arcobacter* strain isolated from marine surface water (Eilers *et al.*, 2000) and was more closely related (95% similarity) with an environmental clone sequence (AY225610) recovered from a Mid-Atlantic ridge hydrothermal system (Lopez-Garcia *et al.*, 2003). Five sequence types clustered within the group F defined by Corre *et al*. (2001) (Fig. 2). Three of them (L50-WB13, L50-WB17, and L50-WB20) had clones obtained from the hydrothermal environment

as closest relatives (97-99% similarity) (Lopez-Garcia *et al.*, 2002) (K.A. Kormas, M.K. Tivey, K. von Damm, and A. Teske, unpublished data). Their closest cultivated relative was a yet uncharacterized mesophilic microaerophile (strain 49MY) isolated from a yellow mat sample from the Okinawa Trough (Takai *et al.*, 2003). The closest relatives of the two other clone sequences from group F (L-50WB2 and L63-WB1) were an environmental clone sequence obtained from tubes of *A. pompejana* (M. A. Cambon-Bonavita, V. Riou, K. Alain, V. Cueff, F. Lesongeur, G. Barbier, and J. Querellou, unpublished data) and *Sulfurovum lithotrophicum*, respectively. The latter organism, recently isolated from a deep-sea hydrothermal sediment core, is a mesophilic chemolithoautotroph capable of sulphur and thiosulphate oxidation and nitrate reduction (Inagaki *et al.*, 2004).

The remaining sequence type (L50-WB11) clustered into the family '*Helicobacteraceae*' (Corre *et al.*, 2001) and had *Sulfurimonas autotrophica* as closest relative (98% similarity); this strain was recently isolated from hydrothermal vent sediment and is a microaerophilic chemolithoautotroph capable of growth on sulphur, thiosulphate and sulphide (Inagaki *et al.*, 2003).

To get more insight into the ecophysiological characteristics of microbial components of the mat sample community, the presence and the diversity of *aclB* genes were evaluated. A total of 51 *aclB* clones were obtained and they showed high identity level between them (> 95% on protein level). All were affiliated to the *'Epsilonproteobacteria'* (Fig. 3) and clustered with sequences of *Nautilia* strain Am-H, *Lebetimonas acidophila,* and environmental clones from alvinellid tubes (Inagaki *et al.*, 2003) regardless the reconstruction methods used. Although Takai *et al*. (2005) recognized that phylogenies for the 16S rRNA and *aclB* genes are incongruent, no significant differences could be made between tree topologies when the 16S rRNA sequence dataset matched that of the available *aclB* sequences (cultured

'Epsilonproteobacteria') (Nye *et al*., 2006). This suggests that the *aclB* sequences retrieved from the mat sample were not that of the *Arcobacter*-like populations.

3.3 Design and specificity of probe EPSI682

The designed probe EPSI682 (5'-CGGATTTTACCCCTACACM-3') matched perfectly all the sequences identified in the study (Fig. 2). The specificity of the probe encompassed the 16S rRNA sequences of environmental clones and cultured strains of families *Campylobacteraceae*, *Helicobacteraceae* and '*Hydrogenimonaceae*'. The probe EPSI682 also matched all the sequences affiliated to the group F defined by Corre *et al.* (2001), except that of the epibionts of hydrothermal invertebrates (one M:A mismatch at position 682; *E. coli* numbering). Members of the new family '*Nautiliaceae*' (Miroshnichenko *et al.*, 2004) and of group G defined by Takai *et al*. (2003) contained a T:G mismatch at position 693.

The specificity of radioactively-labeled probe EPSI682 was also ensured by optimization of experimental hybridization conditions. The *Td* value for probe EPSI682 experimentally measured at 46°C was used as the posthybridization washing temperature to test its specificity by dot-blot hybridization of nucleic acids from 27 target and nontarget species and environmental clones (Fig. 4). Confirming the *in silico* analysis, probe EPSI682 gave positive signals for environmental clones and strains belonging to families *Helicobacteraceae* and *Campylobacteraceae*. As expected, no hybridization signals were obtained for members of the family '*Nautiliaceae*' (Fig. 4c, blots A4 and B4) and non-target strains (Fig. 4c, line 9).

The specificity of the fluorescently labeled probe EPSI682 was tested by whole-cell hybridization of paraformaldehyde-fixed cells of reference strains. The formamide concentration was optimized by using mixtures of target (*Arcobacter skirrowii* and *Campylobacter jejuni*) and non-target strains (*Nautilia lithotrophica* and *Desulfurobacterium thermolithotrophum*). The hybridizations were performed simultaneously with either bacterial

probe EUB338 or *Arcobacter*-specific probe ARC94 (Snaidr *et al.*, 1997). The probe ARC94 was also tested as a majority of the phylotypes retrieved in both libraries clustered within the *Arcobacter* group. The new designed probe showed the brightest fluorescence when hybridization buffers with 20% formamide were used. Only *C. jejuni* and *A. skirrowii* cells, specifically as target strains, had positive hybridization signals with probe EPSI682 (Fig. 5). The simultaneously hybridization with probes EPSI682 and ARC94 distinguished *Arcobacter* cells from that of *C. jejuni* (Fig. 5c).

3.4 Quantification of cells in the mat sample

The probes EPSI682 and ARC94 were used simultaneously with bacterial specific EUB338 or the archaeal specific probe ARCH915 to identify and to quantify the microorganisms in the mat sample. No signals were obtained when paraformaldehyde-fixed cells were hybridized with probe ARCH915. This result and the failure to amplify archaeal 16S rRNA genes from the mat DNA suggest strongly that *Archaea* were absent, or are present in amounts undetectable by the currently applied methods.

FISH experiments (Fig. 6a and 6b) revealed that ~ 95% of the bacterial cells reacted with probe EPSI682. Cells hybridizing with probe EPSI682 were morphologically diverse. Simultaneous hybridization with probes EPSI682 and ARC94 (Fig. 6c-6e) showed that cells of the *Arcobacter* group represented around 60% of the cells detected by probe EPSI682. The cells that had positive hybridization signals with both probes had the same morphologies.

4 Discussion

One of the objectives of the oceanographic cruise PHARE was to identify the microbial communities involved in the first steps of colonization processes on virgin surfaces exposed to the deep-sea hydrothermal vent fluids at the 13°N (Le Bris *et al.*, 2002). Visible structures, macroscopically comparable to that observed at 9°N and Guaymas Basin hydrothermal sites (Taylor *et al.*, 1999) formed in a few days during our colonization experiments. Our goal in this study was to examine the chemical composition of this material and the diversity of the microbial community involved in its formation. The microscopic and chemical analyses of the sample revealed that it consisted in a network of microorganisms embedded in a mucous sulphur-rich matrix. Given that hydrothermal chemical precipitates from the 13°N hydrothermal site do not generally contain such amounts of elemental sulphur (Y. Fouquet, unpublished results), we hypothesized that the sulphur formation had a microbial origin.

Our goal in this study was to determine the diversity of the microbial community involved in the mat formation. We applied the full-cycle rRNA approach, including the construction of 16S rRNA gene libraries and FISH experiments to identify the components of the mat community and to assess quantitatively the dominant microbial populations. The libraries were entirely composed of clones assigned to the *'Epsilonproteobacteria'*. These results were in line with many phylogenetic molecular surveys that showed that *'Epsilonproteobacteria'* can represent 40 to 98% of the clone libraries from hydrothermal environments sampled throughout the world's oceans (Haddad *et al.*, 1995; Moyer *et al.*, 1995; Polz and Cavanaugh, 1995; Cary *et al.*, 1997; Reysenbach *et al.*, 2000; Corre *et al.*, 2001; Longnecker and Reysenbachach, 2001; Alain *et al.*, 2002; Lopez-Garcia *et al.*, 2002; Teske *et al.*, 2002; Lopez-Garcia *et al.*, 2003; Takai *et al.*, 2003; Alain *et al.*, 2004; Goffredi *et al.*, 2004). However, contrary to previous studies that showed that the

'*Epsilonproteobacteria*' of groups F and B (family '*Hydrogenimonaceae*') were the most frequently retrieved phylotypes (Corre *et al.*, 2001; Alain *et al.*, 2002; Lopez-Garcia *et al.*, 2002; Lopez-Garcia *et al.*, 2003; Takai *et al.*, 2003; Alain *et al.*, 2004; Goffredi *et al.*, 2004), the majority of our sequences grouped into a phylotype related to the genus *Arcobacter*. This finding was somewhat unusual as no *Arcobacter* isolates have been reported from deep-sea vents and *Arcobacter*-related phylotypes account generally for a minor fraction of the sequences obtained from this environment (Lopez-Garcia *et al.*, 2002; Huber *et al.*, 2003; Lopez-Garcia *et al.*, 2003; Alain *et al.*, 2004).

The genus *Arcobacter* is unique among the '*Epsilonproteobacteria*' as it contains validly published species isolated from both animal and environmental sources, including marine habitats (McClung *et al.*, 1983; Vandamme *et al.*, 1992; Mansfield and Forsythe, 2000; Donachie *et al.*, 2005; Houf *et al.*, 2005). Furthermore, a number of potentially novel species have been described from environments such as oil fields, marine waters and sediments (Eilers *et al.*, 2000; Gevertz *et al.*, 2000; Kaeberlein *et al.*, 2002; Wirsen *et al.*, 2002). *Arcobacter* species are generally known as common human and animal pathogens or free-living organisms that respond poorly in conventional biochemical and growth tests (On, 1996). Typical features of characterized species are optimal growth under microaerophilic conditions at room temperature (Vandamme *et al.*, 1991). Given that the 16S rRNA sequence of the microorganisms dominant in the mat libraries shared ~ 92% similarity with that of validly published *Arcobacter* species, we can only speculate about their ecophysiology. However, because XRD analyses identified elemental sulphur as a main component of the mat, it is tempting to suggest that our dominant group might be sulphur-oxidizing bacteria. Consistent with this hypothesis, the ability to oxydize sulphur compounds and to use oxygen as electron acceptor is a common trait in various members of the '*Epsilonproteobacteria*' isolated from deep-sea hydrothermal vents (Inagaki *et al.*, 2003; Takai *et al.*, 2003; Inagaki *et*

al., 2004; Takai *et al.*, 2004; Nakagawa *et al.*, 2005). Although direct oxidation of hydrogen sulphide has been demonstrated for a restricted number of '*Epsilonproteobacteria*' isolated from hydrothermal vents (Inagaki *et al.*, 2003; Takai *et al.*, 2003), it seems reasonable to suggest that the mat dominant organisms might also share this metabolic capacity as "*Candidatus* Arcobacter sulfidicus", that is the only described organism able to excrete sulphur filaments comparable to that of our mat, is a sulphide-oxidizer (Wirsen *et al.*, 2002).

Given that autotrophic CO_2 fixation through the rTCA cycle has been recently shown in *'Epsilonproteobacteria'*, the mat sample was analysed for the presence and diversity of the ATP citrate lyase gene. Although the recovered *aclB* environmental clones were also all affiliated to *'Epsilonproteobacteria'*, phylogenetic analyses showed that they did not correspond to that of *Arcobacter*-like populations. It could suggest that the main microbial components of the mat sample may use a different carbon fixation pathway. This hypothesis seems however unlikely as it has been demonstrated that the rTCA cycle operates in a significant number of representative strains from widespread phylogenetic subgroups and physiological types of *'Epsilonproteobacteria'* (Campbell *et al*. 2003; Hügler *et al*., 2005; Takai *et al*., 2005). Since the primers used in this study were designed using a limited number of *aclB* sequences, it is also possible that a range of *aclB* sequence types have escaped molecular detection. These hypotheses need to be evaluated in the future to clarify the main microbial pathways used in the hydrothermal sulphur mat formations.

The ecological dominance of the '*Epsilonproteobacteria*' in the hydrothermal environment has been mostly hypothesized on the basis of their distribution in 16S rRNA gene libraries. However, for a variety of reasons (copy number, bias during DNA extraction, PCR, cloning), the frequency of 16S rRNA gene clone appearance does not reflect the actual microbial community structure (Farrelly *et al.*, 1995; von Wintzingerode *et al.*, 1997). Several studies have reported that biases in PCR amplification could just explain the overwhelming

abundance of '*Epsilonproteobacteria*' (Corre, 2000; Watanabe *et al.*, 2002). Therefore quantitative characterization was required to test if this dominance was also reflected in the makeup of the mat-associated populations *in situ*. Oligonucleotide probes specific to uncultured groups of '*Epsilonproteobacteria*' have been designed over recent years (Polz and Cavanaugh, 1995; Cary *et al.*, 1997; Snaidr *et al.*, 1997; Longnecker and Reysenbach, 2001; Engel *et al.*, 2003). However, some 16S rRNA sequences identified in this study were not targeted by the existing probes, justifying the development of a probe with a wider coverage. Although probe EPSI682 demonstrated the actual omnipresence of '*Epsilonproteobacteria*', it is likely that non targeted members of this lineage (Fig. 2) may also have contributed to the bacterial cells detected with the probe EUB338.

It is interesting to notice that striking differences exist between our results and that of Alain *et al.* (2004) although both colonization experiments took place on the same diffuse vent for the same time period. The very thin microbial veils that grew on the colonization device deployed by Alain *et al*. (2004) were macroscopically different from our thick sulphur mats. The absence of *Archaea* or their very low representation is one of the only common findings between both studies. This is probably due to the fact that biofilms had formed in environmental conditions (low temperatures, presence of oxygen) that are not compatible with the lifestyle of most known archaea (Alain *et al.*, 2004). Although *'Epsilonproteobacteria'* also prevailed in the clone libraries derived from the veils, the dominant phylotypes belonged to group F and no *Arcobacter*-related sequences were retrieved. Several indications suggest that the two biofilm communities had settled under distinct environmental parameters (such as temperature, oxygen and sulphide concentrations). Just before the deployment of our collector, the mechanical removing of the overlying sulphide structure resulted in an increase of the vent emission (Fig. 1). This explains why the fluid temperatures measured with the ROV thermocouple and probably the sulphide concentrations to which our collector was

exposed were generally higher than that recorded earlier (14-860 μM) (Alain *et al.*, 2004). Consistent with this hypothesis, filamentous sulphur formation by "*Candidatus* Arcobacter sulfidicus" required high sulphide concentrations (> 400 μM) and its sulphide optimum was higher (1.5 mM) than those reported for other sulphur-oxidizers (Wirsen *et al.*, 2002). The capacity to grow optimally and selectively at high sulphide concentrations may also explain how these bacteria can locally out-compete other sulphur-oxidizers as we observed in our sample.

This report further expands the known diversity of the '*Epsilonproteobacteria*' from the initial reports of the prevalence of this group at deep-sea vents. Given that the ecological dominance of these microorganisms had been hypothesized on the only basis of their distribution in 16S rRNA gene libraries, we provide here a first demonstration of the numerical abundance of these microorganisms *in situ*. We showed that bacteria taxonomically different from "*Candidatus* Arcobacter sulfidicus" are probably involved in filamentous sulphur formation. This process can be ecologically significant in environments where opposing oxygen-sulphide gradients are available at the necessary concentrations and can contribute significantly to the overall organic matter production at hydrothermal vents. It would therefore be significant now to delineate the environmental parameters that govern this efficient sulphide oxidation and the extent of the diversity and distribution of the responsible organisms.

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Table 1. Phylogenetic affiliations of bacterial type sequences detected in the hydrothermal mat sample.

Sequence type[a]	Other representatives	Clone numbers in libraries		Closest cultivated strains (% identity)
		50F-1407R	63F-1407R	
L50-WB6	L50-WB9, L50-WB56, L63-WB2, L63-WB6	48	15	*Arcobacter* strain KT0913 (AF235110) (92%)
L50-WB53		1		*Arcobacter* strain KT0913 (AF235110) (93%)
L50-WB13	L63WB19	1	1	Epsilonproteobacterial strain 49MY (AB091293) (96%)
L50-WB17		1		Epsilonproteobacterial strain 49MY (AB091293) (94%)
L50-WB20	L50-WB49	5		Epsilonproteobacterial strain 49MY (AB091293) (95%)
L50-WB2		1		*Nitratifractor salsuginis* (AB175500) (93%)
L63-WB1	L63-WB8, L63-WB16		3	*Sulfurovum lithotrophicum* (AB091292) (96%)
L50-WB11	L50-WB7	2		*Sulfurimonas autotrophica* (AB088431) (98%)

[a]Sequence types were identified by RFLP analysis and phylogenetic analysis. All sequence types had generally different RFLP profiles. However, clones L50-WB20 and L63-WB1 displayed the same RFLP profile and were categorized in distinct sequence types on the basis of the phylogenetic analysis. Clones L50-WB6, L50-WB9 and L50-WB56 that had different RFLP types were categorized in one sequence type as their sequence similarity was ≥ 97%. For the same reason, L50-WB20 and L50-WB49 were assigned to a unique sequence type.

2

Legends to figures

Fig. 1. Photographs of the *in situ* collector deployed for 5 days at Genesis vent site. (A) Deposition of the collector near a *Riftia pachyptila* colony; the syntactic foam float (FL) is attached to the collector; white tubes of *Alvinella pompejana* (AL) are visible around the vent orifice (VE); bar, 10 cm (copyright Ifremer/PHARE 2002). (B) Recovery of the *in situ* collector after 5 day incubation; the dense white biofilm formed on the syntactic foam float was scrapped once onboard.

Fig. 2. Phylogenetic position of the epsilonproteobacterial sequences retrieved from the hydrothermal mat sample (in bold). The tree topology was obtained using the neighbour-joining method. Two '*Gammaproteobacteria', Escherichia coli* and *Riftia pachyptila* endosymbiont, and *Bacillus subtilis* were used as outgroup. The reference 16S rRNA sequences were obtained from GenBank; all environmental clone sequences presented in the tree have been retrieved from hydrothermal vents. The numbers at the nodes represent the bootstrap values (≥50%) obtained with 500 resamplings. Scale bar indicates the expected number of changes per sequence position. Environmental clone group F and group G are according to Corre *et al*. (Corre *et al.*, 2001) and Takai *et al*. (Takai *et al.*, 2003), respectively. The light grey and dark grey boxes encompass sequences targeted by probes EPSI682 and ARC94, respectively.

Fig. 3. Phylogenetic position of translated *alcB* clone sequences retrieved from the hydrothermal mat sample (in bold). Other *aclB* sequences were obtained from GenBank. The tree topology was obtained using the maximum likelihood method. The numbers at the nodes represent the bootstrap values (≥50%) obtained respectively with 500 resamplings in

neighbour-joining and 100 resampling in maximum likelihood method. Scale bar indicates the expected number of changes per amino acid position.

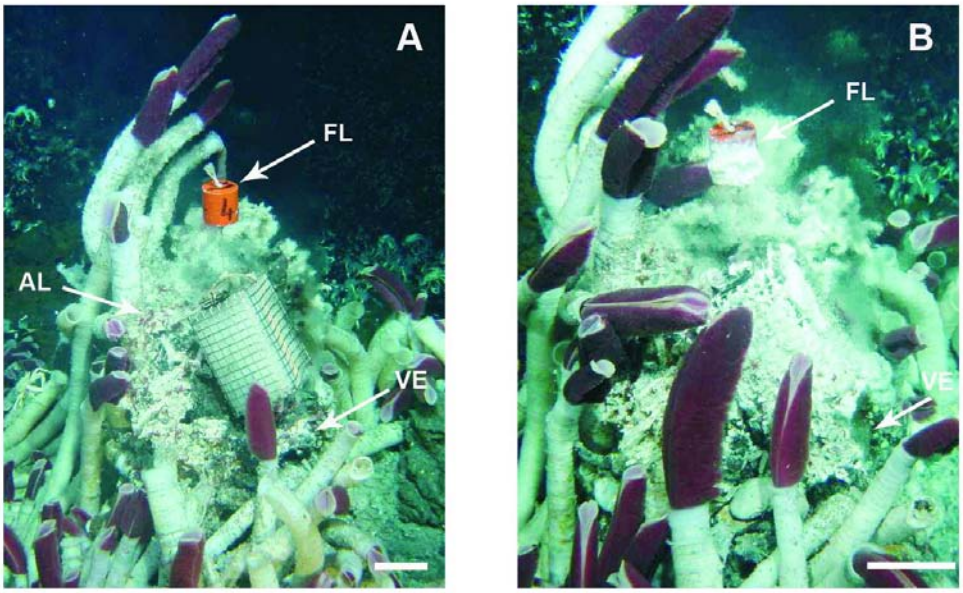
Fig. 4. Dot blot analysis of the probe specificities. Hybridization responses of radioactively labeled probes to nucleic acids from 27 target and nontarget DNAs (left) of environmental clones (light grey background) and strains (dark grey background). The blots were hybridized with the following probes BACT338 (middle) and EPSI682 (right).The layout of nucleic acid samples on the blot is the following: 1A, VC1.2 clone 01; 1B, VC1.2 clone 10; 1C, VC1.2 clone 42; 2A, VC1.2 clone 26; 2B, VC1.2 clone 32; 2C, VC1.2 clone D; 3A, VC1.2 clone 21; 3B, VC1.2 clone 57; 3C, VC1.2 clone 31; 4A, VC1.2 clone 69; 4B, VC2.2 clone 01; 4C, VC2.2 clone 02; 5A, VC2.2 clone 03; 5B, *Alvinella* epibiont APB13B; 5C, *Campylobacter jejuni*; 6A, *Campylobacter coli*; 6B, *Geospirillum barnesii*; 6C, *Arcobacter nitrofigilis*; 7A, *Thiomicrospira denitrificans*; 7B, *Wolinella succinogenes*; 7C, *Helicobacter pylori*; 8A, *Escherichia coli*; 8B, *Zobellia galactanivorans*; 8C, *Bacillus* sp. strain SG9).

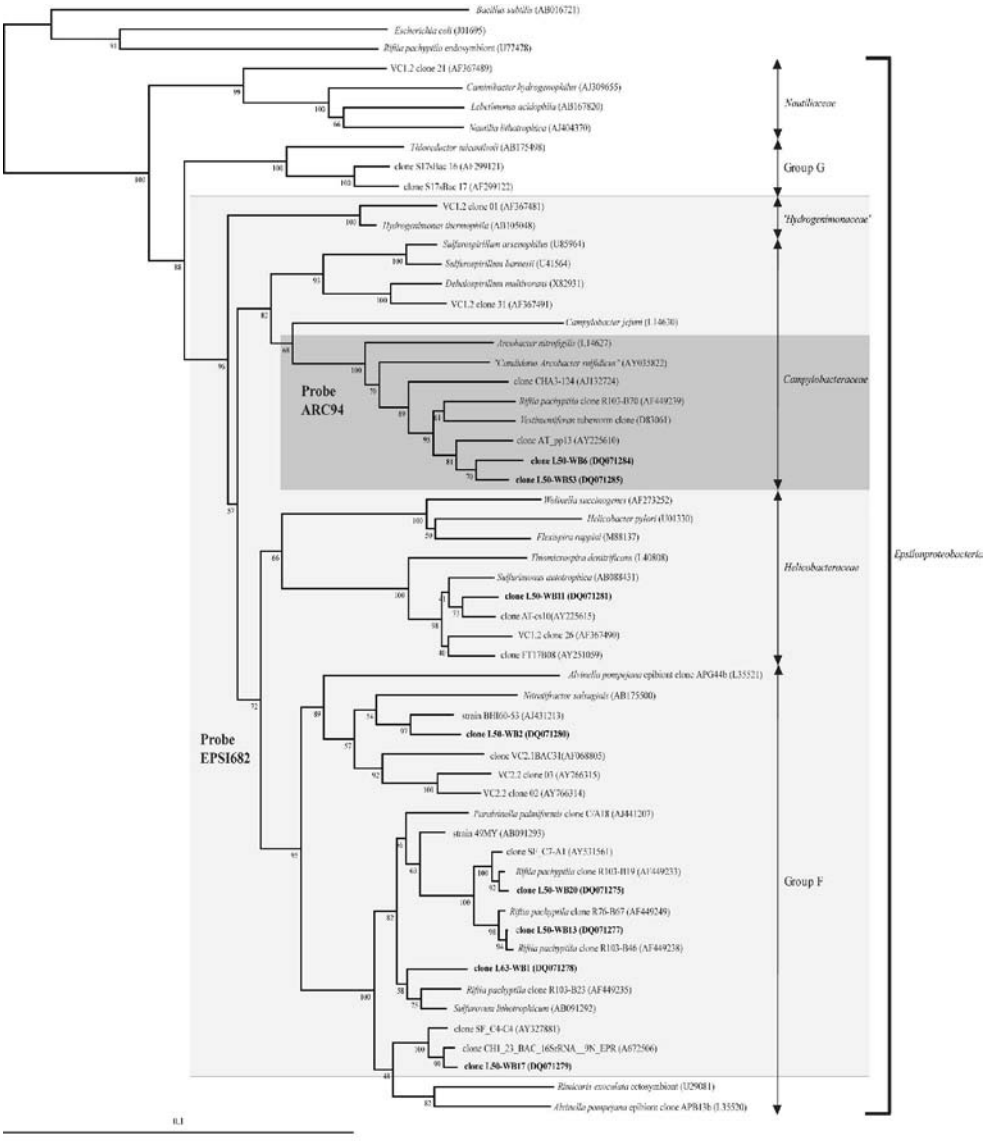
Fig. 5. (top four panels). Whole cell hybridizations of different mixtures of fixed cells hybridized simultaneously with fluorescein-labeled (green fluorescence) and a rhodamine- or Cy3-labeled probe (red fluorescence). Epifluorescence photomicrographs (double exposures) are shown at a magnification of X 1,140 (scale bar, 10 µm). Cells that hybridized with both types of probes appeared yellowish or orange on the double-exposed film. (A) Cells of *Campylobacter jejuni* and *Nautilia lithotrophica* simultaneously hybridized with probes EPSI682 (green fluorescence) and EUB338 (red fluorescence). (B) Cells of *Arcobacter skirrowii* and *Desulfurobacterium thermolithotrophum* simultaneously hybridized with probes EPSI682 (red fluorescence) and EUB338 (green fluorescence). (C) Cells of *A. skirrowii* and *C. jejuni* simultaneously hybridized with probes EPSI682 (green fluorescence) and ARC94

(red fluorescence). (D) Cells of *A. skirrowii* and *C. jejuni* simultaneously hybridized with probe EUB338 (green fluorescence) and ARC94 (red fluorescence).

Fig. 6. (bottom five panels). Whole-cell hybridizations of cells from the mat collected on the 13°N EPR with probes EPSI682 (green fluorescence) and EUB338 (red fluorescence) (A-B), with probes EPSI682 (green fluorescence) and probe ARC94 (red fluorescence) (C) and with probes ARC94 (red fluorescence) and EUB338 (green fluorescence) (D and E). Epifluorescence photomicrographs (double exposures) are shown at a magnification of X 1,140 (scale bar, 10 μm).

Supplementary Fig. 1. Scanning electron microphotographs of the bacterial mat. (A) Micrograph showing the dense network of cells; bar: 5 μm; (B) Enlargement showing mostly rod cells embedded in a polymeric matrix, bar: 2μm.

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Lebetimonas acidiphila (AB167820)
Nautilia lithotrophica (AJ404370)
Thioreductor micantisoli (AB175498)
clone SI7dBac 16 (AF299121)
clone S17dBac 17 (AF299122)
Nautiliaceae
Group G
VC1.2 clone 01 (AF367481)
Hydrogenimonas thermophila (AB105048)
'Hydrogenimonaceae'
Sulfurospirillum arsenophilum (U85964)
Sulfurospirillum barnesii (U41564)
Dehalospirillum multivorans (X82931)
VC1.2 clone 31 (AF367491)
Campylobacter jejuni (L14630)
Arcobacter nitrofigilis (L14627)
'Candidatus Arcobacter nitfidicus' (AY035822)
clone CHA3-124 (AJ132724)
Riftia pachyptila clone R103-B70 (AF449239)
Nautiliaceae tubercolum clone (DQ83061)
clone AT_pp13 (AY225610)
clone L50-WB6 (DQ071284)
clone L50-WB53 (DQ071285)
Campylobacteraceae
Probe ARC94
Wolinella succinogenes (AF273252)
Helicobacter pylori (U00330)
Flexispira rappini (M88137)
Thiomicrospira denitrificans (L40808)
Sulfurimonas autotrophica (AB088431)
clone L50-WB11 (DQ071281)
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VC1.2 clone 26 (AF367490)
clone FT17B08 (AY251059)
Helicobacteraceae
Epsilonproteobacteria
Alvinella pompejana epibiont clone APG44b (L35521)
Nitratifractor salsuginis (AB175980)
strain BH860-53 (AJ431213)
clone L50-WB2 (DQ071280)
clone VC2.1BAC3H(AF068805)
VC2.2 clone 03 (AY766315)
VC2.2 clone 02 (AY766314)
Probe EPSI682
Parafrutiella palmiformis clone C/A1E (AJ441207)
strain 40MY (AB091295)
clone SF_C7-A1 (AY531561)
Riftia pachyptila clone R103-H19 (AF449233)
clone L50-WB20 (DQ071275)
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clone L50-WB13 (DQ071277)
Riftia pachyptila clone R103-B46 (AF449238)
clone L63-WB1 (DQ071278)
Riftia pachyptila clone R103-B23 (AF449235)
Sulfurovum lithotrophicum (AB094292)
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Group F
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Alvinella pompejana epibiont clone APB43b (L35520)
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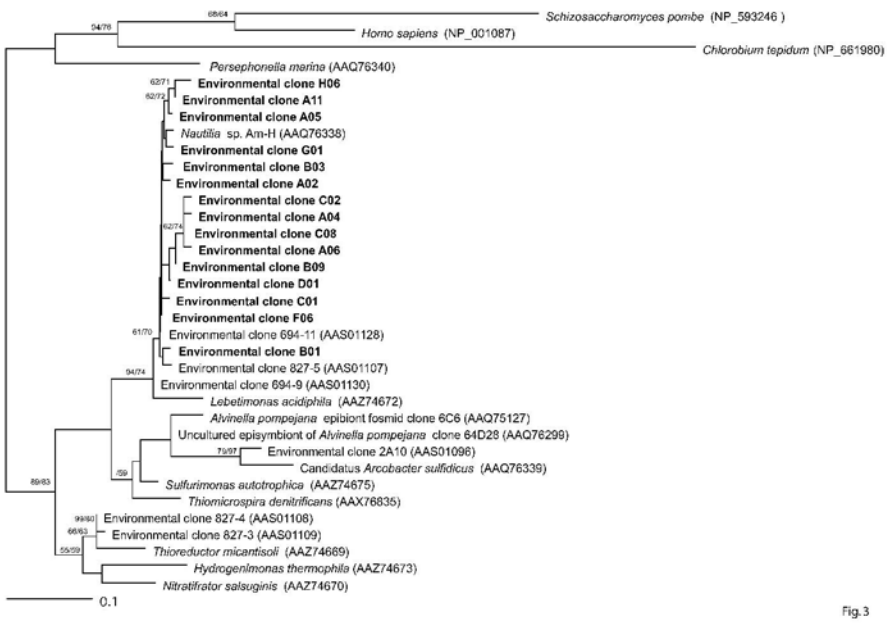
Fig. 2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Persephonella marina (AAQ76340)
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Nautilia sp. Am-H (AAQ76338)
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Environmental clone A04
Environmental clone C08
Environmental clone A06
Environmental clone B09
Environmental clone D01
Environmental clone C01
Environmental clone F06
Environmental clone 694-11 (AAS01128)
Environmental clone B01
Environmental clone 827-5 (AAS01107)
Environmental clone 694-9 (AAS01130)
Lebetimonas acidiphila (AAZ74672)
Alvinella pompejana epibiont fosmid clone 6C6 (AAQ75127)
Uncultured episymbiont of Alvinella pompejana clone 64D28 (AAQ76299)
Environmental clone 2A10 (AAS01096)
Candidatus Arcobacter sulfidicus (AAQ76339)
Sulfurimonas autotrophica (AAZ74675)
Thiomicrospira denitrificans (AAX76835)
Environmental clone 827-4 (AAS01108)
Environmental clone 827-3 (AAS01109)
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Fig.3

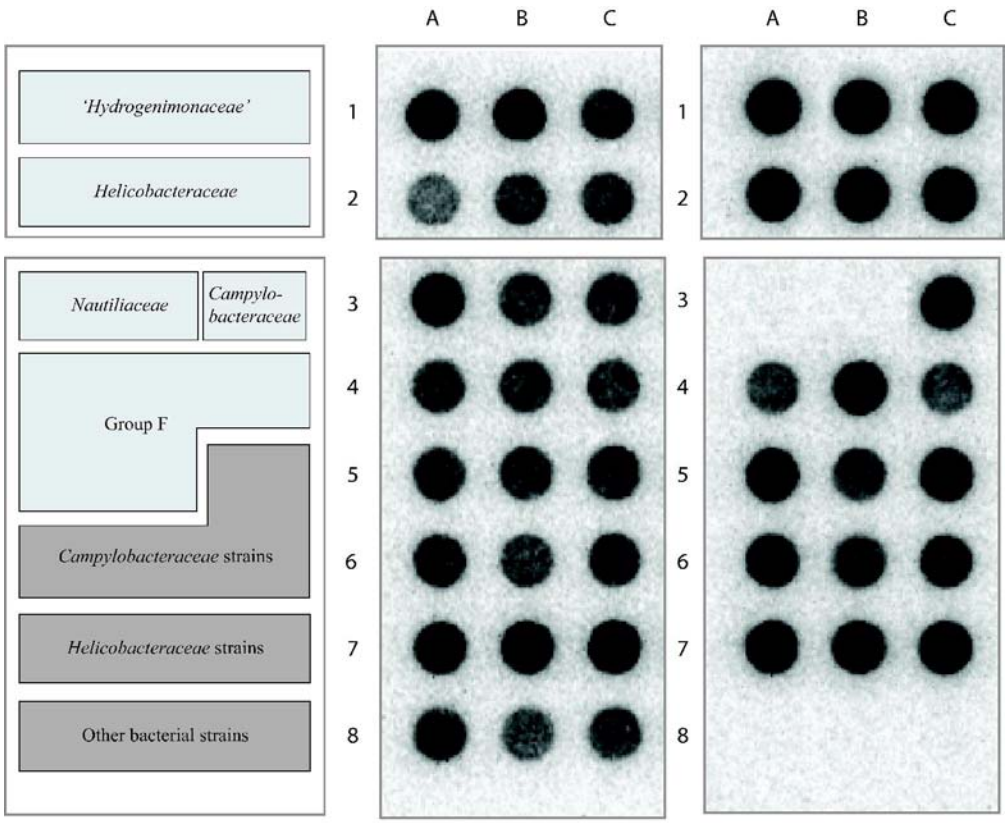
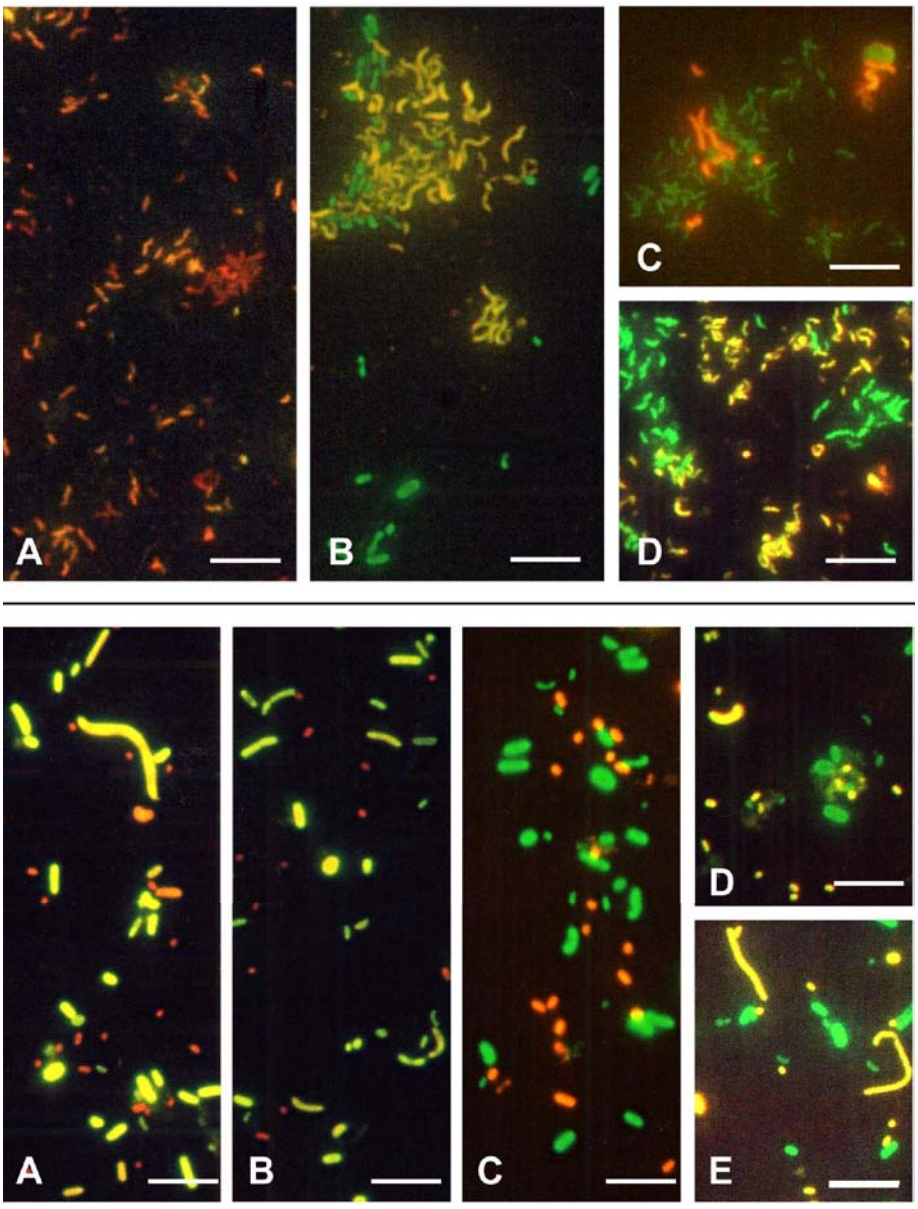
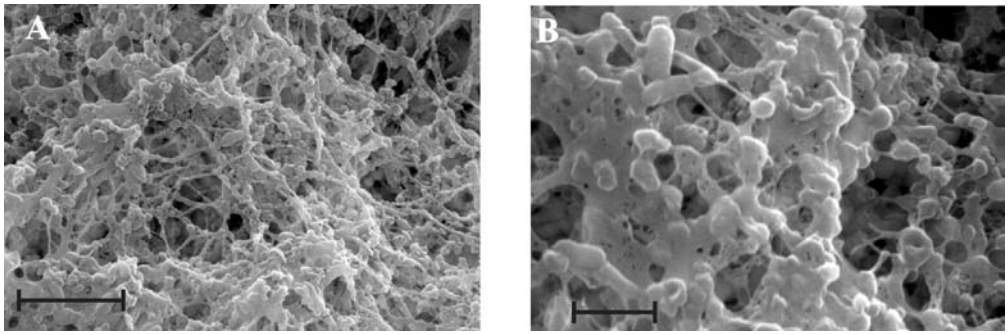


Fig. 4

Dot blot analysis of the probe specificities.


A
B
C
D
A
B
C
D
E



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